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Function of CD4-associated p56^{lck} in T cell activation

by

Keting Chu

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

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in

Immunology

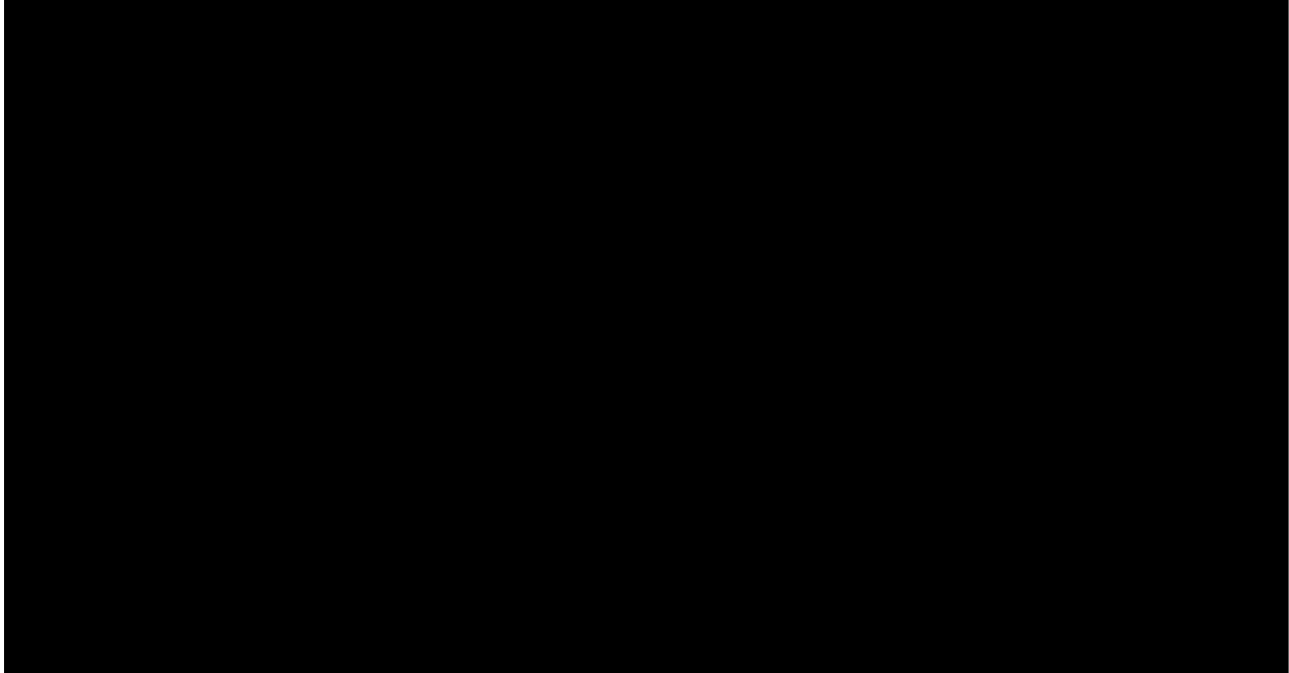
in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco



DEDICATION

**This work
is
dedicated to
my husband, Bill Robinson
and my parents**

PREFACE

I would like to thank my adviser, professor Danial R. Littman, for his generous support of my four-year graduate work, for his critique of my work and for his help in writing this thesis. I would also like to express my appreciation to my thesis committee members, professors Keith Yamamoto, Lewis Williams and Rudolf Grosschedl, for their inspiration and guidance. I feel especially grateful for the encouragement and guidance provided by professor Keith Yamamoto, in my struggle to cope with numerous problems. I could not have completed my graduate study without all of them. During the four years of my graduate study in professor Littman's laboratory, I received great scientific and emotional support from all colleagues in the laboratory, and I thank them all. I especially want to thank Julia Turner and Hua Xu for the cell lines they made. These made this study possible. Of course, without the love and support of my beloved husband, Bill, without the love and high expectations of my parents, I could not have reached this goal.

Function of CD4-associated p56^{lck} in T cell activation

By

Keting Chu

Abstract

Lck (p56^{lck}) , a lymphoid specific *src* family protein tyrosine kinase (PTK), is non-covalently associated with the cytoplasmic tail of CD4 and is required for T cell activation. T cell ligand binding is followed by rapid PTK activation and a number of cellular proteins are tyrosine phosphorylated, including phospholipase C γ 1 (PLC) and CD3 ζ . CD3 ζ is one of the T cell receptor (TCR) associated polypeptides involved in the signal transduction process, and is also specifically associated with a PTK (ZAP70) after TCR stimulation. Tyrosine phosphorylation of PLC γ 1 results in its activation and in subsequent phosphatidylinositol (PI) turnover. To study the effects of CD4/lck on the PTK activation pathway following TCR/CD3 stimulation, we utilized an antibody crosslinking approach in an antigen specific CD4 dependent murine T hybridoma. We found that crosslinking with anti-TCR $\alpha\beta$ antibodies did not activate the PTK pathway, whereas co-crosslinking of CD4 to TCR $\alpha\beta$ enabled PTK activation. In contrast, crosslinking of CD3 resulted in PTK activation and co-crosslinking of CD4 to CD3 facilitated the process. The tyrosine kinase activity of p56^{lck} was essential for the co-crosslinking effects. CD4/lck increased ζ chain tyrosine phosphorylation *in vivo* and *in vitro* and facilitated the initiation of PTK activation when co-crosslinked to a CD8/ ζ chimeric molecule at suboptimal antibody concentration.

Furthermore, the effects of CD4/lck on tyrosine phosphorylation and activation of PLC correlated well with the effects on PTK activation. Antibody crosslinking of CD4 alone failed to induce tyrosine phosphorylation and activation of PLC. The results suggest that p56^{lck} positively regulates activation of the TCR/CD3-coupled PTK pathway by tyrosine-phosphorylating CD3 ζ . Through recognition of a single MHC class II molecule by CD4 and TCR, the CD4 associated p56^{lck} is thus brought adjacent to the TCR complex and facilitates the signaling process during T cell activation by antigens.

Dan Littman

6/9/93

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INTRODUCTION

Activation of T lymphocytes requires not only recognition of major histocompatibility molecule (MHC) and antigen by the T cell receptor (TCR), but also the interaction of several other molecules on the T cell surface with molecules on the surface of antigen presenting cells (1-3). The interactions result in signal transduction through the TCR complex and activation of T cell effector function. The other T cell surface molecules, commonly referred to as accessory molecules, are thought to increase the avidity of interaction between T cells and antigen presenting cells (2-5). The CD4 and CD8 glycoproteins have been included among the accessory molecules. However, the association of a lymphoid specific protein tyrosine kinase (PTK), p56^{lck}, with the cytoplasmic domains of CD4 and CD8 has suggested that these molecules could deliver signal(s) across the cell membrane (6-9). More evidence has argued that these T cell surface glycoproteins perform a critical function in signal transduction, serving as co-receptors along with the TCR (9). This thesis was intended to study the role of the CD4/lck complex as an integral component of the cell signaling machinery during T cell activation.

There is extensive evidence that CD4 and CD8 participate in the signal transduction process through the TCR/CD3 complex during both T cell activation and thymocyte development. However, the mechanism of CD4/CD8 function in the signal transduction process is not fully understood. In order for CD8 to function during thymocyte maturation, CD8 and the TCR have to interact with the same MHC

molecule (202, 204). This strongly suggests that an integration of signal(s) generated from CD4/CD8 and the TCR/CD3 is required for a productive result. The PTK activation is considered to be the first step in signal transduction during T cell activation and is involved in activation of PLC γ 1, which can, in turn, initiate PI turnover and deliver signals into the nucleus. To determine the mechanism by which p56^{lck} affects signal transduction from the TCR, we utilized an antibody crosslinking approach to examine the effects of a preformed CD4/lck complex on PTK activation and on subsequent PLC tyrosine phosphorylation/activation.

Structure of the CD4 and CD8 proteins and genes

Both CD4 and CD8 are T cell surface glycoproteins and members of the immunoglobulin superfamily (10). The CD4 glycoprotein is a 55-58 kd monomer consisting of four distinct extracellular immunoglobulin family domains, a hydrophobic transmembrane domain, and a highly conserved 38 amino acid cytoplasmic tail. Complementary DNA (cDNA) encoding human CD4 was initially isolated by subtractive hybridization techniques using cDNA libraries constructed from mRNA of mouse fibroblasts (L cells) that had been transfected with total human genomic DNA and selected for cell surface expression of human CD4 (11). Mouse (12, 13) and rat (14) cDNA's were subsequently isolated by cross-hybridization to human probes. The mature CD4 polypeptide chain consists of 435 amino acids (AA) in humans and 431 AA in mice.

The extracellular portion of CD4 contains 374 AA in humans and 368 AA in mice. This is followed by a hydrophobic transmembrane segment (26 AA in humans, 25 AA in mice) and a highly basic cytoplasmic tail (38 AA in both species). The human molecule has two potential N-linked glycosylation sites, while the mouse has four. Each has six cysteine residues external to the cell, and biochemical studies on both mouse and sheep CD4 have shown that these form three intrachain disulfide loops connecting adjacent cysteines (15). There are six additional cysteines in human (two in the transmembrane region and four in the cytoplasmic tail) (11). The cytoplasmic domain is the most highly conserved domain of CD4 between mouse and man, with 79% identical residues, while the external domains share only 55% identical residues between these two species (11-13).

CD4 is encoded by a single gene on human chromosome 12 (16, 17) and mouse chromosome 6 (18). The mouse CD4 gene spans 26 kb of DNA and consists of 10 exons that encode distinct domains of the protein (19). An untranslated 5' exon is followed by exons encoding a signal peptide (exon 2), an amino-terminal domain that most closely resembles Ig light chain variable regions (exons 3,4), three other Ig family domains (exons 5-7), a transmembrane domain (exon 8), and a cytoplasmic domain (exons 9,10).

The crystal structure of the N-terminal two domains of CD4 has been reported (20). Both domains have the configuration of immunoglobulin folds, even though the second domain is truncated, and the connection between the domains is quite stiff,

resulting in an elongated molecule with extensive interdomain interaction.

The CD8 glycoprotein exists either as an disulfide-linked aa homodimer/homomultimer or as an ab heterodimer (10) and the disulfide-linkage involves cysteines in the transmembrane region and/or cytoplasmic tail (21-23). Both subunits are members of the immunoglobulin superfamily and have molecular weights of 32-37 kd. The a and b chains each consists of a single Ig-family domain followed by a spacer region of 40-50 residues. The two chains of CD8 have similar overall organization (10, 24): a leader peptide is followed by a domain of approximately 100 AA that is related to Ig light chain variable regions; and a domain rich in serines, threonines, and prolines, containing O-linked glycosylation, links the Ig-like domain to a hydrophobic transmembrane domain and a short cytoplasmic tail (28 residues in CD8a and 19 residues in CD8b). In different species, alternative splicing results in multiple isoforms of both chains of CD8 (25-27); in addition, splicing out of the transmembrane domain results in secretion of the human CD8a homodimer.

Cell surface expression of the b chain requires co-expression of the a chain (28, 29). The cDNA of CD8a in humans, mice and rats has been cloned in the same fashion as that of the CD4 cDNA (26, 30-33). cDNA encoding CD8b was first isolated in the rat (OX8, 37kd) (34). Both CD8a and CD8b are encoded by single copy genes and do not require rearrangement for expression (10, 28, 35). Classical genetic studies led to the early conclusion that mouse

CD8a (Ly-2) and CD8b (Ly-3) genes are closely linked both to each other and to the Ig K light chain locus on chromosome 6 (36-40). These two genes are in the same transcriptional orientation and are separated by 36kb of DNA, with the Ly-3 gene located to the 5' side of the Ly-2 gene (28). The Ly-3 gene spans approximately 16kb of DNA, hence is much larger than the Ly-2 gene which is 5.4kb (28). There are two forms of murine CD8a chain on the cell surface, a (38kDa) or a' (34kDa), encoded by the same gene (10, 32). A 31bp deletion in the cytoplasmic domain results in an early termination and a 4 AA (a') rather than a 29 AA (a) cytoplasmic tail. Two alleles have been defined serologically for both murine CD8a (Ly-2.1 and Ly-2.2) and CD8b (Ly-3.1 and Ly-3.2) (10). There is only one base pair substitution and consequent amino acid change at residue 78 that accounts for the difference in serological properties between Ly-2.1 and Ly-2.2 (27).

Expression of CD4 and CD8

CD4 and CD8 are expressed primarily on mature peripheral T cells and subsets of thymocytes (rev. 10). In general, expression of CD4 or CD8 in peripheral T cells divides them into two subpopulations with distinct properties. The T cells expressing CD4 (65%) are helper or inducer cells while those expressing CD8 (35%) are cytotoxic or suppresser cells. However, this subdivision is not entirely exclusive. There are CD4 expressing cells found to have cytotoxic activity and vice versa. The best correlation of expression of CD4 or CD8 on T cells is with the ability of their TCR to recognize

antigen presented by class II or I molecules of major histocompatibility complexes (MHC) on antigen presenting cells (APC). With few exceptions, T cells expressing CD4 recognize antigen with MHC class II molecules and those expressing CD8 recognize antigen with MHC class I (41). Although the specificity is determined by the specific interaction of TCRs on T cells with antigen plus MHC molecules on APCs, CD4 and CD8 are very important in the process through their direct binding of the non-polymorphic region of MHC molecules (Rev. 42) and their cytoplasmic association with a lymphoid specific tyrosine kinase (PTK), p56^{lck} (6-9).

More interestingly, expression of CD4 and CD8 in thymocytes marks various developmental stages during T cell development. The most immature thymocytes (5%) express neither CD4 nor CD8 and are referred to as "double negative". The majority of thymocytes (80%) expressing both molecules and termed "double positive" cells are intermediate in development. The most mature cells in the thymus express either CD4 (10%) or CD8 (5%) like peripheral T cells and are named "single positive" cells (10). While thymocytes proceed through the different stages, a T cell repertoire that can discriminate between self and non-self antigen is generated through positive and negative selection processes. The interaction between TCR on the surface of thymocytes and antigen peptide plus/ or MHC molecule on the surface of thymic stromal cells is critical for the selection processes (43). CD4 and CD8 also play critical roles in the processes as will be discussed later in detail (10, 42- 44).

Another interesting observation is that, unlike the mouse CD8 α chain, the α' chain is expressed only on the surface of immature thymocytes (double positive and immature single CD8 positive thymocytes which are in the transient transition from the double negative stage to the double positive stage) and not on the mature thymocytes (single positive) and peripheral T cells (10) despite proper transcription, translation, glycosylation and association with the β chain (45). A novel sorting mechanism results in only (or predominantly) $\alpha\beta$ but not $\alpha'\beta$ heterodimers transported from the Golgi to the cell surface in peripheral T cells (45). Recent study (46) in a mouse T cell hybridoma showed that the α' molecule, when expressed as either a homodimer alone or a heterodimer together with CD8 β , could not interact with p56^{lck}. The molecule also showed a decreased ability to restore immune response in this hybridoma system. Although those results were obtained in an artificial situation, it is tempting to speculate that the expression of CD8 α' in immature thymocytes might have a function when these thymocytes are going through negative selection and positive selection. It should be noted, however, that α' chains have not been described in either human or rat (rev. 10).

Although CD4 protein has only been identified on thymocytes and peripheral T cells in mice, it can be found on macrophages and the related Langerhans cells in both human and rat (47). In mouse brain(12, 48, 49), a low level of a smaller (2.7kb) polyadenylated CD4 mRNA has been detected without surface expression of CD4 protein. This RNA contains sequences only from the 3' half of the mouse CD4-coding sequence and the 3'

untranslated region, which is the result of usage of an alternative transcription start site. The brain transcript potentially encodes a truncated form of mouse CD4 beginning with an in frame ATG in exon VI, at AA 214. Whether the truncated protein is expressed on the cell surface has not been determined. It is not yet known in which cells the brain specific transcript is expressed.

In contrast to findings in the mouse, human brain tissue has been found to express not only a smaller form of CD4 transcript, but also a readily detectable quantity of full-length CD4 mRNA that co-migrates with human T cell CD4 transcripts (48). The short transcript does not correspond to the mouse brain transcript and moreover the equivalent ATG in exon VI is absent. The smaller mRNA in human brain appears to be a result of an alternative polyadenylation site usage. The equivalent of the mouse brain transcript is absent in human. Immunocytochemistry of human brain sections with CD4 specific antibodies has shown that CD4 protein is expressed (50, 51). Since human macrophages are known to express CD4 protein, they are most likely the predominant class of CD4⁺ cells in brain. Some neuronal and glial cells in human brain have also been found to express CD4 (51). The full-length CD4 transcripts have also been demonstrated in human granulocytes, which are not known to express cell surface CD4 (49). The physiological role of CD4 on non-T cells is unknown. However, since human CD4 is the receptor for human immunodeficiency virus (HIV), the retrovirus that causes acquired immune deficiency syndrome (AIDS), the expression of CD4 in human brain is of great interest. Central nervous system

involvement, especially a subacute encephalopathy, occurs with high frequency in AIDS' patients (52, 53).

Because of the importance of regulated expression of CD4 and CD8 in development, the regulatory mechanisms controlling these genes have been of great interest. The hope is that knowledge of the mechanism will give insight to the molecular basis of thymocyte development. A lymphoid specific transcriptional enhancer element located 13kb upstream of the mouse CD4 gene has been identified recently in our laboratory (54). In transgenic mice this element, in combination with a genomic DNA fragment of the CD4 gene that contains the promoter region and the first large intron, is able to direct the developmental stage and subset specific expression of reporter cDNA's (55). Studies on the element have shown that two basic helix-loop-helix (bHLH) protein binding sites are mainly responsible for the lymphoid specific transcriptional enhancer activity (54). Further studies on the transcriptional factor(s) bound to the sites in thymocytes and T cells will help a great deal in our understanding of T cell development, as some HLH proteins play very important roles in the development of muscle cell lineage in mouse and human (56), and in the development of sexual and nervous systems in *Drosophila* (57, 58).

Knowledge of CD8 gene expression regulation is relatively poor. Studies to date have shown that mRNAs encoding both CD8 chains were found only in cells that express the protein on the cell surface (26, 27, 32). Sequences at the 5' end of the mouse CD8 gene are more heavily methylated in cells that do not express the

mRNA and protein as compared to cells that do (59). It is likely, as in the case of CD4 gene, that the major level of CD8 expression regulation is transcriptional. As described later in the appendix (unpublished) this author attempted to identify the cis-acting sequences involved in CD8 gene expression.

Interaction of CD4 and CD8 with MHC molecules

The early observation that CD8⁺ cells react specifically with antigen presented by class I MHC molecules prompted the suggestion that CD8 binds to MHC-I molecules on antigen presenting cells (60). This hypothesis was extended to include CD4 binding to MHC-II after the finding that CD4⁺ cells specifically recognize antigen plus class II MHC molecules (61). Evidence for binding of CD4 and CD8 to MHC molecules has been obtained using transfected cells expressing high levels of the various components at the cell surface (62). CV-1 cells infected with an SV40-CD4 recombinant virus and expressing high levels of human CD4 were shown to specifically bind only B cells bearing surface MHC class II molecules (62). This binding could be completely blocked by antibodies to either CD4 or MHC class II molecule. Similarly, radiolabelled MHC class I-expressing B cells were bound to CHO cells cotransfected with an amplifiable DHFR minigene and human CD8a (63). This CD8a-class I MHC interaction was specifically and completely inhibited by antibodies to class I MHC and to CD8a. Artificial cells composed of beads coated with lipid containing either human CD8a or class I MHC were also shown to specifically

bind to each other (64). Taken together, these results demonstrated clearly that CD4 and CD8a can directly bind to class II and class I MHC molecules, respectively.

Using the human CD8a-expressing CHO cell system described above, binding of B cells transfected with naturally occurring or engineered point mutants of a human class I MHC molecule was determined (65). The binding site for CD8a was thus mapped to the membrane-proximal nonpolymorphic $\alpha 3$ domain of this class I molecule. Cells expressing mutants of class I which do not bind CD8a were also found to be poor targets for cytotoxic T cells, presumably because CD8a could not participate in activation of the CTLs. Potter et al. (66) have similarly shown that cells expressing a murine class I molecule with a mutation in the $\alpha 3$ domain are not recognized by CD8-dependent CTLs. These studies provide strong evidence for a direct interaction of CD8 with the $\alpha 3$ domain of class I MHC molecules. Recently, studies from two laboratories (67, 68) showed that both human and mouse CD4 binds to a region of human or mouse MHC class II molecules analogous to the MHC class I binding site for CD8. The mouse CD4 specifically interacted with the $\beta 2$ domain of mouse class II $A\beta$, structurally analogous to the $\alpha 3$ domain of MHC class I, in a cell-cell interaction system similar to that used to test MHC class I binding to CD8 (67). The human recombinant soluble CD4 could bind specifically to recombinant soluble peptide 134-148 of HLA-DR β , which is highly homologous to a sequence of the $\alpha 3$ domain of HLA-class I (residue 223-229) (68). It can also be inferred from the data that, since the TCR interacts with peptides within the

groove formed by the $\alpha 1$ and $\alpha 2$ domains of MHC class I molecules or the $\alpha 1$ and $\beta 1$ domains of MHC class II, CD8 or CD4 and the TCR can simultaneously bind to a single class I or class II molecule. This has significance for the mechanism of CD4/CD8 cooperation with the TCR, as discussed below.

Using the COS transfection system or an MHC-I specific hybridoma, mutant human CD4 molecules were tested for their ability to interact with MHC-II molecules (69, 70). Both studies found that mutations in the CDR-1 region of the N-terminal CD4 domain compromise interaction with MHC-II, but results on mutations within CDR-2 and CDR-3, as well as in domain 2, were not consistent in the two studies. With the 3-D structure of the outermost CD4 domains in hand, it should now be possible to more accurately identify residues that are involved in contacting class II MHC molecules.

Association of p56^{lck} with the cytoplasmic tail of CD4 and CD8

In 1988, it was first discovered that CD4 is complexed with p56^{lck} when immunoprecipitated from cell lysates made from human peripheral T cells (71). Soon after, it was found that both mouse CD4 and CD8 were able to be co-immunoprecipitated with p56^{lck} in mouse peripheral T cells and murine T cell clones (72). Approximately 50% of the p56^{lck} in non-activated CD4⁺ antigen-specific murine T cells (but not other *src* family members such as p60^{c-src}, p62^{c-yes} and p59^{fyn}) was associated with most, if not

all, of the surface CD4 (72). A year later, human CD8 was proven to be able to associate with p56^{lck} in human T cells, and interestingly, both CD4 and CD8 were associated with p56^{lck} in a human CD4⁺CD8⁺ T cell tumor line (MOLT 4) (73). However, it appeared that only 25% of the detectable p56^{lck} in different murine CD8⁺ T cell clones was found in CD8 immunoprecipitates (73). CD8 aa homodimers had a markedly reduced level of associated p56^{lck} activity relative to CD4 (73). From different studies using various cell lines, it has been estimated that approximately 10 to 90% of p56^{lck} exists in a CD4/CD8-associated form (8).

It was first noted that this specific association requires the cytoplasmic domain of CD4 (74). Molecular genetic and biochemical studies showed that a cysteine motif, +--X-Cys-X-Cys-(Pro) (where + means basic residue and X a nonconserved residue), in the cytoplasmic domain of both CD4 and CD8 directly interacts with the first 33 AA in the unique domain of p56^{lck} (75-77). The cytoplasmic domain of CD4 has higher affinity for p56^{lck} than that of CD8a (8). The significance of this is not clear, although both CD4 and CD8 are expressed on immature thymocytes which are going through TCRA gene rearrangement, positive selection and /or negative selection.

Evidence that p56^{lck} and CD4 protein are associated in the intact cell was provided by studies which demonstrated that both proteins could be specifically co-modulated in T cells (7). When CD4⁺ T cells were treated with anti-CD4 antibodies, CD4 molecules were down-regulated by crosslinking-dependent endocytosis and

the abundance of detectable p56^{lck} (but not p59^{fyn}) in the detergent cell lysates decreased proportionally with that of CD4. As a control, crosslinking of other T cell surface proteins such as TCR, Thy1.2 and T200 did not diminish the detectable amounts of p56^{lck} (7). Although the destination of the p56^{lck} is not clear, the result suggested that this might be one way that the CD4-p56^{lck} complex transduces signal(s) into cells. This finding may be related to the observation that thrombin receptor ligand binding in platelets leads to a transient increase of p60^{c-src} activity, followed by a redistribution of p60^{c-src} into the detergent insoluble cytoskeleton (78). The significance of these findings remains to be determined. Both anti-CD4 antibody crosslinking of surface CD4 and HIV gp120 binding of CD4 result in increased p56^{lck} kinase activity and increased autophosphorylation of Tyr394, which is the positive regulatory site for the kinase (79, 80). Concepts about the function of the CD4/CD8-p56^{lck} complex will be discussed later.

Structure and Function of p56^{lck}

p56^{lck} is a member of the *src* family of cytoplasmic PTK's, which consists of at least eight closely related proteins (*c-src*, *c-yes*, *c-fgr*, *fyn*, *lck*, *hck*, *blk* and *lyn*) (rev.7). The genes within this family are considered proto-oncogenes based upon the observation that mutations, whether naturally occurring in retroviruses or resulting from molecular manipulation in the laboratory, can render these genes competent for oncogenic transformation. The tissue distribution of the different *src* family members is varied.

Many cell types express *c-src*, *c-yes* and *fyn*, while *lck*, *c-fgr*, *lyn*, *blk* and *hck* have a more restricted pattern of expression; principally in cells of hematopoietic origin (rev. 7). Unlike growth factor receptors that possess protein kinase activity, the *src* family kinases do not have extracellular or transmembrane domains. However, they are associated with the internal portion of the plasma membrane as a consequence of post-translational myristoylation at their N termini. Only two members of the family have thus far been found specifically associated with cell surface receptors. One is p56^{lck}, which is specifically associated with CD4/CD8 (7, 8), CD2 (81) and interleukin 2 receptor (IL-2R) b chain (82, 83). Another is p59^{fyn}, which was co-precipitated with TCR in a murine T hybridoma line in a mild detergent at low stoichiometry (84) and in human T cells (153). p59^{fyn} is also physically associated with CD23 (FceRII) in natural killer cells and could be activated through stimulation of CD23 (114).

Expression of p56^{lck}

p56^{lck} is normally expressed exclusively in lymphoid cells, predominantly in T cells (85, 86). It was first detected in the Moloney murine leukemia virus-induced T cell lymphoma line LSTRA (85, 87), which overexpresses the *lck* gene due to insertion of viral promoter sequences. As a result, the corresponding mRNA is transcribed and translated more efficiently, and the amount of p56^{lck} is about 50-fold higher than in other T cell lines (87-89). The cDNA encoding p56^{lck} was cloned and sequenced from LSTRA

and murine thymocytes (85, 87, 90), and later from human T cells (91).

In both mouse and human, *lck* gene expression is regulated through T cell development by two different promoters, resulting in heterogeneity in the 5' untranslated sequences (92-95). The proximal promoter is active only in thymocytes, while the distal one is active in both thymocytes and peripheral T cells, and each can function independently (95). The exact significance of the differential regulation is not clear. $p56^{lck}$ is important in both thymocyte development and peripheral T cell activation. In mice genetically ablated in expression of functional $p56^{lck}$, there was a profound block of T cell development (96). In transgenic mice overexpressing $p56^{lck}$, there was a reduction in TCRb rearrangement, specifically the joining of Vb and Db, but there were normal rearrangement of TCRA and normal expression of RAG-1 and RAG-2 gene (both are required for TCR gene rearrangement) (152). In transgenic mice overexpressing a kinase deficient form of $p56^{lck}$, there was a severe defect in the development of ab T cells, and the developmental arrest of thymocytes occurred at an early stage with successful TCRb gene rearrangement but no TCRA gene rearrangement (152). In a mutant T cell tumor line (Jurkat), lack of functional $p56^{lck}$ led to unresponsiveness to antibody against TCR (97). These observations strongly argue that $p56^{lck}$ is involved during development, regulating TCR gene rearrangement and thymocyte maturation, and in activation of mature T cells. It is likely that the dual promoter system evolved as a mechanism to differentially

regulate gene expression under different developmental conditions: i.e., it might be necessary to control *lck* gene expression in peripheral T cells in a manner different from that in thymocytes undergoing gene rearrangement, selection and maturation.

Structure and functional regulation of p56^{lck}

The structure of the p56^{lck} protein is very similar to that of other *src* family members (rev. 7). The N terminal myristoylation site is followed by a unique domain which, in the case of p56^{lck}, contains the CD4/CD8 binding site. Then there are one *src* homology 3 domain (SH3) and one *src* homology 2 domain (SH2), adjacent to each other, followed by the catalytic domain (kinase domain) and a C terminal regulatory segment, which is involved in governing the specific activity of the enzyme. The specific features worthy of note include the myristoylation site at glycine residue 2, several unidentified serine phosphorylation sites (which might affect interactions with other proteins and potentially regulate enzyme activity), the ATP binding site at Lys273, the autophosphorylation site at Tyr394 (defined following *in vitro* immunocomplex kinase assays), and the regulatory phosphorylation site at Tyr505. Replacement of Tyr505 with phenylalanine (an aromatic amino acid incapable of being phosphorylated) results in constitutive activation of p56^{lck} kinase activity (98, 99). CD45, a plasma membrane tyrosine phosphatase, is expressed on all hematopoietic cells except mature erythrocytes and accounts for 90% of protein tyrosine phosphatase (PTP)

activity in T cells (rev. 100). CD45 can dephosphorylate Tyr505 in p56^{lck} *in vitro* and *in vivo*, and activates the kinase activity. In CD45-deficient cells, the Tyr505 of p56^{lck} was hyperphosphorylated relative to wild-type cells (101), the kinase activity was reduced (101, 111) and cellular p56^{lck} was not able to bind to a phosphopeptide containing Tyr505, possibly because the molecule was in a different conformation than that in wild type cells (102). CD45 can also increase p56^{lck} kinase activity when added into the *in vitro* kinase assays (103, 104). Taken together, these data all support the notion that Tyr505 is a negative regulatory site. The proposed model is that the SH2 domain of p56^{lck} binds to phospho-Tyr505 resulting in a molecule having an inactive conformation (102, 106). In contrast, the phosphate occupancy at Tyr394 is detected *in vivo* only following enzyme activation (e.g. in Tyr505 to Phe mutants, when p56^{lck} is grossly overexpressed or in antibody crosslinkings of CD4 and CD8) (79, 80, 98, 99, 105). Tyr394 is thought to be a positive regulatory site, but this has not been conclusively demonstrated.

SH3 and SH2 domains are also found in many other signaling molecules including phospholipase C α 1, GTPase-activating protein (GAP), phosphatidylinositol 3 kinase (PI3-K), growth factor receptor activation binding factor 2 (GRB2), p47^{gag-crk} and many others (rev.106). SH2 domains mediate interaction among signaling molecules by direct binding to phosphotyrosines (106). SH3 domains are also present in the cytoskeletal components, e.g. spectrin, several isoforms of myosin 1b and a yeast actin binding protein. SH3 domains are believed to be responsible for the cell

morphological changes in growth factor receptor mediated signal transduction, probably through binding of GAP's of certain GTP-binding proteins (G protein) (107). A yeast gene (BEM1), which encodes a protein containing two SH3 domains, is required for cell polarization demonstrated by genetic studies (108). More recently, SH3 domains have been shown to bind proline rich sequences in molecules involved in guanine nucleotide exchange (e.g. Sos) (107).

Mutations in p56^{lck} showed that deletions of either or both SH3 and SH2 domains increased kinase activity and led to enhanced autophosphorylation at Tyr394 (109, 110). These data, in combination with data regarding the condition of p56^{lck} in CD45-deficient cells (hyperphosphorylation of Tyr505, reduced kinase activity and inability to bind a Tyr505 containing phosphopeptide), support the intramolecular model of negative regulation of p56^{lck}, in which the SH2 domain binds the phospho-Tyr505 and reduces the kinase activity (102, 106). The kinase that phosphorylates Tyr505 of p56^{lck} has not been identified. A strong candidate is the C-terminal *src* kinase (CSK), which phosphorylates Tyr527 of p60^{src} (equivalent of Tyr505 in p56^{lck}) and can also phosphorylate Tyr505 of p56^{lck} in *in vitro* kinase assays (112, 113). CSK is expressed in T cells, but whether it phosphorylates Tyr505 under physiological conditions remains to be studied. Since p56^{lck} plays an important role in both thymocyte development and T cell activation, identification of the relevant Tyr505 kinase(s) will contribute to a fuller understanding of signal transduction during these processes.

The kinase domain of p56^{lck} is not only the catalytic domain but is also responsible for the direct binding to the IL-2R β chain (82). This physical association is necessary for p56^{lck} activation upon IL-2R ligand binding (83). However, the physiological significance is not understood because the association is not required for mitogenic signal transduction through the receptor when examined in certain cell lines which do not express the receptor normally (83). It is thus expected that the kinase domain will have roles in addition to only catalyzing phosphate transfer.

The association of p56^{lck} with CD4 (not CD8) can be regulated by protein kinase C (PKC) activation, which results in serine/threonine phosphorylation of CD4 and dissociation of p56^{lck} from CD4 (115, 116). In human peripheral T cells and a variety of leukemia T cell lines, a serine/threonine kinase Raf-1 related protein, p110, was detected to be associated with the CD4-p56^{lck} complex and was predominantly phosphorylated on serine residues (117). Treatment of cells with phorbol ester, a potent PKC activator, resulted in a transient increase in the detection of p110 associated with CD4-p56^{lck}, concomitant with the surface loss of CD4 (117). During T cell activation under physiological conditions, there is activation of serine/threonine kinases and PKC, as well as surface loss of CD4 (rev. 2, 7). It will be very interesting to know the significance of p110 in this process.

It has been postulated that p56^{lck} is involved in the G protein and the phosphatidyl-inositol (PI) pathways. A 32kd G protein was co-immunoprecipitated with CD4-p56^{lck} and CD8-

p56^{lck} in human thymocytes, peripheral T cells and T cell lines (118). The ras GAP, which can regulate the GTPase activity of p21^{ras}, was able to serve as substrate for p56^{lck} in *in vitro* kinase assays (119). The tyrosine phosphorylated form of ras GAP could bind to p56^{lck} *in vitro* (119). p56^{lck} was also co-precipitated with phospholipase Cg1 (PLCg1) and a TrpE fusion protein containing the SH2 domain of PLCg1 after activation of a human T cell tumor line (120). As will be discussed later, after TCR stimulation, p21^{ras} activation is the result of reduced ras GAP activity, which is sensitive to PTK inhibitors, and/or the result of activated guanine nucleotide releasing factor (GRF), such as p95^{vav}, whose GRF activity is increased upon tyrosine-phosphorylation (121). PLCg1 is also activated as a consequence of PTK activation (122, 123). p95^{vav} could serve as a substrate for p56^{lck} in the *in vitro* kinase assay (121). Further study on the role of p56^{lck} in the G protein pathway and PLCg1 initiated PI pathway should advance the understanding of signal transduction processes during T cell activation.

Signal transduction and T cell activation

T cell receptor complex and T cell activation

T cells are activated following interactions of surface molecules between T cells and antigen presenting cells (APC). The TCR and CD4/CD8 complexes on T cells interact specifically with the peptide antigen and class II or I major histocompatibility

(MHC) molecules on APCs. Subsequent signal transduction and T cell activation leads to lymphokine production, cell differentiation and proliferation. Other factors, the accessory molecules or co-stimulators, contribute greatly to the process by increasing the avidity of cell-cell interaction, transducing signal(s) directly into T cells, or both (rev. 2, 3).

A simplified scheme of the T cell activation and signal transduction process is shown in Fig. 5. The TCR is a multicomponent complex consisting of a disulfide-linked α and β heterodimer and the non-covalently linked CD3 invariant chains (g, d, e, z and h) (124-126). The antigen specificity of T cells is determined by the TCR $\alpha\beta$ heterodimer, which binds to peptide antigen plus MHC molecules directly (127-133). The $\alpha 1$ and $\alpha 2$ domains of MHC class I molecules form a groove that presents peptide to T cells and are also involved in direct interactions with TCR $\alpha\beta$ heterodimers (124, 134). The CD3 complex is not only non-covalently linked to the $\alpha\beta$ heterodimer, but is also required for the heterodimer's cell surface expression, and believed to be responsible for signal transduction in T cell activation (124). Direct evidence for CD3 signal transducing function comes from recent studies (135-138). The cytoplasmic tail of a single CD3 chain, e.g. CD3z or CD3e, was sufficient to transduce T cell activation signals when expressed as a chimeric molecule on the surface of T cells and a basophil cell line (135-138). Another interesting feature is that each of the CD3 chains contains at least one well conserved putative signaling motif (YxxLxxxxxxYxxL, where Y denotes tyrosine residue, L leucine and X non-conserved residues) (139).

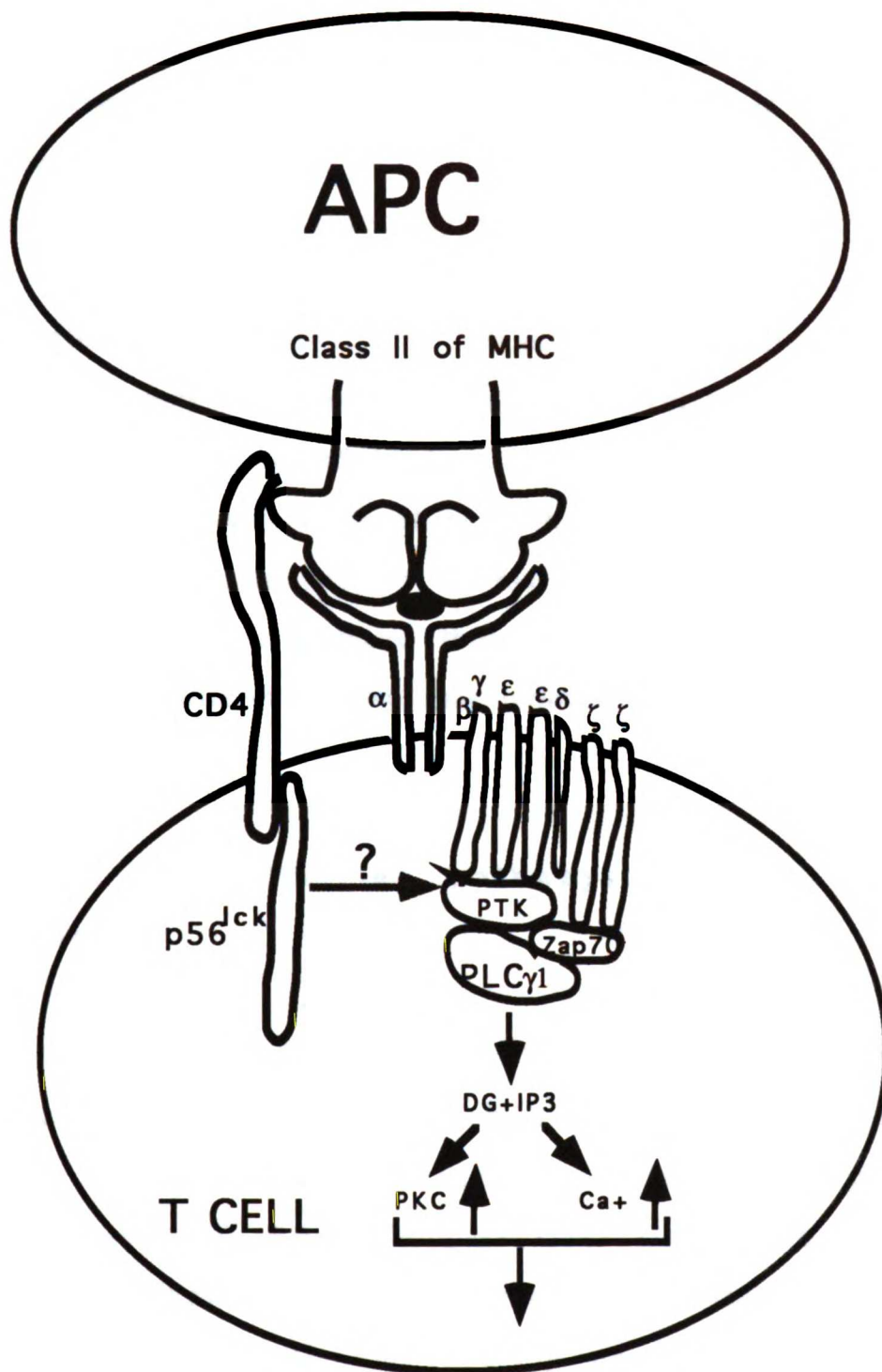


Fig. 5 Scheme of signal transduction in T cell activation by antigen and APC

This begs the question why the seven-chain complex is required if one chain can transduce the complete signal. One possibility is that redundancy increases the sensitivity of T cells to stimuli. Another one is that different chains actually deliver different signal(s) under different conditions. For example, different signaling through different chains may account for the different consequences of TCR ligand binding, such as T cell activation for mature T cells and either clonal deletion or clonal expansion for immature thymocytes. In support of this hypothesis, the pattern of tyrosine-phosphoproteins induced through the cytoplasmic tail of CD3 ϵ chain is different from that induced by the tail of CD3 ζ chain (136).

Signal transduction pathways during T cell activation

As shown in Fig. 5, after T cell ligand binding, a cascade of biochemical events occurs resulting in a variety of cellular responses. They involve generally the activation of protein tyrosine kinases (PTK), phospholipase C (PLC), protein tyrosine phosphatases (PTPase), protein serine/threonine kinases and the G protein pathway (rev. 2). Earlier study on signal transduction during T cell activation showed that the process can be divided into two stages: the early events including phosphatidylinositol (PI) turnover, Ca⁺⁺ influx and PKC activation, and the late nuclear events including activation of transcriptional factors, transcriptional activation of early response genes and, later, IL-2

and IL-2R gene transcriptional activation resulting in cellular activation responses (rev. 140). Activation of phospholipase C is responsible for initiating PI turnover generating diacylglycerol (DG), a potent PKC activator, and inositol 1, 4, 5 trisphosphate (IP3), which can bind to its receptor to release stored intracellular Ca^{++} (140). The activation of PKC and calmodulin-dependent kinases/phosphatases can, in turn, modify a series of transcription factors (124, 141). It appears that PI turnover is the critical step and might be sufficient for T cell activation, as treatment of almost all T cells, including various T cell tumor lines, T cell clones and resting peripheral T cells, with phorbol ester (a potent PKC activator) and calcium ionophore could lead to T cell activation (124, 141). In addition, a muscarinic subtype-1 receptor, which transduces signals by coupling to PI metabolism through a G protein pathway, could activate T cells alone when expressed on the surface following transfection (142). However, when the epidermal growth factor receptor (EGF-R) was expressed on T cells, stimulation of the receptor failed to activate T cells despite a measurable Ca^{++} influx (143). The question whether PI turnover is sufficient for T cell activation therefore remains unanswered.

Protein tyrosine kinases

The importance of tyrosine kinase activation has been suggested by the complete blockade of TCR-mediated signalling events, both the early events (e.g. Ca^{++} influx) and the subsequent T cell activation, when tyrosine kinase inhibitors were used (144,

145). It is believed now that the earliest signal transduction events detected are increased protein tyrosine kinase (PTK) activity and tyrosine phosphorylation of a number of cellular proteins, including of CD3z and one isoform of PLC, PLCg1 (146, 147). There are six tyrosine residues in the cytoplasmic tail of CD3z, which are located in three "signaling motifs" and are critical for the signal transducing capacity of CD3z chain shown by mutagenesis studies (139, 148-150). Tyrosine phosphorylation of PLCg1 leads to PLC activation, which accounts for more than 95% of the PLC activity to initiate PI turnover during T cell activation (122). It is through this mechanism that the PTK pathway is coupled to the PI pathway in T cell signal transduction. There is additional evidence that PLCg1 can be co-precipitated with TCR after T cell activation (151). However, the precise mechanism for PLCg1 tyrosine phosphorylation occurring following TCR stimulation, including the nature of the PTK(s) involved, remains unknown.

There is no known intrinsic enzymatic activity in the TCR/CD3 complex. There have been a number of cytoplasmic PTKs reported to be associated with TCR/CD3 complexes (rev. 125, Fig. 5). One is a *src* family tyrosine kinase, p59^{fyn}, which has been shown to be associated with the TCR/CD3 complex in a murine T cell hybridoma and human T cells at very low stoichiometry(84). p59^{fyn} was able to bind specifically to cytoplasmic domains of all the CD3 subunits (e, g, z, and h chains) through its unique A-terminal domain 10 AA (84). Recently, a PTK named ZAP70 (z associated protein p70) was reported being associated with the

CD3z chain only in activated Jurkat cells, a human leukemia T cell line (154, 155). p56^{lck} is specifically associated with the co-receptors of CD4 and CD8.

The activation of these associated PTKs may account for the protein tyrosine phosphorylation after TCR ligand binding. As discussed before, p56^{lck} is critical in T cell activation and thymocyte development, as demonstrated by both biochemical or genetic studies in T cell lines and transgenic mouse experiments. In transgenic mouse experiments, it has also been shown that p59^{fyn} is involved in T cell activation and development. In mice over-expressing an active form of p59^{fyn}, peripheral T cells were hyperresponsive to TCR stimulation, while in mice over-expressing an inactive form of p59^{fyn} thymocyte response to TCR mediated stimulation was substantially reduced (156). In mice lacking expression of p59^{fyn}, thymocytes showed markedly reduced responses although peripheral T cells were normal (157). In addition, biochemical study has shown that the kinase activity of p59^{fyn} could be increased upon engagement of the TCR/CD3 complex (156).

ZAP70 is a T cell specific non-src family PTK with two SH2 domains and as yet unidentified tyrosine-phosphorylation site(s) (155). ZAP70 is specifically associated to CD3z chain and the association requires an activation signal through the TCR in T cells and co-expression of either p56^{lck} or p59^{fyn} in non-T cells. This suggests that ZAP70 is recruited into the TCR/CD3 complex and is thus activated following TCR ligand binding, in which p59^{fyn} or p56^{lck} (or both) is (are) involved. Other chains of the CD3 complex,

which, like the CD3z chain, have been shown to be able to transduce T cell activation signal(s) (e.g. CD3e), cannot associate with ZAP70 and may function through other cytoplasmic PTKs specifically associated with those chains. It is therefore important to understand the function and interaction of the various PTKs involved during signal transduction in T cells.

Protein tyrosine phosphatases

PTPases are another sets of enzymes critical for signal transduction during T cell activation (rev. 2, 10, 100, 125, 160). The T cell activation process is transient and probably requires a shut-down device. Theoretically, PTPase's could function to oppose the activity of PTK's, i.e. protein tyrosine phosphorylation by PTKs activates the signal cascade then protein tyrosine dephosphorylation by PTPases would inactivate it. Up to now, no experimental results have shown directly that this is true and we know very little about the shut-down mechanism in T cells. However, there is data suggesting that PTPases might play a role in keeping T cells in the resting state and that inhibition of PTPase activity might be required for T cell activation. Membrane associated PTPase activity is high in resting T cells, but is decreased during mitogen-induced blast transformation (158). When the blasts reverted to resting lymphocytes, the PTPase activity increased more than twofold concomitantly with an elevated surface expression of CD45 (a PTPase, see below). When the T blasts were cultured in the presence of a PTPase inhibitor

(vanadate), they maintained their lymphoblastic proliferation and did not revert to resting lymphocytes. Inhibitors of PTPase (vanadate and hydrogen peroxide) could also induce tyrosine phosphorylation of cellular substrates, elevated cytosolic free calcium, and induced IL-2R α chain expression but not IL-2 secretion in normal human T cells. Anti-CD28 antibody in combination with vanadate and hydrogen peroxide induced IL-2 secretion, consistent with the requirement for a co-stimulatory signal in the induction of this gene through the TCR. As stated before, PKC activation and cytoplasmic Ca^{++} elevation is an early consequence of TCR stimulation in T cell activation. Both the Ca^{++} elevation and PKC activation could also induce a transient decreased CD45 PTPase activity in T cells (159a, 159b). The increased Ca^{++} induced the decreased PTPase activity accompanied by decreased serine phosphorylation of CD45 (159a). The PKC activation resulted in decreased PTPase activity and decreased surface expression of CD45 (159b). These results indicated that transient PTPase inhibition could be a consequence of early TCR/CD3 signal transduction during T cell activation, which would theoretically permit enhanced tyrosine phosphorylation of cellular proteins. However, no direct evidence has been obtained in support of this hypothesis. There are down sides with those results obtained from studies using PTPase inhibitor drugs as we do not fully understand the effects of the drugs on living cells. These results are difficult to interpret in light of other studies using PTPase inhibitors and CD45-deficient cell lines.

Other studies on PTPases during T cell activation have also suggested that they have critical roles in transducing activation signals. Partial inhibition of PTPases by phenyl arsine oxide was shown to augment TCR-mediated signal transduction although higher concentration of the drug were inhibitory (167). The CD45 PTPase has been the focus of these studies and has been suggested to play an important role in activation by regulating p56^{lck} or/and p59^{fyn} PTK activity (159-166). Cell lines that lack expression of CD45 and retain apparently normal TCR expression could not be activated through stimulation of the TCR. There was no detectable activation of PTK and PLC nor any increase in intracellular Ca⁺⁺ level (161-166). p56^{lck} in some of the CD45 deficient cell lines and p59^{fyn} in some others showed specifically increased tyrosine phosphorylation of the negative regulatory tyrosine and reduced PTK activity in the *in vitro* kinase assays (102, 103, 111, 168). The activity returned to normal level upon re-expression of CD45 following transfection, and this also restored the ability of cells to be activated through the TCR. Combined with the finding that CD45 can specifically dephosphorylate p56^{lck} on the negative regulatory site *in vitro* and activate its PTK activity, these results suggest that CD45 can activate p56^{lck} or p59^{fyn} or both in T cells. CD45 is physically complexed with CD4 or CD8 after antigen stimulation of TCR in human T cells (169), and CD45 was co-precipitated with p56^{lck} in resting human T cells (170), providing a structural basis for the regulation of p56^{lck} activity by CD45. In some cell lines, the requirement for CD45 in T cell activation could be by-passed by co-crosslinking CD4 and CD3 (165). The exact mechanism

involved is not clear and some possibilities will be discussed in the discussion.

It has been suggested that ligand binding to CD45 may change PTPase activity and inhibit PTK activation upon TCR stimulation or IL-2 stimulation of T cells (159). Antibody ligation of CD45 before T cell stimulation with anti-CD3 antibodies changed the pattern of tyrosine phosphorylation of specific substrates (159). The same ligation of CD45 did not alter the pattern in resting T cells responding to IL-2, except for a mobility shift of a 55kd protein and an increased phosphorylation of a 112kd substrate. However, proliferation in response to both anti-CD3 and IL-2 was inhibited by ligating CD45. Specific ligand(s) of CD45 have not yet been identified. There are other cytoplasmic PTPases also present in T cells, and their roles in T cell activation have not yet been determined.

Protein serine/threonine kinases

Protein serine/threonine kinases are also coupled to TCR signal transduction pathways. One of these, PKC, carries the signal(s) into the nucleus by activating/modifying transcriptional factors. Transcriptional activation of IL-2 or IL-2R genes in response to the TCR ligand binding are the results of activation of cis-acting enhancers in the IL-2 and IL-2R gene loci (141). There are a number of transcription factors reported to be able to bind to the IL-2 enhancer element and thus responsible for its activation, including activator protein 1 (AP1), an octamer binding protein

(OBP) or octamer binding factor 1 and 2 (Oct-1 or 2), nuclear factor of κ box binding (NF κ B) and nuclear factor of activated T cell (NFAT), which are modified, activated and translocated from the cytoplasm to the nucleus as a consequence of signal transduction in T cell activation (141). Treatment of almost any cells with PMA alone, which is a potent PKC activator, can activate AP1, Oct-2 and NF κ B. AP1, Oct-2 and NF κ B are considered to function downstream in the PKC pathway. It has been known that serine phosphorylation and tyrosine dephosphorylation of AP1 are necessary for its activation. NFAT is a complex of proteins in activated T cells containing *fos* related activator 1 (Fra-1) and *jun* B (JunB), which are members of the AP1 complex (141). NFAT function requires both activation of calcineurin, a calmodulin dependent phosphatase, and of PKC (141). There is currently no direct evidence that PKC directly phosphorylates these transcription factors during T cell activation.

The product of the *c-raf* proto-oncogene is a serine/threonine kinase termed raf-1 that is required for growth factor receptor PTK-stimulated proliferation of fibroblasts (171). Genetic studies in *Drosophila* showed that the function of a PTK encoded by the *torso* gene requires the *polehole* gene which encodes a *c-raf1* homologue (171). Stimulation of TCR was shown to induce raf-1 kinase activity that was maximal within 5 min of cellular stimulation, was accompanied by hyperphosphorylation on serine residues, and was dependent on PKC (172). The mechanism of this antigen-induced and PKC-dependent raf-1 serine phosphorylation in T cells is not understood. Although one of the

raf-1 related proteins, p110, was physically associated with CD4-p56^{lck} after TCR stimulation, the function of raf-1 in the signal transduction process needs further study.

Another serine/threonine kinase is microtubule-associated protein 2 kinase (MAP-2K), which is activated within minutes after TCR ligand binding by both tyrosine and threonine phosphorylation (173). CD4 co-crosslinking to TCR/CD3 enhanced the process (174). MAP-2K is critical for growth factor receptor signal transduction (rev. 175). Again, its role in T cell activation is not understood.

G proteins

The G protein pathway is a very important signal transduction pathway involved in a variety of responses, e.g. growth factor stimulated proliferation, neurotransmitter response, olfactory receptors, *C. elegans* vulval and *Drosophila* eye development, etc (176-183). The *ras* family of G proteins are ubiquitously expressed and are required for control of cell cycle progression. Genetic studies of *C. elegans* vulval development indicated that the product of the *let-60* gene, a *ras* protein, is essential for the function of a receptor PTK encoded by the *let-23* gene (183). Two genes encoding a *ras* homologue and a CDC25 (an activator of guanine nucleotide exchange by *ras* protein) homologue were both essential for signaling by the *sevenless* PTK during *Drosophila* eye development (177). Both injection of anti-*ras* antibody and dominant negative *ras* alleles can block signal

transduction through growth factor receptor PTK with blocking of raf-1 and MAP-2K (153). It seems that the *ras* G protein pathway is downstream of the PTK pathway, upstream of raf-1 and MAP-2K serine/threonine kinases, and critical for signal transduction from PTK pathways in other systems (rev. 210).

The function of p21^{ras} in T cell activation is not well understood. There are data strongly arguing that p21^{ras} function is downstream of TCR signal transduction and very important for T cell activation. P21^{ras} was found to be activated in primary T cells and T cell tumor lines within minutes of TCR stimulation because of the reduced GAP activity and/or the activation of guanine nucleotide exchange factor (121). A T cell tumor line (EL4) overexpressing a constitutively active form of p21^{ras} was shown to be hyperstimulatable through the TCR (186). This active p21^{ras} overexpression could partially replace the requirement for PKC activation in synergizing IL-2 production with calcium ionophore. Overexpression of a dominant negative mutant of p21^{ras} caused an inhibition of T cell activation through both the TCR and the PKC pathway (186).

There are multiple mechanisms through which p21^{ras} could be activated through the TCR. One is via PKC activation, and the other is independent of PKC activation but sensitive to PTK inhibitor (185). Treatment of T cells with PKC inhibitors blocked activation of p21^{ras} by PMA and Ca⁺⁺ ionophore, but not by TCR stimulation (185). Other T cell activating surface molecules, such as CD2 (which can activate T cells in a TCR dependent manner upon anti-CD2 crosslinking) and IL-2 receptor (which can induce T cell

progression through the cell cycle and proliferation upon ligand binding to IL-2), were also able to activate p21^{ras} in the same fashion as anti-TCR treatment (121, 185). Ras activation via multiple pathways is not unique to T cells. Insulin receptor-coupled p21^{ras} activation does not require PKC activation, and is very sensitive to a putative tyrosine phosphatase inhibitor (phenylarsine oxide) (185). The mechanism of p21^{ras} activation (how the activation of PTKs is coupled to the pathway and whether PTPases are involved), and the role of p21^{ras} activation (its relation to serine/threonine kinases such as raf-1 or MAP-2K) during T cell activation, remains to be determined. Until this mechanism is understood, it will remain unclear whether p21^{ras} is essential in signal transducing pathways during T cell activation or if it is just an alternative way to activate T cells.

Some small G proteins, such as *rac* and *rho* (a *ras* related G protein), have been shown to be responsible for cytoskeletal changes induced by growth factors (e.g. PDGF) (184). Since T cell activation is a consequence of cell-cell interaction, cytoskeletal changes, which might result in successful cell-cell contact and also require G protein function, could be a very important aspect of the activation process.

Role of CD4 and CD8 in signal transduction

CD4 and CD8 are very important for both peripheral T cell activation and thymocyte maturation. Two views have been advanced as to the role of CD4 and CD8 in T cell activation (rev.

10). It was first proposed that CD4 and CD8 function to increase the avidity of interaction between the T cell and the antigen presenting cell by binding to class II or class I MHC, respectively; this would be particularly important in those T cells having a TCR with low affinity for antigen. Support for this hypothesis came from early studies showing that inhibition of T cell activation or target cell lysis with antibodies against CD4 or CD8 varied between different T cell lines. Some T cells, particularly those generated in secondary immune responses, were less sensitive to antibody treatment (rev. 10).

It was later concluded that CD4/CD8 and TCR/CD3 are one complex (which could be functional or physical, and could be preexisting or induced), and CD4/CD8 might be involved directly or indirectly in the transmission of signals to activate T cells. Other adhesion molecules, such as LFA-1 and CD2, have been shown to play the major role in the initial, antigen-independent conjugate formation between T cells and APCs or target cells (rev. 4, 187). In contrast, CD4 and CD8 are thought to have a critical function at the post-binding steps that are dependent on antigen recognition. For example, monoclonal antibodies (Mab) specific for CD8 blocked cytotoxicity of CTLs not only during the phase of conjugate formation, but also at a postbinding step (188). Similarly, a Mab specific for CD4 was shown to inhibit cytotoxicity of class II-specific CD4⁺ CTL clones at a postbinding step and had only a small effect upon the initial formation of conjugates (189).

There is extensive evidence indicating that CD4/CD8 molecules are involved in signal transduction during T cell

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activation. The ability of anti-TCR Mabs to stimulate a T cell clone was correlated with their ability to co-cap CD4 and TCR or co-modulate both molecules off the cell surface in a mouse T cell hybridoma line (rev. 9, 10). It was calculated that two CD4 molecules were lost for every one TCR molecule based upon the knowledge of the number of binding sites for the respective Mabs (190). A similar result was obtained in human T cell clones for both co-modulation of CD4 and TCR and that of CD8 and TCR (191), and anti-CD4 Mab also induced co-modulation of CD4 and CD3 in a molar ratio of two to one (192). There is also evidence that CD4 can become physically complexed with the TCR/CD3 for CD4 was co-precipitated with the TCR/CD3 in mild detergent lysates of normal T cells and was physically associated with the TCR/CD3 on T cell membranes as shown by antibody staining in permeablized cells upon antibody stimulation of the TCR/CD3 (193).

There is also extensive evidence that CD4 and CD8 are involved in signal transduction processes in T cells. The association of p56^{lck} with the cytoplasmic tails of CD4/CD8 provides the structural basis for both molecules to serve as cell surface receptors that transduce signals across cell membrane and participate in the T cell activation process. It was shown that pretreatment of T cell clones or hybridomas by Mabs to CD4 or CD8 could block intracellular Ca^{++} influx and subsequent T cell activation by lectin, antigen or Mabs to TCR or CD3 (194). Although steric hindrance was not excluded, these results suggested a possible role for CD4/CD8 in signal transduction. More evidence supporting the notion that CD4 and CD8 are capable of

transducing signal(s) in T cells came from findings that separate ligation of CD4 and TCR in peripheral T cells can induce cell death or proliferative arrest. Pretreatment of the cells with anti-CD4, followed by treatment with anti-TCR antibodies in the presence of T cell depleted irradiated splenocytes, resulted in cells going into activation dependent cell death (apoptosis). This was not observed in the absence of anti-CD4 (195). It has also been reported that HIVgp120, which can bind to human CD4, could also induce apoptosis of human peripheral T cells when preincubated with the T cells prior to stimulation of the TCR (196). Like signal transduction in other receptor tyrosine kinase (R-PTK) systems (PDGF-R and FGF-R), biochemical studies showed that both anti-CD4 antibody and HIVgp120 binding of CD4 increased the kinase activity of p56^{lck} via autophosphorylation of the positive regulatory site, Tyr394 (79. 80). The inhibitory signal delivered through CD4 crosslinking is different from that observed upon co-crosslinking CD4 to the TCR/CD3 complex. Co-crosslinking either CD4 or CD8 together with TCR/CD3 delivered a much more potent signal than anti-TCR/CD3 crosslinking alone in both early Ca⁺⁺ influx and later cellular responses (rev. 10).

There are a number of studies indicating that association of p56^{lck} with CD4 or CD8 is essential for effective T cell activation. A murine antigen specific T hybridoma line studied in our laboratory, 171.3, expresses a TCRab (b chain is a Vb3.1) specific for hen egg lysozyme peptides presented by A^b class II MHC molecule. This cell cannot be activated by the peptide presented by A^b in the absence of surface CD4 expression (197). This

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dependence on CD4 also requires the association of CD4 with p56^{lck}, as mutant CD4 with point mutations in the cysteines of the cytoplasmic tail, which abolish association with p56^{lck}, could not replace wild type CD4 when expressed on the cell surface at an equivalent level. Similar results for CD8a function were obtained in a murine CD8-dependent T hybridoma expressing a TCRab specific for class I H-2K^b molecules, where the association of CD8 with p56^{lck} is required for the cells to be activated by APCs (47, 198). This requirement of p56^{lck}, a PTK, for the function of CD4/CD8 during T cell activation by specific antigens strongly suggests a role for the coreceptor/PTK complex in the signal transduction process.

Function of CD4 and CD8 in thymocyte development

It is widely accepted now that CD4 and CD8 are critical for the development of CD4⁺ T cells and CD8⁺ T cells, respectively. Thymocytes with a TCR specific for class I MHC can normally mature only into CD8⁺ T cells and those with the TCR for class II MHC only into CD4⁺ cells (rev. 10, 42). Administration of anti-CD4 antibodies to mice blocked maturation of CD4⁺ T cells resulting in loss of peripheral CD4⁺ T cells, and anti-CD8 antibodies had the same effect on CD8⁺ T cells (rev. 10). Recently, gene targeting experiments showed that mice genetically ablated for expression of CD4 had a defect in CD4⁺ T cell development. In these mice, the peripheral CD4⁺CD8⁻ T cell subset, accounting for about 65% of the TCRab⁺ cells normally, was reduced to about 10% CD8⁻ T cells (55).

Re-expression of both mouse CD4 and human CD4 transgenes restored the profile of both thymocytes and peripheral T cells completely (55). Similarly, loss of the CD8 gene resulted in defective development of CD4⁻ cytotoxic T cells (200).

Other transgenic experiments have suggested that the interaction of TCR and CD8 with the same class II or class I MHC molecule on thymic stromal cells is important for thymocyte positive selection and negative selection processes. In transgenic mice expressing a TCRab specific for the male H-Y antigen plus the H-2D^b class I MHC molecule, CD8⁺ T cells with the TCR transgene (TCR^{tg}) were absent in H-2D^b male mice (negatively selected) and were expanded in H-2D^b female mice (positively selected) (201). When the TCRab transgenic mice were crossed with mice expressing a mutant form of H-2D^b, in which a point mutation in the a3 domain specifically abolished its interaction with CD8 but did not affect presentation of antigen to the TCR, positive and negative selection of CD8⁺TCR^{tg}⁺ thymocytes were not observed in female and male mice respectively. This developmental defect was observed even though the mice expressed other class I MHC molecules which could bind both CD8 and endogenous TCR but were not able to interact with the TCR^{tg} (202). Similar results have been obtained by using other TCR transgenes (204), suggesting that the requirement for CD8 and the TCR to interact with the same MHC molecule is a general rule.

The transmembrane and/or cytoplasmic domains of CD4 or CD8 are also important for the function of CD4/CD8 in thymocyte development. When H-Y specific TCRab^{tg} mice were crossed to

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mice expressing a fusion molecule with the CD8 extracellular domain and the transmembrane and cytoplasmic domains of CD4, the TCR^{tg+} thymocytes were directed to differentiate into CD4⁺ lineage cells after bone marrow cell transfer into irradiated H-2D^b female mice (203). This suggests that the transmembrane and cytoplasmic domains of CD4 and CD8 can deliver distinct signals during thymocyte development. Overexpression of a tailless form of a CD4 transgene restored the development of CD4⁺ T cells in mice with a defective CD4 gene (211). This indicates that the adhesion function of CD4 is also important during T cell development. This issue will be discussed in more detail in the discussion section.

All of the evidence described above strongly suggests that CD4 and CD8 participate in the signal transduction process through the TCR/CD3 complex during both T cell activation and thymocyte development. However, the mechanism of CD4/CD8 function in the signal transduction process is not fully understood. As described above, in order for CD8 to function during thymocyte maturation, CD8 and TCR have to interact with the same MHC molecule (202, 204). This strongly suggests that an integration of signal(s) generated from CD4/CD8 and the TCR/CD3 is required for a productive result.

As detailed above, PTK activation is the first step in signal transduction during T cell activation and is involved in activation of PLC γ 1, which can, in turn, initiate PI turnover and deliver signals into the nucleus. To determine the mechanism by which

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p56^{lck} affects signal transduction from the TCR, we utilized an antibody crosslinking approach to examine the effects of a preformed CD4/lck complex on PTK activation and on subsequent PLC tyrosine phosphorylation/activation (shown by the question mark in Fig. 5). In the CD4-dependent antigen specific murine T hybridoma line, 171.3, it was found that fusion molecules of the extracellular and transmembrane domains of CD4 and the intracellular Lck molecule or other *src* family kinases (*src* and *fynT*) were able to functionally replace CD4 in the activation of cells by antigen and APC (212). We utilized these fusion molecules and found that PTK activation and PLC phosphorylation/PLC activation could be induced by antibody crosslinking with anti-CD3 alone but not with anti-TCRab antibody alone. However, co-crosslinking of CD4/lck and TCRab enabled PTK activation. Furthermore, mutagenesis of the p56^{lck} molecule showed that the kinase function was essential for activation of the tyrosine kinases by CD4 and TCR co-crosslinking. We also found that CD4/lck could change the kinetics of protein tyrosine phosphorylation induced by CD3 crosslinking. The events peaked earlier and higher when CD4/lck and CD3 were co-crosslinked. Crosslinking of CD4 alone could not induce the same pattern of protein tyrosine phosphorylation nor PLC tyrosine phosphorylation/activation. Using a CD8/z chimeric molecule, we found that CD4/lck can rescue tyrosine kinase activation via CD8/z when co-crosslinked to this chimeric protein at suboptimal antibody conditions. We also found that CD4/lck phosphorylated CD8/z on tyrosines when crosslinked to it in T cells or co-expressed with it in *COS* cells. This suggests

that one mechanism by which CD4/lck facilitates PTK activation through the CD3 complex might be by tyrosine-phosphorylation of the CD3z chain, thus facilitating coupling of the complex with a downstream tyrosine kinase pathway.

MATERIALS AND METHODS

Cells and antibodies

The CD4 dependent antigen specific T cell hybridoma 171.3 and 171.3 derived cell lines were grown as previously described (197). 171.3 CD4/lck was generated by introducing a chimeric molecule of the extracellular and transmembrane domains of murine CD4 and the full length p56^{lck} molecule into 171.3CD4-cells by retroviral infection as described previously (197, 211). 171.3 CTmL273A cells express a mutant form of the chimeric molecule, which was mutated at the ATP binding site (Gly273) and deficient in kinase function. When the mutant chimeric molecule was transfected into COS cells, it was confirmed that the kinase activity was completely abolished (211). 171.3 CD8/z express a chimeric molecule of the extracellular domain of human CD8 and the transmembrane and cytoplasmic domains of human CD3z chain in 171.3CD4/lck background (211). Cell lines used were pools of the transfectants and screened for comparable surface expression of various forms of CD4 and TCR/CD3 complexes by FACS IV (Beckton-Dickinson) analysis as described previously (197) (shown in Fig. 1).

145-2C11 (2C11) and H57.97 are hamster monoclonal antibodies and bind the murine CD3e chain and murine TCRab heterodimer respectively (Pharmingen). The purified L3T4 rat monoclonal antibody (anti-murine CD4) was purchased from Beckton-Dickinson. The OKT8 mouse monoclonal antibody is

[illegible]

specific for human CD8 (Ortho). The rabbit anti-hamster affinity purified antibody (cross reacting with human, mouse and rat Ig) was used as secondary crosslinking antibody (Jackson). The anti-phosphotyrosine antibody (4G10) used was supernatant from the hybridoma cell which is a gift from Dr. Debbie Morrison.

Cell activation, anti-phosphotyrosine (4G10) western blotting and *in vitro* PLC assay of 4G10 immunoprecipitates

Cells were activated by antibody crosslinking as described previously () with some modification. In summary, 2×10^7 cells in 100ml of RPMI1640 (3%FCS) were incubated with 0.5mg of 2C11 or H57.97 or OKT8 or/L3T4 at 4°C for 10', washed with PBS, crosslinked by adding 2mg of rabbit anti-hamster Ig at 37°C for an appropriate time and lysed by adding equal amount of 2x lysis buffer (40mMTris PH8, 274mM NaCl, 2% triton x-100 and proteinase/PTPase inhibitors) (197). The cell lysates were assayed for protein concentration by Bradford method (BioRad). Equal amounts of protein (200mg/lane) were loaded on 10% SDS-PAGE and electrophoresed for 4G10 western blotting as described previously (155). Most of the western blots were developed by using ECL method (Amersham) except a few titration for antibody concentrations, which were by alkaline phosphatase method (BRL). Usually, 10% of the lysates were used for 4G10 western blotting and the remainder for 4G10 immunoprecipitation (IP) and *in vitro* PLC assay as described before (146, 147). Briefly, the *in vitro* PLC

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assay was done by using ^3H -PIP₂ (phosphatidylinositol biphosphate) as substrate (Sigma), the 4G10 IPs as PLC enzyme, mixing the two together at 37°C for 15min, and assaying the amount of ^3H -IP₃ (inositol triphosphate) generated by a scintillation counter (Packard). 4G10 IP were done by first incubating 1ml of 50% protein-A sepharose beads suspension with 10ml of 4G10 hybridoma cell supernatant at 4°C for 1.5hr with constant rotating, washing the 4G10 bound protein-A beads twice with 1X lysis buffer/vanadate+proteinase-inhibitors, and then mixing the washed beads with cell lysates at 4°C for 2hr.

Immunoprecipitation of CD4,CD3,CD8 and CD4+CD8 and *in vitro* kinase assay

Immunoprecipitations of CD4, CD3, CD8 and CD4+CD8 were performed as described previously (75, 197, 155) with slight modification. 171.3CD8/z cells (4×10^7 cells / sample) were crosslinked by appropriate antibodies as described above, at 2' after adding the second antibody the cells were washed with ice-cold medium (0% FCS) and then lysed as stated above. The cell lysates were precipitated with appropriate antibodies (L3T4 for CD4 IP, 2C11 for CD3 and OKT8 for CD8) at 4°C for one hour and with protein A sepharose 4B (Pharmacia) or/and anti rat Ig conjugated agarose beads (the later one is used specifically for L3T4) (Sigma) for another hour. All the IPs were done in the presence of phosphatase inhibitor (Na orthovanadate), kinase inhibitor(EDTA) and proteinase inhibitor (PMSF). The beads were

washed. 75% of the beads were used for 4G10 western blotting and 25% for *in vitro* kinase assay as described previously with modifications (197). IPs were incubated with 10mCi ^{32}P -ATP (adenosine triphosphate) at room temperature for 20min, washed three times with water and run on 10% SDS-PAGE. The gel was dried and exposed to X-ray film as described before (197).

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RESULTS

Up-regulation of PTK activation by co-crosslinking of CD4 and CD3

To further study the role of CD4 and the associated p56^{lck} in signal transduction during T cell activation, cellular proteins were analyzed by immunoblot with anti-phosphotyrosine antibodies following anti-CD3 and anti-CD4 antibody crosslinkings in 171.3 cells expressing various forms of CD4. As shown in fig. 1A, a number of proteins became phosphorylated on tyrosine (p145, p120, p110, p100, p85, p70-72, p63-65 and p37) at 30 seconds after anti-CD3 crosslinking in 171.3CD4⁻ cells. The phosphorylation reaction peaked at 2 min and persisted for 10 min after the crosslinking. This shows that the PTK pathway can be activated by antibody crosslinking of CD3 alone and that it is independent of CD4 surface expression. A similar pattern of protein tyrosine phosphorylation was observed upon anti-CD3 crosslinking in 171.3 cells expressing either CD4 or CD4/lck (fig. 1B, C). This correlates well with previous results that antibody crosslinking of CD3 can induce IL-2 secretion independent of CD4 expression in this cell and is consistent with previous studies demonstrating activation of a PTK pathway upon treatment with anti-CD3 (197, 209).

When CD3 and CD4 were co-crosslinked in 171.3CD4/lck and 171.3CD4⁺ cells, the kinetics of protein tyrosine phosphorylation were altered, although the pattern of tyrosine phosphoproteins was similar to that induced by CD3 crosslinking alone (fig. 1 B, C).

Fig.1 Expression of CD3 and CD4 on the surface of different cell lines used in this study.

Different cell lines were incubated with different fluorescent labelled anti-CD3 (2C11) and anti-CD4 (L3T4) antibodies. The labelled antibodies used are biotin-2C11+ avidin-FITC or L3T3-PE as described in materials and methods. Cells then were subjected to analysis by FACS IV (Beckman). The results are shown in this figure.

Fig. 1A-E Effects of CD4/lck on CD3 crosslinking.

Anti-phosphotyrosine (4G10) western blot. Cells were incubated with 2C11 or L3T4 or 2C11+L3T4 at 4°C for 15 min, washed by PBS and then crosslinked by a secondary antibody at 37°C. Cells were lysed at various time points as indicated in the figures and equal amount of whole cell lysates were used for electrophoresis in non-reducing SDS-PAGE. The gels were transferred to nitrocellulose paper and blotted by 4G10 antibodies (Materials & Methods). A, 171.3 CD4⁻ cell line. B, 171.3CD4/lck cell. C, 171.3CD4⁺ cell. D, 171.3CD4Ti cell. E, 171.3CTmL273A cell.

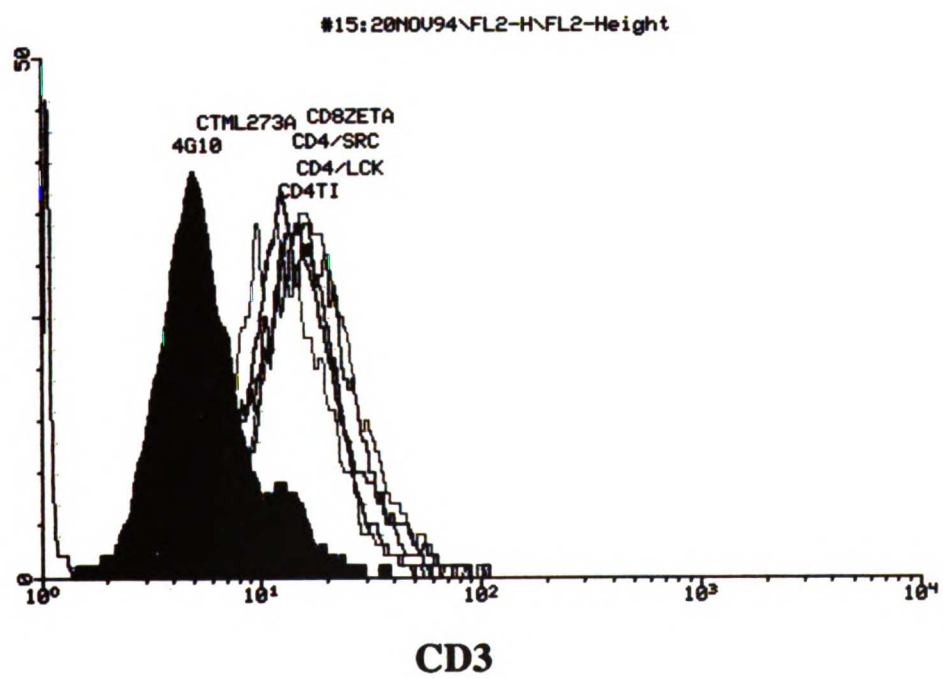
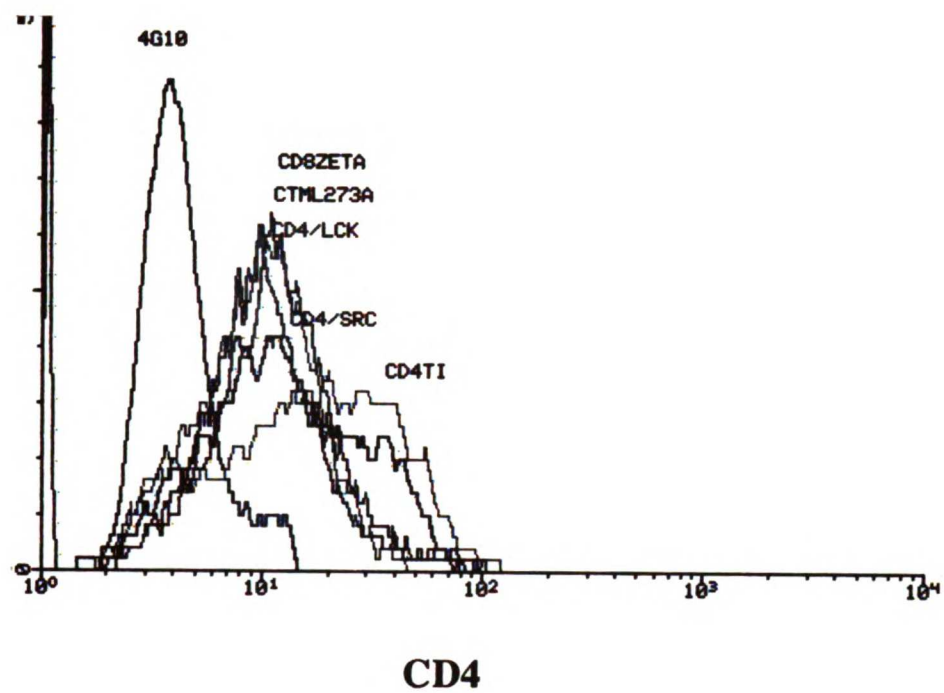


Fig. 1

Fig. 1A

171.3CD4-

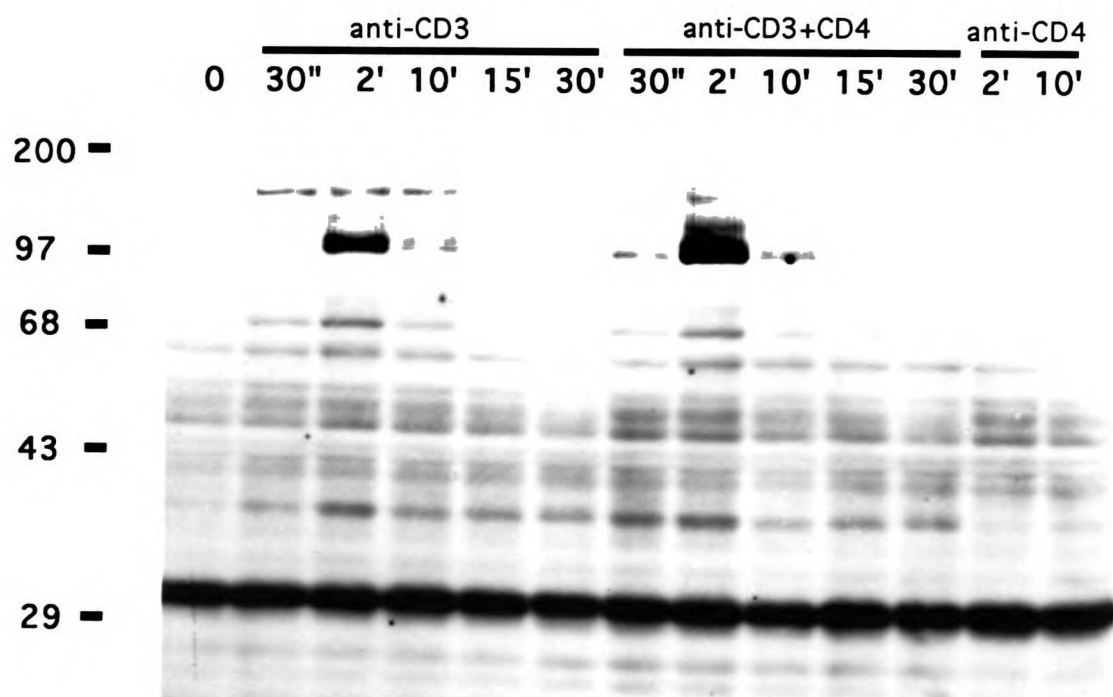


Fig. 1B

171.3CD4/lck

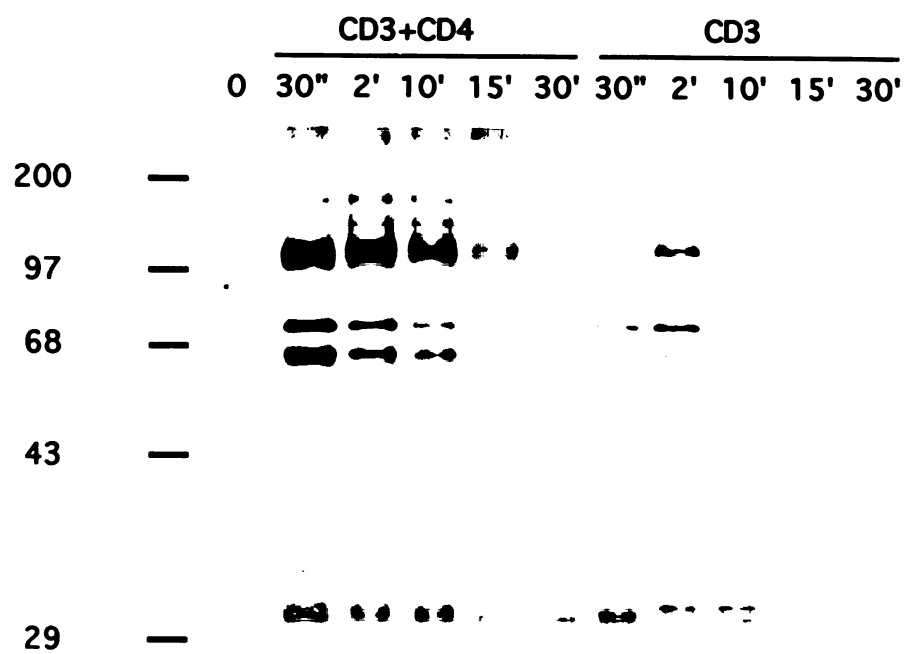


Fig. 1C

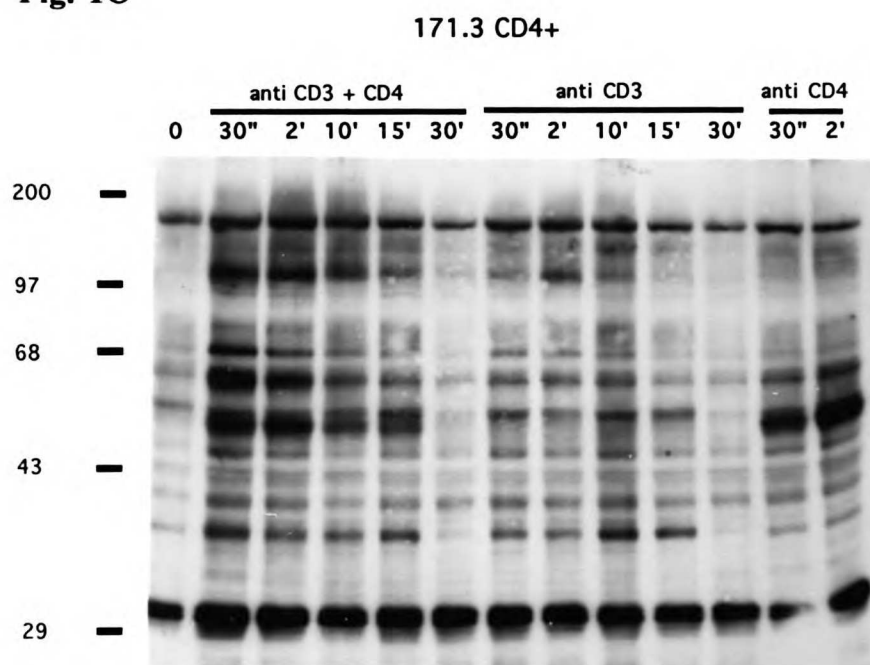
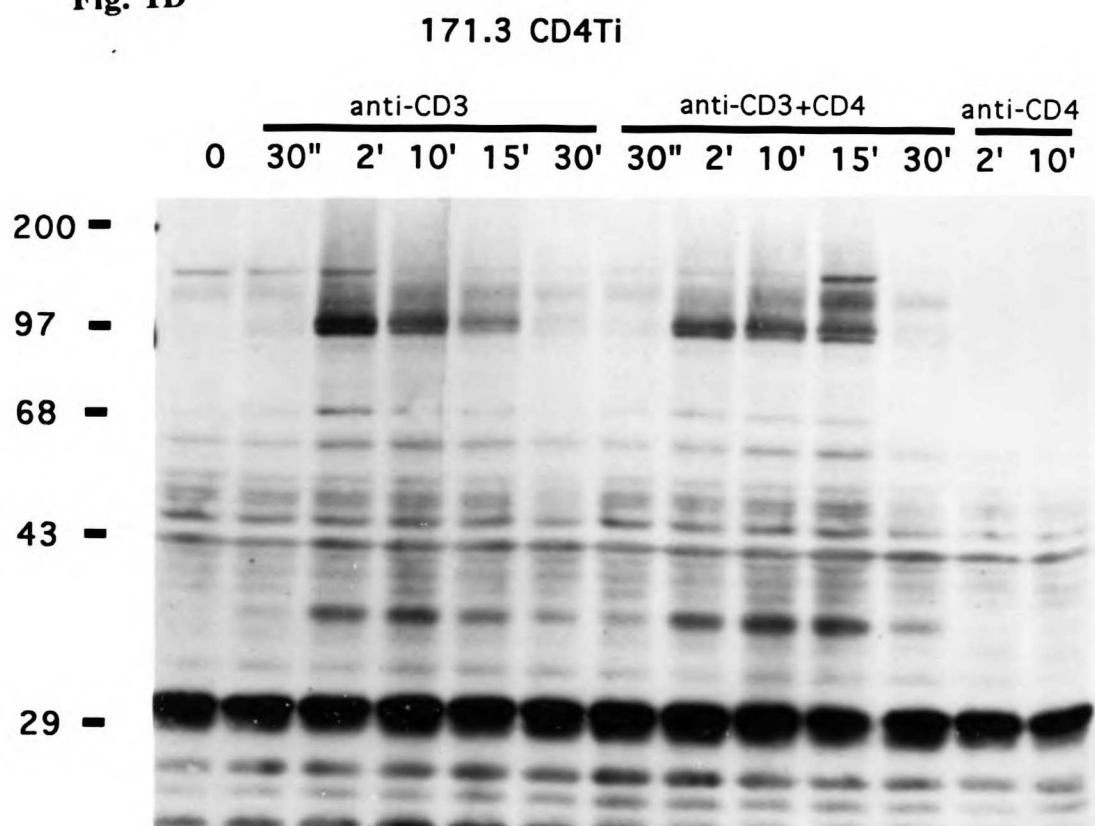
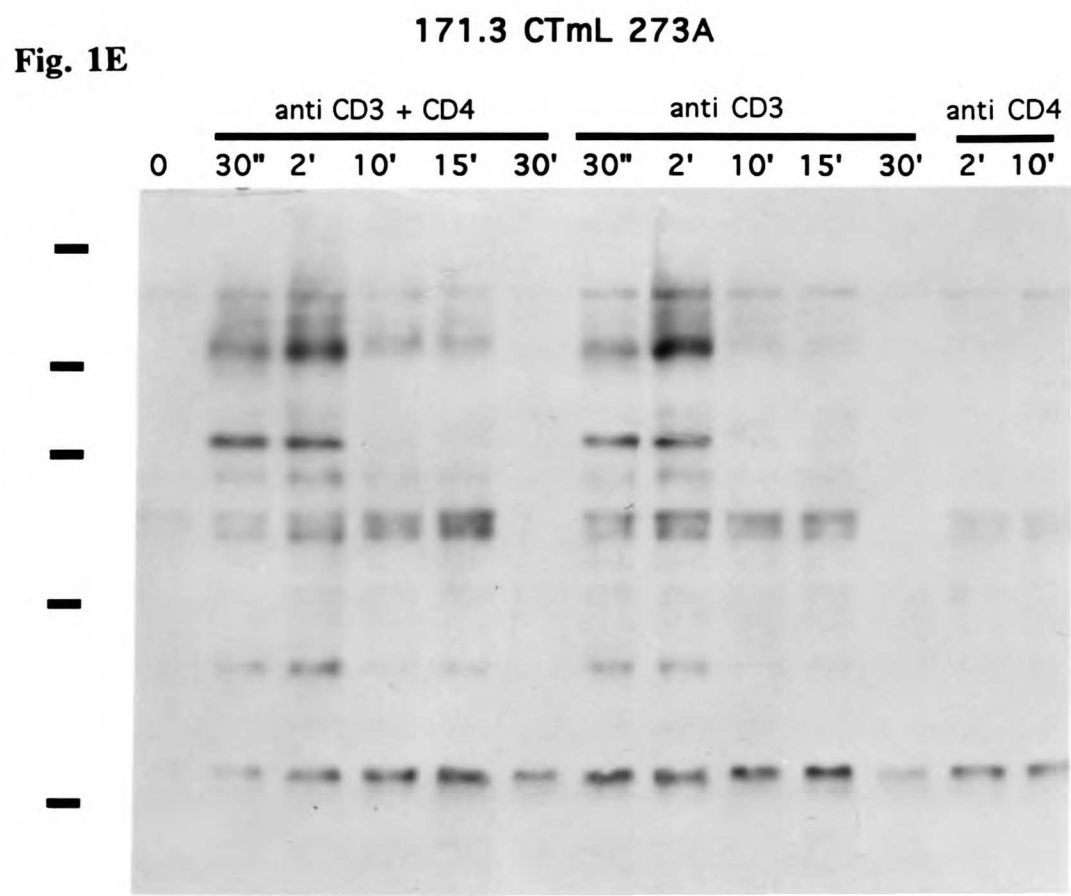


Fig. 1D





The tyrosine phosphoproteins appeared earlier, peaked at 30 seconds after the co-crosslinking, and persisted longer (15 min after the co-crosslinking). This indicates that CD4 can positively affect PTK activation through CD3 and thus changes the kinetics of the PTK activation process when it is brought into proximity of the CD3 complex.

When CD4 was crosslinked alone in 171.3 CD4⁺ cells, the most heavily phosphorylated protein had the same electrophoretic mobility as p56^{lck} (fig. 1B). It was shown before that CD4 crosslinking can result in increased tyrosine phosphorylation of p56^{lck} (79, 80). There were other less prominent tyrosine phosphoproteins induced by CD4 crosslinking alone. In comparison with cellular protein tyrosine phosphorylation after CD3 crosslinking, the most obvious difference was that no cellular proteins of approximately 70-72KD molecular weight were detected in the anti-phosphotyrosine western blot after CD4 crosslinking alone (fig. 1B). This suggests that the CD4 molecule itself is not directly coupled to the PTK pathway in the same way as the CD3 complex, to which the 70-72KD molecules appear to be specifically coupled.

To determine whether the altered kinetics was due to p56^{lck} associated with CD4, similar co-crosslinking experiments were carried out in 171.3CD4Ti cells and 171.3CTmL273A cells. 171.3CD4Ti cells express a tailless form of CD4, which cannot associate with p56^{lck}, at an equivalent level as described in the Materials and Methods. 171.3CTmL273A cells express a kinase-deficient CD4/lck chimeric molecule at an equivalent surface level.

In both cell lines, co-crosslinking of CD4 and CD3 failed to alter the kinetics of PTK activation compared to treatment with anti-CD3 alone (Fig. 1 D. E). Therefore, it is p56^{lck}, noncovalently linked to the cytoplasmic tail of CD4 in 171.3CD4+ cells and covalently fused to the transmembrane domain of tailless CD4 in 171.3CD4/lck cells, that acts as a tyrosine kinase and up-regulates the PTK activation process generated through CD3 complexes.

The PTK pathway cannot be activated by crosslinking TCRab alone but requires co-crosslinking of CD4 and TCRab

During T cell activation by antigen and APCs, it is the interaction between TCRab heterodimers on the T cell surface and peptide antigen plus MHC molecules on the APC surface that leads to signal transduction by CD3 complexes and activation of T cells (as reviewed in the introduction). To study the effect of CD4/lck on signal transduction from TCRab across the cell membrane, the same antibody crosslinking approach as described above was taken using antibodies directed against TCRab heterodimers instead of antibodies to CD3. Surprisingly, the PTK pathway could not be activated by antibody crosslinking of TCRab alone in 171.3 cells with or without CD4 expression (fig. 2 A-E) at the same antibody concentration as that of anti-CD3. However, co-crosslinking of CD4 and TCRab in 171.3CD4/lck cells induced the same pattern of protein tyrosine phosphorylation as that observed after CD4 and CD3 co-crosslinking

Fig. 2 Effects of CD4/lck on TCRab crosslinking.

Anti-phosphotyrosine (4G10) western blot. The same as in Fig. 1A-E excepting that the lysates are from cells crosslinked by H57.97 or L3T4 or H57.97+L3T4. A, 171.3CD4⁻ cell. B, 171.3CD4/lck. C, 171.3CD4Ti. D, 171.3cTmL273A cell. E, D11.10 cell. F, EL4 cell. H. Surface expression of CD3 and TCRab in 171.3 cells, D11.10, and EL4 cells stained by biotin-2C11 and biotin-H57.97 plus avidin-FITC respectively. Results obtained by FACS VI as described in the materials and methods. I. 4G10 western blot of 171.3 cell lysates. H57.97 titration. Crosslinking as in materials and method. J. The same as fig. 2I except that this is KJ25 titration experiments.

Fig. 2A

171.3 CD4-

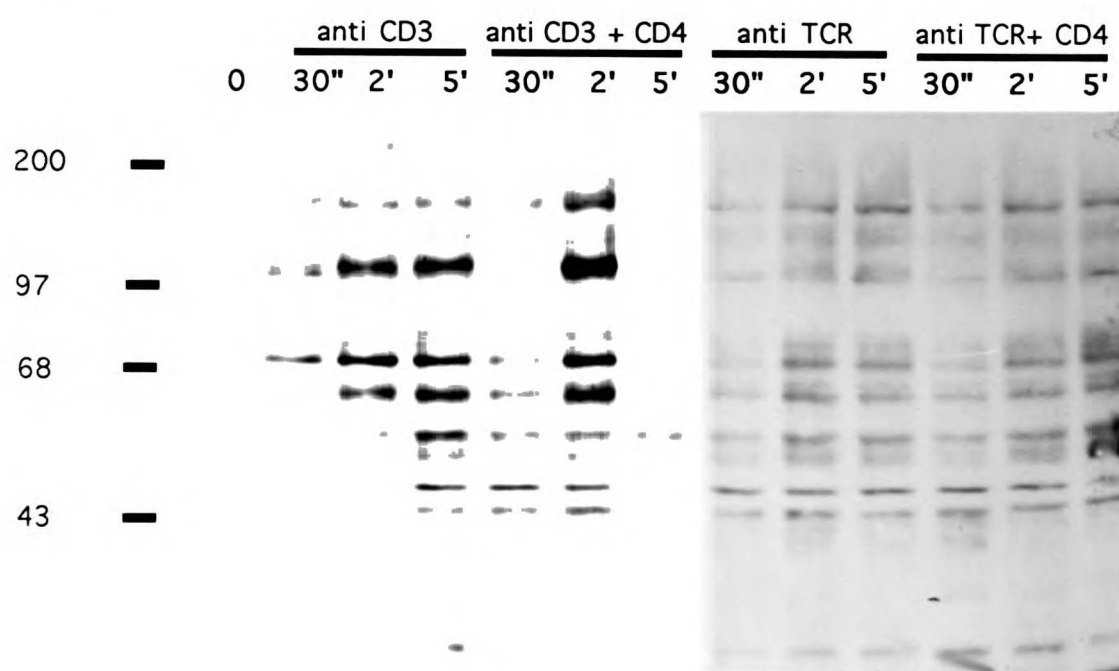
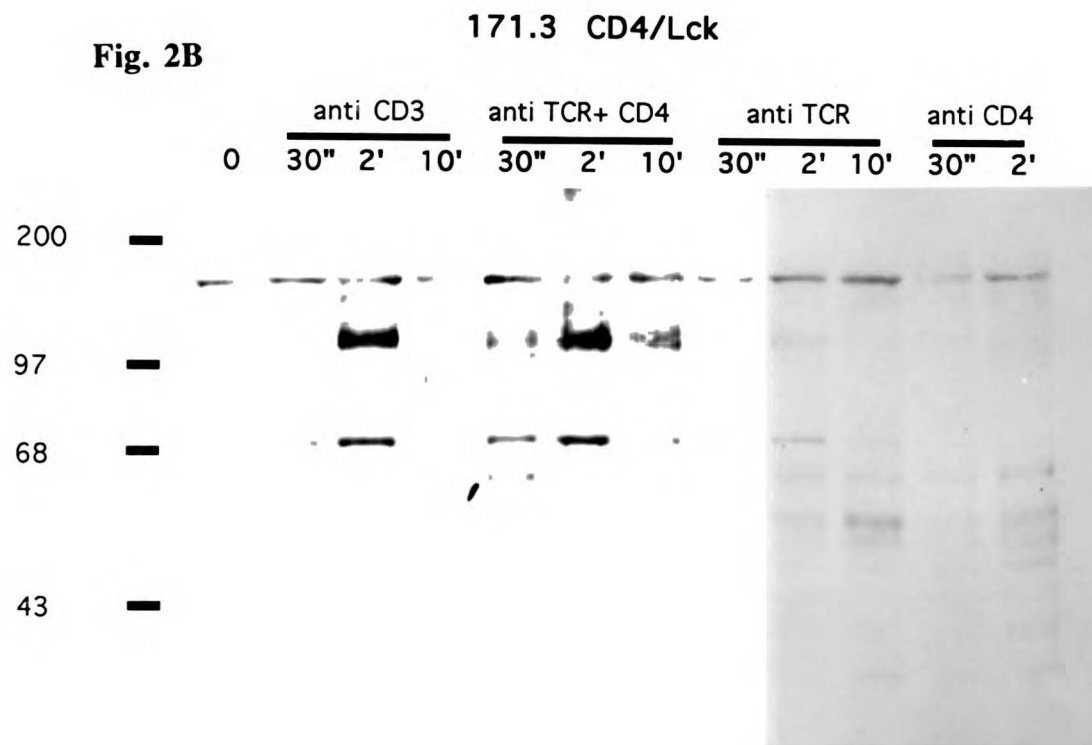


Fig. 2B



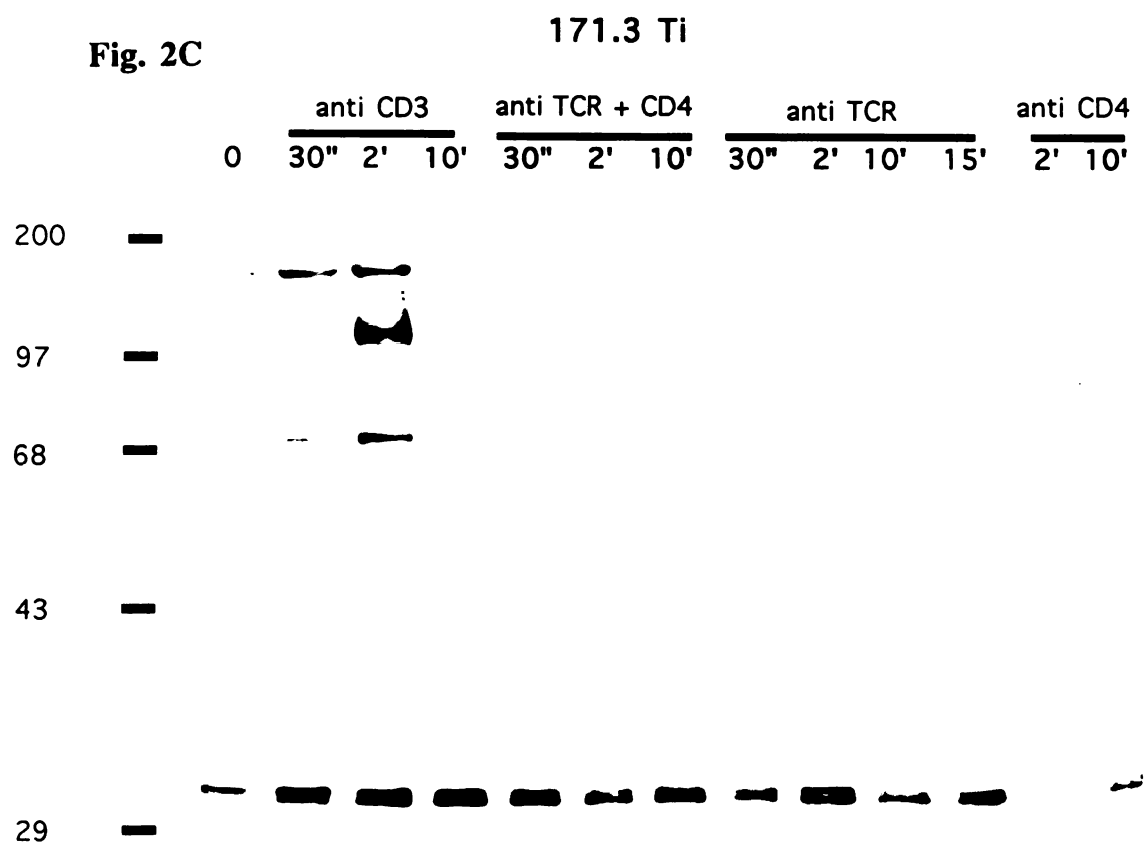


Fig. 2D

171.3 CTmL 273A

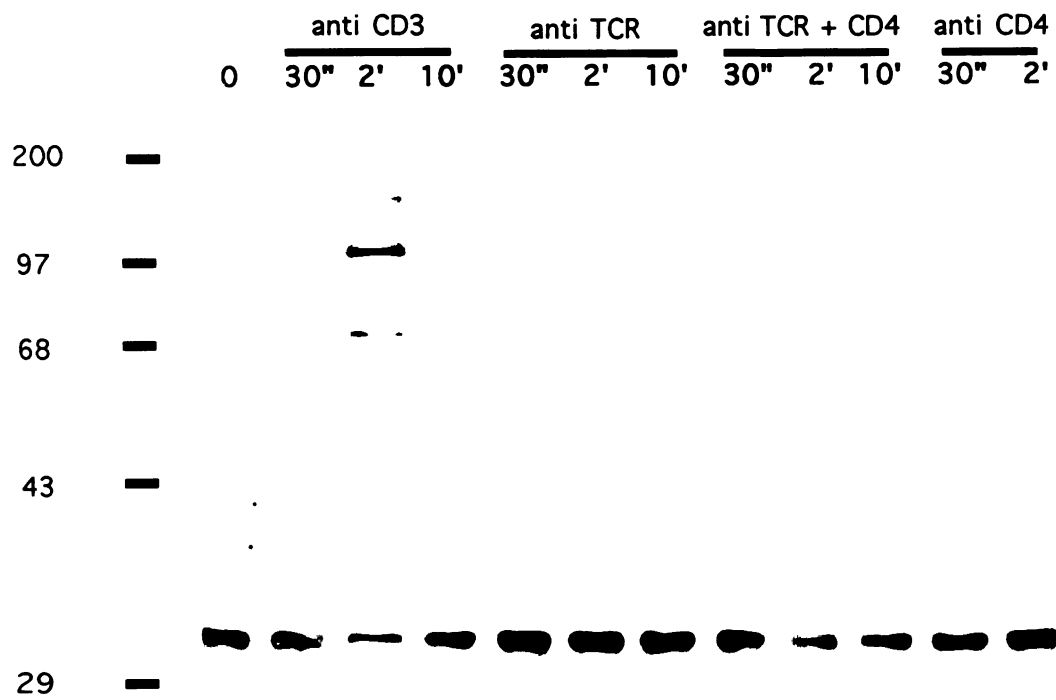


Fig. 2E

D11.10

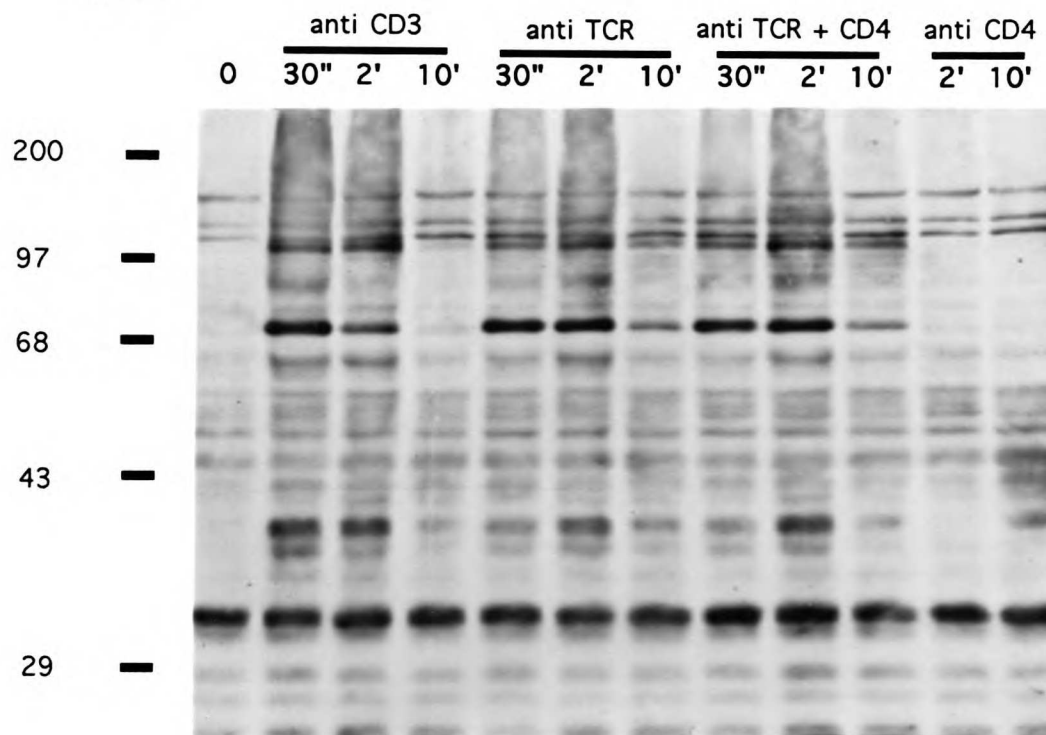


Fig. 2F

EL4

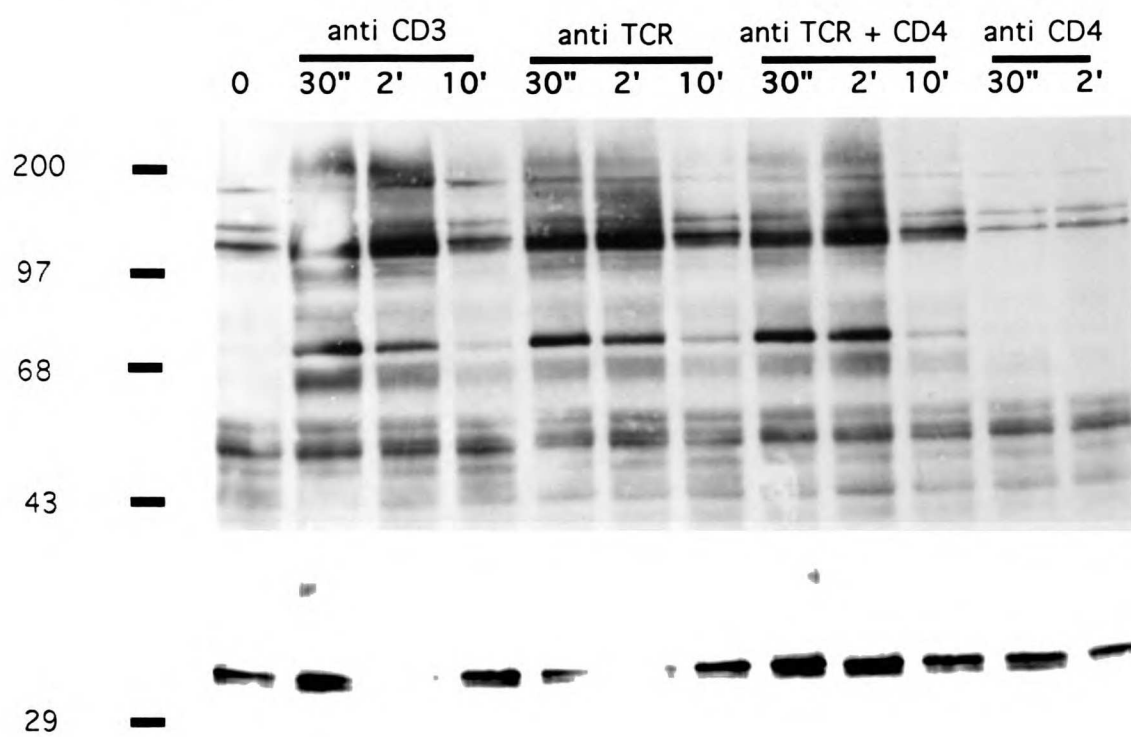
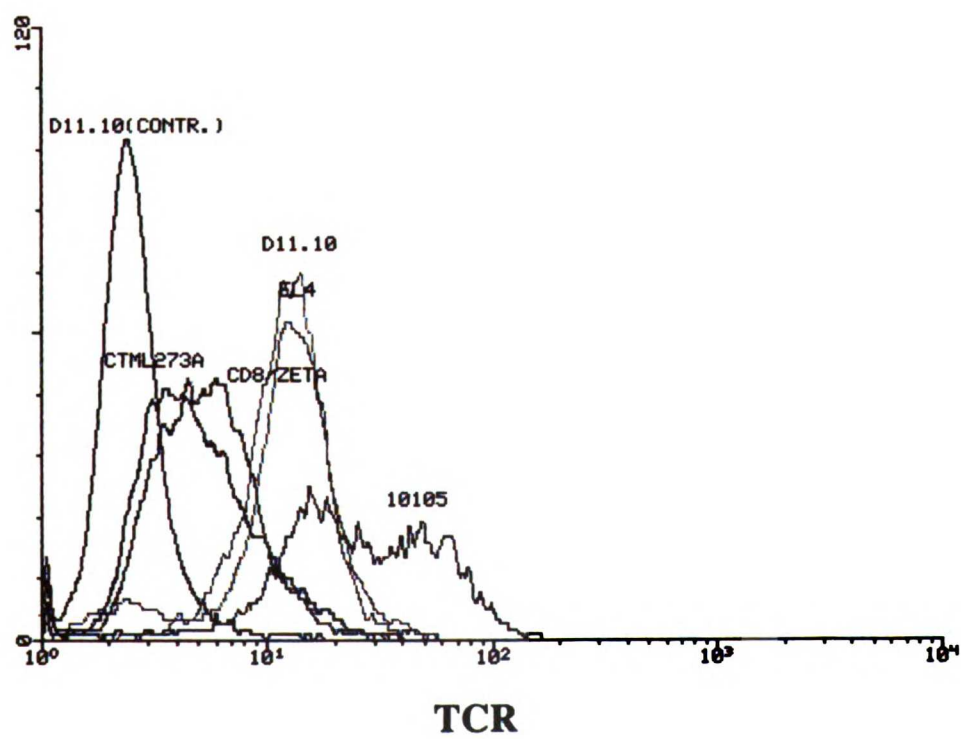
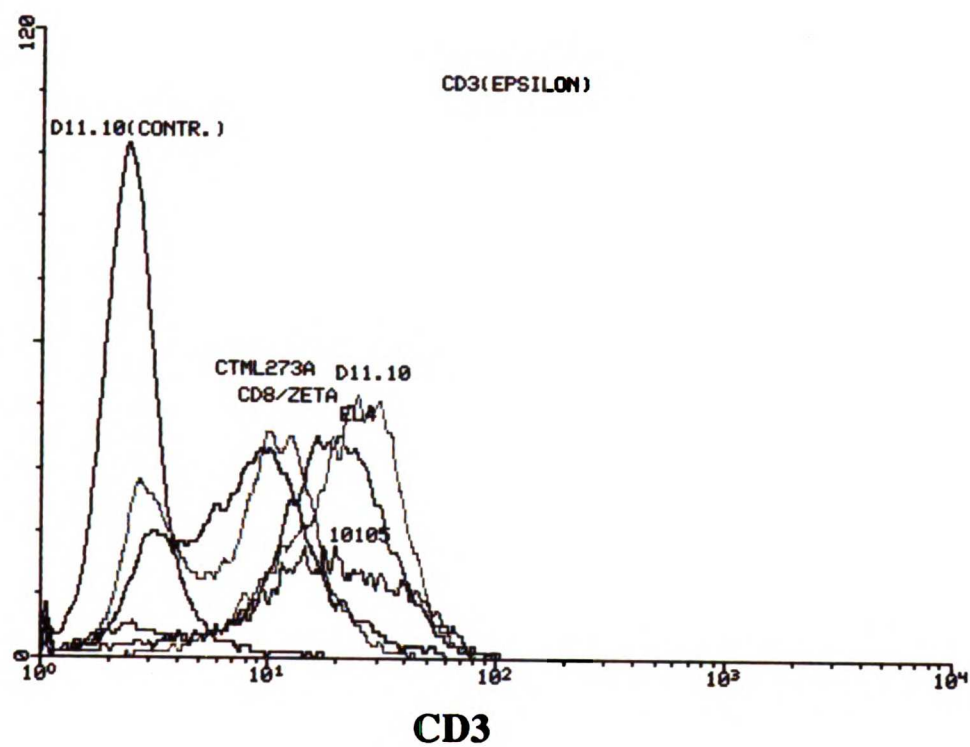
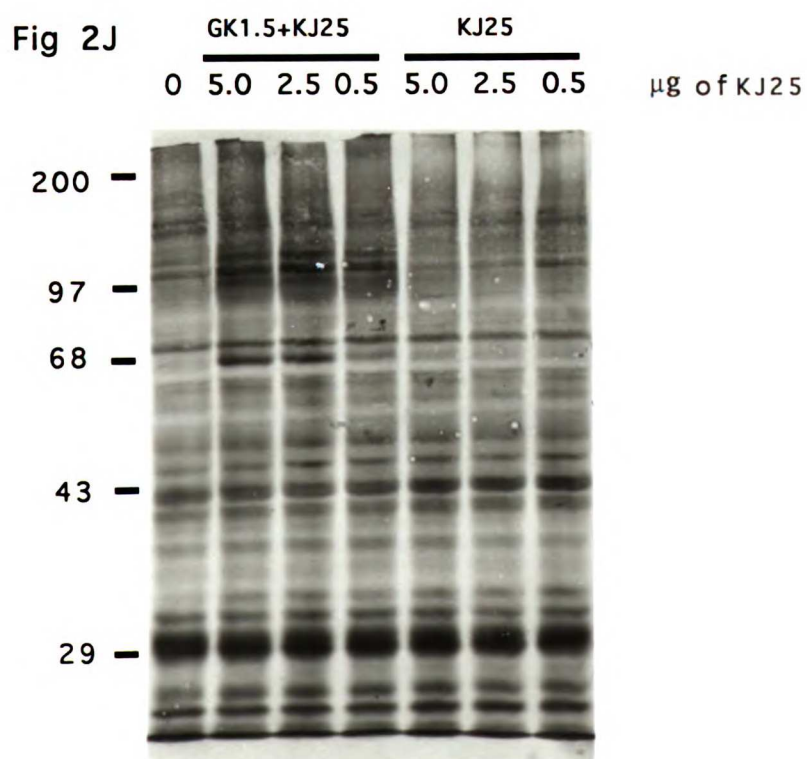
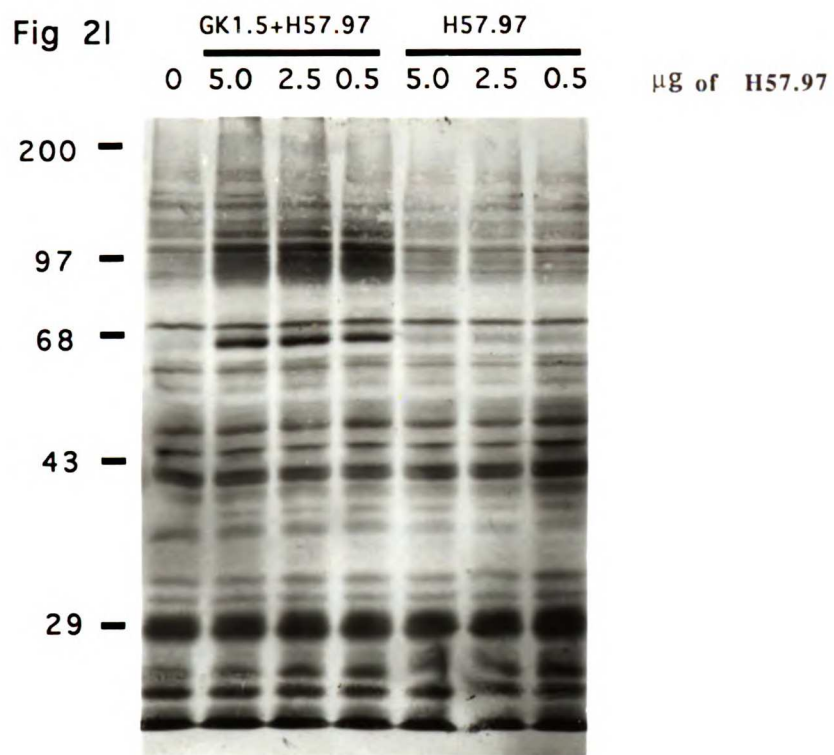


Fig. 2H





(fig. 2 B). The PTK activation could not be achieved by co-crosslinking CD4 and TCRab in 171.3CD4Ti and 171.3CTmL273A cells (Fig. 2 C, D). Therefore, in contrast to antibodies against CD3e, those to TCRab are strictly dependent on CD4-associated p56^{lck} kinase activity to initiate the PTK pathway upon crosslinking.

To determine whether the antibodies used for TCRab crosslinking (H57.97) are able to activate other T cells under the same conditions, another murine T hybridoma line (D11.10) and a T cell tumor line (EL4) were subjected to similar treatments. Both cell lines could be activated by TCRab crosslinking alone at the same antibody concentration (fig. 2 E, F). FACS analysis for surface expression of TCR and CD3 in 171.3 cells, EL4 and D11.10 cells showed that the later two cell lines have about four to five fold higher TCR and CD3 surface expression than do 171.3 cells (fig. 2H).

A series of antibody titration experiments were done without successful PTK activation by antibodies to TCRab alone (fig. 2 I). Because the b chain of TCR expressed on the surface of 171.3 cells is Vb3.1, antibody against Vb3.1 (KJ25) was also used in a series of titration experiments. Again, the PTK pathway was not successfully activated unless CD4 was co-crosslinked (fig. 2 J).

CD4/lck can tyrosine-phosphorylate CD8/z both *in vivo* and *in vitro*, and initiates PTK activation when co-crosslinked to CD8/z

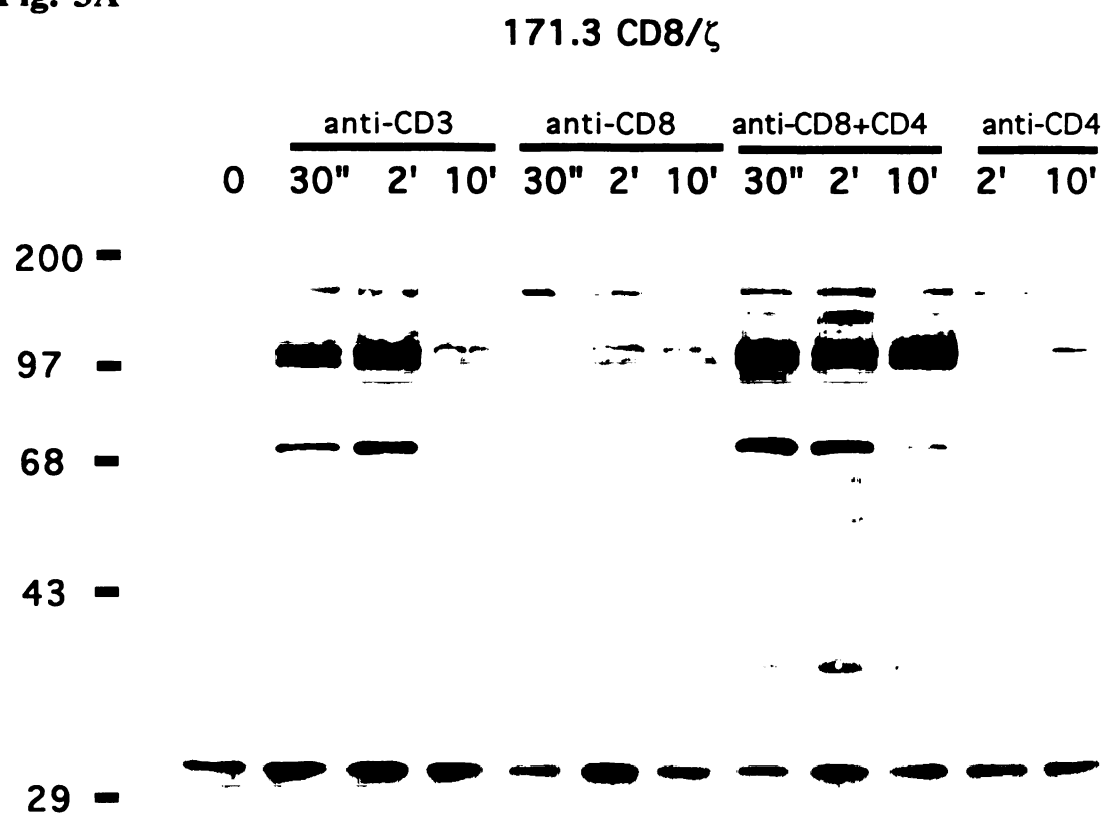
The TCR/CD3 complex consists of at least seven different chains. Previous studies showed that the cytoplasmic domain of CD3z or ϵ alone could deliver signals across the membrane to activate T cells when expressed as chimeric molecules on the cell surface (135-138). Recent results showed that a tyrosine kinase, ZAP70, is associated with CD3z chain in activated Jurkat cells and that this association is strictly activation-dependent (154, 155). In order to understand the mechanism by which CD4/lck affects PTK activation through TCR/CD3 complexes, the CD8/z chimeric molecule was used to simplify the system. CD8/z, a fusion molecule consisting of the extracellular and transmembrane domains of human CD8a and the full cytoplasmic domain of the CD3z chain, has been shown to reconstitute the signal transducing properties of the CD3 complex in Jurkat cells (135). The CD8/z molecule was expressed in 171.3CD4/lck cells (now called 171.3CD8/z) in our laboratory as described in Materials and Methods. These cells were used for similar antibody crosslinking experiments as above.

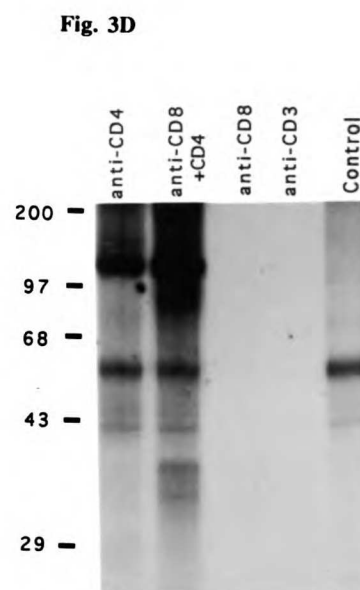
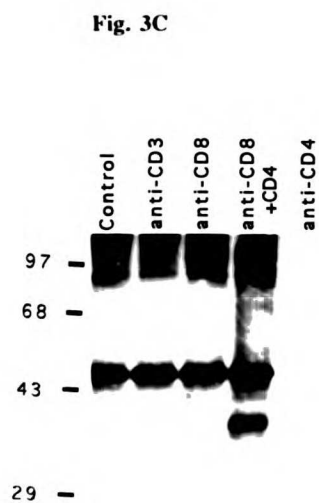
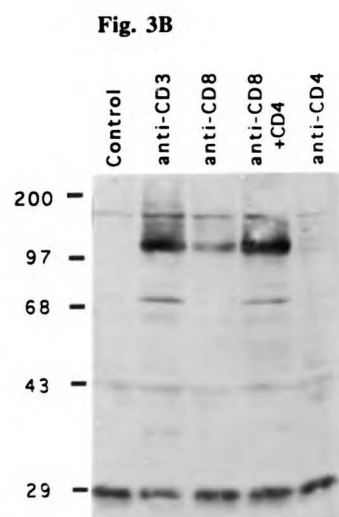
The PTK pathway could not be activated by crosslinking of CD8/z alone at a low antibody concentration (OKT8) ($0.5\text{mg}/2 \times 10^7$ cells) (fig. 3 A), although protein tyrosine phosphorylation was observed at a higher antibody concentration ($5\text{mg}/2 \times 10^7$ cells) (fig. 3 H).

Fig. 3 Effects of CD4/lck on CD8/z crosslinking.

A & B. Anti-phosphotyrosine western blot. The lysates (200mg protein/lane) here are from 171.3CD8/z cells crosslinked by OKT8 or L3T4 or OKT8+L3T4 at a suboptimal concentration of OKT8. **C,** anti-phosphotyrosine western blot of immunoprecipitates by 2C11 or OKT8 or OKT8+L3T4 or L3T4 from the lysates (2mg protein/lane) described in Fig. 3A. **D,** *in vitro* kinase assay of 25% of the immunoprecipitates from Fig. 3B as described in Materials and Methods. **E.** 4G10 western blot of lysates from *cos* cells transiently transfected with plasmids as indicated in the figure. **F.** 4G10 blot of immunoprecipitates by antibodies indicated in the figure from 171.3 CD8/z cell lysates (15mg protein/lane). **H.** 4G10 blot of 171.3 CD8/z cell lysates (200mg protein/lane) after crosslinking by antibodies indicated in the figure (High OKT8 concentration-5mg/2x10⁷ cells in 100ml).

Fig. 3A





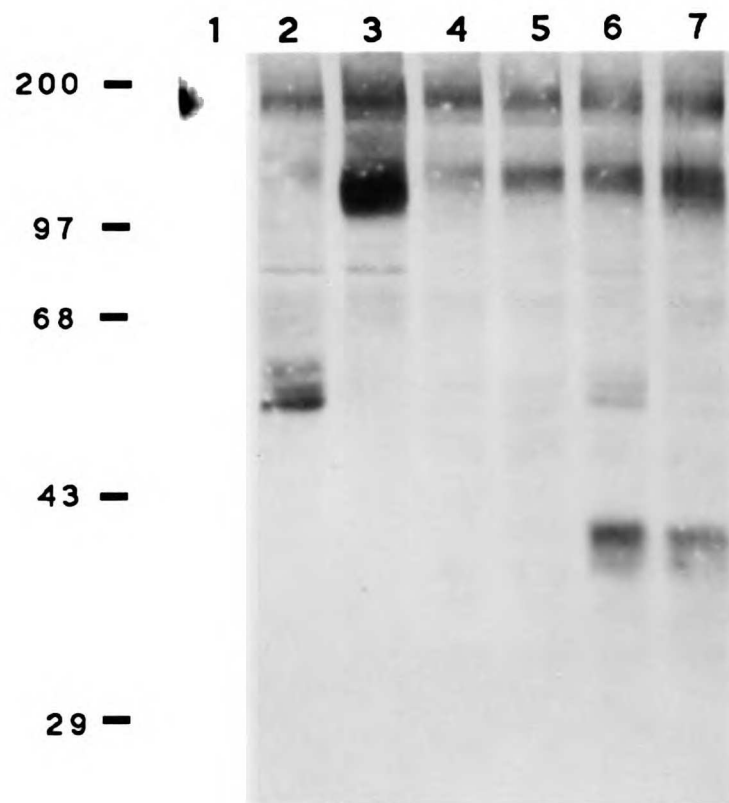
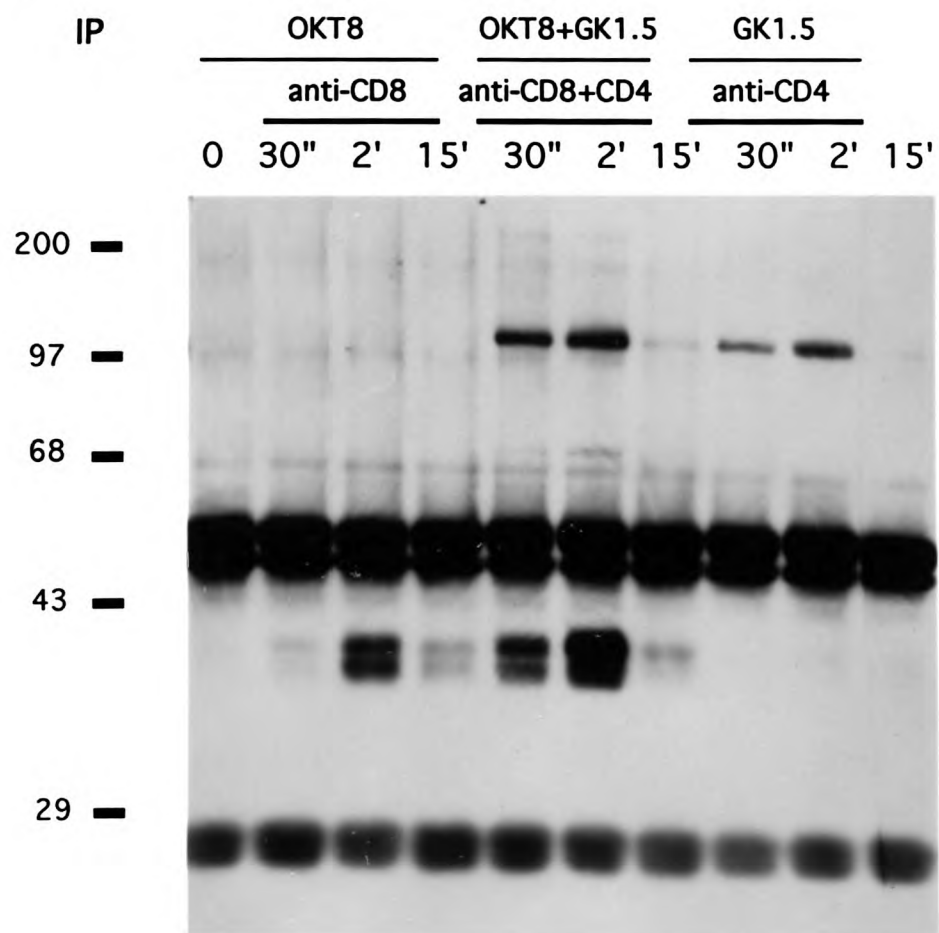


Fig. 3E 4G10 western blot of cell lysates from *cos* cells transfected by psm vector alone (1), psmICK (2), psmCD4/ICK (3), psmCD8/ζ (4), pCDitk (5), psmICK+psmcd8/ζ (6), and psmCD4/ICK+psmCD8/ζ (7).

Fig. 3F



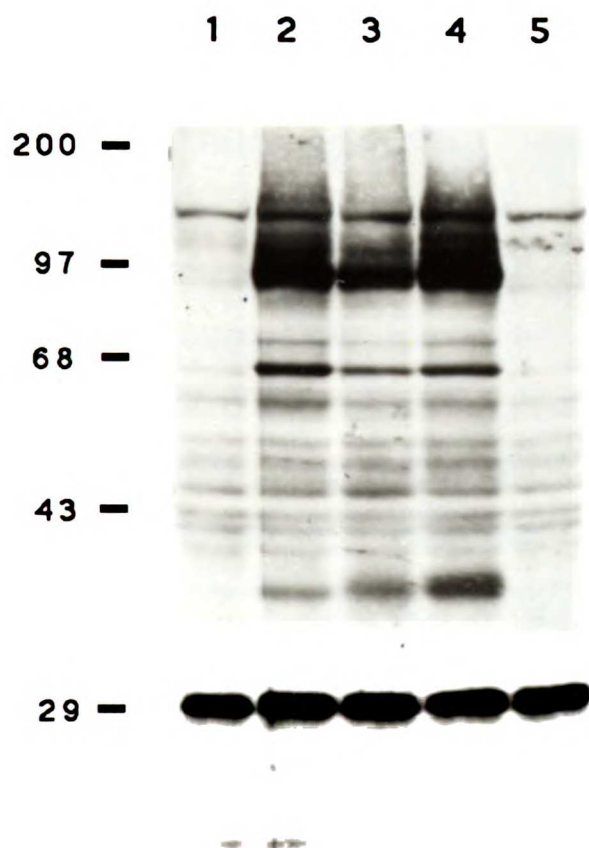


Fig. 3H 4G10 western blot of cell lysates from 171.3CD8/ζ cells crosslinked by 2nd antibody alone (1), 2C11 (2), OKT8 (3), OKT8+L3T4 (4), and L3T4 (5) at high OKT8 concentration (5μg).

Interestingly, at the suboptimal concentrations of OKT8 (0.5mg/100ml), co-crosslinking of CD4/lck and CD8/z on the cell surface resulted in the same pattern of protein tyrosine phosphorylation as that obtained after CD3 crosslinking (fig. 3 A).

As discussed in the Introduction, previous studies showed that both tyrosine-phosphorylation of CD3z and ZAP70 association with z chain occurred after TCR stimulation. The tyrosines in the cytoplasmic tail of CD3z were shown to be important for its signal transducing property. It is not understood how ZAP70 associates with CD3z. When expressed transiently in *cos* cells, the association of CD8/z with ZAP70 requires co-expression of p56^{lck} or p59^{fyn}. Co-expression of the PTKs also resulted in increased tyrosine phosphorylation of CD3z chain and ZAP70 (155). ZAP70 is a non-*src* family cytoplasmic tyrosine kinase, containing two SH2 domains (155). It can be speculated that the association of CD3z and ZAP70 might be via the binding of the SH2 domains of ZAP70 to phosphotyrosines of CD3z, and the binding of tyrosine kinase to z might be a critical step for PTK activation and ultimately T cell activation. One possibility is that CD4/lck could help initiate the PTK pathway by phosphorylating the tyrosine(s) of z and thus providing binding site(s) for z associated PTK(s), e.g. the SH2 domains of ZAP70 could bind to the phosphotyrosines in CD8/z. Alternatively, CD4/lck could phosphorylate PTK(s) associated with z chain to regulate the kinase function.

To determine how CD4/lck affects the PTK pathway activated through CD8/z, cells were stimulated as described previously and lysed 2 min after antibody crosslinking. The cell lysates were used

for immunoprecipitation assays. Part of each precipitate was used for anti-phosphotyrosine blotting and another part for an *in vitro* kinase assay. After co-crosslinking of CD4 and CD8, increased tyrosine-phosphorylation of CD8/z was detected when it was immunoprecipitated (fig. 3 C, F). As shown in Fig. 3F, CD8/z was phosphorylated at 30 sec and reached a peak at 2 min after CD8 crosslinking alone; after CD4 and CD8 co-crosslinking, the phosphorylation of CD8/z chain was much more intense at both 30 sec and 2 min, when CD8/z molecules were immunoprecipitated. To determine if the increased tyrosine-phosphorylation of CD8/z *in vivo* was due to CD4/lck directly, *in vitro* kinase assays were performed using CD8 and/or CD4 immunoprecipitates. After CD8 crosslinking alone, no detectable kinase activity was co-precipitated by OKT8 in an *in vitro* kinase assay (fig. 3 D). Interestingly, CD8/z molecules were phosphorylated in the *in vitro* kinase assay in the presence of CD4/lck immunoprecipitates (fig. 3 D). In anti-CD4 immunoprecipitates alone, the CD4/lck chimeric molecule was both auto-phosphorylated in the *in vitro* kinase assay and tyrosine-phosphorylated *in vivo*, as shown by anti-phosphotyrosine (4G10) western blot, after CD4 crosslinking alone (fig. 3 C, D). There was no other protein co-precipitated with CD4/lck that was phosphorylated in these assays (fig. 3 C, D). No other protein co-precipitated with CD4 or CD8 alone in both *in vitro* kinase assays and in the 4G10 blot of lysates after *in vivo* antibody crosslinking. These results suggest that CD4/lck can phosphorylate CD8/z *in vitro* and that the increased tyrosine phosphorylation of CD8/z upon co-crosslinking of CD4/lck and

CD8/z *in vivo* might be the consequence of p56^{lck} being brought into the proximity of the z chain.

In order to show that CD4/lck can tyrosine-phosphorylate CD8/z *in vivo* in the absence of other T cell specific tyrosine kinases, transient transfections in *cos* cells were carried out. As shown in Fig. 3E, CD8/z molecules showed significantly increased tyrosine phosphorylation when co-expressed with CD4/lck or p56^{lck}. Surface expression of CD8 and CD4 on each transfected population was determined by FACS analysis, and the results showed equal levels of CD8 surface expression among the different populations of transfectants (data not shown)

When the amount of lysate used for CD4 and CD8 IPs from the T cell hybridoma was increased from 2mg proteins (as used above) to 15mg, it was shown in 4G10 western blot that a phosphoprotein of approximately 72KD was co-precipitated with CD8 only from lysates of cells being co-crosslinked by anti-CD4 and anti-CD8 but not from lysates of cells being crosslinked by anti-CD8 alone (fig. 3F). This co-precipitation was transient, as it was observed 30 seconds after co-crosslinking, reached a peak at 2min, and disappeared after that. It correlated very well with the extent of CD8/z chain phosphorylation as shown in fig. 3F. This result thus supports the notion that CD4/lck facilitates z chain association with its PTK(s) possibly through tyrosine-phosphorylation of z chain directly.

CD4/lck increases tyrosine phosphorylation and the activity of Phospholipase C (PLC)

As a result of PTK pathway activation during T cell activation, PLC γ 1 is phosphorylated on tyrosines and is activated, resulting in PI turnover. To determine whether CD4/lck modulates this process, the effects on tyrosine phosphorylation and activation of PLC by antibody crosslinking of CD4/lck to TCR α β , CD3 and CD8/z were studied. As described in Materials and Methods, cell lysates were immunoprecipitated with 4G10, an anti-phosphotyrosine Mab, and assayed for PLC activity *in vitro*. The same samples were analyzed for PTK activation by 4G10 western blotting shown in Fig.1 A-E. The results are shown in Fig. 4A. There were rapid increases in the PLC activity in 4G10 immunoprecipitates from both 171.3CD4Ti and 171.3CD4/lck cells with CD3 crosslinking alone. The increases were detected 30 seconds after antibody crosslinking, peaked at 2min and returned to base levels at 10min. As was observed with PTK activation, co-crosslinking of CD4 and CD3 caused a change in the kinetics of PLC activation in 4G10 immunoprecipitates from 171.3CD4/lck cell but not 171.3CD4Ti cell lysates. The PLC activation peaked at a higher level and lasted longer (15min after antibody crosslinking) (fig. 4A). There was no detectable change in PLC activity upon CD4 crosslinking alone in 171.3CD4/lck cells (fig. 4B). This suggests that CD4/lck itself is not coupled to the PLC pathway, but it can enhance PLC tyrosine phosphorylation and activation in the same fashion as it regulates the PTK activation through CD3.

Fig. 4 Effects of CD4/lck on PLC tyrosine phosphorylation/activation by antibody crosslinking of CD3, TCRab, and CD8/z.

The time course of fold of increased PLC activity in an *in vitro* PLC assay after immunoprecipitated (IP) by 4G10 as described in Materials and Methods. Cells were crosslinked by different antibodies and then lysed as described before. Part of the lysates were used for anti-phosphotyrosine western blotting shown in Fig. 1-3. Part of the lysates was immunoprecipitated by 4G10. The 4G10 IP's were then used for assays of PLC activity. A, 171.3CD4/lck and 171.3CD4Ti cells crosslinked by 2C11 alone or 2C11+L3T4. B, 171.3CD4/lck and 171.3CD4Ti cells crosslinked by L3T4 alone. C, 171.3CD4/lck cells crosslinked by H57.97 alone or H57.97+L3T4. D, 171.3CD4Ti cells crosslinked by H57.97 or H57.97+L3T4. E, 171.3CD8/z cells crosslinked by OKT8 or OKT8+L3T4.

Fig. 4A PLC ACTIVATION IN 171.3 CELLS

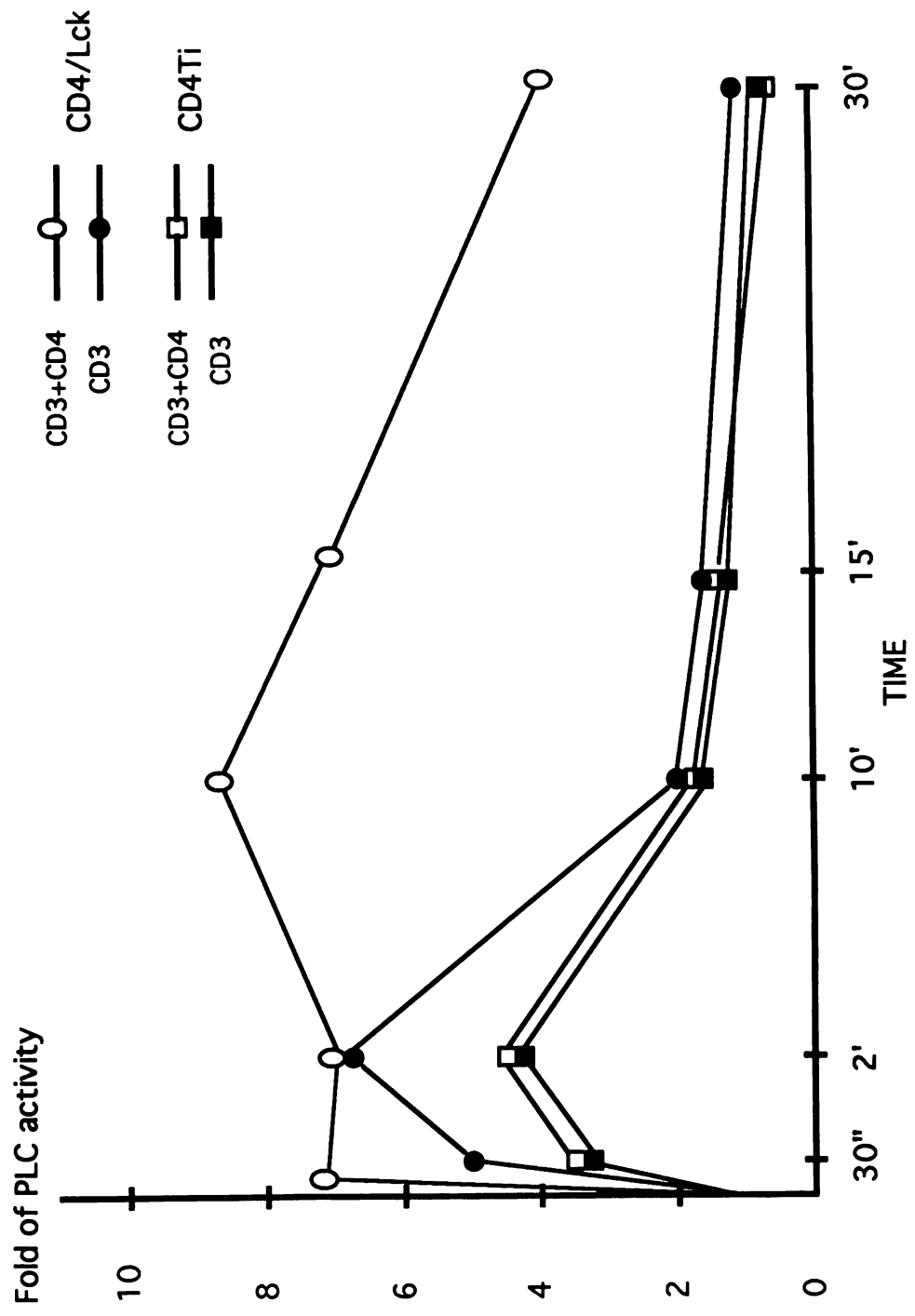
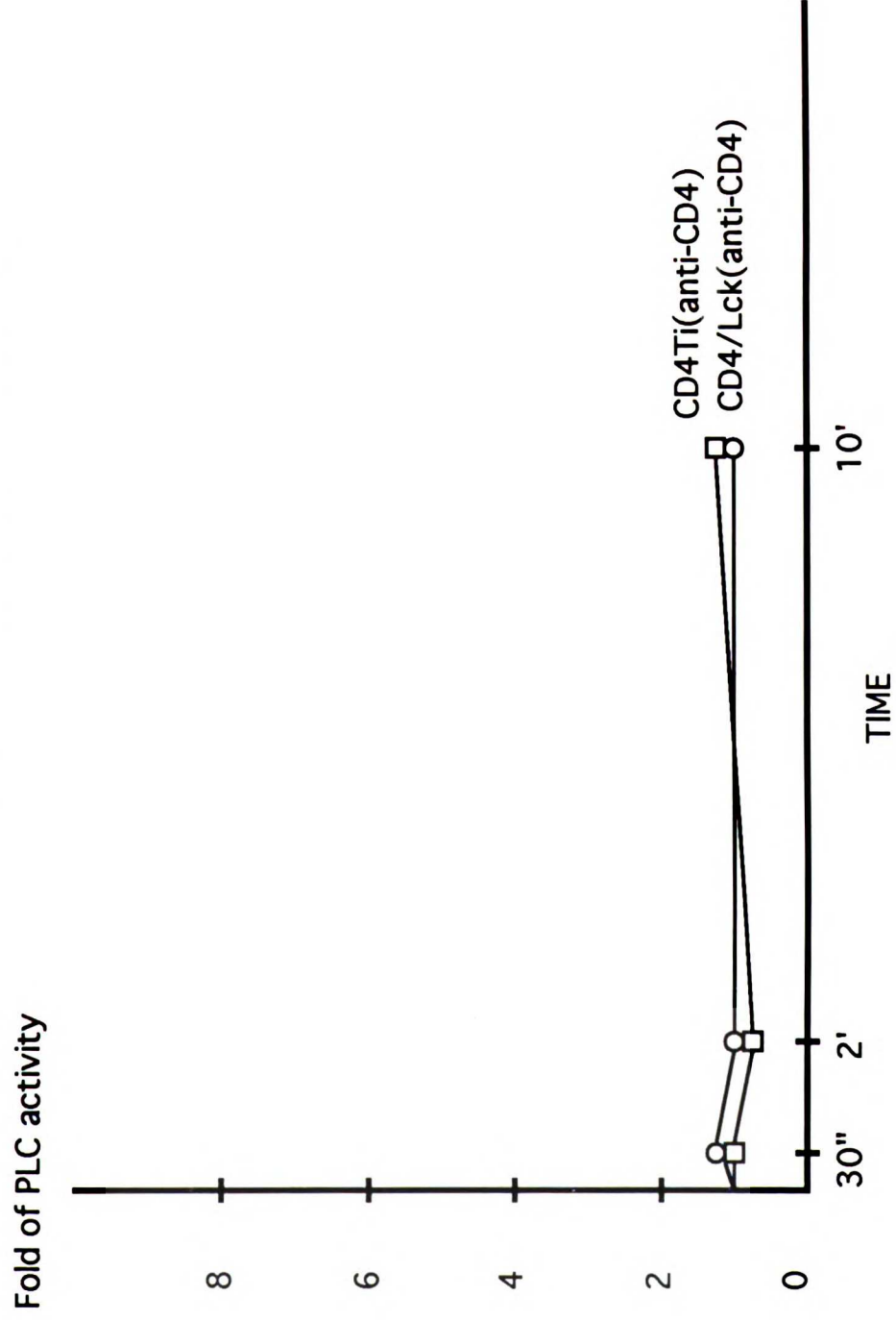


Fig. 4B

PLC ACTIVATION IN 171.3 CELLS



PLC ACTIVATION IN 171.3 CD4/LCK CELLS

Fig. 4C

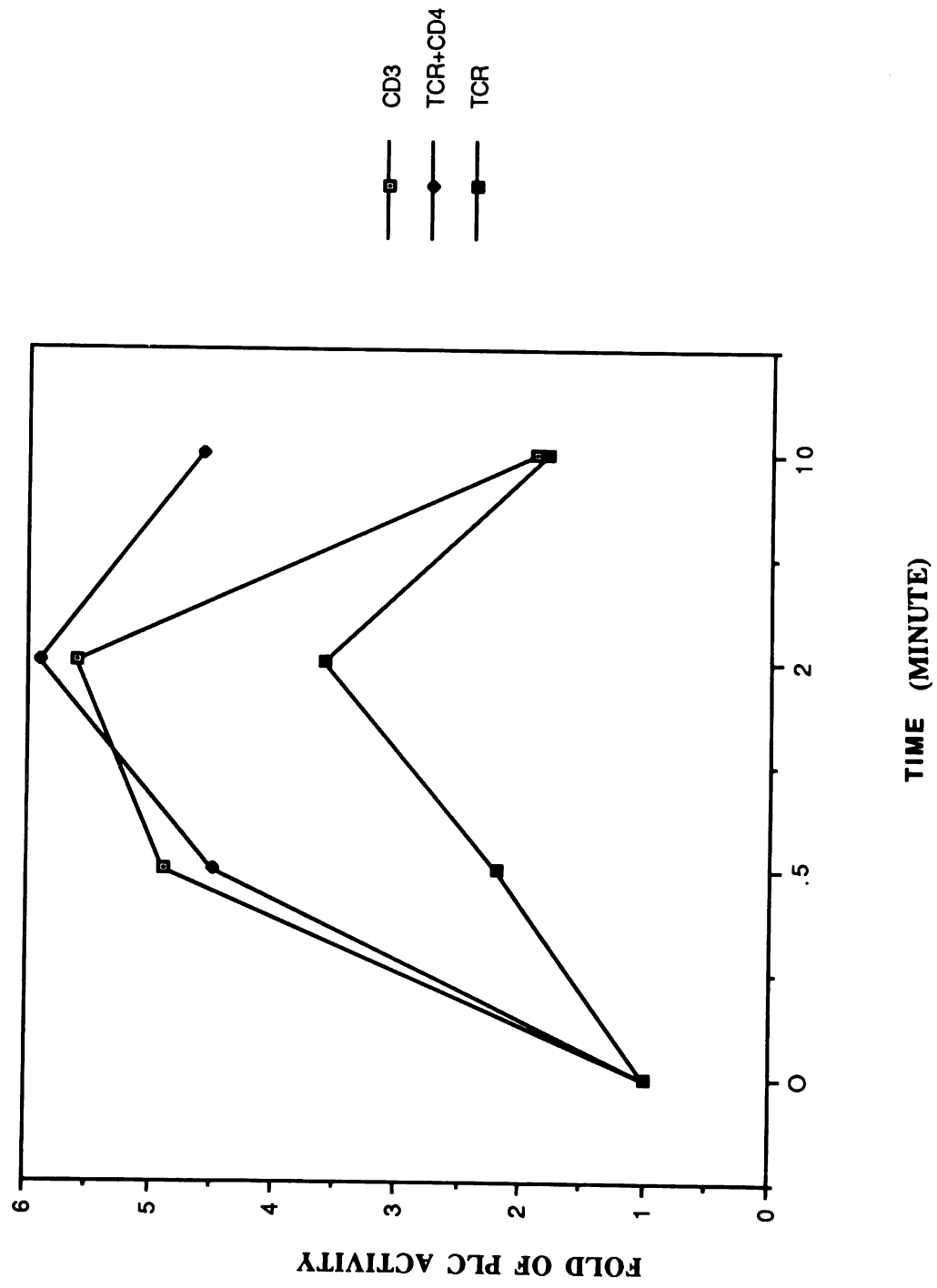


Fig. 4D

PLC ACTIVATION IN 171.3 CD4Ti CELLS

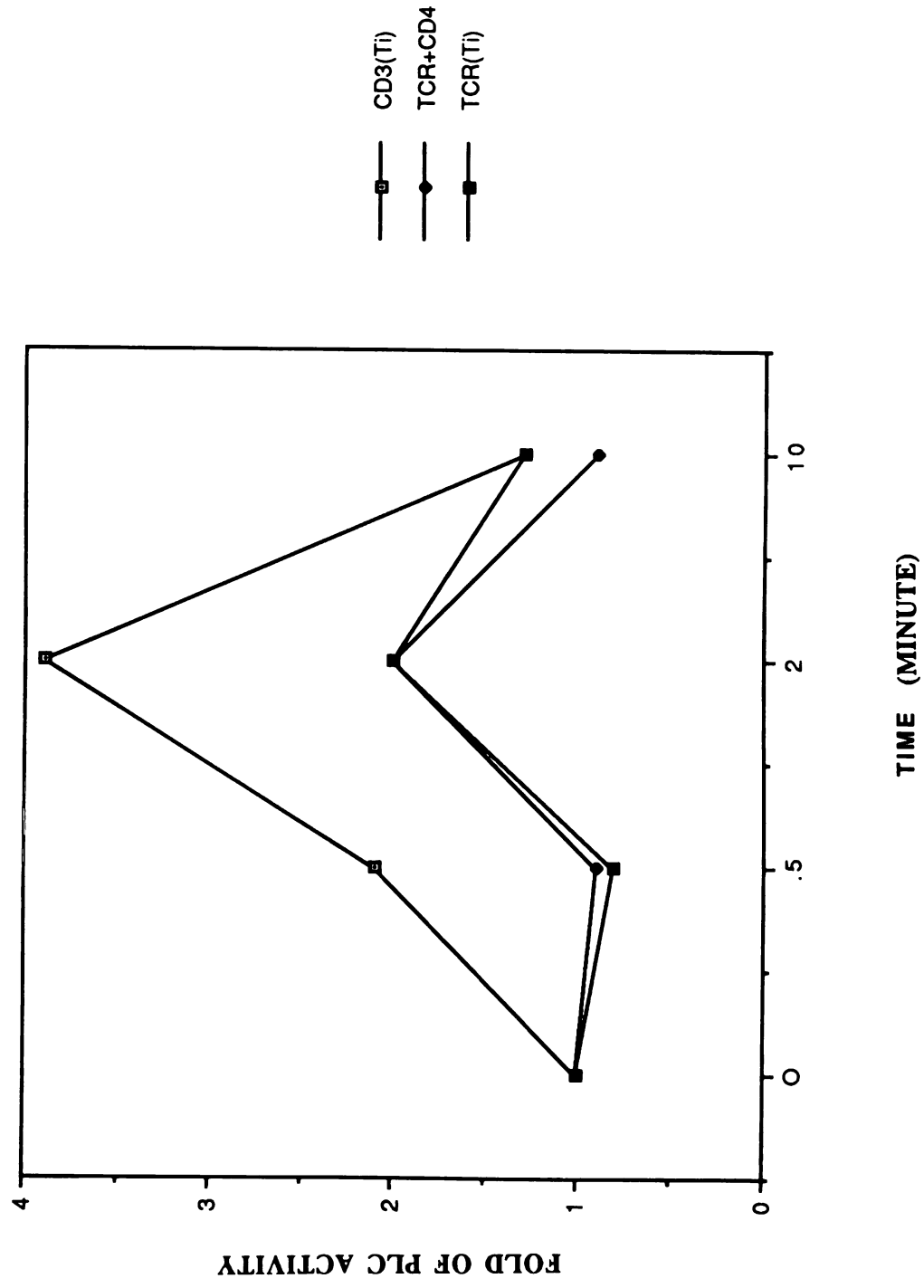
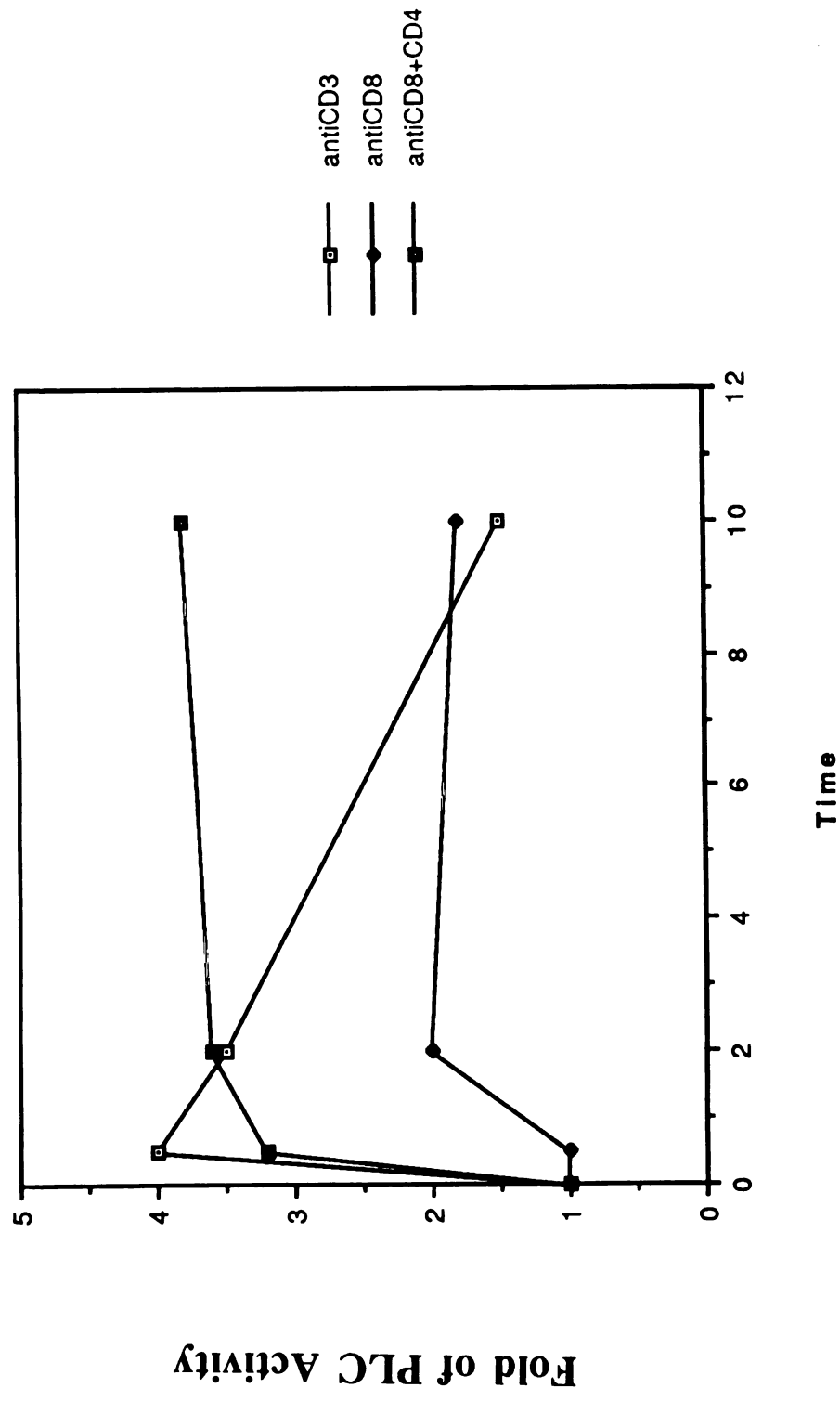


Fig. 4E

171.3 CD8/ ζ



Less than 2-fold increases in the PLC activity were observed 2min after TCRab crosslinking alone in 171.3CD4/lck and 171.3CD4Ti cells (fig. 4C, D). Co-crosslinking of CD4 and TCRab resulted in activation of PLC similar to that observed after CD4 and CD3 co-crosslinking in 171.3CD4/lck cells (fig. 4C) but not in 171.3CD4Ti cells (fig. 4D). The PLC was still activated 10min after the co-crosslinking, whereas, after CD3 crosslinking alone, the PLC activity returned to base level after 10 min (fig. 4C).

Similar results were obtained by crosslinking CD8 alone and co-crosslinking CD4 and CD8 in 171.3 CD8/z cells (fig. 4E). Co-crosslinking of CD4 and CD8 altered the kinetics of PLC tyrosine phosphorylation and activation as shown in fig. 4E. Overall, CD4/lck enhanced tyrosine phosphorylation and activation of PLC when co-crosslinked to CD3, TCRab, and CD8/z. This confirmed that CD4/lck can positively affect the PTK activation pathway generated through TCR/CD3 complexes.

Discussion

There is extensive evidence that CD4 and CD8 have important functions in T cell activation and thymocyte maturation. CD4 and CD8 could act as adhesion molecules *via* binding to MHC molecules to increase the avidity of interactions of T cells with APCs and of thymocytes with thymic stromal cells. This is unlikely to be the sole function of CD4 and CD8. It is also believed that these molecules are able to transduce signals into cells, and this function might be essential under certain conditions.

The lymphoid-specific protein tyrosine kinase, p56^{lck}, is specifically associated with CD4/CD8. This interaction provides the structural basis for CD4 and CD8 to transduce signals that may be comparable to those mediated by growth factor receptor PTKs. Like receptor PTKs (e.g. PDGF-R, FGF-R or EGF-R), whose kinase activity is activated by transphosphorylation upon ligand binding, the kinase activity of p56^{lck} is increased via autophosphorylation of Tyr394 upon antibody crosslinking of CD4 or CD8. Antibody crosslinking of CD4 or the binding of human immunodeficiency virus (HIV) gp120 to CD4 transduce signal(s) into cells such that subsequent stimulation through TCR/CD3 results in apoptosis of mature T cells instead of normal T cell activation.

Protein tyrosine kinase activation is the first step in the cellular signal cascade, since PTK inhibitors can block all the biochemical events measurable for T cell signal transduction, including early signaling events such as Ca⁺⁺ influx and subsequent T cell activation. There are a number of tyrosine kinases which are

candidates of the PTK pathway in T cell activation. p56^{lck} has been proven to be essential for both thymocyte development and T cell activation. In mice genetically ablated in expression of functional p56^{lck}, there was almost no T cell development. In JCAM1, a mutant derivative of the T cell tumor line Jurkat, lack of functional p56^{lck} led to loss of responses to antibody against TCR. This thesis was designed to study the role of the CD4-p56^{lck} complex in the PTK pathway during T cell activation elicited by anti-TCR/CD3 antibodies.

Local aggregation of PTK's is the critical step for PTK pathway activation during T cell activation

The complete blockade of signaling during T cell activation through TCR/CD3 by PTK inhibitors indicated that PTK activation is the initial step in this signaling process. Our results suggested that there is a threshold of local PTK aggregation for initiation of PTK pathway during T cell activation. In the 171.3 cell line, the PTK pathway can be activated by antibody crosslinking of the CD3 complex alone. However, crosslinking of TCRab alone or of a CD8/z chimeric molecule alone did not result in PTK activation under similar conditions, although a ten fold increase in antibody concentration induced PTK activation in the CD8/z cells. In other T cells (EL4 and D11.10) with about four to five fold higher TCR/CD3 surface expression, the same anti-TCRab antibody was able to activate the PTK pathway at the same antibody concentration.

CD3 consists of six chains (ϵ δ ϵ_2 $\zeta_2/\zeta\eta$) that contain the ARAM, antigen related activation motif, and two of them, CD3 ϵ and CD3 ζ chain, were able to transduce signals independently. In a sense, one CD3 complex can be viewed as multiple ζ chains. Since the individual chains of CD3 might be coupled to different PTKs, it is also possible that the CD3 complex is coupled to multiple PTKs rather than just ZAP70 associated with the ζ chain. Theoretically, CD3 crosslinking would increase local aggregation of associated PTKs to reach a threshold for PTK activation at a lower level than that of CD8/ ζ crosslinking alone. Thus, the CD3 complex is more potent for PTK pathway activation than a single CD3 chain alone.

One way to reach the threshold is by accumulation of more TCR/CD3 chains, which can be achieved by higher level of surface expression of the complex or higher concentration of crosslinking antibodies in the case of antibody crosslinking experiments. When T cell lines with four to five fold higher surface expression of the TCR/CD3 complex were used, anti-TCR crosslinking alone could activate the PTK pathway. When we increased the concentration of anti-CD8 in the crosslinking experiments, the PTK pathway was also activated by crosslinking of CD8 alone. Other factors, such as higher expression levels of the PTKs or a higher percentage of CD3 molecules associated with the PTKs, could also contribute to the threshold. The latter is one way through which p56^{lck} may function to affect the PTK pathway, as discussed below.

It could be speculated that, when the local aggregation of CD3 associated PTKs reaches a threshold, intramolecular or/and intermolecular phosphorylations of these PTKs occur, which results

in coupling to the downstream signal transduction pathways. In receptor tyrosine kinase systems, it is the ligand binding which leads to dimerization, autophosphorylation and activation of the receptors. The autophosphorylation of receptors is a means to couple the receptor PTK to other signal transduction pathways, e.g. the PI pathway is coupled to FGF-R through the direct binding of the SH2 domain of PLC γ 1 to phosphotyrosine on FGF-R.

In T cell activation mediated by antigen and APC, the amount of antigen will affect the amount of the TCR/CD3 engaged on the T cell surface. This may be why very low amounts of antigen could not activate T cells, a phenomenon that was observed long ago (205). It can be speculated that co-receptors (the CD4/CD8-p56^{lck} complexes) will be more important for T cell activation or thymocyte maturation when only low amounts of antigen are presented by MHC molecule; or when the TCR affinity for antigen is low.

CD4/lck plays an important role in the PTK pathway during T cell activation

Previous studies showed that in order for CD8 to function during thymocyte maturation, CD8 and TCR have to interact with the same MHC molecule. This strongly suggests that an interaction between signal(s) generated from CD4/CD8 and from TCR/CD3 is required for a productive result. However, the mechanism by which CD4/CD8 carries out this function is not clear. p56^{lck} is a PTK, and a PTK pathway is directly coupled to TCR/CD3 and also very critical in

T cell activation as discussed in the introduction. The PTK activity of p56^{lck} can be increased upon antibody crosslinking of CD4/CD8. There is evidence that indicates CD4/CD8 and TCR/CD3 are one physical and functional complex during T cell activation. It is thus important to study the effect of p56^{lck} on PTK activation through TCR/CD3 when these molecules are brought into proximity with each other.

In our study, when CD4/lck was co-crosslinked with TCRab or CD8/z, activation of PTK's was obtained. The PTK pathway failed to be initiated when the same conditions were used on crosslinking TCRab or CD8/z alone. There were previous reports that both PTK activation and intracellular Ca⁺⁺ influx were enhanced if CD4 and TCR/CD3 were co-crosslinked in peripheral T cells, thymocytes and T hybridoma cell lines (rev. 10), indicating that this is a general function of CD4/lck. However, the mechanism through which CD4 and the associated p56^{lck} molecules affects the PTK pathway has not been elucidated.

The binding of ZAP70 to CD3z chain might be through the interaction of ZAP70's SH2 domains and phosphotyrosines of CD3z, and is likely be a critical step for activation of the PTK pathway after TCR/CD3 ligand binding. Tyrosine phosphorylation of CD3z chain follows ligand binding to the TCR during T cell activation and thymocyte maturation. This is accompanied by binding of ZAP70, which is co-precipitated with z chain only in activated T cells. There are six tyrosines in the cytoplasmic domain of z chain, located in three well defined "signaling motifs". These motifs can signal independently, and their function requires both tyrosines to be

present. Phosphorylation of these tyrosines may result in binding of ZAP70 SH2 domains and may be regulated by PTKs, such as p56^{lck}. In support of this notion, previous *Cos* cell transient expression experiments showed that ZAP70 can not associate with CD3z in the absence of p56^{lck} or p59^{fyn}, or in the absence of tyrosine phosphorylation of the z chain. This study also indicated that p56^{lck} and p59^{fyn}, which are specifically associated with CD4/CD8 and TCR/CD3 respectively, may play a role in the regulation of ZAP70 interacting with the z chain. Our results showed that p56^{lck} can phosphorylate CD3z chain on tyrosines both *in vitro* and *in vivo* when brought into its proximity, and promoted the association of z chain and a tyrosine-phosphoprotein with a molecular weight of 70kd, probably ZAP70, resulting in successful PTK activation. These results strongly suggest that one of the mechanisms for CD4/lck function during T cell activation may be to tyrosine-phosphorylate CD3z chain, thus facilitating greater association of ZAP70 with the z chain and resulting in a threshold of local PTK aggregation. In support, the effects of CD4 and CD3 co-crosslinking on PTK activation were not observed in a mutant T cell line defective in z chain expression and having a comparable level of CD3 expression to a control cell line (206). The CD3z chain thus seems to be required for the enhancing effect of CD4/lck on signal transduction upon TCR/CD3 stimulation.

The threshold for activation of the PTK pathway might be the consequence of local CD3 chain (e.g. z chain) tyrosine phosphorylation leading to the local accumulation of CD3-associated PTKs (e.g. ZAP70). p56^{lck} exists in T cells as at least two different

forms, one free in the cytoplasm and the other membrane associated via either myristoylated N-terminus anchoring to the cell membrane or specific binding to CD4 and CD8. In T cells, there may be a dynamic interaction between p56^{lck} or p59^{fyn} and CD3 chains and/or their associated PTKs, i.e. a certain percentage of CD3z chains are constantly phosphorylated on their tyrosines by p56^{lck} or p59^{fyn} and dephosphorylated by some PTPases without TCR ligand binding. There would, therefore, be a certain percentage of CD3 chains associated with their specific PTKs. In T cell activation, the threshold of CD3 phosphorylation could be achieved either by the CD4/CD8 associated membrane form as the consequence of CD4/CD8 and TCR/CD3 binding to the same MHC molecule and of co-crosslinking by anti-CD4/CD8 and anti-TCR/CD3, or by the N-terminal membrane-anchored and cytoplasmic free forms of p56^{lck} as the result of random molecular movements. Sufficiently high local accumulation of CD3 chains associated with PTKs would then lead to activation of the PTK pathway. This basal percentage of PTK-associated CD3 chains may be one explanation as to why CD3 crosslinking alone can activate the PTK pathway at saturating antibody concentrations. Co-aggregation of CD4/CD8-p56^{lck} and less TCR/CD3 complexes could also result in more CD3 associated PTKs locally because of the increasing tyrosine-phosphorylation of z chains.

It can also be speculated that another way to reach the threshold could be either by prolonging the duration of TCR/CD3 aggregation at low levels of receptor occupancy or by increasing the stability of the interaction between the TCR/CD3 and APC under

limiting conditions. This may increase the chance of achieving the threshold of local PTK accumulation because of the dynamic interactions between p56^{lck} or p59^{fyn} and CD3 chains. This would explain why T cells with high affinity TCRs are more readily activated by antigen and APCs. Adhesion molecules could also thus facilitate this process by increasing the avidity of the interactions between T cells and APCs. Overexpression of the tailless CD4 transgene in mice defective for CD4 gene expression can thus restore the development of CD4⁺ T cells by increasing the avidity of interactions between thymocytes and thymic stromal cells.

When the local threshold of CD3 associated PTKs is achieved, bringing the PTKs into proximity with each other, phosphorylation may occur intramolecularly or/and intermolecularly, leading to the activation of downstream signal transduction pathways, e.g. the PI, G protein and serine/threonine kinase pathways.

Early studies on T cell activation by antigen and APCs or anti-TCR/CD3 antibodies indicated that capping of cell surface TCR/CD3 occurs upon ligand binding (rev. 124). Antibody staining for CD4 on the cell surface and for p59^{fyn} and p56^{lck} in mild detergent permeabilized intact cells showed that all three molecules could be co-capped with TCR/CD3 during this process (84, 207). This result suggests that the local aggregation mechanism and the physical interaction of CD4 and CD3 exists in intact cells physiologically.

When CD4/lckCTmL273A was expressed in 171.3 cells, the effects of CD4 and TCR/CD3 co-crosslinking on PTK activation were not observed. However, the kinase deficient chimera could partially replace CD4 in assays measuring response to specific peptide

antigen. This suggests that another function of p56^{lck} associated with CD4 is mediated by the SH2 and SH3 domains and may be to regulate the avidity of CD4-dependent TCR interaction with MHC class II molecules. It was previously reported that association of CD8 with p56^{lck} increased the binding avidity of CD8 for MHC class I molecules (213). The binding avidity of CD8 to MHC class I can be increased as a result of TCR signalling in a PTK dependent fashion (213). No similar data have been reported with CD4, but following TCR activation there is redistribution of CD4 towards the TCR complex shown by fluorescent energy transfer studies. There are also differences in cell surface expression of CD4, CD8ab heterodimer and CD8aa homodimer during T cell activation, e.g. CD4 and CD8ab heterodimer are down-regulated as the result of TCR ligand binding, while CD8aa homodimer is not (rev. 10). Whether the association of p56^{lck} with CD4, independent of its kinase activity, could increase the affinity of the molecule for MHC class II remains to be determined. Another possibility that the kinase deficient CD4/lck functions during T cell activation by interacting with other signaling molecules, such as PLCg1, GAP, or even endogenous PTK's, through its SH2 or SH3 domains. The phenomenon that the potency of anti-TCR antibodies correlated with their ability to co-cap CD4 also supports the notion that CD4 may be able to enhance the signal transduction process by interacting with the CD3 complex. Our demonstration that CD3 and CD4/lck co-crosslinking altered the kinetics of the PTK activation process (peaking earlier and higher, lasting longer) also is consistent with CD4/lck functioning by promoting the PTK association with the CD3 complex. The result of

co-crosslinking of CD4/lck and CD8/z at suboptimal anti-CD8 antibody concentration, which successfully initiated a PTK pathway accompanying early, faster and more prominent tyrosine phosphorylation of CD8/z, also supports this view.

Although our results were obtained by antibody crosslinking, CD4 is recruited into TCR/CD3 complex during T cell activation by antigen and APC or even by some potent anti-TCR/CD3 antibodies as mentioned before. There is no direct evidence that CD4 and TCR are interacting with the same class II MHC molecule yet. However, it is clear that CD8 and TCR/CD3 are interacting with the same MHC class I molecule on the thymic stromal cells, i.e. CD8 and TCR/CD3 could be crosslinked on the cell surface by the same MHC class I molecule, and this interaction is essential for positive and negative selection during thymocyte maturation. Both CD8 and CD4 interact with similar regions of MHC molecules, nonpolymorphic membrane proximal domains. This suggests that CD4 interacts with the same MHC class II molecule as TCRab does and is crosslinked to the TCR complex on the T cell surface under physiological conditions. The antibody co-crosslinking results are therefore likely physiologically relevant.

Our results argue strongly that CD4/lck affects PTK activation by tyrosine phosphorylating CD3z chains, thus promoting the association of CD3z with its PTK. Tyrosine phosphorylation is also an important mechanism for regulating the kinase activity of *src* family tyrosine kinases, or for activating receptor PTKs (R-PTK) and coupling the R-PTKs with downstream signaling pathways. It is also possible that p56^{lck} could activate other TCR-associated PTKs after

they bind to their specific CD3 chains. The CD3-bound PTKs could also phosphorylate CD3 chains close to them and further amplify the whole process. This is likely to be the case in cells that can be activated even in the absence of CD4 and CD8. All these possibilities should be considered in future studies.

CD4/lck affects coupling of the TCR to the CD3 signal-transducing machinery

Our data suggests that CD4/lck can affect transduction of signals by the CD3 complex after engagement of TCRab. Our results are consistent with findings of several other previous studies. In a mutant Jurkat cell line, JCAM1, which is defective in the expression of a functional p56^{lck}, PTK and ultimately T cell activation could not be achieved by antibodies against the TCR. However, antibodies directed at a CD3 polypeptide did partially activate the PTK pathway. Expression of the p56^{lck} cDNA in JCAM1 restored the ability of the cells to respond to TCR stimulation. In another murine T hybridoma system (208), antibodies to TCRab could activate only CD4⁻ variants but not CD4⁺ cells. Co-crosslinking of CD4 and TCR by antibodies or by antigen plus MHC molecules on the surface of APC was required for the activation of the CD4⁺ cells. In this cell line, T cell activation by antibodies to CD3 is not affected by expression of CD4. These results suggest that, for the signal to be transduced from the TCRab polypeptide to the CD3 complex, CD4-p56^{lck} function is required. This function does not appear to be absolutely required in signaling generated by anti-CD3.

In T cell activation, ligand binding to the TCR may induce some conformational changes in both the TCR and CD3 chains, making the tyrosines on the CD3 chains more accessible for phosphorylation by CD4 associated p56^{lck}, non-CD4 associated p56^{lck}, or TCR associated p59^{fyn}. Studies on antibodies specific for different TCRab epitopes have shown that only some of these could activate T cells, and even some of their monovalent Fab fragments were capable of T cell activation (209). These findings strongly support the conformational change model. In the CD4⁻ cell line discussed above (208), p56^{lck} exists either as a membrane associated form through its N-terminal myristoylation site or as a cytoplasmic form, either of which may gain access to tyrosines on CD3 chains. In the CD4⁺ cells, p56^{lck} exists as a CD4 associated form which may not be able to move freely to gain access to the tyrosines on CD3 chains upon TCR crosslinking alone. However, if co-crosslinked with TCR simultaneously by the same MHC molecule or by a second antibody, the signal transducing process can be amplified. This is particularly important in T cells when p56^{lck} is a limiting factor, due to its low expression level or its low kinase activity, as in the CD45⁻ mutant T cells. In those CD45⁻ cells (165), there is an increase of phosphorylation of Tyr505 (the negative regulatory site) of p56^{lck} and a decrease of the kinase activity of p56^{lck}. Crosslinking with anti-TCR failed to activate the cells but co-crosslinking of CD4 and TCR activated the cells successfully. Again, crosslinking by anti-CD3 did not require co-crosslinking of CD4 for T cell activation in the mutant cells, suggesting that anti-CD3 antibody

might be more potent to induce the right conformational changes in the CD3 cytoplasmic domains.

Another possibility is that p56^{lck} might be able to interact with TCRab directly or indirectly and that this interaction has much lower affinity or lower stoichiometry than the interaction between p56^{lck} and CD4/CD8. This could also explain some of the phenomena stated above, but does not explain the difference in results between anti-TCR and anti-CD3 stimulation. This difference could also simply be due to anti-CD3 antibodies having higher affinity than anti-TCR antibodies; however, in multiple systems, using different anti-TCR antibodies, this has been a consistent finding.

CD4/lck influences activation of the PI pathway by the TCR/CD3 complex

The fact that crosslinking CD4 alone did not result in PLC tyrosine phosphorylation and activation suggested that p56^{lck} alone is not directly linked to the PLC pathway during T cell activation (fig. 5B). Our data indicate a good correlation between PTK activation and PLC tyrosine phosphorylation/activation after co-crosslinking CD4 with CD3, TCR, or CD8/z. It is likely that p56^{lck} can indirectly up-regulate PLC activation, probably by enhancing PTK activation through TCR/CD3 complexes. p56^{lck} is still very important for PI pathway activation via the TCR alone, since in JCAM1 cells, which lack functional p56^{lck} expression, the PI pathway could not be activated by anti-TCR.

The observation that both anti-PLCg1 antibody and bacteria expressed fusion proteins containing an SH2 domain of PLCg1 could co-precipitate p56^{lck} in activated Jurkat cells suggested that p56^{lck} could play some role in the activation of PLCg1 and in regulating the PI pathway. However, anti-p56^{lck} antibody failed to co-precipitate PLCg1 from these lysates. It is not clear whether p56^{lck} and PLCg1 are directly associated or part of a large complex formed with TCR/CD3 during T cell activation.

The most obvious difference between CD4 crosslinking alone and CD3 crosslinking alone in 4G10 western blots was the absence of tyrosine phosphoproteins in the molecular weight range of 70-72kD with anti-CD4 (fig.1C). This suggests that the 70-72kD phosphoproteins might be a linkage between CD3 complexes and PLCg1, and that their tyrosine phosphorylation is important for PLC activation. In other words, p56^{lck} might facilitate the signaling pathway by phosphorylating ζ chain leading to activation of CD3 associated PTKs (e.g. ZAP70) which, in turn, tyrosine phosphorylate and activate PLC directly. The other evidence supporting this notion is that PLCg1 was found to be co-precipitated with CD3 complexes only after T cell activation (151), which makes it possible for CD3 associated PTKs to phosphorylate PLCg1 directly.

Summary

In the current study, we were able to show that CD4/lck could regulate protein tyrosine phosphorylation events induced by CD3 antibody crosslinking and could initiate the process induced by

TCRab crosslinking when brought into proximity of TCR/CD3 complexes by second antibodies. One possible mechanism by which CD4/lck may function in this process is by phosphorylating tyrosine(s) of the CD3z chain and thus facilitating the association of z chain with its specific PTK. When local accumulation of PTKs reaches a threshold, the PTK pathway can be activated resulting in T cell activation.

It has been previously reported that surface crosslinking of CD4 alone by antibodies resulted in increased tyrosine phosphorylation of the z chain (79). Our results, showing increased CD8/z tyrosine phosphorylation both *in vivo* in the co-crosslinking experiments in the T cell hybridoma and in *cos* cells co-expressing CD8/z and CD4/lck and *in vitro* after immunoprecipitation of CD8/z and CD4/lck, provide evidence that the CD3z chain could be a substrate for p56^{lck} in T cells. Since co-precipitation of a 70kd tyrosine-phosphoprotein with CD8/z is only observed after CD4/lck and CD8/z co-crosslinking, it appears that a consequence of the interaction between p56^{lck} and CD3z is the increased association of ZAP70 with z chain in T cells.

Future directions

Many of future directions on the function of CD4/CD8 and the mechanism of TCR/CD3 signal transductions during T cell activation and thymocyte development have been discussed in the introduction. Among them the most interesting ones are briefly discussed here.

The mechanism by which signal transduction upon MHC/antigen recognition by TCR/CD3 complex on mature T cells or the immature thymocytes can result in completely different consequences. The interaction between the mature T cell and the APC leads to T cell activation, while that between the immature thymocyte and thymic stromal cell leads to negative or positive selection. It is clear that p56^{lck} is required for thymocyte development. It has been proposed that p56^{lck} is not required for the negative selection process since PTK inhibitors did not block the process (214). It is questionable, however, whether PTK inhibition was complete in that study. Further study of the effects of p56^{lck} on signaling pathways upon TCR ligand binding during specific stages of T cell development will give more insight into the molecular and biochemical basis of T cell activation and thymocyte maturation, and as well as signal transduction in general.

There are other signaling pathways which can be activated through TCR/CD3 complexes during T cell activation, e.g. the G protein pathway. As discussed in the introduction, the G protein pathway is downstream of PTK activation and absolutely required for R-PTK signal transductions in *C. elegans* vulval development, *Drosophila* eye development and growth factor receptor PTK-induced mitogenesis (PDGF-R, FGF-R and EGF-R). As mentioned in the introduction, the *ras* G protein pathway is activated, may be through both PKC and PTK activation, and is a critical step during T cell activation. The ras-GAP served as a substrate for p56^{lck} in an *in vitro* kinase assay and a tyrosine phosphorylated form of ras GAP was co-precipitated with p56^{lck}. The SH3 domain in p56^{lck} might

be able to associate with GAPs like the SH3 domain of *c-abl* , thus coupling p56^{lck} to the G protein pathway. Our results indicated that p56^{lck} promoted PTK pathway activation through TCR/CD3. In addition, a 32kd G protein was previously reported to be co-precipitated with CD4 (118). Taken together, these findings suggest that p56^{lck} may play a role in regulating the G protein pathway during T cell activation. Small G proteins are responsible for cytoskeletal changes caused by R-PTKs. Cytoskeletal changes during T cell interaction with APC or thymocyte interaction with thymic stromal cells are likely to be important factors in the differentiation and function of these cells. The contribution of p56^{lck} in this regard remains to be explored.

As summarized in the Introduction, the serine/threonine kinase pathway also has an important role in signal transduction in R-PTK systems and in T cell signal transduction. There is a p110, a Raf-1 serine/threonine kinase related protein, reported to be complexed with CD4/p56^{lck}. Both CD4 and CD3 chains are phosphorylated on serine and threonine residues after TCR stimulation. MAP-2K, a serine kinase, was found to be phosphorylated on both tyrosine and serine/threonine residues, and was activated after T cell activation. The effect of p56^{lck} on this pathway is another interesting subject to be studied.

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APPENDIX

Characterazation of Lyt-2 and Lyt-3 gene luci

Cosmid library of genomic DNAs from the liver of Balb/C mouse (a gift from Dr. Glen Evans) was screened with Lyt-3 cDNA as prob. Three distinct clones, containing Lyt-3 coding sequence, were obtained. They span 45Kb of the genomic DNA, including 17.5Kb of the Lyt-3 coding region, 19.5Kb upstream and 9Kb downstream of Lyt-3 gene. A 1 phage clone was kindly given by Dr. Elithabeth Lacy, which contains the 5.4Kb of Lyt-2 coding region and 12.9Kb upstream of Lyt-2 gene. Restriction mapping of these genomic DNA fragementts was carried out by restriction enzym digestion, subcloning and southern blot. The restriction maps are in Fig. 6A.

DNase I hypersensitive sites (DHS) mapping in Lyt-2 and Lyt-3 gene luci

Nuclei from mouse thymocytes, 1200M (CD4⁻CD8⁺ T cell line), 10105 (CD4⁺CD8⁻ T cell line), MoLT4 (CD4⁺CD8⁺ T cell line) and M12 (mature B cell line) cells were isolated gentally according to previously described method, and digested by increasing amount of DNase I. DNAs were then purified from the neuclei, digested by certain restriction enzymes completely and subjected to southern blot analysis. There are 12 DHS's in total which were found to be T cell specific (Fig. 6B).

Testing of the tissue specific enhancer activity of Lyt-2 and Lyt-3 gene promoters, and of each of the DHS

Fragments of DNAs around the DHS's were cut out and subcloned into a vector with a promoter from Lyt-2, Lyt-3, L3T4 or the thymidine kinase (TK) gene and a reporter gene, such as CAT gene, as a enhancer element. These fragments were cloned either upstream or downstream of the promoter-reporter gene in both orientations. These constructs were tested for their tissue specific expression in different cell lines, as mentioned above, by transient transfections. The results are as follows:

For Lyt-2, Lyt3, or L3T4 promoter, there are no tissue specificity

Lyt-2 promoter used is a 500bp fragment upstream of the transcription starting site in Lyt-2 gene; Lyt-3 promoter, a 1Kb fragment upstream of Lyt-3 gene; and L3T4 promoter, a 500bp fragment upstream of L3T4 gene. These promoter fragments were subcloned into PBL-CAT to direct CAT gene expression. The constructs were then transiently transfected into different T, B, or nonT-nonB cell lines by DEAE-Dextran Method. Cell lysates from those transfectants were assayed for CAT activity.

There was no enhanced CAT activity in lysates from T cell transfectants compare to that from B and nonT-nonB cells, suggesting that there is no tissue specificity for promoters of Lyt-

2, L γ t-3 and L3T4 genes. This is consistent with previous results that genomic DNAs containing those promoter fragments failed to direct tissue specific expression in transgenic mice.

No tissue specific enhancer activity were detected from fragments containing the tissue specific DHS's in L γ t-2 and L γ t-3 gene luci

When those constructs, containing fragments around the DHS's as enhancers in the context of different promoters, were transiently transfected into different cell lines as the promoter testing, there was also no T cell specific enhancer activity found. There was one fragment, about 1Kb in length, which had very strong non T cell specific enhancer activity. The results are summarized in table-1.

Tabl-1 Tissue specific enhancer activity of the DHS's

Promoter	DHS	1200M	MoL-T4	M12	3T3	Hela
lyt-2 or lyt-3	1(4.3kb)	-	-	-	ND	ND
	2(1.0kb)	-	-	-	ND	ND
	3(4.2kb)	-	-	-	ND	ND
	4(5.0kb)	-	-	-	ND	ND
	5(3.9kb)	-	-	-	ND	ND
	6+7(7.2Kb)	-	-	-	ND	ND
CD4	1(4.3kb)	-	-	-	ND	ND
	2(1.0kb)	+	+	+	-	-
	3(4.2kb)	-	-	-	ND	ND
	6+7(7.2kb)	-	-	-	ND	ND

It is interesting that this 1Kb fragment having a non-T cell specific enhancer activity in the context of L3T4 promoter. This indicates that there might be some transcriptional factors being able to bind this fragment and to interact with L3T4 promoter to enhance transcription. It is located 7.2Kb upstream of Lyt-2 gene. If the transcription factors could bind this fragment under physiological conditions, there might be some interactions between them and the Lyt-2 promoters. The outcome might not be transcription activation considering that there was no enhancer activity detected in the context with Lyt-2 promoter. During thymocyte development, both CD8 and CD4 genes were expressed in the immature double positive stage, CD8 gene continued to be expressed in mature CD8⁺CD4⁻ thymocytes and CD4 gene expression was shut-off in the same cells, and *vesi versa*. The significance of the fragment in Lyt-2 gene expression regulation during thymocyte development remain to be determined.

There was no enhancer activity found in this search. It is possible that the enhancer is located 3' downstream of Lyt-2 gene or further away from Lyt-2 and Lyt-3 genes, which have not been searched. Another possibility is that a collaborative effect among the DHS's is required for CD8 gene expression regulation, similar to that of the β globin gene. Further studies should be carried out.



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