STRUCTURE-FUNCTION ANALYSES OF THE TYROSINE PROTEIN KINASE $p56^{lck}$ IN T LYMPHOCYTES.

by

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ABSTRACT

$p56^{lck}$ is a member of the Src-family of tyrosine protein kinases which is abundantly expressed in T lymphocytes. Increasing evidence indicates that $p56^{lck}$ participates in T-cell receptor-mediated signalling. Firstly, this enzyme is physically associated with the CD4 and CD8 T-cell surface glycoproteins. CD4 and CD8 are respectively involved in the recognition of major histocompatibility complex class II and class I, that are expressed on antigen presenting cells. Secondly, expression of activated $p56^{lck}$ polypeptides in CD4- and CD8-negative T cells has been documented to enhance antigen-induced T-cell responsiveness. To understand the mechanism(s) by which $p56^{lck}$ participates in this process, the effects of mutations in known regulatory domains of $p56^{lck}$ on the ability of activated $p56^{lck}$ to enhance antigen-induced T-cell responses have been examined. Our results indicate that $p56^{lck}$ regulates T-cell antigen receptor signalling through a complex process requiring multiple distinct structural domains of the enzyme.

RÉSUMÉ

p56^{lck} est une protéine tyrosine kinase de la famille Src abondamment exprimée dans les lymphocytes T. De nombreuses observations indiquent que p56^{lck} est impliquée dans le processus de transduction du signal médié par le recepteur des lymphocytes T. Premièrement, cette enzyme est associée aux glycoprotéines CD4 et CD8, qui sont exprimées à la surface des cellules T. CD4 et CD8 sont impliquées dans la reconnaissance respective des complexes d'histocompatibilité majeurs de classe II et I. Deuxièmement, on a démontre que l'expression d'une forme activée de p56^{lck}, dans des lymphocytes T CD4- et CD8- négatifs, augmente la réponse lymphocytaire aux antigenes. Afin de mieux comprendre le(s) mécanisme(s) par le(s)quel(s) l'activation de cette enzyme contribue à l'augmentation de la réponse aux antigenes, les effets de mutations additionelles dans des domaines régulateurs identifiés de la protéine ont été examinés. Les résultats démontrent que la regulation des signaux intracellulaires induits par l'activation des lymphocytes T est un processus complexe qui nécessite l'apport particulier de plusieurs domaines régulateurs de la protéine.

PREFACE

The experimental work presented in this thesis is comprised in **Chapter 2**. The option provided to the candidate by section 2 of the Guidelines Concerning Thesis Preparation has been utilized "Candidates have the option, subject to the approval of their department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. In this case, connecting texts, providing logical bridges between the different papers, are mandatory The thesis must still conform to all other requirements of the Guidelines Concerning Thesis Preparation and should be in a literary form that is more than a mere collection of manuscripts published or to be published

The thesis must include, as separate chapters or sections. (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis

In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, supervisors must attest to the accuracy of the claims. Since the task of the Examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of authors of co-authored papers".

Chapter 2 has been published in **Molecular and Cellular Biology, vol. 12, no. 6, p. 2720-2729**, and has its own Abstract, Introduction, Materials and Methods, Results, Discussion and References. Apart from my supervisor, Dr. Andre Veillette, additional co-authors in Chapter 2 are Ninan Abraham and Dr. Tony Pawson. Ninan Abraham generated the cells expressing A2F505 and F394F505 double mutants *lck* cDNAs and performed the experiments presented in figures 1, 2, 3A, 7A and 7B. Dr. Tony Pawson provided glutathione-S-transferase (GST) fusion proteins containing the SH2 domains of phospholipase C-γ 1(PLC-γ 1) and provided anti PLC-γ1 antibodies.

As required by the Guidelines, Chapter 1 is a General Introduction to the subject of the thesis. Chapter 3 includes a General Discussion and General Conclusion.

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Chapter 1

General Introduction

INTRODUCTION

Phosphorylation and dephosphorylation of proteins allow efficient regulation of important cellular activities (Krebs and Beavo, 1979). Protein phosphorylation is mediated by amino acid-specific kinases which are enzymes recognizing hydroxyl groups on precise amino acid residues. In mammalian cells, the phosphoamino acids most commonly found involve histidine, serine and threonine residues, and to a lesser extent, arginine, lysine and tyrosine (Hunter and Cooper, 1985). For reasons that will become clear, the protein kinases most extensively studied are the serine/threonine kinases and the tyrosine kinases.

Tyrosine phosphorylation and growth regulation

In the normal cell, tyrosine phosphorylation is a rare modification, constituting approximately 0.02% of total phosphoamino acids (Hunter and Cooper, 1985). Nevertheless, several findings suggest that tyrosine protein phosphorylation plays an important role in the regulation of cell proliferation and cell differentiation. Initial evidence originated with the identification of $pp60^{v\text{-}src}$, the protein product of the Rous sarcoma virus *src* gene which was found to encode a tyrosine-specific protein kinase (Hunter and Cooper, 1985). Accumulating data now supports the involvement of tyrosine protein phosphorylation in both oncogenesis and growth factor-dependent mitogenesis (Rayter *et al.*, 1987). Half of all reported oncogenes code for tyrosine protein kinases (Bishop, 1985, Bishop, 1991), which explains the considerable interest in understanding the mechanisms by which these enzymes react to external stimuli and transduce these stimuli into changes in cell proliferation and cell differentiation.

In providing a rapid and reversible mecnanism of protein modification, tyrosine protein phosphorylation signalling should be envisaged as a dynamic equilibrium process involving both protein tyrosine kinases and protein tyrosine phosphatases. The importance of tyrosine protein phosphatases has been recently established, with several observations reporting their implication in the control of cell growth (Alexander and Cantrell, 1989; Tonks and Charbonneau, 1989)

Tyrosine protein kinases form two major groups

Tyrosine protein kinases are categorized in two major groups. **1.** Cytoplasmic tyrosine protein kinases, which include the products of the *src, fps* and *abl* gene families. **2.** Receptor tyrosine protein kinases for a variety of polypeptide growth factors, such as the receptors for epidermal growth factor (EGF-R), platelet-derived growth factor (PDGF-R), nerve growth factor (Trk, NGF-R), insulin (Ins.-R) or colony-stimulating factor-1 (CSF-1-R)(for a review, see Cantley *et al* , 1991).

Dimerization-induced activation model for growth factor receptors

Binding of growth factors to the extracellular portion of their respective receptors is thought to induce receptor dimerization, which brings receptor tyrosine kinase domains into close proximity Dimerization promotes intermolecular tyrosine phosphorylation (cross autophosphorylation), resulting in receptor activation. Downstream signalling requires the interaction of intermediate signal transducers with the autophosphorylated growth factor receptor A number of these signal intermediates have been identified including phospholipase C-γ 1(Morrison *et al* , 1990; Rotin *et al* , 1992), phosphatidyl inositol 3' kinase (Otsu *et al* , 1991) and Src kinases (Kypta *et al*., 1990).

The association of activated receptor tyrosine kinases with these intermediate signal transducers involves SH2-mediated recognition of the autophosphorylated receptors. These interactions are shown to generally result in the tyrosine phosphorylation of these receptor targets, which correlate with their activation. Apparently, this model of ligand-induced receptor-mediated signal transduction may also be applied to non-receptor tyrosine protein kinases that are physically linked to cell surface receptors. Consequently, this model will be used to understand the molecular mechanism by which the Src-family kinase, $p56^{lck}$ contributes to T-cell receptor-mediated signal transduction.

THE SRC-FAMILY OF TYROSINE PROTEIN KINASES

Origin

Src was the first viral transforming gene encoding a tyrosine protein kinase to be identified. This retroviral oncogene was later found to be a mutated form of a normal cellular gene (proto-oncogene) denoted *c-src*. Complete sequence analysis of both chicken *c-src* and *v-src* genes (Takeya and Hanafusa, 1983) revealed that the phosphoprotein $pp60^{v\text{-}src}$ contained several scattered point mutations and lacked the carboxy-terminal regulatory domain, which is normally present in the proto-oncogene product. Indeed, the last 19 amino acids of chicken c-Src are replaced with 12 unrelated amino acids in v-Src. These structural alterations are responsible for the constitutive upregulation of $pp60^{v\text{-}src}$ kinase activity and its ability to induce cellular transformation.

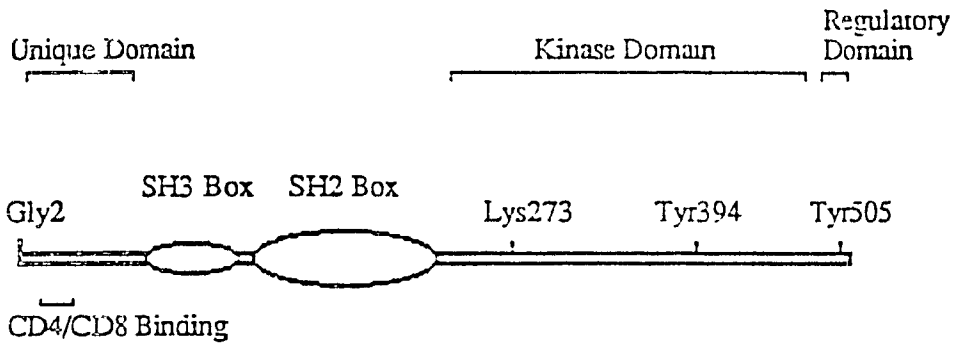
Since the discovery of *v-src*, other related genes have been identified either as viral oncogenes, (such as *v-yes* and *v-fgr*), or on the basis of structural homology [*fyn, lck, lyn, hck, blk* (*tkl* being the avian homolog of mammalian *lck*; Chow *et al.*, 1992)] (for reviews, see Veillette and Bolen, 1989; Cooper, 1990). This family of *src*-related genes comprises 8 distinct members which all encode closely related proteins with tyrosine protein kinase activity.

Primary sequence features

Although they share several structural similarities, each member of the Src-family possesses a unique amino-terminal domain that may be required for specific cellular activities (see Figure 1). Common attributes of Src-related polypeptides include a conserved

amino-terminal glycine residue at position 2 which is critical for protein myristylation. A modulatory region encompassing the Src Homology (SH) domains 3 and 2 is juxtaposed to the kinase domain (also denoted as the SH1 domain). Finally, Src-family members share a small carboxy-terminal regulatory domain.

Figure 1. Primary sequence features of a prototypical Src-like kinase: p56^{lck}



p56 lck

Protein myristylation

Experiments with Src protein chimeras have shown that the first 7 amino-acid residues of this tyrosine protein kinase are required for myristylation (for a review, see Resh, 1990) It has been proposed that this region constitutes a recognition site for N-myristyltransferase. This enzyme allows the addition of myristic acid in amide linkage to the alpha amino group of glycine 2 This cotranslational modification appears to be critical for plasma membrane association Although the occurence of myristylation and membrane association has not been tested for all family members (only for Src, Lck, Fgr and Hck) (Perlmutter, 1990), it is reasonable to assume that all these cytoplasmic enzymes are recruited to cellular plasma membrane through amino-terminal myristylation.

Uniqueness among the family members results from the presence of different amino-terminal domains, which may confer distinct abilities. However, few examples for this proposed function have been reported to date including the association of $p56^{lck}$ with the CD4 and CD8 T-cell glycoproteins (Rudd *et al* , 1988, Veillette *et al.*, 1988a) and the association of $p59^{fyn}$ with the T-cell receptor for antigen (A. Shaw, unpublished).

SH2 and SH3 modulatory regions

The remaining portion of the amino-terminal half of Src-like kinases contains proposed modulatory sequences, denoted as the Src Homology 2 and 3 (SH2 and SH3) regions (for reviews, see Pawson, 1988; Koch *et al* , 1991). The SH3 region is a sequence of roughly 50 amino acids, juxtaposed to the unique domain. The SH2 domain is located downstream from the SH3 region and consists of about 100 amino acids, which include five areas of highly conserved sequences

This modulatory tandem is present in the Src and Abl families of cytoplasmic tyrosine protein kinases (Takeya and Hanafusa, 1983; Shtivelman *et al.*, 1986) as well as in an expanding number of proteins such as phospholipase C (PLC)-γ 1 and 2 (Stahl *et al.*, 1988; Suh *et al* , 1988, Emori *et al.*, 1989), the guanosine triphosphate (GTP)ase

Activating Protein of $p21^{ras}$ (GAP) (Trahey *et al* , 1988: Vogel *et al.*, 1988), the p85 subunit of phosphatidyl inositol (PI) 3' kinase (Escobedo *et al* , 91; Otsu *et al* , 91), the oncoproteins v-Crk (Matsuda *et al.*, 1991), Vav (Katzav *et al* , 1989) and Nck (Lehman *et al.*, 1990). The SH2 and SH3 domains are also present in a transcription factor complex (Fu, 1992), the protein kinase Csk (Nada *et al* , 1991) and the putative adaptor protein Sem-5 (Clark *et al.*, 1992) [with its human homolog GRB2 (Lowenstein *et al* , 1992)]

The SH2 domain (without SH3) is found in the Fps family of tyrosine protein kinases (Huang *et al* , 1985), tensin (Davis *et al* , 1991), other classes of cytoplasmic tyrosine protein kinases (Mano *et al* , 1990; Taniguchi *et al* , 1991) and some cytoplasmic tyrosine protein phosphatases (Shen *et al* , 1991; Plutzky *et al* , 1992, Yi *et al* , 1992).

The importance of the SH2 and SH3 modulatory function has been established from several lines of evidence, including mutational analyses of Src family members (Wendler and Boschelli, 1989, Hirai and Varmus, 1990; O'Brien *et al* , 1990, Brott *et al* , 1991; Cheng *et al* , 1991; Fukui and Hanafusa, 1991; Fukui *et al* , 1991, Kanner *et al* , 1991; Matsuda *et al.*, 1991; Wages *et al* , 1992). Because SH2 domains can bind tyrosine phosphorylated proteins *in vitro*, it is proposed that these domains are important for directing protein-protein interactions.

The SH3 domain is also present independantly of SH2 sequences in several cytoskeleton-associated proteins such as non-erythroid alpha spectrin (Wasenius *et al* , 1989), myosin 1b (Jung *et al* , 1987), the yeast actin binding protein (Drubin *et al* , 1990) and the yeast bem-1 protein (Chenevert *et al* , 92). This finding suggests that SH3 sequences may have a role in mediating interactions with the cytoskeleton The SH3 domain can also be found in proteins such as the yeast fus1(Mc Caffrey *et al* , 1987, Truehart *et al* , 1987) and CDC25 (Broek *et al* , 1987) proteins, a major palmitoylated erythrocyte membrane protein (Ruff, 1991) and two neutrophil NADPH oxidase-associated proteins (Lomax *et al.*, 1989; Leto *et al* , 1990)

The catalytic domain

The second half of a typical Src-like molecule comprises the kinase domain, which includes ATP-binding and autophosphorylation sites. The ATP-binding site is centered around a lysine residue, while the autophosphorylation site is a tyrosine residue located approximately 120 amino acids further downstream. Phosphorylation of this tyrosine residue occurs during *in vitro* protein kinase reactions and can be observed *in vivo* when the tyrosine protein kinase is activated. Hence, *in vivo* tyrosine phosphorylation at the site of autophosphorylation is a marker for activation.

The regulatory domain

At the very carboxyl end of the molecule, there is a small regulatory domain containing a conserved tyrosine residue. Extensive *in vivo* phosphorylation of this tyrosine residue is implicated in the negative regulation of the catalytic function of Src-like products, consistent with the molecular mechanism by which $pp60^{c\text{-}src}$ is activated (Cartwright *et al.*, 1987, Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987). Truncation or replacement of this region by other sequences impairs an important mechanism of tyrosine protein kinase activity regulation, and unmasks the oncogenic potential of several Src-related kinases.

A LYMPHOCYTE-SPECIFIC SRC-FAMILY MEMBER: p56^{lck}

There have been extensive studies of the functional requirements for the oncogenic activity of Src-derived products (Veillette and Bolen, 1989). Based on these studies, there is strong evidence that strict regulation of these kinases is required to allow normal cellular growth. The exact physiological function of these products is however still unresolved Fortunately, a better understanding of the physiological functions of Src-like kinases has been made possible due to important discoveries involving a lymphocyte-specific Src-family member: p56^{lck}.

Characterization

p56^{lck} is a 56 kilodalton (kDa) Src-related tyrosine protein kinase which is normally expressed exclusively in cells of lymphoid origin, most predominantly in T lymphocytes and natural killer cells. Low levels can also be found in B lymphocytes (for reviews, see Bolen and Veillette, 1989; Perlmutter, 1990; Abraham and Veillette, 1991, Sefton, 1991, Veillette *et al*, 1991) p56^{lck} was initially discovered and characterized in the Moloney Murine Leukemia Virus (MoMuLV)-induced mouse thymoma cell line LSTRA (Voronova *et al.*, 1984; Marth *et al.*, 1985; Voronova and Sefton, 1986, Garvin *et al*, 1988, Marth *et al.*, 1988b). LSTRA cells overexpress *lck* transcripts, generating high levels of this membrane-associated phosphotyrosine-containing protein The homology to c-Src was originally suggested by the analysis of trypsin-digestion products of the 56 kDa protein (Casnellie *et al.*, 1983) Similar properties were observed between the c-Src tryptic fragment containing the autophosphorylation site and a tryptic fragment resulting from the digestion of the autophosphorylated 56 kDa phosphoprotein The cDNA for *lck* was

obtained by screening a LSTRA , as well as a normal thymus cDNA library, using degenerate oligonucleotide probes based on the amino acid sequence of c-Src autophosphorylation site (Marth *et al* , 1985, Voronova and Sefton, 1986). As expected for any member of the *src*-related gene family, the *lck* nucleotide sequence was found to have strong homology to *c-src*.

Control of p56^{lck} expression

In the human and mouse, *lck* transcripts are derived from two separate promoters resulting in two types of *lck* mRNA which differ in their 5' untranslated regions (Voronova *et al.*, 1987, Garvin *et al* , 1988, Perlmutter *et al* , 1988; Takadera *et al.*, 1989). Type I transcripts originate from the proximal (or downstream) promoter, which lies 5' proximal to the coding region for the *lck* gene Type II transcripts arise from the distal (or upstream) promoter In humans, the *lck* distal promoter is located 34 kilobases upstream from the proximal promoter (Wildin *et al* , 1991). The exact role for the existence of two different 5' untranslated regions is not known

lck expression during T-cell ontogeny

The *lck* gene is transcribed in both mature and immature T lymphocytes (for reviews, see Perlmutter, 1990, Abraham and Veillette, 1991). In humans, the appearance of *lck* transcripts in the thymus correlates with the colonization of the thymus by bone marrow-derived T-cell precursors Because the proximal *lck* promoter is mainly active in the thymus, immature thymocytes predominantly express type I transcripts (Reynolds *et al.*, 1990) Maturation of thymocytes is paralleled by a progressive increase in type II *lck* transcripts, while levels of type I *lck* transcripts decrease. At the end of the maturation process, when T lymphocytes leave the thymus for other lymphoid organs such as the

spleen and lymph nodes, they predominantly express type II *lck* mRNA transcripts

The mechanisms responsible for the developmental regulation of *lck* transcription is currently unknown. A recent study (Allen *et al* , 1992) using transgenic mice bearing truncations in the proximal *lck* promoter has shown that this promoter element contains several binding sites for nuclear proteins, suggesting a molecular mechanism for differential modulation of *lck* transcription

lck expression during T-cell activation

Changes in promoter usage have also been reported during T-cell activation (Takadera *et al* , 1989; Leung and Miyamoto, 1991) Stimulation of resting T cells with lectins or activating anti-TCR antibodies results in a decline of *lck* RNA levels, corresponding to a decrease of transcription from the distal promoter

Implication of $p56^{lck}$ in oncogenesis

In lymphocytes

Various observations support the view that overexpression of $p56^{lck}$ may be implicated in the oncogenesis of lymphomas. In the thymoma cell lines LSTRA and Thy 19, increased levels of *lck* message (7 fold in LSTRA) and protein (50 fold in LSTRA)(Hurley and Sefton, 1989) are likely to result from a combination of elevated *lck* transcription and more efficient *lck* translation (Marth *et al* , 1985, Marth *et al* , 1988b) The genetic basis for Lck overexpression in these cell lines includes the insertion of the Moloney murine leukemia virus (MoMuLV) long terminal repeats (LTR) in the upstream (5') portion of the *lck* gene (Marth *et al* , 1985, Voronova and Sefton, 1986, Garvin *et al* , 1988). *Lck* transcripts arising from the upstream insertion of viral LTR possess a novel 5' untranslated region (UT), that is believed to confer more efficient translation (Voronova and Sefton, 1986, Garvin *et al* , 1988, Marth *et al* , 1988b).

That overexpression of intact Lck protein may contribute to lymphoid transformation is further supported by reports that overexpression of either wild type or activated (F505) Lck polypeptides in transgenic mice induces thymic tumorigenesis (Abraham, K M *et al*, 1991b) Thus altered *lck* transcriptional and translational controls may contribute to the transformation of lymphoid cells.

The recent findings of chromosomal translocations joining *lck* [which is located on human chromosome one (Marth *et al*, 1986)] to the T-cell receptor (TCR) β gene in two human T-cell leukemias (Burnett *et al*, 1991; Tycho *et al*, 1991) provides additional evidence that overexpression of $p56^{lck}$ may participate to lymphoid neoplasia. In these cell lines, the chromosomal breakpoint is either located upstream or between the two *lck* promoters, juxtaposing the constant region of the TCR β locus (with its associated transcriptional enhancer) with the protein-coding region of the *lck* gene. These rearrangements result in enhancement of *lck* transcription.

In other cell types

Aberrant tissue expression of *lck* (mRNA or protein) can also play a role in the process of oncogenesis, as suggested by the reports of *lck* expression in several human cancers, including colon, lung and breast (Veillette *et al*, 1987; Sartor *et al*., 1989; Koster *et al*, 1991)

Based on studies in fibroblasts, it has recently been proposed that Lck overexpression may not be sufficient to induce transformation, and that alterations in $p56^{lck}$ carboxy-terminus may contribute to oncogenicity (Adler and Sefton, 1992). This view is mainly supported by studies reporting that mutation of the carboxy-terminal tyrosine residue 505 to a non-phosphorylatable amino acid can induce cellular transformation in fibroblasts (Amrein and Sefton, 1988; Marth *et al*., 1988a). It is, however, not clear whether these mechanisms occur in lymphocytes

In general, various observations suggest that deregulation of the mechanism of *lck* gene expression, (at the levels of transcription and translation) and/or expression of activated Lck proteins are likely to result in increased Lck tyrosine protein kinase activity, and therefore may contribute to the elevated levels of cellular phosphotyrosine typically observed in transformed cells.

Structure-function analyses of p56^{lck}

A better understanding of the role of p56^{lck} in normal cellular physiology is critical for evaluating its implication in cellular transformation. Most of our knowledge regarding this enzyme comes from sequence comparison of Src-family members and structure function analyses. Expression of various mutant Lck polypeptides in NIH3T3 cells (murine fibroblasts) has provided in-depth insights into post-translational mechanisms by which this enzyme is regulated. Overall, these findings have shown that protein phosphorylation is an important regulator of tyrosine protein kinase activity. Indeed p56^{lck} is extensively phosphorylated on tyrosine and serine residues *in vivo* (Veillette *et al*., 1988c).

Regulation by tyrosine phosphorylation

As is the case for other members of the Src-family of protein tyrosine kinases, the major site of p56^{lck} *in vivo* tyrosine phosphorylation is located within the carboxy-terminal regulatory domain, on a tyrosine residue at position 505. Replacing tyrosine 505 by a non phosphorylatable amino acid such as phenylalanine results (unlike wild type) in a constitutively activated molecule, that is capable of cellular transformation when ectopically expressed in fibroblasts (Amrein and Sefton, 1988, Abraham and Veillette, 1990). These results demonstrate that phosphorylation at tyrosine 505 is implicated in the negative regulation of p56^{lck}.

In fibroblasts, activation of $p56^{lck}$ molecules through mutation of tyrosine 505 requires the participation of other functional domains, such as intact sites of autophosphorylation (tyrosine 394) and myristylation (glycine 2), as well as a functional kinase domain (Abraham and Veillette, 1990; Veillette and Fournel, 1990). The importance of phosphorylation at tyrosine 394 for activation of $p56^{lck}$ has also been documented in T cells. Indeed, significant *in vivo* tyrosine 394 phosphorylation is observed upon activation of $p56^{lck}$ through CD4 stimulation (Veillette *et al.*, 1989a; Luo and Sefton, 1990). This site was identified as the autophosphorylation site because it becomes extensively phosphorylated during *in vitro* protein tyrosine kinase reactions (Abraham and Veillette, 1990). Hence, this major site of autophosphorylation may be involved in positive regulation of $p56^{lck}$ tyrosine protein kinase activity *in vivo*

In contrast to tyrosine 394, it appears that tyrosine 505 phosphorylation does not result from autophosphorylation. This is supported by the observation that a kinase-deficient variant of $p56^{lck}$ (carrying a non-functional ATP binding site) is still extensively phosphorylated on tyrosine 505 when expressed in fibroblasts (A.Veillette, unpublished). Reports of a protein kinase that specifically phosphorylates the carboxy-terminal tyrosine 527 residue of $pp60^{c\text{-}src}$ (Okada and Nakagawa, 1988; Okada *et al* , 1989; Nada *et al.*, 1991, Okada *et al* , 1991, Thomas *et al* , 1991) have provided evidence for the involvement of other tyrosine protein kinases in the regulation of Src-like kinases. This enzyme has been identified as $p50^{csk}$ (for c-Src kinase)

Csk is a cytosolic tyrosine kinase which is ubiquitously expressed in cells and tissues, especially in cells of hematopoietic origin (Okada *et al.*, 1991; Partanen *et al.*, 1991). Interestingly, $p50^{csk}$ contains one SH3 and one SH2 domain, but lacks a myristylation signal and carboxy-terminal regulatory domain. In addition, $p50^{csk}$ does not show autophosphorylation. These characteristics may be important for its regulation and/or

substrate specificity. *In vitro*, $p50^{csk}$ appears to phosphorylate all Src-like tyrosine protein kinases including Src, Lck, Fyn and Lyn (Okada *et al*., 1991; Bergman *et al*., 1992) However, *in vivo*, no stable complex formation between Csk and c-Src or Csk and Lck has been demonstrated (Sabe *et al*., 1992; L.Chow, unpublished). Whether $p50^{csk}$ phosphorylates tyrosine 505 and down regulates $p56^{lck}$ activity *in vivo* remains to be established.

In summary, various experiments suggest that tyrosine phosphorylation is a key mechanism for regulation of $p56^{lck}$ enzymatic activity. Phosphorylation of $p56^{lck}$ involves two major sites, tyrosine 394 and tyrosine 505 While tyrosine 505 phosphorylation is implicated in the negative regulation of $p56^{lck}$ kinase activity, phosphorylation at tyrosine 394 appears to positively regulate Lck tyrosine protein kinase activity

Serine phosphorylation

Stimulation of T cells with anti-TCR antibodies, mitogenic lectins or activators of protein kinase C (PKC) results in a marked increase in amino-terminal serine phosphorylation of $p56^{lck}$. This phosphorylation event involves serine residues located presumably in the unique or modulatory (SH3 or SH2) domains of the molecule and correlates with a shift in electrophoretic mobility, from 56 kDa to approximately 60 kDa in SDS-PAGE gels (Veillette *et al.*, 1988b, Veillette *et al*., 1988c, Danielan *et al*., 1989, Marth *et al.*, 1989). It has been suggested that the serine/threonine protein kinase C (PKC) may be responsible for this event. However, treatment of cells with an inhibitor of PKC (H7) did not prevent serine phosphorylation of $p56^{lck}$, therefore implying that PKC may not be the only serine kinase involved in this process (Marth *et al*., 1989) Although the role of serine phosphorylation of $p56^{lck}$ has not been determined, it may modify the ability of the enzyme to take part in protein-protein interactions

Modulatory functions, the role of the SH3 and SH2 domains

A further characterization of the processes regulating the enzymatic activity of $p56^{lck}$ has been obtained by examining the role of the two conserved non-catalytic domains of $p56^{lck}$, namely the SH3 and SH2 sequences. Previous experiments have shown that the SH2 domain can direct two types of protein-protein interactions: first, it appears to target signal transducing molecules such as GAP, PI 3' kinase or PLC-γ 1 to autophosphorylation sites of activated growth factor receptors; second, the SH2 domain of Src-related enzymes appears to bind intra-molecularly to its carboxy-terminal phosphotyrosine residue (for reviews, see Pawson, 1988, Moran *et al*, 1990; Cantley *et al.*, 1991; Koch *et al.*, 1991).

A detailed study of the functions of $p56^{lck}$ SH2 and SH3 regions (Veillette *et al.*, 1992) reveals that the oncogenic potential (evaluated in murine fibroblasts) of $p56^{lck}$ molecules lacking SH2 sequences is markedly enhanced, as is the case for mutant Lck polypeptides carrying a tyrosine to phenylalanine substitution at tyrosine 505. Most importantly, deletion of the SH2 domain produces similar results whether it is performed alone or in combination with the mutation of tyrosine 505. In contrast, when compared with other activating mutant versions of $p56^{lck}$, sole removal of the SH3 region results in a modest enhancement of $p56^{lck}$ oncogenic potential and biochemical functions. Together, these results suggest that down-regulation of $p56^{lck}$ kinase activity through tyrosine 505 phosphorylation is impaired by removal of the SH2 domain but appears operational in the absence of the SH3 domain.

Similar experiments with Src (Seidel-Dugan, 1992) reveal that deletion of parts of the SH2 domain (B or C box) or the SH3 domain in c-Src is sufficient to fully activate wild-type c-Src and produce transforming Src molecules. Although deletion of SH2 or SH3 sequences in the I-527 activated background is not evaluated, this report suggests that the induction of cellular transformation (by a G378 activated variant of c-Src) is not affected by

deletion of either the SH2 or the SH3 domain. In conclusion, both Lck and Src deletional studies provide indirect *in vivo* evidence for a cooperative regulation involving the SH2 domain and the phosphorylated carboxy-terminal tyrosine residue of Src like enzymes.

This model is further supported by studies showing the selective binding of activated (F527) c-Src by immobilized carboxy-terminal phosphopeptides (Roussel *et al*., 1991). More specifically, the cooperative regulation model would involve physical association between the negatively regulating phosphotyrosine residue and the SH2 sequences. An inactive enzyme would have a "closed protein conformation" possibly allowing more stable tyrosine phosphorylation at the carboxy-terminal site, by preventing the action of tyrosine protein phosphatases. Deletion of the SH2 domain would result in an "opened protein conformation" that could promote tyrosine dephosphorylation of the inhibitory phosphotyrosine residue and thus activate the enzyme. This mechanism would explain the absence of phenotypic differences between the effects of SH2 deletion in wild type or F505 polypeptides. Based on this model, activation of Src like kinases through dephosphorylation at the carboxy-terminal tyrosine residue might provoke the destabilization of the inactive protein conformation and, by allowing the SH2 domain to bind to other tyrosine phosphorylated proteins, might promote substrate interactions.

Substrate interaction

As is the case for all SH2 domains studied, the SH2 domain of $p56^{lck}$ can stably associate *in vitro* with phosphotyrosine-containing proteins. Interactions of Lck-SH2 domain with tyrosine phosphorylated substrates can be observed in NIH3T3 cell lines expressing activating mutants of $p56^{lck}$ (Veillette *et al*., 1992). In T cells, the SH2 domain of Lck appears to bind *in vitro* to a specific set of tyrosine phosphorylated proteins (L. Caron, unpublished). From these results, it is proposed that the SH2 domain of $p56^{lck}$ allows interactions with putative Lck substrates *in vivo*.

Recent reports of the three-dimensional structure of the SH2 domain (Booker *et al.*, 1992, Overduin *et al* , 1992, Waksman *et al* , 1992) provide evidence for the covalent nature of the interactions between SH2 domains and phosphotyrosine-containing proteins. Collectively, these results demonstrate the importance of the conserved SH2 subdomains. More specifically, they confirm the critical role of three positively charged, conserved amino acid residues (R155, R175 and K203 in v-Src) in binding to tyrosine phosphorylated peptides. The chemical basis for this interaction is that binding of the SH2 domain and the phosphotyrosine-containing peptide overcomes unfavourable electrostatic interactions arising from the proximity of positively charged amino acid side chains in the unbound protein. Also, these results offer an explanation for the phosphotyrosine specificity of SH2 domains Apparently, the complex between the SH2 domain and the tyrosine phosphorylated protein can be visualized as a "sandwich", the phosphotyrosine side chain interacting with the conserved arginine and lysine residues buried in the SH2 pocket As a result, it is believed that phosphoserine and phosphothreonine, which possess smaller side chains, are unable to interact with the critical residues and thus, are not recognized by SH2 domains

Association with the T-cell surface molecules CD4 and CD8

Interactions of $p56^{lck}$ with the CD4 and CD8α T-cell surface molecules have been documented in murine, human and avian T lymphocytes, and exist in both mature and immature T cells (Rudd *et al* , 1988, Veillette *et al* , 1988a; Veillette *et al.*, 1989c; Veillette and Ratcliffe, 1991). The emergence of CD4 or CD8α-bound $p56^{lck}$ correlates with the appearance of these molecules at the surface of immature thymocytes (Veillette *et al.*, 1989c) The stoichiometry of these complexes has not been rigorously established, but quantitative analyses have shown that in normal T cells, approximately 50-90% and 15-

25% of p56^{lck} molecules are associated with CD4 and CD8α respectively (Veillette *et al* , 1988a; Veillette *et al* ., 1989c; Luo and Sefton, 1990). These proportions may vary in transformed T cells (Luo and Sefton , 1990)

Various studies have shown that the association of p56^{lck} with the CD4 and CD8α glycoproteins involves the interaction of the cytoplasmic tails of CD4 and CD8α with sequences in the unique domain of p56^{lck} (Shaw *et al* , 1989; Zamoyska *et al* , 1989, Shaw *et al*., 1990; Turner *et al*., 1990, Veillette and Ratcliffe, 1990) Minimal sequences sufficient for the formation of these complexes have been defined between residues 2 to 34 of p56^{lck}, residues 394 to 421 of murine CD4 and residues 194 to 203 of murine CD8α (Shaw *et al* , 1989; Shaw *et al*., 1990; Turner *et al* , 1990) More specifically, these interactions require two critical cysteine residues located at positions 20 and 23 in the amino terminal region of p56^{lck} as well as another pair of cysteines located in the CD4 and CD8α cytoplasmic tails. Myristylation of p56^{lck} at glycine 2 is not required for association with CD4 (Shaw *et al* , 1990; A Veillette, unpublished). However, it appears to facilitate this interaction, possibly by locating Lck polypeptides on cellular membranes, thus promoting CD4/CD8α and p56^{lck} colocalization. Since CD4-p56^{lck} and CD8α-p56^{lck} are rapidly dissociated in ionic detergents (such as sodium dodecyl sulfate) or by treatment with alkylating agents (such as N-ethyl maleimide or iodoacetamide) (Shaw *et al* , 1990; A.Veillette, unpublished.), these non-covalent interactions may involve a metal ion coordination complex.

Based on this structural association, it has been proposed that p56^{lck} transduces an intracellular signal for the CD4 and CD8 receptors. Studies evaluating the contribution of CD4-bound tyrosine protein kinase activity to normal T-cell physiology will be discussed in the following sections.

OVERVIEW OF T-LYMPHOCYTE FUNCTION

General structure organization of T cells

Lymphocytes are hematopoietic cells found circulating in the blood and lymph, as well as infiltrating lymphoid organs such as the thymus, the spleen and the lymph nodes. Based on their origin and their immunological function, lymphocytes have been classically divided in two groups, B cells and T cells. The former develop in the bone marrow and regulate humoral responses through antibody production, whereas the latter originate from the thymus and account for cell-mediated immunity

Through the antigen receptor, T cells recognize foreign antigens as processed peptide fragments in association with major histocompatibility complex (MHC) molecules, which are displayed on the surface of antigen presenting cells (APC), (typically B cells and macrophages) This recognition procedure is highly specific as a result of TCR clonality (whereby one TCR clone is specific for a given peptide/MHC combination).

Major events in T-cell activation

Upon binding of the TCR to the proper antigen/MHC combination, a cascade of biochemical events is triggered, leading to T-cell activation. This process is analogous to growth factor receptor stimulation, where ligand binding triggers receptor activation and recruitment of signal amplifiers to produce an associated cellular response (for a review, see Klausner and Samelson, 1991)

Tyrosine phosphorylation

Tyrosine phosphorylation of several cellular proteins occurs rapidly after stimulation of the T-cell antigen receptor complex (Samelson *et al.*, 1986b; Hsi *et al.*,

1989). Because none of the components of the T-cell receptor complex has tyrosine protein kinase activity, the mechanism involved in the generation of this signal is uncommon However, several observations suggest that Src-related tyrosine protein kinases participate in this signal transduction process.

The critical role of TCR-induced tyrosine protein phosphorylation has been documented with the use of tyrosine protein kinase inhibitors (Garcia-Morales *et al* , 1990; June *et al.*, 1990; Stanley *et al* , 1990; Trevillyan *et al* , 1990) These studies have demonstrated that in the absence of tyrosine protein phosphorylation, antigen-induced or antibody-mediated TCR stimulation is impaired. More specifically, phosphatidyl inositol hydrolysis and successive rise in intracellular calcium are prevented, blocking IL 2 secretion as well as IL-2 receptor expression This is not due to T-cell unresponsiveness since T-cell activation can be rescued with the use of calcium ionophores

Another clue to the importance of tyrosine phosphorylation events is provided by observations that TCR/CD3-induced stimulation of T cells is also impaired in the absence of a major T-cell surface tyrosine protein phosphatase (CD45) (Pingel and Thomas, 1989; Koretsky *et al* , 1990, Koretsky *et al* , 1991) One possibility is that CD45 is required for activation of Src-like kinases, by dephosphorylating the negative regulatory carboxy terminal tyrosine residue.

Together, these results indicate that protein tyrosine phosphorylation signals are regulated both by tyrosine kinase and tyrosine phosphatase activities, and are critical for antigen-induced TCR-mediated activation of specific second messenger pathways leading to T-cell activation.

Activation of phospholipase C-γ 1

In addition to generating tyrosine phosphorylation signals, binding of the TCR by antigen or anti-TCR antibodies results in important biochemical changes including the breakdown of phosphatidyl inositols (PI), changes in cytoplasmic calcium levels and

activation of protein kinase C (PKC) (Ledbetter *et al* , 1987). Increasing evidence suggests that these events result from a common receptor-mediated signal transduction mechanism involving the activation of the enzyme phospholipase C–γ 1 (Desai *et al.*, 1990).

Various studies indicate that the functional coupling of T-cell receptor stimulation to the PI signalling pathway requires tyrosine phosphorylation of phospholipase C–γ 1 (Mustelin *et al* , 1990, Nishibe *et al.*, 1990; Park *et al* , 1991; Secrist *et al.*, 1991; Weiss *et al.*, 1991). This modification results in the activation of the enzyme, promoting the hydrolysis of phosphatidyl inositol bisphosphate (PIP_2) to inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a water soluble molecule that has specific intracellular receptors regulating the mobilization of intracellular calcium (Shears, 1991). The initial rise in levels of cytoplasmic calcium observed upon T-cell receptor stimulation results from intracellular calcium store release Maintenance of this intracellular calcium increase comes from a transmembrane flux involving a voltage-gated channel which is also regulated by IP_3 and its metabolites.

DAG is a potent second messenger which functions to regulate a calcium/phospholipid dependant PKC (for a review, see Bell, 1986). PKC represents a large family of closely related serine/threonine kinases that share common structural features as well as requirement for calcium, phospholipids and DAG. DAG regulates PKC activation by increasing its affinity for calcium and phospholipids. PKC activation is associated with its translocation from the cytosol to the membrane

Although the distribution of the various PKC isozymes as well as their substrate specificities are still undefined in T cells, various studies reveal that PKC mediates the regulation of a number of TCR-induced secondary signal transduction responses(Ledbetter *et al* , 1987, Patel *et al* , 1987; Siegel *et al.*, 1990, Izquierdo *et al.*, 1992).

Little is known regarding the subsequent steps of intracellular signal transduction. These cellular responses involve cytoskeletal changes, early gene activation, lymphokine release and expression of new T-cell surface molecules. $p95^{vav}$ (Katzav *et al* , 1989; Bustelo *et al.*, 1992; Margolis *et al* , 1992), a TCR-induced tyrosine phosphorylated substrate containing SH2 and SH3 domains, a DAG/Phorbol Ester binding motif, and having significant similarity to guanine nucleotide dissociation stimulators (GDS) of small ras-like GTPases (Boguski *et al* , 1992) is proposed to play a role in signal transduction of cytoskeletal organization (Adams *et al* , 1992)

Various observations indicate that the SH3 domain may serve as a link between the tyrosine phosphorylation signal and cytoskeletal rearrangements This view is supported by experiments documenting that a protein similar to GAP-rho, binds to the SH3 region of the abl tyrosine kinase (Cicchetti *et al* , 1992) Interestingly, rho, a small ras-like GTPase has been shown to regulate the reorganization of actin into stress fibers in response to growth factors (Ridley and Hall, 1992) The nature and function of these protein interactions remain to be clarified.

Structure of the TCR/CD3 complex

Immunoprecipitation with anti-TCR antibodies following gentle solubilization of T cells has shown that the TCR is a multicomponent polypeptide complex (Samelson *et al* , 1985). The structural organization of this complex involves a disulfide linked heterodimer of the polymorphic α and β chains that is non-covalently associated with a cluster of three distinct invariant proteins γ, δ and ε, which constitute the CD3 complex In addition, the $\alpha\beta$ - CD3 complex is associated with a dimer of small intracellular disulfide-linked ζ chains The ζ family now extends to three related proteins denoted as ζ, η and γ (Orloff *et al* ,

1990).

There has been great interest in determining the structure of the TCR/CD3 complex and the relationship between its components. It has been assumed that each αβ heterodimer is associated with two ε chains (associated with one γ chain and one δ chain) and one ζ homo or heterodimer (Koning *et al* , 1990). This model however fails to take into account other experimental results and the balance of electric charges (Kappes and Tonegawa, 1991). To address these difficulties, alternative models for this multicomponent receptor have been proposed, suggesting a complex stoichiometry which is outside the scope of this overview and will not be further discussed

Signalling role of the TCR/CD3 complex

As mentioned previously, effective recognition of foreign antigen requires that the physical interaction of the TCR be converted into a biochemical signal for proper cellular activation and proliferation Studies using TCR/CD3 variant cell lines (Wegener *et al.*, 1992) have demonstrated the existence of two dissociable functional signal transduction modules inherent to the TCR structure These units represent the CD3 component and the ζ chains respectively Dissociation of the TCR signal has been demonstrated from the ability of each module to trigger late activation events It is not clear if both modules function through specific and/or common signalling circuits However, studies of early T-cell signalling events have suggested the activation of at least two protein phosphorylation pathways, as reflected by tyrosine phosphorylation of the ζ chain and serine phosphorylation of the CD3 γ chain (Samelson *et al* , 1986b; Samelson *et al.*, 1987).

The importance of the ζ chain for the conversion of binding events to intracellular signals is well established This is supported by studies showing that chimeric proteins consisting of T-cell surface receptors (such as the CD8α or the α subunit of the IL-2 receptor) and the intracellular portion of ζ are able to trigger typical T-cell activation responses (Irving *et al* , 1991, Letourneur and Klausner, 1991). The recent observation that

a GTP-binding function may be linked to TCR-ζ suggests additional interesting avenues for studies of antigen receptor signalling (Cenciarelli *et al*., 1992; Ohmura *et al*., 1992, Peter *et al*., 1992).

CD4 and CD8 T-cell surface molecules

CD4 and CD8 are transmembrane molecules with a large extracellular domain (structurally related to immunoglobulins), a typical hydrophobic transmembrane domain and an intracellular domain containing a binding site for the tyrosine protein kinase $p56^{lck}$. CD4 exists as a monomer and is known as the receptor for the human immuno-deficiency virus (HIV) (Silberman *et al*., 1991). In contrast, CD8 naturally exists as a heterodimer of an α and a β chain. The α chain contains the binding site for $p56^{lck}$ (Shaw *et al*., 1990) and is able to function as a homodimer. A variant of the α chain (α') that is essentially devoid of intracellular sequences has also been reported (Zamoyska *et al*., 1989).

CD4 and CD8 expression was initially correlated with distinct T cell populations, where CD4-positive T cells would usually provide helper function and CD8 positive T cells were mostly cytotoxic T lymphocytes (for a review, see Bierer *et al*., 1989). More specifically, CD4 and CD8 designate T-cell surface glycoproteins that respectively bind non polymorphic regions of the MHC class II and class I products. The functions of CD4 and CD8 have been described as adhesion molecules, enhancing the interactions between the T cell and the APC, as signalling molecules, through their association with $p56^{lck}$, and as co receptors for the TCR, whereby CD4 or CD8 bind the same MHC molecule as TCR and potentiates signalling (Parnes *et al*., 1989, Janeway, 1991, Miceli and Parnes, 1991, Miceli *et al*., 1991; Dianzani *et al*., 1992).

Signalling properties of CD4 and CD8

Since CD4 and CD8 are physically associated with $p56^{lck}$, Lck tyrosine protein kinase activity may in part contribute to the antigen-induced changes in tyrosine protein phosphorylation. This model is supported by multiple observations (Barber *et al.*, 1989; Chalupny *et al*., 1991, Veillette *et al*., 1988c). Firstly, stimulation of CD4 receptors induces a rapid activation of $p56^{lck}$ as well as parallel changes in intracellular tyrosine protein phosphorylation (Veillette *et al*., 1989a, Veillette *et al*., 1989b; Veillette *et al.*, 1989c). This activation process may be obtained through antibody-mediated aggregation of CD4 molecules or by MHC class II determinants. In activated T cells, the resulting tyrosine phosphorylated substrates includes $p56^{lck}$ itself, the ζ subunit of the TCR complex and still undefined cellular products of approximately 120 kDa, 70-80 kDa and 36 kDa.

Secondly, CD4 and CD8 molecules that cannot bind $p56^{lck}$ cannot enhance T-cell responsiveness (Zamoyska *et al*., 1989, Veillette *et al*., 1990, Miceli *et al.*, 1991; Haughn *et al*., 1992). Thirdly, expression of constitutively activated Lck (F505) molecules can at least partially substitute for the function of CD4 in an antigen-specific T-cell line (Abraham, N. *et al*., 1991).

Experiments in which the physical association of CD4 and $p56^{lck}$ was reconstituted in fibroblasts also support a functional interaction between CD4 and $p56^{lck}$ (Simpson *et al.*, 1989, Veillette and Fournel, 1990). In a manner analogous to ligand-induced receptor activation, the CD4 $p56^{lck}$ complex can be viewed as a receptor tyrosine protein kinase that becomes activated through dimerization or oligomerization. Activation of $p56^{lck}$ results in prominent *in vivo* tyrosine phosphorylation of the enzyme, which seems to specifically involve the autophosphorylation site, tyrosine 394. As mentionned previously, phosphorylation of tyrosine 394 is thought to be critical for activation of $p56^{lck}$. Consistently, substitution of this amino acid by a phenylalanine abrogates CD4-induced

activation of $p56^{lck}$ (Veillette and Fournel, 1990). In addition to phosphorylation at tyrosine 394, activation of $p56^{lck}$ (in fibroblasts) is accompanied by a variable increase in phosphorylation at tyrosine 505. This observation may be surprising, since tyrosine 505 phosphorylation is involved in the negative regulation of $p56^{lck}$ enzymatic activity. However, it may represent a feed back mechanism aimed at rapid down-regulation of the function of activated $p56^{lck}$ molecules.

In T cells, antibody-mediated aggregation of CD8 also induces changes in tyrosine protein phosphorylation, though perhaps less markedly (Veillette *et al* , 1989c, L. Caron and A. Veillette, unpublished). In fibroblasts, results from reconstitution experiments involving $p56^{lck}$ and CD8 α demonstrate that in contrast to CD4, antibody mediated aggregation of CD8α does not activate $p56^{lck}$ tyrosine protein kinase activity (L. Caron, unpublished). The functional differences observed between the CD4-$p56^{lck}$ and the CD8α-$p56^{lck}$ complexes both in T cells and in experimental systems such as NIH3T3 cells may be explained by the known structural differences between CD4 and CD8, or by the different extents at which the cellular Lck protein is associated with these receptors

<u>The co-receptor function of CD4 in TCR signalling</u>

The mutual binding of CD4 and TCR to MHC class II determinants upon antigen presentation results in the colocalization of two receptor complexes (involving TCR/CD3 $p59^{fynT}$ and CD4-$p56^{lck}$), thus providing a positive signalling response. This positive function can also be obtained by antibody-mediated co-aggregation of CD4 and TCR/CD3, and is exemplified by enhanced tyrosine protein phosphorylation, increased intracellular calcium levels and DNA synthesis. In contrast, pretreatment of T cells with aggregating CD4 antibodies can abrogate antigen or anti-TCR antibody-induced T-cell activation (for a review, see Julius *et al* , 1991)

To reconcile these contradictory results, it has been proposed that CD4 provides

prerequisite signals which are critical for antigen receptor-induced T-cell proliferation. Hence, the molecular basis for the generation of an apparent negative signal upon TCR stimulation following CD4 antibody-mediated aggregation may be the sequestration of CD4-p56^{lck} complexes from TCR/CD3 complexes. Sequestration would be induced by pretreatment with anti-CD4 antibodies, abrogating the co-receptor function of CD4. However, as the TCR can function in the absence of CD4 or CD8, it is more likely that p56^{lck} itself is responsible for these signalling functions. This hypothesis has been supported by experiments using double cysteine mutant CD4 molecules that are incapable of interacting with p56^{lck} (Haughn *et al.*, 1992). In these experiments, expression of the mutant CD4 failed to confer enhanced T-cell responsiveness. This argues that the capacity of CD4 to enhance antigen receptor -mediated T-cell functions is in large part related to its association with p56^{lck}

At this point, it appears relevant to examine possible effectors of p56^{lck} signalling function Thus, this section ends with a rapid overview of major T-cell surface molecules that have been suggested to interact with p56^{lck}.

CD45: a T-cell phosphatase

CD45, also designated as the leucocyte common antigen, is a family of high molecular weight glycoproteins expressed on the surface of all nucleated cells of hematopoietic origin In lymphocytes, CD45 constitutes approximately 10% of the cell surface and possesses various protein isoforms ranging from 180 kDa to 240 kDa. These arise by alternative splicing of three exons encoding the amino-terminal portion of the extracellular domain (for a review, see Thomas, 1989).

Interestingly, CD45 has a large highly conserved cytoplasmic domain of approximately 700 amino acids, that encloses two related subdomains with tyrosine protein phosphatase activity.

Regulation of T-cell activation by CD45

The importance of CD45 in T-cell physiology has been mainly elucidated from studies involving T lymphocytes lacking CD45 expression (Pingel and Thomas, 1989; Koretsky *et al.*, 1990; Koretsky *et al.*, 1991, Weaver *et al* , 1991) These studies conclude that in the absence of CD45, TCR-mediated signal transduction is impaired T-cell proliferation could nevertheless be achieved using calcium ionophores suggesting that the site of action of CD45 is localized upstream of this second messenger pathway Apparently, CD45 expression would be essential for effective coupling of the antigen-induced signal to the phosphatidyl inositol (PI) pathway (Koretsky *et al* , 1991, Marvel *et al* , 1991) Other experiments suggest that CD45 loss results in constitutive tyrosine phosphorylation of several proteins, including the TCR ζ chain (Volarevic *et al* , 1992) In addition, TCR-induced stimulation of CD45-negative T cells reveals the lack of phosphatidyl inositol hydrolysis, a loss in the early rise of intracellular calcium and the appearance of a novel pattern of late intracellular calcium oscillation The exact mechanism by which CD45 regulates these major signalling pathways remains to be elucidated

Interestingly, coaggregation of CD4 and TCR/CD3 leads to activation of T cells despite the absence of CD45 (Deans *et al* , 1992). As TCR-mediated T-cell activation is impaired in the absence of CD45, these observations suggest that activation of $p56^{lck}$ induced by coaggregation of TCR/CD3 and CD4 may compensate for the defect in TCR/CD3 signalling. Since the activity of tyrosine protein kinases such as $p56^{lck}$ can be regulated by tyrosine dephosphorylation, CD45 may be a direct regulator of $p56^{lck}$ activity This view is supported in part by the observation that the *in vivo* state of tyrosine 505

phosphorylation of $p56^{lck}$ is noticeably increased in cells lacking CD45 (Ostergaard *et al.*, 1989). Alternatively, the recent identification of a trimolecular complex involving CD45, $p56^{lck}$ and pp32 (a putative tyrosine phosphorylated substrate) in resting T lymphocytes (Schraven *et al* , 1991) suggests that regulation of $p56^{lck}$ by CD45 might involve a protein intermediate.

Other T-cell signalling molecules

IL-2 receptor

Interleukin-2 or T-lymphocyte growth factor is produced by a population of activated T cells Stimulation of IL-2 dependent T cells with interleukin-2 results in the activation of tyrosine phosphorylation signals culminating in cellular proliferation (Mills *et al.*, 1990). As the IL-2 receptor is not itself a tyrosine protein kinase, the functional coupling of a non-receptor tyrosine protein kinase with the IL-2 receptor has been proposed Recent reports suggesting that $p56^{lck}$ interacts with the IL-2 receptor and is modified following IL-2 treatment (Hatekayama *et al.*, 1990; Horak *et al.*, 1990), raise the possibility that $p56^{lck}$ can participate in IL-2 receptor-induced signal transduction.

CD2 and Thy-1

CD2 and Thy-1 are also part of a growing number of cell surface molecules that are able to trigger signal transduction that leads to T-cell activation (He *et al.*, 1988; Volarevic *et al.*, 1990) Interestingly, there have been reports of association of Thy-1 with CD45 (Volarevic *et al* , 1990), $p56^{lck}$ (Stefanova *et al.*, 1991) and $p59^{fyn}$ (Thomas and Samelson, 1992) On the other hand, some reports show that activation of T cells through CD2 results in $p56^{lck}$ serine hyperphosphorylation and activation (Danielan *et al.*, 1989; Danielan *et al.*, 1991). The nature and significance of these interactions are presently unknown.

REGULATION OF T-CELL ACTIVATION BY MEMBERS OF THE SRC-FAMILY OF TYROSINE PROTEIN KINASES

Studies of the molecular mechanisms of signal transduction in T cells reveal that the major biochemical events following T-cell activation are similar to the signalling processes of activated tyrosine kinase growth factor receptors. Upon stimulation of receptor tyrosine protein kinases, a wide variety of proteins become phosphorylated on tyrosine residues. It has been suggested that this critical event induces the pleiotropic effects required for cellular activation (Klausner and Samelson, 1991) To better understand how tyrosine phosphorylation is linked to downstream biochemical signals, various studies have focused on the identification of the physiological substrates of tyrosine protein kinases Recent progress has been made in establishing the causal relationship between receptor activation and changes in cytoplasmic activities Several groups have demonstrated that upon ligand binding, the platelet-derived growth factor (PDGF, a model receptor tyrosine protein kinase) shows tyrosine autophosphorylation and becomes physically associated with a number of cytoplasmic proteins. The ability of activated growth factor receptors to phosphorylate these signalling proteins on tyrosine residues provides strong evidence that tyrosine protein phosphorylation is required to induce the pleiotropic effects leading to cell growth.

Interestingly, the identified cellular substrates of Src-family tyrosine protein kinases are mostly similar to those targeted by receptor tyrosine kinases (Fukui and Hanafusa, 1989; Ellis *et al* , 1991). As low level of immediate *in vivo* tyrosine phosphorylation produces rapid pleiotropic effects, it is proposed that the critical cellular targets of protein tyrosine kinases are signal amplifiers It is noteworthy that many signal amplifiers possess SH2 and SH3 domains, and appear to be regulated at least in part by tyrosine phosphorylation. This observation is important in the light of recent findings suggesting

that tyrosine phosphorylation creates binding sites for recruitment of signal amplifiers through SH2-mediated protein-protein recognition.

Activated versions of two Src-related tyrosine protein kinases abundantly expressed in T cells ($p56^{lck}$ and $p59^{fynT}$) can enhance antigen receptor-mediated functions (for a review, see Veillette and Davidson, 1992). As non-receptor tyrosine protein kinases are also found associated with several signal amplifiers, T-cell antigen receptor signal transduction may be taking place as a result of similar key recognition signals. The contribution of $p56^{lck}$ and $p59^{fyn}$ to the generation of these signals is now being discussed.

Studies involving $p59^{fyn}$

$p59^{fyn}$ is a Src-related tyrosine protein kinase existing as two isoforms, due to alternative use of exons 7A and 7B. Interestingly, the Fyn isoforms have a different pattern of tissue expression $p59^{fynB}$ is highly expressed in the brain whereas $p59^{fynT}$ is found predominantly in T cells (Cooke and Perlmutter, 1989).

The view that $p59^{fynT}$ may play a specialized role in T-cell physiology has been supported by studies reporting the association of $p59^{fynT}$ with the TCR/CD3 complex in digitonin lysates of T cells (Samelson *et al* , 1991; Gassmann *et al.*, 1992). However, the lack of evidence for the activation of $p59^{fynT}$ tyrosine protein kinase activity upon TCR stimulation raises questions about the functional significance of this association. Further studies of the role of Fyn is documented by the overexpression of both Fyn isoforms in transgenic mice (Cooke *et al* , 1991). Overexpression of either form of the Fyn protein in T cells (under the control of the proximal Lck promoter) enhances TCR-induced tyrosine protein phosphorylation, calcium response, and IL-2 production. No significant difference between overexpression of $p59^{fynT}$ and $p59^{fynB}$ is noted. Interestingly, overexpression of

Fyn has also been reported in the T cells of mice with a lymphoproliferative disorder (Katagiri *et al.*, 1989), which appear to demonstrate abnormal tyrosine phosphorylation of their T-cell receptors (Samelson *et al* , 1986a).

The introduction of constitutively activated versions of $p59^{fynT}$ or $p59^{fynB}$ (tyrosine 528 to phenylalanine) into a T-cell hybridoma (Davidson *et al* , 1992) shows that both 528 mutants of $p59^{fynB}$ and $p59^{fynT}$ efficiently enhance T-cell responses upon antibody-mediated aggregation of TCR Interestingly, only $p59^{fynT}$ efficiently increases T-cell responses to antigen stimulation, supporting the view that $p59^{fynT}$ have a lymphocyte-specific function As $p59^{fynT}$ and $p59^{fynB}$ only differ within a stretch of 51 amino acids (encompassing the end of the SH2 domain and the beginning of the catalytic domain), the observed difference may relate to substrate specificity and/or catalytic efficiency. It has been proposed that through its unique sequence, $p59^{fynT}$ may regulate specific cellular pathways, which would modulate T-cell responsiveness to antigen This is supported by a study demonstrating that the sequences involved in the binding of $p59^{fynT}$ to TCR-ζ are located within the amino-terminal region of $p59^{fynT}$ (A. Shaw, unpublished)

Studies involving $p56^{lck}$

The involvement of Src-like kinases in TCR-mediated signalling is further supported by studies addressing the role of $p56^{lck}$ in T cell development and activation Mice lacking $p56^{lck}$ show a considerable thymic atrophy which is associated with a major decrease in the double positive (CD4 and CD8) cell population (Molina *et al* , 1992) In addition, peripheral T cells (lacking Lck) exhibit a reduced proliferative response to TCR/CD3 stimulation, despite normal responses to Il-2, phorbol esters and calcium ionophores. These observations suggest that $p56^{lck}$ is not essential for TCR/CD3 or IL-2-

mediated signalling, but may participate as a signal amplifier. Importantly, the profound perturbation of thymocyte development that is observed in Lck-deficient mice raises the possibility that $p56^{lck}$ is required for appropriate proliferative response of double positive thymocytes (Molina *et al* , 1992)

Further investigation of the role of $p56^{lck}$ in lymphocyte function has been performed with the generation of transgenic mice expressing $p56^{lck}$ (Abraham, K.M. *et al.*, 1991a; Abraham, K M. *et al* , 1991b) These experiments show that a moderate increase in $p56^{lck}$ expression induces a delay in thymocyte development In this system, both wild type and activated (F505) versions of the gene produce similar effects, albeit with differing efficiencies In addition, overexpression of either wild type or activated versions of the *lck* gene induces the development of thymic tumors Collectively, these observations suggest that thymocyte maturation is affected by Lck-mediated signalling, and that overexpression of this kinase may contribute to thymic tumorigenesis.

The role of $p56^{lck}$ in T-cell activation has been examined by the introduction of a constitutively activated form of the lymphocyte-specific tyrosine protein kinase in a CD4 - negative, T-cell hybridoma (Abraham, N *et al.*, 1991) This study demonstrates that expression of activated $p56^{lck}$ polypeptides (generated by the mutation of the carboxy-terminal tyrosine residue 505 to a phenylalanine) enhances T-cell responsiveness as indicated by increased TCR-induced tyrosine protein phosphorylation and elevated antigen-induced IL-2 production These observations suggest that activation of $p56^{lck}$ molecules can directly (in the absence of CD4 or CD8 expression) enhance antigen receptor-mediated signals

SUMMARY

The Src-family of tyrosine protein kinases comprises structurally related enzymes, which have been proposed to perform specialised functions as a result of their respective unique amino-terminal domains. $p56^{lck}$ is a lymphocyte-specific member of the Src-family of tyrosine protein kinases that is predominantly associated through its unique amino-terminal region to the CD4 and CD8 T-cell surface glycoproteins

Recent studies have conferred a central role for tyrosine protein kinase activity in early events of TCR-mediated signal transduction Indeed, tyrosine phosphorylation of a limited set of substrates is essential for TCR-induced T-cell activation, although the TCR itself is devoid of tyrosine protein kinase activity. Since both $p56^{lck}$ and $p59^{fyn(T}$ are able to enhance antigen receptor-mediated T-cell responsiveness, it is a possibility that these cytoplasmic kinases interact either directly or through a cellular intermediate with the TCR complex.

Structure-function analyses of $p56^{lck}$, have revealed important aspects of $p56^{lck}$ regulation. Given that the SH2 domain of $p56^{lck}$ may interact in *cis* with its own carboxy-terminal regulatory domain to modify catalytic activity or substrate specificity, and that positive signalling by $p56^{lck}$ may involve cellular amplifiers, further studies of the effects of non-catalytic portions of $p56^{lck}$ are required to address the role of this kinase in T-cell activation.

The studies reported in the next chapter investigate the involvment of $p56^{lck}$ in signal transduction events following T-cell receptor-mediated activation of T lymphocytes The experimental work to be presented examines the effects of important structural domains of $p56^{lck}$ on the ability of the activated kinase to enhance T-cell responsiveness.

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Chapter 2

Structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase $p56^{lck}$

ABSTRACT

To understand the mechanism(s) by which $p56^{lck}$ participates in T-cell receptor (TCR) signalling, we have examined the effects of mutations in known regulatory domains of $p56^{lck}$ on the ability of F505 $p56^{lck}$ to enhance the responsiveness of an antigen specific murine T-cell hybridoma. A mutation of the amino-terminal site of myristylation (glycine 2), which prevents stable association of $p56^{lck}$ with the plasma membrane, completely abolished the ability of F505 $p56^{lck}$ to enhance TCR-induced tyrosine protein phosphorylation. Alteration of the major site of *in vitro* autophosphorylation, tyrosine 394, to phenylalanine diminished the enhancement of TCR-induced tyrosine protein phosphorylation by F505 $p56^{lck}$. Such finding is consistent with the previous demonstration that this site is required for full activation of $p56^{lck}$ by mutation of tyrosine 505. Strikingly, deletion of the non-catalytic Src Homology (SH) domain 2, but not the SH3 domain, markedly reduced the improvement of TCR-induced tyrosine protein phosphorylation by F505 Lck. Additional studies revealed that all the mutations tested, including deletion of the SH3 region, abrogated the enhancement of antigen-triggered IL-2 production by F505 $p56^{lck}$, thus implying more stringent requirements for augmentation of antigen responsiveness by F505 Lck. Finally, it was also observed that expression of F505 $p56^{lck}$ greatly increased TCR-induced tyrosine phosphorylation of phospholipase C (PLC)-γ 1, raising the possibility that PLC-γ 1 may be a substrate for $p56^{lck}$ in T-lymphocytes. Our results indicate that $p56^{lck}$ regulates T-cell antigen receptor signalling through a complex process requiring multiple distinct structural domains of the protein.

INTRODUCTION

Evidence is increasing that tyrosine protein phosphorylation plays a critical role in T-lymphocyte activation (for reviews, see references 20 and 49). Indeed, tyrosine phosphorylation of a limited number of partially characterized substrates occurs rapidly after stimulation of the T-cell receptor (TCR) for antigen by either antigen/major histocompatibility complex (MHC) or anti-TCR antibodies (17). This biochemical signal is critical for T-lymphocyte activation, as supported by the finding that tyrosine-specific protein kinase inhibitors (such as genistein or herbimycin A) prevent TCR-induced phosphatidyl inositol (PI) turnover, rise in intracellular calcium and lymphokine release (18,30,40,43). As the TCR complex does not possess an intrinsic tyrosine protein kinase activity, it has been postulated that this process is mediated through the recruitment of membrane-associated tyrosine protein kinases.

The Src family of internal membrane tyrosine protein kinases comprises eight well characterized members named c-Src, c-Fgr, c-Yes, Lck, Fyn, Hck, Lyn and Blk (for a review, see reference 14). The members of this family have highly conserved structural features including (from the amino-terminus to the carboxy-terminus): 1) an amino-terminal glycine residue (glycine 2), which is required for myristylation and membrane association; 2) a unique domain, which differs in sequence for each member of the family, therefore potentially providing distinguishing properties to these various products; 3) the Src Homology 3 (SH3) domain, a motif also found in a number of cytoskeletal constituants such as fodrin and the yeast actin binding protein, and presumed to mediate interactions with the cytoskeleton (for a review, see references 8 and 21); 4) the Src Homology 2 (SH2) region, which is additionally present in several signal transducers and amplifiers such as the γ isoform of phospholipase C (PLC-γ) and the GTPase-activating protein (GAP) of $p21^{ras}$, and which has been proposed to modulate enzyme-substrate interactions

through its ability to bind phosphotyrosine-containing proteins (for a review, see references 8 and 21); 5) a prototypical catalytic domain, containing sites of ATP binding and *in vitro* autophosphorylation and 6) the short carboxy-terminal regulatory domain, which encompasses a conserved negative regulatory site of *in vivo* tyrosine phosphorylation

The demonstration that the lymphocyte-specific *lck* gene product (25,53), $p56^{lck}$, is associated with and regulated by the CD4 and CD8 T-cell surface antigens provided the first strong evidence for the involvement of Src-related tyrosine protein kinases in normal T-cell physiology (23,34,45-47; for reviews, see references 37 and 44) It has since become progressively clear that the ability of CD4 and CD8 to enhance T-cell responsiveness to low doses of antigen or sub-optimal antigen stimulation is largely mediated through interactions with $p56^{lck}$ (26). Lck may also be involved in other T-cell signalling pathways, as suggested by descriptions of physical complexes between this polypeptide and other T-cell surface molecules like the β chain of the IL-2 receptor (16) and glycophosphatidylinositol (GPI)-anchored molecules such as Thy-1 (41)

Direct indication of the role of $p56^{lck}$ in T-cell physiology was lent by experiments in which a constitutively activated version of this tyrosine protein kinase [carrying a mutation of the negative regulatory carboxy-terminal site of tyrosine phosphorylation, tyrosine 505, to a non-phosphorylatable phenylalanine (F505 $p56^{lck}$)] was introduced in a CD4-negative and CD8-negative, class II MHC-restricted, antigen-specific murine T-cell hybridoma (BI-141) (1). These studies showed that expression of F505 $p56^{lck}$, but not wild-type or kinase-deficient Lck polypeptides, significantly enhanced T-cell responsiveness, as indicated by increased TCR-induced tyrosine protein phosphorylation and interleukin-2 (IL-2) production (1) In addition to implying a biochemical mechanism by which CD4 and CD8 (and possibly other T-cell surface molecules) improve T cell function, these observations also indicated that activated $p56^{lck}$ can participate in antigen

receptor signalling in the absence of CD4 and CD8 expression.

As $p56^{lck}$ is not stably associated with the TCR complex (34,46), the mechanism by which this enzyme contributes to TCR signalling is not implicitely evident. To better understand this process, we have evaluated the role of known structural domains of $p56^{lck}$ in the enhancement of TCR signalling by an activated version of this polypeptide (F505 $p56^{lck}$). Specifically, we have tested the roles of the sites of myristylation (glycine 2) and autophosphorylation (tyrosine 394), as well as of the non-catalytic SH3 and SH2 domains of $p56^{lck}$. The results of our experiments imply that $p56^{lck}$ enhances TCR signalling through a complex process which requires multiple independent structural domains of the protein

MATERIALS AND METHODS

Cells. BI-141 is a CD4-negative and CD8-negative, class II MHC-restricted T-cell hybridoma specific for beef insulin (33) The BI-141 cell line was grown in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), streptomycin and penicillin. ψ-2 retrovirus packaging cells transfected with cDNAs encoding A2I-505 Lck, F394F505 Lck, ΔSH2F505 Lck and ΔSH3F505 Lck were described elsewhere (2,48) Briefly, the deletions of the SH3 or SH2 motif removed amino-acids 67-122 and 122-234 of $p56^{lck}$, respectively (48). The process of these deletions did not introduce any additional alteration in the Lck amino-acid sequence (48). The ψ-2 derivatives were maintained in α minimal essential medium (αMEM) containing 10% heat-inactivated FCS, penicillin, streptomycin, and the aminoglycoside G418 (500 μg/ml). BI-141 derivatives expressing the neomycin phosphotransferase gene (*neo*) alone or F505 $p56^{lck}$ have been described previously (1). The $A_\alpha{}^bA_\beta{}^k$ class II MHC-expressing L-cells (FT5 7) were kindly provided by Dr Ron Germain, NIH, Bethesda, MD The IL-2-dependent HT-2 T-cell clone was maintained in culture as described (29)

Retroviral infection. Retroviral infection of BI-141 cells was performed as outlined (4), using retroviral supernatants obtained from established ψ-2 packaging cell lines BI-141 derivatives expressing the various mutant Lck proteins were generated by selection in medium containing 750 μg/ml G418 Monoclonal cell lines were established by limiting dilution. All cell lines selected for our studies displayed levels of TCR CD3, Thy 1.2 and CD45, as well as growth characteristics (morphology and growth rate) that were identical to those of parent BI-141 cells Moreover, all cells remained CD4-negative (data not shown).

Antibody-mediated aggregation of the T-cell receptor (TCR) for antigen. Antibody-mediated aggregation of TCR was performed as outlined previously (1) Briefly,

$5X10^6$ BI-141 T-cells were incubated for 30 minutes on ice in serum-free RPMI-1640 medium containing saturating amounts (12 μg/ml) of the anti-TCR $V_\beta 8$ mouse monoclonal antibody (MAb) F23.1 (38). After washing the excess free antibody, aggregation was performed by addition of excess amounts (60 μg/ml) of a second-step antibody [rabbit anti-mouse (RAM) or sheep anti-mouse (SAM) IgG; Organon-Teknika] and incubation at 37°C for the indicated periods of time. Controls were with the addition of the second-step antibody alone. Cells were then immediately lysed in boiling sample buffer or NP-40 containing buffer. Lysates were processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or immunoprecipitation (see below).

Immunoblots. Immunoblots were performed as explained elsewhere (19,46,51). For Lck immunoblotting, we used antisera generated either against a synthetic peptide corresponding to amino-acids 29-54 of $p56^{lck}$ (51), or against a fusion protein containing amino-acids 2-148 of this polypeptide (1). Anti-phosphotyrosine immunoblotting was conducted using affinity-purified polyclonal rabbit anti-phosphotyrosine antibodies (our unpublished data). Anti-PLC-γ 1 immunoblots were performed using a previously described rabbit anti-PLC-γ 1 serum (42). This antibody does not recognize PLC-γ 2. Detection of immunoreactive products was accomplished using ^{125}I-Protein A (Amersham) and subsequent autoradiography. For quantitation, bands were cut from the nitrocellulose membranes and counted in a γ counter. The presence of equivalent amounts of cellular proteins in each lane was confirmed by amido-black staining of nitrocellulose filters (data not shown).

PLC-γ 1 immunoprecipitation. Cells were stimulated for the indicated periods of time with anti-TCR MAb F23.1 and sheep anti-mouse (SAM) IgG (Organon-Teknika), as described above. Cells were lysed in 2X TNE buffer (IX TNE = 50 mM Tris pH8.0, 1% NP-40, 2 mM EDTA pH8.0) supplemented with 20 μg each of the protease inhibitors

leupeptin, aprotinin, N-tosyl-L-phenylalanine chloromethyl ketone, N-p-tosyl-L-lysine chloromethyl ketone and phenylmethylsulfonyl fluoride per ml, as well as the phosphatase inhibitors sodium fluoride (100 mM) and sodium orthovanadate (2 mM). Post-nuclear supernatants were first precleared with *Staphylococcus aureus* protein A (Pansorbin, Calbiochem) and then immunoprecipated with the polyclonal rabbit anti-PLC-γ1 serum mentioned above (42). Immune-complexes were collected with *S. aureus* protein A and washed three times with 1X TNE buffer containing 1 mM sodium orthovanadate. Proteins were eluted in sample buffer, boiled and electrophoresed in 8% SDS-PAGE gels. After transfer to nitrocellulose membranes, immunoprecipitates were probed by immunoblotting with either anti-phosphotyrosine or anti-PLC-γ1 antibodies.

Antigen stimulation assays. BI-141 T-cells ($1X10^5$ cells) were plated in triplicate in 96-well Falcon tissue culture plates with $5X10^4$ irradiated (4500 rads) $A_\alpha^b A_\beta^k$ class II MHC-expressing L cells and serial dilutions of the antigen beef insulin (Sigma). After incubation at 37°C for 24 hours, supernatants were collected and frozen for one hour at -70°C to destroy carry-over cells. IL-2 production was measured by testing the ability of these supernatants to stimulate ^{3}H-thymidine incorporation in $1X10^4$ IL-2 dependent HT-2 indicator cells. This assay has been described in more details elsewhere (1,27).

RESULTS

The site of Lck myristylation, glycine 2, is absolutely required for enhancement of TCR signalling by F505 p56^{lck}

We have previously reported that expression of a constitutively activated version of p56^{lck} [tyrosine 505 to phenylalanine 505 Lck mutant (F505 p56^{lck})] in the CD4-negative and CD8-negative, class II MHC-restricted and beef insulin-specific murine T-cell hybridoma BI-141 results in enhanced T-cell responsiveness (1). This functional improvement was indicated by augmented TCR-induced tyrosine protein phosphorylation and increased antigen/MHC-triggered IL-2 production. Preliminary studies showed that F505 p56^{lck} is neither associated with the TCR complex nor enzymatically activated or tyrosine phosphorylated upon TCR stimulation with anti-TCR antibodies (3). Thus, the process by which p56^{lck} is recruited in the TCR signalling pathway appeared fundamentally different from that by which it is involved in CD4- and CD8-induced signal transduction events.

To begin evaluating the mechanism by which the activated version of p56^{lck} (F505 p56^{lck}) enhanced TCR signalling, the effects of a point mutation (glycine 2 to alanine 2) abolishing F505 Lck myristylation and membrane association (2) were tested. BI-141 cells were infected with retroviruses encoding A2F505 p56^{lck} and selected for growth in the presence of the aminoglycoside G418. Clonal cell lines were established by limiting dilution and tested for expression of the mutant Lck polypeptide by a specific anti-Lck immunoblot assay. Multiple independent BI-141 derivatives containing amounts of A2F505 p56^{lck} (Figure 1, lanes 3-6) generally similar (within two-fold) to those of F505 p56^{lck} (lanes 11-15) were selected for further analyses. Quantitative studies showed that both types of cell lines expressed 5- to 12-fold higher levels of p56^{lck} than Neo cells (lanes

1 and 2). As formerly described (2), A2F505 p56^{lck} polypeptides had a slightly retarded electrophoretic mobility in SDS-PAGE gels. Although the basis for this observation remains unclear, we have previously ruled out that it results from additional mutations in the A2F505 *lck* cDNA (2).

Cells were stimulated with anti-TCR MAb F23.1 and RAM IgG for 2 minutes and intracellular tyrosine protein phosphorylation assessed by anti-phosphotyrosine immunoblotting (Figure 2). This assay revealed that, unlike cells expressing F505 p56^{lck} polypeptides (Figure 2, lanes 13-16), cells containing A2F505 p56^{lck} (lanes 5-12) failed to show an enhancement of anti-TCR antibody-induced tyrosine protein phosphorylation over Neo cells (lanes 1-4). Similar observations were made when TCR stimulation was extended up to 30 minutes (data not shown). These findings implied that myristylation and/or membrane association is absolutely required for F505 p56^{lck} to contribute to TCR-induced tyrosine phosphorylation signalling events.

Figure 1. Expression of A2F505 Lck and F394F505 Lck polypeptides in BI-141 T-cells.

Lck immunoblot. Levels of $p56^{lck}$ in several independent BI-141 cell lines expressing a myristylation-defective (A2F505 Lck) or an autophosphorylation site (F394F505 Lck) F505 $p56^{lck}$ mutant were measured by Lck immunoblot using a specific Lck antiserum (1). Lanes 1: Neo 1; 2: Neo.2, 3. A2F505 2, 4: A2F505.3, 5: A2F505.4; 6: A2F505 6, 7 F394F505.8; 8: F394F505.11; 9. F394F505.12; 10: F394F505.22; 11: F505 3; 12 F505 7, 13 F505 9; 14: F505.12 and 15: F505.13. The position of $p56^{lck}$ is indicated on the left Exposure 12 hours

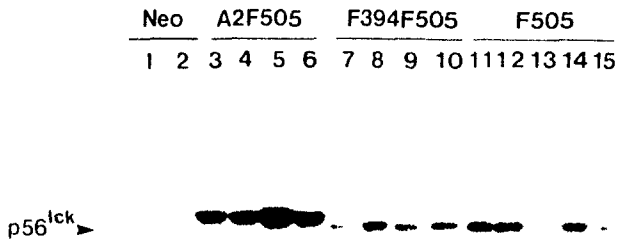
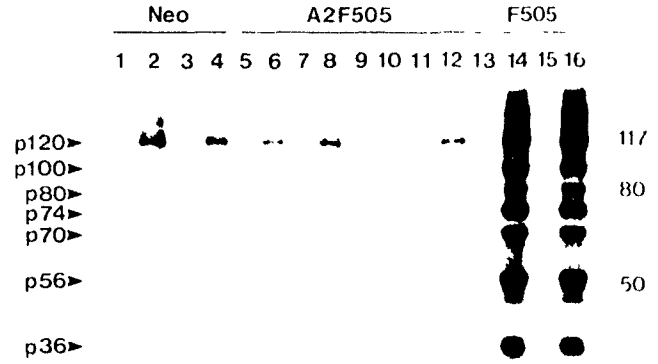


Figure 2. Effects of expression of A2F505 p56^{lck} polypeptides on TCR-induced tyrosine protein phosphorylation.

The abundance of phosphotyrosine-containing proteins in BI-141 cells expressing the neomycin resistance marker alone (Neo, lanes 1-4), A2F505 p56^{lck} (A2F505, lanes 5-12) or F505 p56^{lck} (F505; lanes 13-16) was measured by **anti-phosphotyrosine immunoblotting**. Antibody-mediated aggregation of TCR was performed for 2 minutes at 37°C. Lanes 1,3,5,7,9,11,13 and 15: RAM IgG alone and 2,4,6,8,10,12,14 and 16 MAb F23.1 + RAM IgG. Lanes 1 and 2: Neo 1, 3 and 4: Neo 2, 5 and 6: A2F505 1, 7 and 8: A2F505 2, 9 and 10: A2F505 4, 11 and 12: A2F505 6, 13 and 14: F505 7 and 15 and 16: F505 9. The positions of the major phosphotyrosine containing proteins (on the left) and of prestained molecular mass markers (on the right) are marked. Exposure: 8 hours.



Mutation of the site of Lck autophosphorylation, tyrosine 394, partially reduces the enhancement of TCR signalling by F505 p56^{lck}

Accumulating data suggest that tyrosine phosphorylation by activated tyrosine protein kinase receptors (such as the platelet-derived growth factor receptor) is dependent on binding of phosphorylated tyrosine residues on the receptor to SH2 sequences typically encountered in intracellular substrates (reviewed in 8,21). Tyrosine 394 is the major site of autophosphorylation of p56^{lck} (2,5,10,24,51,52). While not normally phosphorylated *in vivo*, significant *in vivo* occupancy of tyrosine 394 is noted in the context of activation of p56^{lck} by mutation of tyrosine 505 (2,5,24) or antibody-mediated aggregation of CD4 (23,45). In both cases, activation of p56^{lck} is significantly reduced by mutation of tyrosine 394 to phenylalanine (2,50).

Thus, we wished to test the possibility that tyrosine 394 is pivotal for the enhancement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}. F505 Lck polypeptides carrying a tyrosine to phenylalanine substitution at position 394 (F394F505 p56^{lck}) were introduced in BI-141 cells, as described above. Cell lines expressing amounts of Lck protein (Figure 1, lanes 7-10) similar to those noted in cells expressing F505 p56^{lck} (lanes 11-15) were identified by Lck immunoblot, and subjected to antibody-mediated TCR stimulation.

An anti-phosphotyrosine immunoblot of lysates from control or TCR-stimulated cells (Figure 3A) revealed that, in comparison with Neo cells (lanes 1-4), cells containing F394F505 p56^{lck} (lanes 5-10) showed enhanced TCR-induced tyrosine protein phosphorylation (measured after 2 minutes of stimulation). While similar sets of cellular substrates were affected by expression of F394F505 p56^{lck} and F505 p56^{lck}, the enhancement by F394F505 p56^{lck} was less than that conferred by F505 p56^{lck} (lanes 11-14). Time-course experiments using representative cell lines supported these assertions

(Figures 3B and 3C), albeit these quantitative differences tended to diminish after 5 minutes of stimulation. Taken together, these results are consistent with the previous demonstration that the catalytic activity of F394F505 $p56^{lck}$ is lower than that of F505 Lck (2). They also imply that tyrosine 394 is not absolutely required for activated $p56^{lck}$ molecules to participate in TCR-induced signalling events.

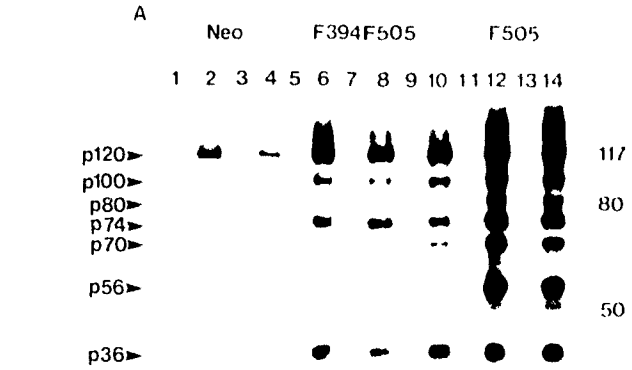
Figure 3. Effects of expression of F394F505 p56^{lck} polypeptides on TCR-induced tyrosine protein phosphorylation.

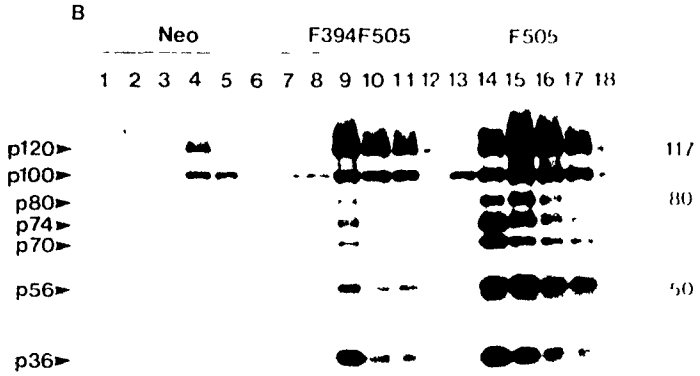
A. Anti-phosphotyrosine immunoblot. Cells expressing the neomycin resistance marker alone (Neo, lanes 1-4), F394F505 p56^{lck} (F394F505; lanes 5-10) or F505 p56^{lck} (F505, lanes 11-14) were tested for TCR-induced tyrosine protein phosphorylation. Antibody-mediated aggregation of TCR was performed for 2 minutes at 37°C. Lanes 1,3,5,7,9,11 and 13 RAM IgG alone and 2,4,6,8,10,12 and 14. MAb F23.1 + RAM IgG. Lanes 1 and 2 Neo 1, 3 and 4 Neo 2, 5 and 6: F394F505.11; 7 and 8 F394F505.12; 9 and 10 F394F505 22, 11 and 12 F505.7 and 13 and 14: F505.9 The positions of major phosphotyrosine containing proteins (on the left) and of prestained molecular mass markers (on the right) are indicated Exposure 8 hours

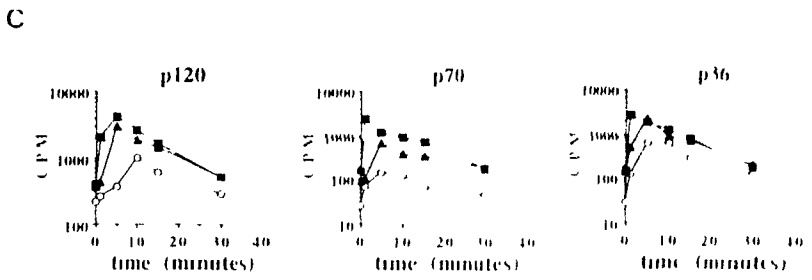
B. Time-course experiment. Monoclonal cell lines expressing the neomycin resistance marker alone (Neo 2, lanes 1-6), F394F505 p56^{lck} (F394F505 22, lanes 7-12) or F505 p56^{lck} (F505 7, lanes 13-18) were stimulated for various time periods at 37°C Lanes 1,7 and 13 RAM IgG alone for 1 minute, lanes 2-6, 8-12 and 14-18 MAb 23.1 + RAM IgG (2,8 and 14 one minute, 3,9 and 15: 5 minutes; 4,10 and 16 10 minutes, lanes 5,11 and 17 15 minutes and 6,12 and 18: 30 minutes) The positions of major phosphotyrosine-containing proteins (on the left) and of prestained molecular weight markers (on the right) are indicated Exposure 15 hours

C. Quantitative analyses. Quantitation of the changes in tyrosine protein phosphorylation of the 120 (p120), 70 (p70) and 36 (p36) kDa polypeptides was performed by cutting the appropriate bands and counting in a γ counter. Ordinate: counts per minute (CPM), logarithmic scale, abscissa time in minutes, linear scale. Neo 2. ○ : F394F505 22 ▲ and F505 7 ■

Figure 3. Effects of expression of F394F505 p56^{lck} polypeptides on TCR-induced tyrosine protein phosphorylation.







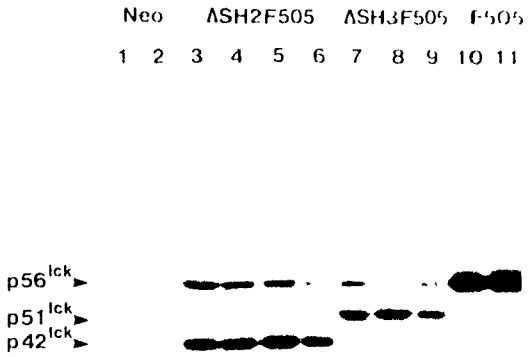
Expression of SH2 and SH3 domain deletion mutants of F505 Lck in BI-141 T-cells

Despite the fact that the SH2 and SH3 domains of cytoplasmic tyrosine protein kinases seem dispensable for the catalytic function of these products, accumulating data suggest that they modulate enzyme-substrate interactions (8,21). Therefore, to evaluate the potential involvement of these non-catalytic sequences in the enhancement of TCR signalling by F505 Lck, BI-141 cells expressing F505 Lck polypeptides lacking either the SH2 (ΔSH2F505 Lck) or the SH3 (ΔSH3F505 Lck) motif were generated, as described in Materials and Methods. Importantly, previous analyses conducted in NIH 3T3 cells revealed that ΔSH2F505 Lck and ΔSH3F505 Lck exhibit enzymatic activities apparently equal to that of F505 $p56^{lck}$ (48).

Cell lines were tested for expression of the mutant Lck polypeptides by immunoblot, using a rabbit anti-Lck serum generated against a synthetic peptide corresponding to amino-acids 29-54 of the murine $p56^{lck}$ sequence. As this sequence is located within the unique domain of $p56^{lck}$, the antiserum is presumed to equally recognize the different Lck polypeptides examined. Deletion of the SH2 and SH3 sequences generated Lck proteins migrating at 42 and 51 kDa in SDS-PAGE gels, respectively (Figure 4). Consequently, these products were termed $p42^{lck}$ and $p51^{lck}$. After taking into consideration the abundance of the endogenous $p56^{lck}$, cell lines expressing amounts of ΔSH2 (lanes 3-6) and ΔSH3 (lanes 7-9) F505 Lck proteins comparable (within two-fold) to those of F505 $p56^{lck}$ (lanes 10 and 11) were selected for additional studies. Importantly, the two different deletion mutants were expressed at equivalent levels in these clones. The small variations (two-fold) in the abundance of endogenous Lck polypeptides in these cells were within the range previously noted in a series of independent neomycin phosphotransferase expressing BI-141 cell lines (1).

Figure 4. Expression of ΔSH2F505 Lck and ΔSH3F505 Lck polypeptides in BI-141 T-cells.

Anti-Lck immunoblot. Levels of Lck protein in several independent BI-141 cell lines expressing the neomycin resistance marker alone (Neo, lanes 1 and 2), ΔSH2F505 Lck (ΔSH2F505, lanes 3-6), ΔSH3F505 Lck (ΔSH3F505, lanes 7-9) or F505 $p56^{lck}$ (lanes 10-11) were assessed as in Figure 1, except that an antiserum generated a synthetic peptide corresponding to amino-acids 29-54 of the murine $p56^{lck}$ sequence (53) was used. Lanes 1: Neo 1; 2: Neo 2; 3: ΔSH2F505.3; 4: ΔSH2F505.7; 5: ΔSH2F505.9; 6: ΔSH2F505.20; 7: ΔSH3F505.12; 8: ΔSH3F505.15; 9: ΔSH3F505.24; 10: F505.7 and 11: F505.9. The relative migrations of $p56^{lck}$, $p51^{lck}$ and $p42^{lck}$ are indicated on the left. Exposure: 2 days. For quantitation, bands were cut from nitrocellulose filters and counted in a γ counter (data not shown). After taking into consideration the abundance of the endogenous Lck polypeptides, we established that the levels of ΔSH2F505 Lck and ΔSH3F505 Lck were approximately 50% those of F505 $p56^{lck}$. Similar quantitations were achieved when serial dilutions of lysates from F505 Lck expressing cells were used for comparison (data not shown).



Deletion of the non-catalytic SH2 domain of p56^{lck} significantly alters the improvement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}

BI-141 cells expressing ΔSH2F505 or ΔSH3F505 Lck polypeptides were tested for TCR-induced tyrosine phosphorylation changes, as outlined above. Although ΔSH2F505 Lck (Figure 5A, lanes 5-12) consistently enhanced TCR-induced tyrosine protein phosphorylation, this improvement was markedly less than that provided by F505 p56^{lck} (lanes 13-16). In contrast, expression of ΔSH3F505 Lck polypeptides (Figure 5B, lanes 5-10) increased the TCR-induced signal in a manner analogous to that mediated by introduction of F505 p56^{lck} (lanes 11-14). It should be noted that neither ΔSH2F505 Lck nor ΔSH3F505 Lck augmented tyrosine protein phosphorylation in unstimulated cells to a greater degree than F505 p56^{lck}. This finding implies that the process by which levels of phosphotyrosine are suppressed prior to TCR stimulation in F505 p56^{lck} expressing BI-141 cells is independent of regulation through the SH2 or SH3 sequences (1).

Kinetic analyses of representative cell lines (Figures 5C and 5D) showed that, in contrast to the enhancement seen in cells expressing F505 p56^{lck} (Figure 5C, lanes 13-18; Figure 5D, lanes 13-18), or ΔSH3F505 Lck (Figure 5D, lanes 7-12), the improvement of TCR-induced tyrosine protein phosphorylation by expression of ΔSH2F505 Lck polypeptides (Figure 5C, lanes 7-12) was short-lived. Indeed, after 5 minutes of TCR stimulation, the pattern of tyrosine protein phosphorylation observed in these cells was essentially similar to that of Neo cells (Figure 5C, lanes 1-6). This is substantiated by quantitative analyses of the 120, 70 and 36 kDa substrates (Figures 5E and 5F). Similar observations were made with other ΔSH2F505 Lck expressing BI-141 cell lines (data not shown). The increase in baseline tyrosine phosphorylation of certain substrates noted in ΔSH3F505 15 cells (Figure 5D, lane 7) was not seen in other experiments (see Figure 5B,

lane 7). A similar increase in baseline phosphorylation was at times also noted in some F505 Lck expressing cell lines (Figure 3A, lane 11; Figure 3B, lane 13, and Figure 5C, lane 13). While the reason for this variation is unclear, such phenomenon does not affect TCR-induced tyrosine protein phosphorylation responses.

Figure 5. Effects of SH2 or SH3 domain deletions on the ability of F505 Lck to enhance TCR-induced tyrosine protein phosphorylation.

A TCR-induced tyrosine protein phosphorylation (**anti-phosphotyrosine immunoblot**) Same as Figure 2, except that cell lines expressing the neomycin resistance marker alone (Neo, lanes 1-4), ΔSH2F505 Lck (ΔSH2F505, lanes 5-12) or F505 $p56^{lck}$ (F505, lanes 13-16) were tested. Moreover, TCR stimulation was performed for one minute. Lanes 1,3,5,7,9,11,13 and 15: RAM IgG alone and 2,4,6,8,10,12,14 and 16: MAb F23.1 + RAM IgG. Lanes 1 and 2: Neo.1, 3 and 4: Neo.2; 5 and 6: ΔSH2F505.3; 7 and 8: ΔSH2F505.7, 9 and 10: ΔSH2F505.9, 11 and 12: ΔSH2F505.20, 13 and 14: F505.7 and 15 and 16: F505.9. The positions of the major phosphotyrosine-containing polypeptides (on the left) and of prestained molecular mass markers (on the right) are indicated. Exposure: 6 hours.

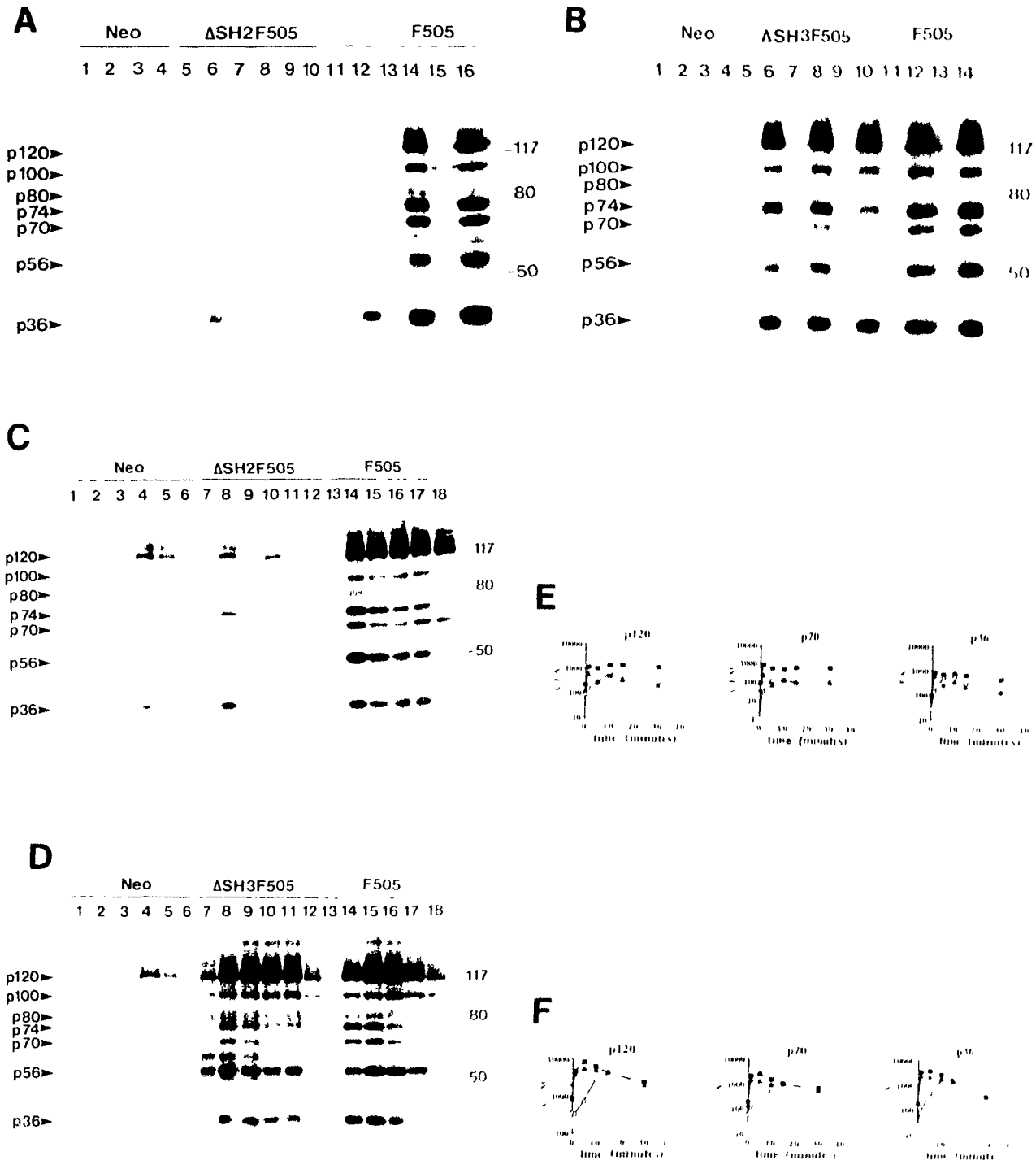
B. TCR-induced tyrosine protein phosphorylation (**anti-phosphotyrosine immunoblot**) Same as in Figure 5A, except that cell lines expressing the neomycin resistance marker alone (Neo, lanes 1-4), ΔSH3F505 Lck (ΔSH3F505; lanes 5-10) or F505 $p56^{lck}$ (F505, lanes 11-14) were tested. Anti-TCR antibody stimulation was for one minute. Lanes 1,3,5,7,9,11 and 13: RAM IgG alone and 2,4,6,8,10,12 and 14: MAb F23.1 + RAM IgG. Lanes 1 and 2: Neo.1, 3 and 4: Neo.2, 5 and 6: ΔSH3F505.12; 7 and 8: ΔSH3F505.15, 9 and 10: ΔSH3F505.24, 11 and 12: F505.7 and 13 and 14: F505.9. The positions of the major phosphotyrosine-containing proteins (on the left) and of prestained molecular weight markers (on the right) are indicated. Exposure: 11 hours.

C. Effects of expression of ΔSH2F505 Lck polypeptides. **Time-course experiment.** As in Figure 3B, the same time-points were examined. Lanes 1-6: Neo.2; 7-12: ΔSH2F505.3 and 13-18: F505.9. Exposure: 24 hours.

D. Effects of expression of ΔSH3F505 Lck polypeptides. **Time-course experiment.** Same as Figure 3B. Lanes 1-6: Neo.1, 7-12: ΔSH3F505.15 and 13-18: F505.9. Exposure: 5 hours.

E and **F**. **Quantitative analyses** of the experiments depicted in Figures 5C and 5D, respectively. As in Figure 3C. Neo: ○; ΔSH2F505.3 or ΔSH3F505.15: ▲ and F505.9: ■.

Figure 5. Effects of SH2 or SH3 domain deletions on the ability of F505 Lck to enhance TCR-induced tyrosine protein phosphorylation.



Effects of activated Lck variants on antigen receptor-induced tyrosine phosphorylation of phospholipase C-γ 1

The identity of the polypeptides undergoing tyrosine phosphorylation upon TCR stimulation is largely undefined However, recent studies indicate that antigen receptor stimulation results in rapid tyrosine phosphorylation of the phosphatidyl inositol (PI)-specific phospholipase C-γ 1 isoenzyme (32,36,54) This biochemical alteration is thought to be responsible for the increase in phospholipase activity and the elevation in PI turnover noted during T-cell activation (30,31,32,36,54).

Consequently, to gain additional understanding of the mechanism by which F505 p56^{lck} enhances T-cell functions, the effects of expression of this polypeptide on TCR-induced tyrosine phosphorylation of PLC-γ 1 were tested (Figure 6). Cells expressing the *neo* gene alone (Neo) or F505 p56^{lck} were stimulated with anti-TCR MAb F23.1 and sheep anti-mouse (SAM) IgG for one minute, lysed in NP-40 containing buffer and PLC-γ 1 isolated by immunoprecipitation using a specific rabbit anti-PLC-γ 1 serum. The extent of PLC-γ 1 tyrosine phosphorylation was evaluated by anti-phosphotyrosine immunoblotting (Figure 6A) Contrary to Neo cells (lanes 1-4), cells containing F505 p56^{lck} (lanes 5-8) demonstrated detectable tyrosine phosphorylation of a 150 kDa polypeptide after TCR stimulation (lanes 6 and 8) This product was not recovered when normal rabbit serum was used as the immunoprecipitating antibody (lanes 9 and 10) and was consistent with PLC-γ 1 (41 44) As is the case for overall tyrosine protein phosphorylation, no significant tyrosine phosphorylation of PLC-γ1 was noted prior to TCR stimulation (lanes 1,3,5 and 7) Importantly, the increased TCR-induced tyrosine phosphorylation of PLC-γ 1 in F505 p56^{lck} expressing cells was not secondary to elevated levels of PLC-γ 1, as indicated by anti-PLC-γ 1 immunoblotting of PLC-γ 1 immunoprecipitates (Figure 6B).

We also explored the impact of mutation of tyrosine 394, or deletion of the SH2 or SH3 domain on the enhancement of TCR-induced PLC-γ 1 tyrosine phosphorylation by F505 $p56^{lck}$ (Figures 6C-6E). Time-course studies were performed on representative F394F505 Lck (Figure 6C), ΔSH2F505 Lck (Figure 6D) and ΔSH3F505 Lck (Figure 6E) expressing cells. Patterns of PLC-γ 1 tyrosine phosphorylation were compared with those of cells expressing the neomycin resistance gene alone (Neo) or F505 $p56^{lck}$. First, in agreement with other reports (32,36,54), these experiments collectively demonstrated a small and reproducible rise (between 2 and 5 minutes) in PLC-γ 1 tyrosine phosphorylation after TCR stimulation on Neo cells (Figure 6 and data not shown). Expression of F505 $p56^{lck}$ resulted in a prominent enhancement (approximately 10-fold) of TCR-induced PLC-γ 1 tyrosine phosphorylation, which was maintained over the time period analysed (up to 10 minutes). The effects of mutations or deletions in F505 $p56^{lck}$ on PLC-γ 1 tyrosine phosphorylation resembled those noted earlier for overall tyrosine protein phosphorylation (Figures 3 and 5). Indeed, F394F505 $p56^{lck}$ expressing BI-141 cells (Figure 6C) showed an intermediate increase in TCR-induced tyrosine phosphorylation of PLC-γ 1. Moreover, the improvement in PLC-γ 1 tyrosine phosphorylation by F505 Lck was greatly reduced by deletion of the SH2 sequence (Figure 6D), while less affected by deletion of the SH3 region (Figure 6E).

Figure 6. TCR-induced tyrosine phosphorylation of phospholipase C-γ 1.

A. Effects of F505 $p56^{lck}$ expression on the extent of TCR-induced tyrosine phosphorylation of phospholipase (PLC)-γ 1. **Anti-phosphotyrosine immunoblot.** Cells were stimulated with anti TCR MAb F23.1 + SAM IgG for 1 minute at 37°C (lanes 2,4,6,8 and 10) or SAM IgG alone (lanes 1,3,5,7 and 9). Lysates were immunoprecipitated with either a specific polyclonal rabbit anti-PLC-γ 1 serum (lanes 1-8) or normal rabbit serum (lanes 9 and 10). Lanes 1 and 2: Neo.1, 3 and 4: Neo 2, 5 and 6: F505.7 and .10. F505.9. Tyrosine phosphorylated proteins were detected by immunoblotting with anti phosphotyrosine antibodies. The position of PLC-γ 1 (PLC) is indicated on the left while those of prestained molecular mass markers are shown of the right. Exposure: 3 days.

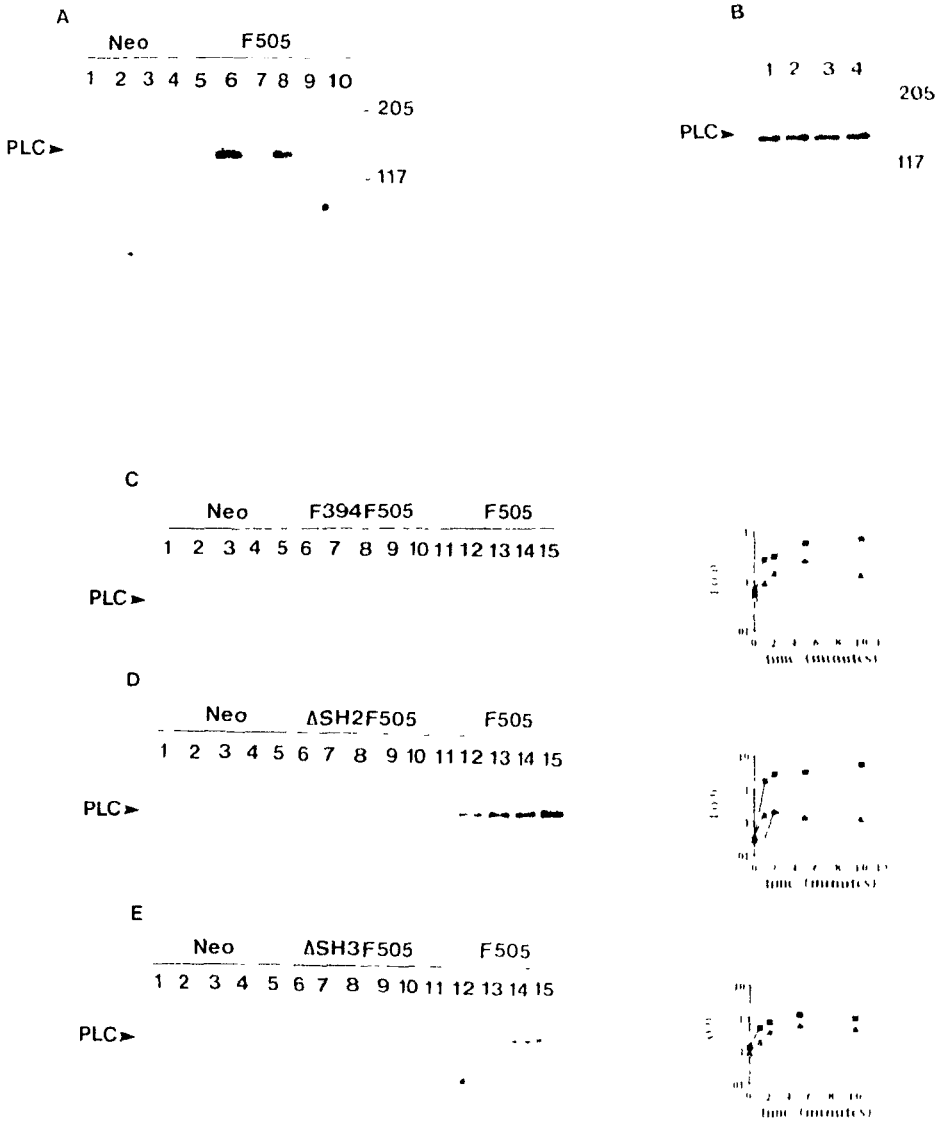
B. Anti-PLC-γ 1 immunoblot. Cells were stimulated as in Figure 6A and PLC-γ 1 recovered by immunoprecipitation. The abundance of PLC-γ 1 in the various immunoprecipitates was assessed by immunoblotting with the rabbit anti PLC-γ 1 serum described earlier. Lanes 1 and 2: Neo 1 and 3 and 4: F505.9. Lanes 1 and 3: SAM IgG alone for one minute and lanes 2 and 4: MAb F23.1 + SAM IgG for one minute. The position of PLC-γ 1 (PLC) is indicated on the left, while those of prestained molecular mass markers are shown of the right. Exposure: 3 hours.

C. Effects of F394F505 $p56^{lck}$ expression on TCR-induced PLC-γ 1 tyrosine phosphorylation. **Time-course experiment.** Lanes 1-5: Neo 1 (○); 6-10: F394F505.8 (▲) and 11-15: F505.9 (■). Lanes 1,6 and 11: SAM IgG alone for one minute, lanes 2-5,7-10 and 12-15: MAb F23.1 + SAM IgG (lanes 2,7 and 12: one minute, 3,8 and 13: 2 minutes, 4,9 and 14: 5 minutes and 5,10 and 15: 10 minutes). Exposure: 3 days. Bands were quantitated by densitometry using a BioImage (Millipore). A graphic representation of this quantitation is shown on the right. Ordinate: integrated optical density (I.O.D), logarithmic scale; abscissa: time (minutes), linear scale.

D. Effects of SH2 deletion on the enhancement of TCR-induced PLC-γ 1 tyrosine phosphorylation by F505 $p56^{lck}$. **Time-course experiment.** Same as Figure 6C, except that lanes 1-5: Neo 1 (○), 6-10: ΔSH2F505.3 (▲) and 11-15: F505.9 (■). Exposure: 3 days.

E. Effects of SH3 deletion on the enhancement of TCR-induced PLC-γ 1 tyrosine phosphorylation by F505 $p56^{lck}$. **Time-course experiment.** Same as Figure 6C, except that lanes 1-5: Neo 1 (○), 6-10: ΔSH3F505.15 (▲) and 11-15: F505.9 (■). Exposure: 4 days.

Figure 6. TCR-induced tyrosine phosphorylation of phospholipase C-γ 1.

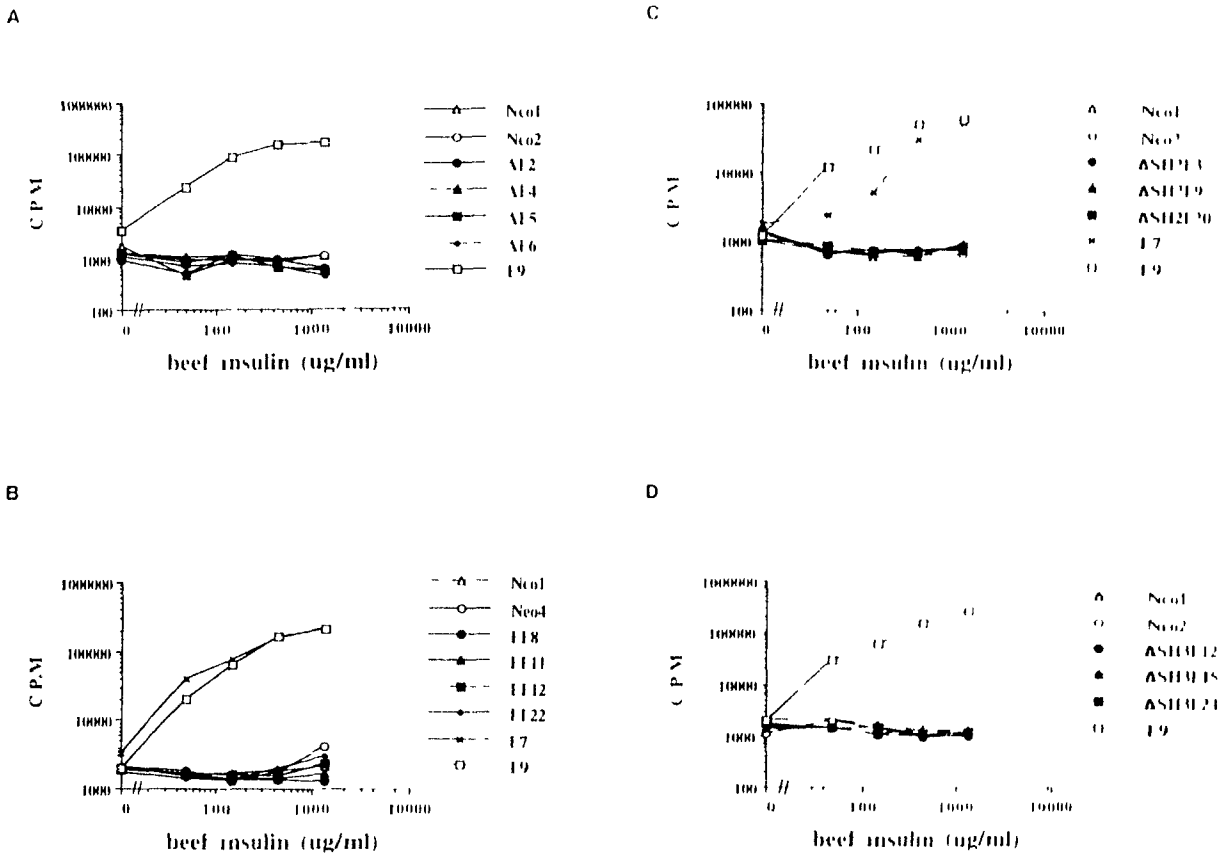


Effects of Lck mutants on antigen responsiveness.

We have previously found that expression of F505 $p56^{lck}$, but not wild-type or kinase-deficient Lck polypeptides, enhanced lymphokine (IL-2) production in response to antigen/MHC stimulation (1). Consequently, we also analysed the antigen responsiveness of BI-141 cells expressing A2F505, F394F505, ΔSH2F505 or ΔSH3F505 Lck. Cells were stimulated with varying concentrations of beef insulin in conjunction with the appropriate class II MHC molecules ($A_\alpha^b A_\beta^k$). After 24 hours, supernatants were collected and tested for IL-2 content using the IL-2 sensitive cell line HT-2. Responsiveness of the various cell lines was compared to that of cells expressing the neomycin resistance marker alone (Neo) or F505 $p56^{lck}$. Each panel of cell lines was analysed in at least three independent assays. Representative assays are shown in Figure 7. These revealed that mutation of the site of myristylation (Figure 7A) or autophosphorylation (Figure 7B), or deletion of the SH2 (Figure 7C) or SH3 (Figure 7D) sequence completely abolished the ability of F505 Lck to enhance lymphokine production in response to antigen/MHC stimulation

Figure 7. Antigen stimulation assays.

The effects of expression of various Lck mutants on antigen responsiveness of BI 141 cells were assessed by measuring lymphokine production in response to various concentrations of the antigen beef insulin presented in the context of the appropriate class II MHC molecules ($A_\alpha{}^bA_\beta{}^k$), as described in Materials and Methods. BI-141 clones expressing the neomycin resistance marker alone (Neo) or F505 $p56^{lck}$ (F505) were compared with several independent cell lines expressing either A2F505 $p56^{lck}$ (**AF; Figure 7A**), F394F505 $p56^{lck}$ (**FF; Figure 7B**), ΔSH2F505 Lck (**ΔSH2F; Figure 7C**) or ΔSH3F505 Lck (**ΔSH3F; Figure 7D**). Ordinate: ^3H thymidine incorporation in counts per minute (C.P.M.), logarithmic scale; abscissa: concentration of beef insulin (μg/ml), logarithmic scale. Spontaneous IL-2 production is shown as the zero point on the abscissa.



DISCUSSION

Mutation of glycine 2 of p56^{lck} to alanine has previously been shown to prevent myristylation and membrane association of this polypeptide (2). Data presented in this report demonstrate that this amino-acid substitution precludes enhancement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}. Thus, myristylation and/or membrane association are required for participation of Lck in the TCR signalling pathway. Even though the exact basis for this requirement remains to be clarified, such modifications would certainly favor the interaction of F505 Lck with membrane-bound molecules, possibly the TCR regulated substrates themselves, or molecular "intermediates" linking p56^{lck} to the TCR signalling pathway [such as the ζ subunit of TCR or the 70 kDa TCR-associated molecule ZAP-70 (11)]. In addition, membrane binding may facilitate further post-translational modifications which improve the efficiency of F505 p56^{lck} at participating in TCR signalling events. Consistently, we have previously shown, in NIH 3T3 cells, that the enzymatic activity of A2F505 p56^{lck} is less than that of F505 p56^{lck}, possibly because myristylation and/or membrane association are required for adequate *in vivo* phosphorylation of F505 p56^{lck} at tyrosine 394 (2).

The major site of *in vitro* autophosphorylation of p56^{lck}, tyrosine 394, is involved in positive regulation of the catalytic activity of p56^{lck}. Substitution of this residue by phenylalanine interferes with activation of Lck by tyrosine 505 mutation (2) or antibody-mediated aggregation of CD4 (50). In keeping with these observations, we noted that the intensity of the TCR-induced tyrosine phosphorylation signal in BI-141 cells expressing F394F505 p56^{lck} was intermediate between those of cells expressing F505 p56^{lck} or the neomycin resistance marker alone. Therefore, the decreased enzymatic function of F394F505 Lck is presumably responsible for its diminished capability to improve TCR signalling. Importantly, these data also indicate that, unlike signal transduction through

tyrosine protein kinase receptors (8,21), p56^{lck}-mediated signals in T-cell activation do not require the interaction of the Lck autophosphorylation site with tyrosine phosphorylation substrates. One caveat to this interpretation is that F505 p56^{lck} may undergo tyrosine phosphorylation at sites other than tyrosine 394. However, this possibility seems unlikely, as metabolic labeling and peptide mapping studies have failed to reveal the presence of such sites in F505 p56^{lck} molecules from resting or TCR-stimulated cells (3).

The SH3 domain of p56^{lck} is not required for enhancement of anti-TCR antibody induced tyrosine protein phosphorylation by F505 Lck (although deletion of this sequence abrogates improvement of antigen-induced IL-2 production, see below). These results are in agreement with former studies conducted in NIH 3T3 cells, which revealed that the catalytic activity and oncogenic potential of ΔSH3F505 Lck are equivalent to those of F505 p56^{lck} (48). In contrast to the limited impact of deletion of the SH3 region on TCR signalling, removal of the SH2 domain greatly affected the ability of F505 Lck to augment TCR-induced tyrosine protein phosphorylation. Indeed, expression of ΔSH2F505 Lck at best allowed a minor and transient improvement of TCR-induced tyrosine protein phosphorylation.

Earlier studies have shown that the SH2 motif of activated tyrosine protein kinases such as v-Src or v-Fps is critical for the full oncogenic potential of these enzymes, although they appear dispensable for the elevated catalytic activity of these products (6,7,22,28). It has been proposed that this function relates to the ability of SH2 domains to associate with phosphotyrosine-containing proteins (8,21). As the tyrosine protein kinase activity of F505 Lck is seemingly not altered by deletion of the SH2 region (48), our findings are highly consistent with the idea that the SH2 sequence does modulate the interaction of activated Lck molecules with tyrosine phosphorylation substrates. Consistently, we have found that bacterial fusion proteins containing the SH2 domain of p56^{lck} bind several of the

phosphotyrosine-containing proteins from stimulated T-cells (9). Based on these findings, we postulate that the SH2 domain may stabilize, or prolong the interaction of $p56^{lck}$ with proteins such as ζ, ZAP-70 and/or TCR-regulated substrates. The SH2 motif may additionally protect these polypeptides from dephosphorylation by tyrosine phosphatases like CD45.

While searching for potentially relevant substrates of $p56^{lck}$ in T-cells, we observed that the TCR-induced tyrosine phosphorylation of PI-specific PLC γ 1 was markedly increased (approximately 10-fold) by expression of F505 $p56^{lck}$. This response was affected by mutation of tyrosine 394 and deletion of the SH2 domain in manners comparable to those reported above for overall TCR-induced tyrosine protein phosphorylation. These observations provide evidence that the activity of PLC-γ 1 can also be regulated by non-receptor tyrosine protein kinases. As we have been unable to detect association of PLC-γ 1 with $p56^{lck}$ (9), it is conceivable that these polypeptides interact through the intermediate of another molecule. In light of the fact that bacterially produced SH2 sequences from $p56^{lck}$ and PLC-γ 1 can bind apparently similar tyrosine phosphorylated proteins from stimulated T-cells (9), one of these polypeptides may serve as the "bridge" between Lck and PLC-γ 1.

Despite the differential effects of the mutations/deletions tested on TCR-induced tyrosine protein phosphorylation, all alterations were found to abrogate enhancement of antigen/MHC-induced lymphokine production by F505 $p56^{lck}$. This is especially surprising in the cases of F394F505 Lck and ΔSH3F505 Lck, which allowed significant enhancement of the anti-TCR antibody-induced signal. Such discrepancy is perhaps indicative of unsuspected qualitative alterations of the augmented TCR-induced signal as a result of these additional mutations. Indeed, tyrosine 394 or the SH3 region may be required for phosphorylation of substrates poorly represented in anti-phosphotyrosine

immunoblots of total cell lysates. Alternatively, the use of high-affinity anti-TCR antibodies for T-cell triggering may obliterate subtle, albeit meaningful, differences in antigen receptor-induced tyrosine phosphorylation. As we have been unable to conclusively study changes in intracellular tyrosine protein phosphorylation after antigen/MHC stimulation of BI-141 cells (9), additional efforts will be necessary to test these different possibilities.

In summary, the data presented herein imply that F505 $p56^{lck}$ molecules enhance TCR signalling by a process which requires myristylation and/or membrane association of $p56^{lck}$. Moreover, this mechanism is dependent on increased $p56^{lck}$ activity, as indicated by the facts that wild-type Lck fails to enhance these signals (1), while F394F505 $p56^{lck}$ allows a partial improvement of TCR signalling. Importantly, the non-catalytic SH2 sequence (but not the SH3 sequence) is required for robust and sustained enhancement of TCR-induced tyrosine protein phosphorylation by F505 $p56^{lck}$. Through its ability to physically associate with tyrosine phosphorylated proteins (9), the SH2 motif may modulate the interaction of $p56^{lck}$ with the TCR signalling complex. Intriguingly, all the mutations tested, including deletion of the SH3 region, abrogated the enhancement of antigen-triggered IL-2 production by F505 Lck, thus implying more stringent requirements for enhancement of antigen responsiveness by F505 Lck. Finally, we observed that expression of F505 $p56^{lck}$ greatly enhanced TCR-induced tyrosine phosphorylation of phospholipase C (PLC)-γ 1, suggesting that PLC-γ 1 may be a substrate for $p56^{lck}$ in T-lymphocytes.

As the TCR complex does not possess an intrinsic tyrosine protein kinase activity, the mechanism by which this receptor transduces an intracellular tyrosine protein phosphorylation signal during T-cell activation has been the object of intense investigations. As discussed above, it is becoming increasing clear that the lymphocyte-specific tyrosine protein kinase $p56^{lck}$ can participate in this process. Other findings

indicate that $p59^{fynT}$, another Src-related enzyme abundantly expressed in T-lymphocytes (13), can also contribute to this signalling pathway (12,15,20,35). It is therefore of great importance that future studies be aimed at defining if these two products regulate T-cell functions through similar, overlapping, or different mechanisms.

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Chapter 3

General Discussion and General Conclusion.

GENERAL DISCUSSION

In contrast to growth factor receptor tyrosine protein kinases, the T-cell receptor (TCR) for antigen does not possess intrinsic tyrosine protein kinase (TPK) activity Nevertheless, TCR stimulation leads to induction of TPK activity and TCR "autophosphorylation" as reflected by tyrosine phosphorylation of the ζ chains Consequently, it is proposed that one or more cytoplasmic TPK are mediating TCR-induced tyrosine protein phosphorylation signals Candidate TPK include $p56^{lck}$, $p59^{fynT}$ and ZAP-70, a 70kDa tyrosine phosphorylated protein which is found associated with the TCR-ζ chains upon TCR stimulation Notwithstanding the complexity of T-cell signalling, this chapter proposes simple molecular mechanisms (based on the model for growth factor receptor signalling) for TCR-induced TPK activation

A TCR-associated TPK complex

The view that the TCR is physically associated with a cytoplasmic TPK is supported in part by the observation that a fraction of $p59^{fynT}$ is communoprecipitated with the TCR complex (Samelson *et al* , 1991; Gassmann *et al* , 1992). Alternatively, the interaction of the TCR with CD4 or CD8 during antigen/MHC recognition presumably brings $p56^{lck}$ in close proximity to the TCR and thus, may allow $p56^{lck}$ to function like a "TCR-associated" kinase Evidence for functional interactions between the TCR and these Src-family kinases is provided by their ability to enhance antigen induced T cell responses (Abraham, N *et al* , 1991; Caron *et al* , 1992. Davidson *et al* , 1992) However, it is currently unknown whether these two enzymes act through common, intersecting or independant signalling pathways.

Several findings suggest that ZAP-70 may also participate in TCR-mediated responses. Firstly, the molecular cloning of ZAP-70 reveals that the 70 kDa protein possesses a kinase domain and two SH2 domains (A Weiss, unpublished). A study reporting the ability of a 70 kDa tyrosine phosphorylated protein (presumably ZAP-70) to bind ATP analogs (Wange *et al*, 1992) further suggests that ZAP-70 has PTK activity. Secondly, a functional interaction with the TCR is suggested by the observation that both tyrosine phosphorylation of ZAP-70 and its association to ζ require TCR stimulation (Chan *et al*, 1991). Similarly, in B cells, antibody-mediated ligation of IgM results in increased tyrosine phosphorylation of a 72 kDa protein (Hutchcroft *et al*, 1991). This 72 kDa phosphoprotein is likely to represent PTK72 and $p72^{syk}$ (Zioncheck *et al*, 1986; Zioncheck *et al*, 1988, Taniguchi *et al*, 1991), a cytoplasmic TPK with two SH2 domains, that is predominantly expressed in lymphoid cells. Nonetheless, no evidence indicates that ZAP-70 is indeed responsible for the observed TPK activity which is associated with activated TCR.

Interestingly, cotransfection studies in cos cells involving ZAP-70 and the ζ subunit of the TCR have revealed that another TPK is required to promote tyrosine phosphorylation and association of ZAP-70 to ζ (A Weiss, unpublished). This observation suggests that TCR-mediated tyrosine protein phosphorylation may involve the cooperation of more than one TPK. A possible scenario is that upon TCR stimulation, $p56^{lck}$ or $p59^{fynT}$ interacts with ZAP-70, inducing tyrosine phosphorylation of this 70 kDa protein and its subsequent binding to the TCR ζ chains. Alternatively, $p56^{lck}$ or $p59^{fynT}$ may mediate tyrosine phosphorylation of the TCR ζ chains, providing binding sites for SH2-containing signalling molecules such as ZAP-70.

Evidence for $p56^{lck}$ contribution in this process is supported by the significant decrease in TCR-mediated tyrosine protein phosphorylation observed in T cells expressing SH2 deletion of $F505p56^{lck}$ (Caron *et al*, 1992). Deletion of Lck SH2 domain is suggested to prevent F505Lck interaction with critical TCR substrates, thus preventing the increase in TCR-induced tyrosine protein phosphorylation. As $p56^{lck}$ is not directly associated with the TCR (N. Abraham, unpublished), TCR signals must be transduced to $p56^{lck}$ via a common effector protein.

Considering that $p59^{fynT}$ may play a similar role, it would be of interest to examine the effects of SH2 deletion on the ability of $F528p59^{fynT}$ to enhance TCR induced tyrosine protein phosphorylation signals. These experiments might provide some indication concerning the specificity of $p56^{lck}$ and $p59^{fyn}$ function in T-cell activation. Clearly, more experiments are required to determine the exact mechanism by which these "TCR-associated kinases" regulate TCR-mediated tyrosine protein phosphorylation.

From the TCR-associated TPK complex to PLC-γ 1.

By analogy with growth factor receptor autophosphorylation, which is thought to regulate the coupling of activated receptors to a variety of signal transducer molecules, one can view tyrosine phosphorylated ζ and/or its associated cytoplasmic TPK as "docking components" for putative SH2-containing signal amplifiers. On the basis that tyrosine phosphorylation of PLC-γ 1 is associated with increased PI turnover (Mustelin *et al*, 1990, Secrist *et al*, 1991), the coupling of second messengers (IP_3 and DAG) with activated TCR complexes is likely to involve the interaction of PLC γ 1 with one or more TCR associated TPK including $p56^{lck}$, $p59^{fynT}$ or ZAP-70.

A study showing that some PLC-γ 1 activity is associated with TCR-ζ (Dasgupta *et*

al , 1992) supports this view. As PLC-γ 1 contains two SH2 domains, the association of tyrosine phosphorylated molecules with PLC-γ 1 SH2 domains has been examined extensively using *in vitro* binding assays (Caron *et al* , 1992, Gilliland *et al*., 1992; Weber *et al* , 1992)

In stimulated T cells, phosphotyrosine containing proteins of approximate molecular weight of 74 and 35/36 kDa are observed to associate with PLC-γ 1 SH2-Ig fusion proteins (Gilliland et al , 1992). Another study using bacterially expressed TrpE-fusion proteins of PLC-γ 1 SH2 domains reports the binding of 36, 38, 58 and 63kDa proteins Finally, Glutathion-S-transferase (GST)-PLC-γ1 SH2 fusion proteins incubated with lysates from anti-TCR antibody-stimulated F505 $p56^{lck}$ expressing T cells bind phosphotyrosine containing proteins of apparent molecular weights of 120, 80, 74, 70, 62, 54-56 and 36 kDa (Caron *et al* , 1992). These products co-migrate with the major phosphotyrosine-containing proteins detected in lysates of TCR-stimulated F505 $p56^{lck}$ expressing cells (Caron *et al* , 1992). However, it is currently unknown whether these products include a TPK

Our results also show that bacterially produced GST-Lck SH2 and GST-PLC-γ 1 SH2 fusion proteins appear to recruit a common set of phosphotyrosine-containing proteins when incubated *in vitro* with lysates of activated T cells (Caron *et al* , 1992) Interestingly, these substrates fail to show marked and sustained enhancement of TCR-induced tyrosine phosphorylation in cells expressing SH2-deleted F505 Lck molecules. In addition, cells expressing F505$p56^{lck}$ demonstrate a marked increase in PLC-γ 1 tyrosine phosphorylation (Caron *et al* , 1992). However, in contrast to a study suggesting that $p56^{lck}$ is transiently associated with PLC-γ 1 SH2 domain (Weber *et al* , 1992), our results and that of others (Gilliland *et al* , 1992) exclude a direct physical association between $p56^{lck}$ and PLC-γ 1

To explain these results, possible avenues may be explored On one hand, it is a possibility that one of these TCR-regulated polypeptides serves as a "bridge" between Lck and PLC-γ 1. This is suggested by the report of 36 and 38 kDa molecules coprecipitating with $p56^{lck}$ in anti-PLC-γ 1immune complexes (Weber *et al*., 1992) This view is further supported by the observation that in anti-TCR antibody-stimulated J505$p56^{lck}$ expressing T cells, p36 and p80 appear to bind the SH2 sequences of Lck and PLC-γ 1 with the highest affinity (A. Veillette, unpublished)

Alternatively, based on results suggesting that ζ may be one of $p56^{lck}$ substrates (Veillette et al., 1989) and that some PLC-γ 1 is found associated with TCR ζ (Dasgupta *et al*., 1992), it is possible that $p56^{lck}$ induces tyrosine phosphorylation of TCR-ζ, generating binding sites for SH2-containing intermediates such as PLC-γ 1 Similarly, the fraction of TCR-associated $p59^{fynT}$ may induce the phosphorylation of TCR-ζ chains Because the expression of both activated forms of $p56^{lck}$ and $p59^{fynT}$ (Abraham, N *et al* , 1991; Caron *et al*., 1992; Davidson *et al* , 1992), increase tyrosine protein phosphorylation of a similar pattern of TCR-regulated substrates, these enzymes may act in a coordinate fashion and/or via a common signalling intermediate (possibly ζ or ZAP-70) to maximize this process.

On the other hand, as PLC-γ 1 contains two SH2 domains, it is possible that more than one TCR-regulated substrate binds to PLC-γ 1 SH2-mediated interactions involving molecules with two SH2 domains have been described in experiments examining the binding of the p85 subunit of PI 3' kinase to the PDGF receptor (Kavanaugh *et al* , 1992) p85 contains two SH2 domains, one which bind tyrosine phosphorylated PDGF receptor with high affinity and one which bind tyrosine phosphorylated PDGF receptor with low affinity. Upon binding of p85 to autophosphorylated PDGF receptor (through its high affinity SH2 domain), p85 is modified, presumably by PDGF receptor-mediated tyrosine

phosphorylation This modification is postulated to provide regulation of the ability of the low affinity SH2 domain to interact with either the receptor or other signalling proteins. More experiments are necessary to determine if this model applies to PLC-γ 1 in T cells, and to distinguish between all possible functional arrangements involving PLC-γ 1 and the TCR-associated TPK complex.

The SH2-mediated intramolecular binding model of Src-family kinases in T cells.

The observation that F505p56^{lck}, but not wild type p56^{lck} enhances T-cell responsiveness in CD4-negative and CD8-negative T cells indicates that mutation of tyrosine 505 to a non-phosphorylatable residue can substitute in part for CD4/CD8-mediated activation of wild type (wt) p56^{lck}. However, as enhancement of T-cell responses in CD4-negative and CD8-negative T cells expressing F505p56^{lck} is strickly dependant on TCR stimulation (Abraham, N *et al* , 1991; Caron *et al* , 1992), critical TCR signals are required to complement the effects of tyrosine 505 dephosphorylation. Based on experiments showing that enhancement of T-cell responsiveness by F505p56^{lck} depends on several structural requirements (Caron *et al* , 1992), these TCR signals are likely to induce the recruitment of F505p56^{lck} in close proximity to the TCR, promoting interactions of F505p56^{lck} with TCR-regulated substrates.

Importantly, this hypothesis suggests that a cooperative interaction between phosphorylated tyrosine 505 and the SH2 domain of p56^{lck} may occur in T cells. According to this model, mutation of tyrosine 505 would provide a readily "opened protein conformation" for possible Lck-SH2-mediated interactions with the TCR-regulated substrates TCR stimulation may then provide a signal promoting SH2-mediated binding of F505p56^{lck} to critical TCR targets. Consequently, an intact SH2 domain and stable

tyrosine 505 dephosphorylation would be essential to allow Lck-mediated enhancement of TCR-induced tyrosine protein phosphorylation. Abrogation of either of these requirements (deletion of SH2 in F505Lck or unstable tyrosine 505 dephosphorylation) would prevent TCR-mediated increase in tyrosine protein phosphorylation.

As a result, the observation that wild type $p56^{lck}$ expressed in CD4-negative and CD8-negative T cells does not enhance T-cell responsiveness may be due to a "closed protein conformation", which would prevent stable tyrosine 505 dephosphorylation Without interactions with CD4 molecules, wild type $p56^{lck}$ would not be able to properly interact with critical TCR-substrates.

A study (Luo and Sefton, 1992) similar to the one presented in chapter 2, suggesting that expression of an activated version of $p56^{lck}$ in T-cells induces spontaneous IL-2 production, may apparently contradict the results obtained in our studies However, a careful examination of these data reveals otherwise Firstly, these authors examined the effects of $F505p56^{lck}$ expressed in CD4-positive T-cells. They found that (in contrast to wild type) $F505p56^{lck}$ expressed in these CD4-positive T-cells induced spontaneous Il-2 production, but did not enhance antigen-induced IL-2 production This is intriguing in light of other findings by these authors suggesting that T-cells expressing $F505p56^{lck}$ do not demonstrate constitutive tyrosine protein phosphorylation Secondly, to determine whether spontaneous IL-2 production was the result of $F505p56^{lck}$ expression , these authors introduced $F505p56^{lck}$ into a CD4-negative T-cell line. Their results showed that $F505p56^{lck}$ expression induced spontaneous IL-2 production and that deletion of the SH2 domain, but not the SH3 domain, abrogated spontaneous IL-2 production These results are however difficult to interpret as expression of wild type $p56^{lck}$ also generated low levels of spontaneous IL-2. Unfortunately, a more accurate determination of the role of SH2 and especially the SH3 domain has not been pursued by studies examining the effects

of SH2 and SH3 deletion in TCR-mediated tyrosine protein phosphorylation or antigen-dependant IL-2 responses.

Nevertheless, part of these results may be explained using the SH2-mediated cooperative interaction model described above. Expression of F505p56^{lck} in CD4-positive T-cells is likely to result in the generation of physical complexes involving F505p56^{lck} and CD4, along with the existing wild type p56^{lck}-CD4 complexes. As mentionned previously, enhancement of T-cell responses (namely IL-2 production) requires two complementary signals, including dephosphorylation of tyrosine 505 and TCR stimulation (for proper induction of TCR-substrtate interactions with Lck). Hence, it is possible that these requirements are already met in T-cells expressing both CD4 and F505p56^{lck}.

Candidate regulators of TCR-associated TPK.

The understanding of TPK contribution to TCR-mediated signalling must take into account the function of possible TPK regulators. An important feature of tyrosine protein kinases is their ability to undergo autophosphorylation. Autophosphorylation of Src-related enzymes is associated with an increase in catalytic activity, as measured *in vitro* by their ability to phosphorylate an exogenous substrate. The importance of tyrosine phosphorylation in the regulation of Src-family kinases has been described (see general introduction) Mechanisms for the regulation of these enzymes, include autoregulation in either an intra or intermolecular fashion and regulation by other effectors such as tyrosine protein phosphatases and tyrosine protein kinases. Candidate regulators of Src-related kinases in T cells include the abundantly expressed tyrosine phosphatase CD45 and the carboxy-terminal c-Src kinase, p50^{csk}.

Although CD45 and p50^{csk} have been demonstrated to modify the tyrosine

phosphorylation state of tyrosine residue 505 *in vitro* , no firm evidence clearly establishes that these enzymes are true effectors of $p56^{lck}$ activity *in vivo*. One possibility is that these Src-related kinases ($p56^{lck}$ and $p59^{fynT}$) are indirectly regulated by CD45 and $p50^{csk}$. This view mainly comes from studies reporting the association of CD45 with $p56^{lck}$ and a 32kDa tyrosine phosphorylated intermediate (Schraven *et al.*, 1991) and studies reporting the association of CD45 with Thy-1 (Volarevic *et al* , 1990) , which is also found associated with $p59^{fyn}$ (Thomas and Samelson, 1992) Further studies examining these functional interactions are necessary to determine the role of these putative TPK regulators in coordinating TCR-mediated tyrosine protein phosphorylation signalling

Summary

Accumulating evidence indicates that antigen-induced TCR-mediated signal transduction is initiated by tyrosine protein phosphorylation and involve SH2-mediated recruitment of signal amplifiers. Several observations support the implication of $p56^{lck}$ and $p59^{fynT}$ in TCR-induced tyrosine protein phosphorylation, however, their exact contribution remains to be determined. Studies exploring the possible cooperation between $p56^{lck}$ and $p59^{fynT}$ in antigen-induced T-cell activation may serve to determine their respective contribution.

The coordinate regulation and overlapping SH2-binding substrate specificity between $p56^{lck}$ and PLC-γ 1 indicate that both molecules are functionnally coupled However, the nature of this coupling is not clear. The view that $p56^{lck}$ is directly linked to PLC-γ 1 (presumably through SH2 interactions) remains controversial Nevertheless, it is conceivable that protein-protein interactions involving SH2 sequences and tyrosine phosphorylated proteins, although very stable *in vitro*, represent a means of transient

interactions *in vivo*. Transient protein interactions may provide an efficient mechanism for signal amplification of TCR-induced stimuli.

Despite major advances in the characterization of SH2 domain interactions (the recent determination of the three-dimensional structure of the SH2 domain), the exact contribution of these interactions *in vivo* needs further investigation. Hence, a better understanding of the protein-protein interactions regulating antigen-mediated T-cell activation awaits further knowledge of the contribution of cytoplasmic tyrosine protein kinases to TCR-induced signalling as well as the identification of TCR immediate targets.

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GENERAL CONCLUSION

This thesis has examined the structural requirements for enhancement of T-cell responsiveness by the lymphocyte-specific tyrosine protein kinase, $p56^{lck}$. Based on studies indicating that expression of a constitutively activated version of this protein [carrying a tyrosine (Y) to phenylalanine (F) substitution at residue 505, a known negative regulatory site] enhanced T-cell responsiveness, this thesis evaluated the role of known structural domains of $F505p56^{lck}$ in the enhancement of TCR signalling.

This thesis has established that $F505p56^{lck}$ proteins enhanced TCR signalling (as reflected by increased TCR-induced tyrosine protein phosphorylation and elevated antigen-induced IL-2 production) by a process which required myristylation and/or membrane association, an intact site of autophosphorylation and the presence of intact SH3 and SH2 sequences.

This thesis has also demonstrated that $F505p56^{lck}$ enhanced tyrosine phosphorylation of PLC-γ 1, a process which also depended on similar Lck structural requirements. As tyrosine phosphorylation is associated with PLC-γ 1 enzymatic activation, these results suggest that PLC-γ 1 is functionally coupled to $p56^{lck}$.

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Enfin, j'exprime mon entière reconnaissance à mon ami Christian Foisy, qui m'a toujours encouragée dans mes entreprises.

APPENDIX

Contribution to other published manuscripts:

Veillette, A., L. Caron, M. Fournel, and T. Pawson. 1992. Regulation of the enzymatic function of the lymphocyte-specific tyrosine protein kinase $p56^{lck}$ by the non-catalytic SH2 and SH3 domains. Oncogene **7**: 971-980.

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Structural Requirements for Enhancement of T-Cell Responsiveness by the Lymphocyte-Specific Tyrosine Protein Kinase p56^{lck}

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To understand the mechanism(s) by which p56^{lck} participates in T-cell receptor (TCR) signalling, we have examined the effects of mutations in known regulatory domains of p56^{lck} on the ability of F505 p56^{lck} to enhance the responsiveness of an antigen-specific murine T-cell hybridoma. A mutation of the amino-terminal site of myristylation (glycine 2), which prevents stable association of p56^{lck} with the plasma membrane, completely abolished the ability of F505 p56^{lck} to enhance TCR-induced tyrosine protein phosphorylation. Alteration of the major site of in vitro autophosphorylation, tyrosine 394, to phenylalanine diminished the enhancement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}. Such a finding is consistent with the previous demonstration that this site is required for full activation of p56^{lck} by mutation of tyrosine 505. Strikingly, deletion of the noncatalytic Src homology domain 2, but not of the Src homology domain 3, markedly reduced the improvement of TCR-induced tyrosine protein phosphorylation by F505 Lck. Additional studies revealed that all the mutations tested, including deletion of the Src homology 3 region, abrogated the enhancement of antigen-triggered interleukin-2 production by F505 p56^{lck}, thus implying more stringent requirements for augmentation of antigen responsiveness by F505 Lck. Finally, it was also observed that expression of F505 p56^{lck} greatly increased TCR-induced tyrosine phosphorylation of phospholipase C-γ_1, raising the possibility that phospholipase C-γ_1 may be a substrate for p56^{lck} in T lymphocytes. Our results indicate that p56^{lck} regulates T-cell antigen receptor signalling through a complex process requiring multiple distinct structural domains of the protein.

Evidence that tyrosine protein phosphorylation plays a critical role in T lymphocyte activation is increasing (for reviews, see references 20 and 49). Indeed, tyrosine phosphorylation of a limited number of partially characterized substrates occurs rapidly after stimulation of the T-cell receptor (TCR) for antigen by either antigen and major histocompatibility complex (MHC) or anti-TCR antibodies (15). This biochemical signal is critical for T lymphocyte activation, as supported by the finding that tyrosine-specific protein kinase inhibitors (such as genistein or herbimycin A) prevent TCR-induced phosphatidylinositol (PI) turnover, increase in intracellular calcium, and lymphokine release (18, 30, 40, 43). As the TCR complex does not possess an intrinsic tyrosine protein kinase activity, it has been postulated that this process is mediated through the recruitment of membrane-associated tyrosine protein kinases.

The Src family of internal membrane tyrosine protein kinases comprises eight well-characterized members named c-Src, c-Fgr, c-Yes, Lck, Lyn, Hck, Fyn, and Blk (for a review, see reference 14). The members of this family have highly conserved structural features including (from the amino terminus to the carboxy terminus) (i) an amino-terminal glycine residue (glycine 2) which is required for myristylation and membrane association, (ii) a unique domain which differs in sequence for each member of the family, therefore potentially providing distinguishing properties to these various products, (iii) the Src homology 3 (SH3) domain, found also in a number of cytoskeletal constituents such as fodrin and the yeast actin-binding protein and presumed to mediate interactions with the cytoskeleton (for a review, see references 8 and 21), (iv) the Src homology 2 (SH2) region, which is additionally present in several signal transducers and amplifiers such as the γ isoform of phospholipase C (PLC-γ) and the GTPase-activating protein of p21^{ras} and which has been proposed to modulate enzyme-substrate interactions through its ability to bind phosphotyrosine-containing proteins (for a review, see references 8 and 21), (v) a prototypical catalytic domain, containing sites of ATP binding and in vitro autophosphorylation, and (vi) the short carboxy-terminal regulatory domain, which encompasses a conserved negative regulatory site of in vivo tyrosine phosphorylation.

The demonstration that the lymphocyte-specific *lck* gene product (25, 53), p56^{lck}, is associated with and regulated by the CD4 and CD8 T-cell surface antigens provided the first strong evidence for the involvement of Src-related tyrosine protein kinases in normal T-cell physiology (23, 34, 45, 47, for reviews, see references 37 and 44). It has since become progressively clear that the ability of CD4 and CD8 to enhance T-cell responsiveness to low doses of antigen or suboptimal antigen stimulation is largely mediated through interactions with p56^{lck} (26). Lck may also be involved in other T-cell signalling pathways, as suggested by descriptions of physical complexes between this polypeptide and other T-cell surface molecules such as the β chain of the interleukin-2 (IL-2) receptor (16) and glycophosphatidylinositol-anchored molecules such as Thy-1 (41).

The role of p56^{lck} in T-cell physiology was directly indicated by experiments in which a constitutively activated version of this tyrosine protein kinase (carrying a mutation of the negative regulatory carboxy-terminal site of tyrosine phosphorylation, tyrosine 505, to a nonphosphorylatable phenylalanine [F505 p56^{lck}]) was introduced in a CD4-nega-

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tive and CD8-negative class II MHC-restricted antigen-specific murine T-cell hybridoma (BI-141) (1). These studies showed that expression of F505 p56 [illegible] but not wild-type or kinase-deficient Lck polypeptides significantly enhanced T-cell responsiveness as indicated by increased TCR-induced tyrosine protein phosphorylation and IL-2 production (1). In addition to implying a biochemical mechanism by which CD4 and CD8 (and possibly other T-cell surface molecules) improve T-cell function, these observations also indicated that activated p56 [illegible] can participate in antigen receptor signalling in the absence of CD4 and CD8 expression.

As p56 [illegible] is not stably associated with the TCR complex (34, 46), the mechanism by which this enzyme contributes to TCR signalling is not implicitly evident. To better understand this process, we have evaluated the role of known structural domains of p56 [illegible] in the enhancement of TCR signalling by an activated version of this polypeptide (F505 $p56^{lck}$). Specifically, we have tested the roles of the sites of myristylation (glycine 2) and autophosphorylation (tyrosine 394) as well as of the noncatalytic SH3 and SH2 domains of $p56^{lck}$. The results of our experiments imply that p56 [illegible] enhances TCR signalling through a complex process which requires multiple independent structural domains of the protein.

MATERIALS AND METHODS

Cells. BI-141 is a CD4-negative and CD8-negative, class II MHC-restricted T-cell hybridoma specific for bovine insulin (33). The BI-141 cell line was grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, streptomycin, and penicillin. ψ2 retrovirus packaging cells transfected with cDNAs encoding A2F505 Lck, F394F505 Lck, ΔSH2F505 Lck, and ΔSH3F505 Lck were described elsewhere (2, 48). Briefly, the deletions of the SH3 and SH2 motifs removed amino acids 67 to 122 and 122 to 234 of $p56^{lck}$, respectively (48). The process of these deletions did not introduce any additional alteration in the Lck amino acid sequence (48). The ψ2 derivatives were maintained in α minimal essential medium containing 10% heat-inactivated fetal calf serum, penicillin, streptomycin, and the aminoglycoside G418 (500 μg/ml). BI-141 derivatives expressing the neomycin phosphotransferase gene (*neo*) alone or F505 $p56^{lck}$ have been described previously (1). The $A^k_\alpha A^k_\beta$ class II MHC-expressing L cells (LT5 [illegible]) were kindly provided by Ron Germain, National Institutes of Health, Bethesda, Md. The IL-2-dependent HT-2 T-cell clone was maintained in culture as described previously (29).

Retroviral infection. Retroviral infection of BI-141 cells was performed as outlined previously (1) by using retroviral supernatants obtained from established ψ2 packaging cell lines. BI-141 derivatives expressing the various mutant Lck proteins were generated by selection in medium containing 750 μg of G418 per ml. Monoclonal cell lines were established by limiting dilution. All cell lines selected for our studies displayed levels of TCR, CD3, Thy-1.2, and CD45 and growth characteristics (morphology and growth rate) that were identical to those of parent BI-141 cells. Moreover, all cells remained CD4 negative (data not shown).

Antibody-mediated aggregation of the TCR for antigen. Antibody-mediated aggregation of TCR was performed as outlined previously (1). Briefly, 5 × 10 [illegible] BI-141 T cells were incubated for 30 min on ice in serum-free RPMI 1640 medium containing saturating amounts (12 μg/ml) of the anti-TCR $V_\beta 8$ mouse monoclonal antibody (MAb) F23.1 (35). After the excess [illegible] antibody was washed off, aggregation was performed by [illegible] of excess amounts (60 μg/ml) of a second-step [illegible] rabbit anti-mouse [RAM] or sheep anti-mouse [SAM] immunoglobulin G [IgG]; Organon Teknika) and incubation at 37°C for the indicated period of time. Controls were [illegible] with the addition of the second-step antibody alone. Cells were then immediately lysed in boiling sample buffer or [illegible] [illegible] immunoprecipitation buffer. Lysates were processed [illegible] sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) or immunoprecipitation (see below).

Immunoblots. [illegible] were performed as [illegible] elsewhere (19) [illegible]. For Lck immunoblotting, we used antisera generated against a synthetic peptide corresponding to amino acids [illegible] of p56 [illegible] or a [illegible] fusion protein [illegible] amino acids 2 to 118 of this polypeptide (1). Antiphosphotyrosine immunoblotting was conducted with [illegible] purified polyclonal rabbit antiphosphotyrosine antibodies [illegible]. Anti-PLC-γ_1 immunoblots were performed with a previously described rabbit anti-PLC [illegible]. This antibody does not recognize PLC [illegible] of [illegible] products was accomplished by [illegible] protein A (Amersham) and subsequent [illegible]. For quantitation, bands were cut from the nitrocellulose membranes and counted in a gamma counter. The presence of equivalent amounts of cellular proteins in each lane was confirmed by amido black staining of nitrocellulose [illegible] (data not shown).

PLC-γ_1 immunoprecipitation. Cells were stimulated for the indicated periods of time with anti-TCR MAb F23.1 and SAM IgG (Organon Teknika) as described above. Cells were lysed in [illegible] TNE buffer (1% [illegible], 50 mM Tris [pH 8.0], 1% Nonidet P-40, and 2 mM EDTA [pH 8.0]) supplemented with 20 μg [illegible] of the protease inhibitors leupeptin, aprotinin, N-tosyl-L-phenylalanine chloromethyl ketone, N-p-tosyl-L-lysine chloromethyl ketone, and phenylmethylsulfonyl fluoride [illegible] as well as the phosphatase inhibitors sodium fluoride (200 mM) and sodium orthovanadate (2 mM). Postnuclear supernatants were first precleared with *Staphylococcus aureus* protein A (Pansorbin; Calbiochem) and then immunoprecipitated with the polyclonal rabbit anti-PLC-γ_1 serum mentioned above (19). Immune complexes were collected with *S. aureus* protein A and washed three times with TNE buffer containing 1 mM sodium orthovanadate. Proteins were eluted in sample buffer, boiled, and electrophoresed in SDS-PAGE gels. After transfer to nitrocellulose membranes, immunoprecipitates were probed by immunoblotting with either antiphosphotyrosine or anti-PLC [illegible] antibodies.

Antigen stimulation assays. BI-141 T cells (1 × 10 [illegible]) were plated in triplicate in 96-well Falcon tissue culture plates with 5 × 10 [illegible] irradiated (4,000 rad) $A^k_\alpha A^k_\beta$ class II MHC-expressing L cells and serial dilutions of the antigen bovine insulin (Sigma). After incubation at 37°C for 24 h, supernatants were collected and frozen for 1 h at −70°C to destroy carryover cells. IL-2 production was measured by testing the ability of the supernatants to stimulate [3H]thymidine incorporation in 10⁴ IL-2-dependent HT-2 indicator cells. This assay has been described in more detail elsewhere (1, [illegible]).

RESULTS

The site of Lck myristylation, glycine 2, is absolutely required for enhancement of TCR signalling by F505 $p56^{lck}$. We have previously reported that expression of a constitu-

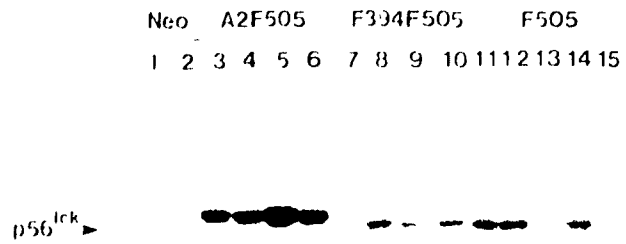

FIG. 1. Expression of A2F505 Lck and F394F505 Lck polypeptides in BI-141 T cells. Shown is an Lck immunoblot. Levels of p56lck in several independent BI-141 cell lines expressing myristylation-defective (A2F505 Lck) or autophosphorylation site (F394F505 Lck) F505 p56lck mutant were monitored by Lck immunoblot with a specific Lck antiserum (4). Lanes: 1, Neo 1; 2, Neo 2; 3, A2F505 2; 4, A2F505 5; 5, A2F505 4; 6, A2F505 6; 7, F394F505 3; 8, F394F505 11; 9, F394F505 12; 10, F394F505 22; 11, F505 12; 12, F505 15; 13, F505 9; 14, F505 13; 15, F505 18. The position of p56lck is indicated on the left. Exposure, 1 h.

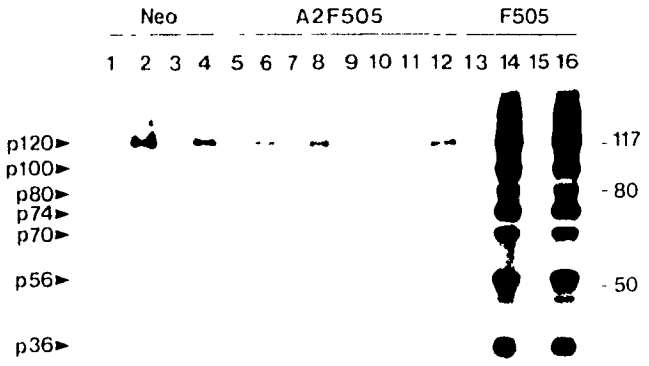

FIG. 2. Effects of expression of A2F505 p56lck polypeptides on TCR-induced tyrosine protein phosphorylation. The abundance of phosphotyrosine-containing proteins in BI-141 cells expressing the neomycin resistance marker alone (Neo; lanes 1 to 4), A2F505 p56lck (A2F505; lanes 5 to 12), or F505 p56lck (F505; lanes 13 to 16) was measured by antiphosphotyrosine immunoblotting. Antibody-mediated aggregation of TCR was performed for 2 min at 37°C. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 contain RAM IgG alone. Lanes 2, 4, 6, 8, 10, 12, 14, and 16 contain MAb C305 plus RAM IgG. Lanes 1 and 2, Neo 1; lanes 3 and 4, Neo 2; lanes 5 and 6, A2F505 1; lanes 7 and 8, A2F505 2; lanes 9 and 10, A2F505 4; lanes 11 and 12, A2F505 6; lanes 13 and 14, F505 7; lanes 15 and 16, F505 9. The positions of the major phosphotyrosine-containing proteins (on the left) and of presumed molecular mass markers (on the right) are marked. Exposure, 8 h.

tively activated version of p56lck tyrosine 505 to phenylalanine 505 Lck mutant [F505 p56lck] in the CD4-negative, CD8-negative, class II MHC-restricted bovine insulin-specific murine T-cell hybridoma BI-141 results in enhanced T-cell responsiveness (11). This functional improvement was indicated by augmented TCR-induced tyrosine protein phosphorylation and increased antigen plus MHC-triggered IL-2 production. Preliminary studies showed that F505 p56lck is neither associated with the TCR complex nor enzymatically activated or tyrosine phosphorylated upon TCR stimulation with anti-TCR antibodies (11). Thus, the process by which p56lck is recruited in the TCR signalling pathway appeared to be fundamentally different from that by which it is involved in CD4- and CD8-induced signal transduction events.

To better characterize the mechanism by which the activated version of p56lck (F505 p56lck) enhances TCR signalling, the effect of a point mutation relevant to disease (abolishing F505 Lck myristylation and membrane association) (3) was tested. BI-141 cells were infected with retroviruses encoding A2F505 p56lck and selected for growth in the presence of the aminoglycoside G418. Clonal cell lines were established by limiting dilution and tested for expression of the mutant Lck polypeptide by a specific anti-Lck immunoblot assay. Multiple independent BI-141-derivative cell lines contained amounts of A2F505 p56lck (Fig. 1, lanes 5 to 6) nearly similar (within twofold) to those of F505 p56lck (lanes 11 to 15) were selected for further analyses. Quantitative studies showed that both types of cell lines expressed 5- to 10-fold higher levels of p56lck than cells expressing the neomycin resistance alone (Neo cells) (lanes 1 and 2). As previously described (3), A2F505 p56lck polypeptides had a slightly retarded electrophoretic mobility in SDS-PAGE gels. Although the basis for this observation remains unclear, we have previously ruled out that it results from additional mutations in the A2F505 *lck* cDNA (3).

Cells were stimulated with anti-TCR MAb C305 and RAM IgG for 2 min, and net cellular tyrosine protein phosphorylation was assessed by antiphosphotyrosine immunoblotting (Fig. 2). This assay revealed that, unlike cells expressing F505 p56lck polypeptides (Fig. 2, lanes 13 to 16), cells containing A2F505 p56lck (lanes 5 to 12) failed to show an enhancement of anti-TCR antibody-induced tyrosine protein phosphorylation over Neo cells (lanes 1 to 4). Similar observations were made when TCR stimulation was extended up to 30 min (data not shown). These findings implied that myristylation and/or membrane association is absolutely required for F505 p56lck to contribute to TCR-induced tyrosine phosphorylation signalling events.

Mutation of the site of Lck autophosphorylation, tyrosine 394, partially reduces the enhancement of TCR signalling by F505 p56lck. Accumulating data suggest that tyrosine phosphorylation by activated tyrosine protein kinase receptors (such as the platelet-derived growth factor receptor) is dependent on binding of phosphorylated tyrosine residues on the receptor to SH2 sequences typically encountered in intracellular substrates (for reviews, see references 5 and 21). Tyrosine 394 is the major site of autophosphorylation of p56lck (2, 5, 10, 24, 51, 52). While tyrosine 394 is not normally phosphorylated in vivo, significant in vivo occupancy of tyrosine 394 is noted in the context of activation of p56lck by mutation of tyrosine 505 (2, 5, 24) or antibody-mediated aggregation of CD4 (23, 45). In both cases, activation of p56lck is significantly reduced by mutation of tyrosine 394 to phenylalanine (2, 50).

Thus, we wished to test the possibility that tyrosine 394 is pivotal for the enhancement of TCR-induced tyrosine protein phosphorylation by F505 p56lck. F505 Lck polypeptides carrying a tyrosine to phenylalanine substitution at position 394 (F394F505 p56lck) were introduced in BI-141 cells, as described above. Cell lines expressing amounts of Lck protein (Fig. 1, lanes 7 to 10) similar to those noted with cells expressing F505 p56lck (lanes 11 to 15) were identified by Lck immunoblot and subjected to antibody-mediated TCR stimulation.

An antiphosphotyrosine immunoblot of lysates from control or TCR-stimulated cells (Fig. 3A) revealed that, in comparison with Neo cells (lanes 1 to 4), cells containing F394F505 p56lck (lanes 5 to 10) showed enhanced TCR-induced tyrosine protein phosphorylation (measured after 2

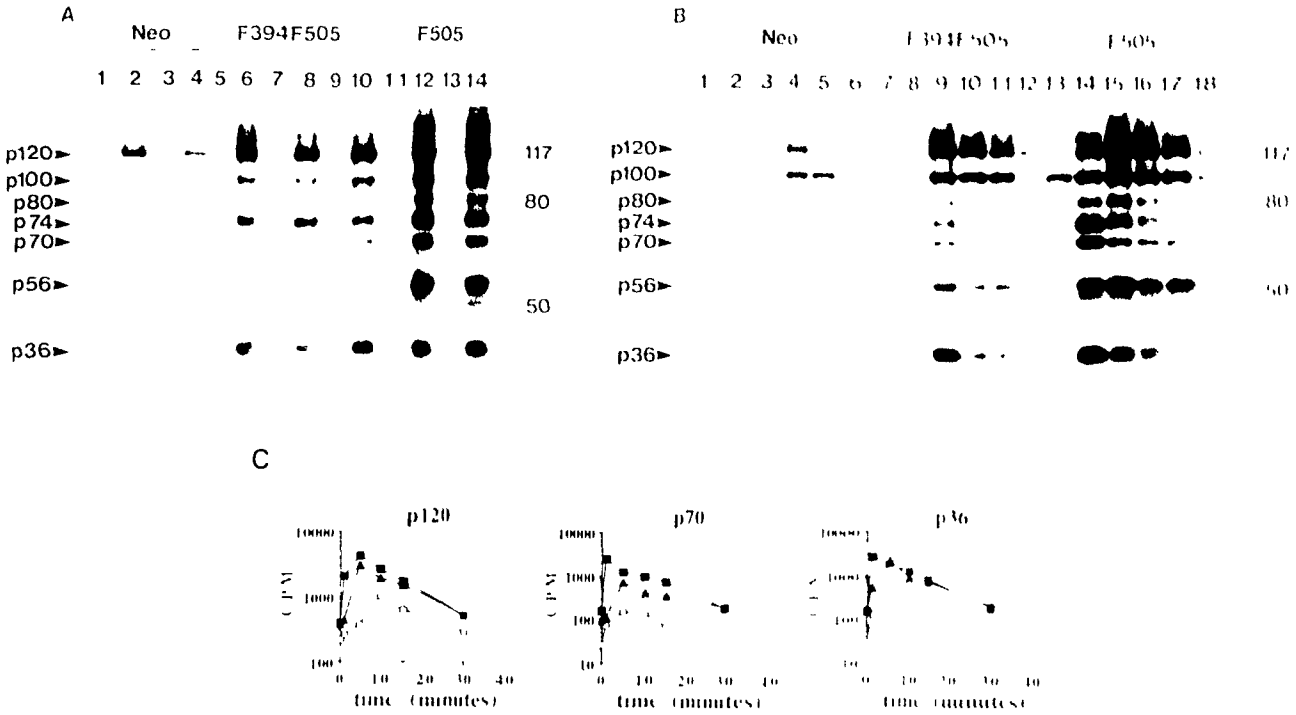

FIG. 3. Effects of expression of F394F505 pp56 polypeptides on TCR-induced tyrosine protein phosphorylation. (A) Antiphosphotyrosine immunoblot. Cells expressing the neomycin resistance marker alone (Neo; lanes 1 to 6), F394F505 (lanes 6 to 10), or F505 pp56^{lck} (F505; lanes 11 to 14) were tested for TCR-induced tyrosine protein phosphorylation. Antibody-mediated aggregation of TCR was performed for 2 min at 37°C (lanes 1, 3, 5, 7, 9, 11, and 13 contain RAM IgG alone). Time = 0 (lanes 2, 4, 6, 8, 10, 12, and 14 contain MAb F23.1 plus RAM IgG; lanes 1 and 2, Neo 1; lanes 3 and 4, Neo 2; lanes 5 and 6, F394F505-11; lanes 7 and 8, F394F505-3; lanes 9 and 10, F394F505-22; lanes 11 and 12, F505-13; and lanes 13 and 14, F505-9. The positions of major phosphotyrosine-containing proteins (on the left) and of prestained molecular weight markers (on the right) are indicated. Exposure, 8 h. (B) Time course experiment. Monoclonal cell lines expressing the neomycin resistance marker alone (Neo 2; lanes 1 to 6), F394F505 pp56 (F394F505.22; lanes 7 to 12), or F505 pp56^{lck} (F505-3; lanes 13 to 18) were stimulated for various time periods at 37°C (lanes 1, 7, and 13, RAM IgG alone [1 min]; lanes 2 to 6, 8 to 12, and 14 to 18, MAb F23.1 plus RAM IgG [2, 3, 9, and 15, 5 min; 4, 10, and 16, 10 min; 5, 11, and 17, 15 min; 6, 12, and 18, 30 min]). The positions of major phosphotyrosine-containing proteins (on the left) and of prestained molecular weight markers (on the right) are indicated. Exposure, 8 h. (C) Quantitative analysis. Quantitation of the changes in tyrosine protein phosphorylation of the 120-(p120), 70- (p70), and 36-kDa (p36) polypeptides was performed by cutting the appropriate bands and counting in a gamma counter. Ordinate, count per minute (CPM) (arithmetic scale); abscissa, time (in minutes) (linear scale). Symbols: ●, Neo 2; ▲, F394F505-22; ■, F505-3.

min of stimulation). While similar sets of cellular substrates were affected by expression of F394F505 p56^{lck} and F505 p56^{lck}, the enhancement by F394F505 p56^{lck} was less than that conferred by F505 p56^{lck} (lanes 11 to 14). Time course experiments with representative cell lines supported these assertions (Fig. 3B and C), although these quantitative differences tended to diminish after 5 min of stimulation. Taken together, these results are consistent with the previous demonstration that the catalytic activity of F394F505 p56^{lck} is lower than that of F505 Lck (7). They also imply that tyrosine 394 is not absolutely required for activated p56^{lck} molecules to participate in TCR-induced signalling events.

Expression of SH2 and SH3 domain deletion mutants of F505 Lck in BI-141.1 cells. Despite the fact that the SH2 and SH3 domains of cytoplasmic tyrosine protein kinases seem dispensable for the catalytic function of these products, accumulating data suggest that they modulate enzyme-substrate interactions (see 21). Therefore, to evaluate the potential involvement of these noncatalytic sequences in the enhancement of TCR signalling by F505 Lck, BI-141.1 cells expressing F505 Lck polypeptides lacking either the SH2 (ΔSH2F505 Lck) or the SH3 (ΔSH3F505 Lck) motif were generated, as described in Materials and Methods. Importantly, previous studies conducted with NIH 3T3 cells revealed that ΔSH2F505 Lck and ΔSH3F505 Lck exhibit enzymatic activities apparently equal to that of F505 p56^{lck} (48).

Cell lines were first tested for expression of the mutant Lck polypeptides by immunoblot by using a rabbit anti-Lck serum generated against a synthetic peptide corresponding to amino acids 39 to 61 of the murine p56^{lck} sequence. As this sequence is located within the unique domain of p56^{lck}, the antiserum is presumed to equally recognize the different Lck polypeptides examined. Deletion of the SH2 and SH3 sequences generated Lck proteins migrating at 53 and 51 kDa in SDS-PAGE, respectively (Fig. 4). Consequently, these products were termed p53^{ΔSH2} and p51^{ΔSH3}. After the abundance of the indicated polypeptides was taken into consideration, cell lines expressing amounts of ΔSH2 (lane 3 to 6) and ΔSH3 (lane 7 to 9) F505 Lck protein comparable (within twofold) to those of F505 p56^{lck} (lane 10 and 11) were selected for additional study. Importantly, the two different deletion mutants were expressed at equivalent levels in the cells. The modification (twofold) in the abundance of endogenous Lck polypeptides in the cell

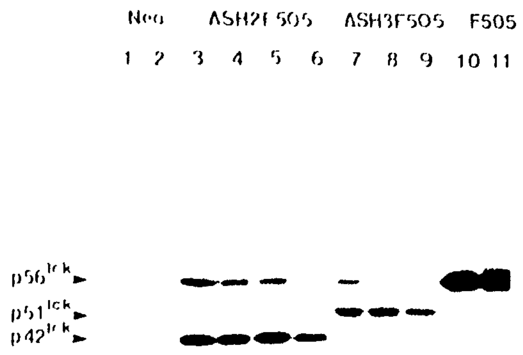

FIG. 4. Expression of ΔSH2F505 Lck and ΔSH3F505 Lck polypeptides in BI-141 T cells. Shown is an anti-Lck immunoblot. Levels of Lck protein in several independent BI-141 cell lines expressing the neomycin resistance marker alone (Neo; lanes 1 and 2), ΔSH2F505 Lck (ΔSH2F505; lanes 3 to 6), ΔSH3F505 Lck (ΔSH3F505; lanes 7 to 9), or F505 p56 (F505; lanes 10 and 11) were [illegible] as in Fig. 1, except that an antiserum generated against a synthetic peptide corresponding to amino acids 39 to 54 of the murine p56 sequence [illegible] Lanes: 1, Neo-1; 2, Neo-2; 3, ΔSH2F505-4; 4, ΔSH2F505-[illegible]; 5, [illegible] ΔSH2F505-9; 6, ΔSH2F505-20; 7, ΔSH3F505-[illegible]; 8, ΔSH3F505-[illegible]; 9, ΔSH3F505-21; 10, F505-7; 11, F505-9. The relative migration of p56lck, p51lck, and p42lck are indicated on the left. Exposure, [illegible] days. For quantitation, bands were cut from nitrocellulose filters and counted in a gamma counter (data not shown). After taking into consideration the abundance of the endogenous Lck polypeptide, we established that the levels of ΔSH2F505 Lck and ΔSH3F505 Lck were approximately 50% those of F505 p56lck. Similar quantitations were achieved when serial dilutions of lysates from F505 Lck-expressing cells were used for comparison (data not shown).

were within the range previously noted for a series of independent neomycin phosphotransferase-expressing BI-141 cell lines (1).

Deletion of the noncatalytic SH2 domain of p56^{lck} significantly alters the improvement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}. BI-141 cells expressing ΔSH2F505 or ΔSH3F505 Lck polypeptides were tested for TCR-induced tyrosine phosphorylation changes, as outlined above. Although ΔSH2F505 Lck (Fig. 5A, lanes 5 to 12) consistently enhanced TCR-induced tyrosine protein phosphorylation, this improvement was markedly less than that provided by F505 p56 (lanes 13 to 16). In contrast, expression of ΔSH3F505 Lck polypeptides (Fig. 5B, lanes 5 to 10) increased the TCR-induced signal in a manner analogous to that mediated by introduction of F505 p56 (lanes 11 to 14). It should be noted that neither ΔSH2F505 Lck nor ΔSH3F505 Lck augmented tyrosine protein phosphorylation in unstimulated cells to a greater degree than F505 p56^{l}. This finding implies that the process by which levels of phosphotyrosine are suppressed prior to TCR stimulation in F505 p56-expressing BI-141 cells is independent of regulation through the SH2 or SH3 sequences (1).

Kinetic analyses of representative cell lines (Fig. 5C and D) showed that, in contrast to the enhancement seen in cells expressing F505 p56 (Fig. 5C, lanes 13 to 18, and Fig. 5D, lanes 13 to 18) or ΔSH3F505 Lck (Fig. 5D, lanes 7 to 12), the improvement of TCR-induced tyrosine protein phosphorylation by expression of ΔSH2F505 Lck polypeptides (Fig. 5C, lanes 7 to 12) was short lived. Indeed, after 5 min of TCR stimulation the pattern of tyrosine protein phosphorylation observed in these cells was essentially similar to that of Neo cells (Fig. 5C, lanes 1 to 6). This is substantiated by quantitative analyses of the 120-, 70-, and 36-kDa substrates (Fig. 5E and F). Similar observations were made with other ΔSH2F505 Lck-expressing BI-141 cell lines (data not shown). The increase in baseline tyrosine phosphorylation of certain substrates noted in ΔSH3F505-15 cells (Fig. 5D, lane 7) was not seen in other experiments (see Fig. 5B, lane 7). A similar increase in baseline phosphorylation was at times also noted in some F505 Lck-expressing cell lines (Fig. 3A, lane 11; Fig. 3B, lane 13; and Fig. 5C, lane 13). While the reason for this variation is unclear, such a phenomenon does not affect TCR-induced tyrosine protein phosphorylation responses.

Effects of activated Lck variants on antigen receptor-induced tyrosine phosphorylation of PLC-γ_1. The identities of the polypeptides undergoing tyrosine phosphorylation upon TCR stimulation are largely undefined. However, recent studies indicate that antigen receptor stimulation results in rapid tyrosine phosphorylation of the PI-specific PLC-γ_1 isoenzyme (32, 36, 54). This biochemical alteration is thought to be responsible for the increase in phospholipase activity and the elevation in PI turnover noted during T-cell activation (30, 31, 32, 36, 54).

Consequently, to gain additional understanding of the mechanism by which F505 p56 enhances T-cell functions, the effects of expression of this polypeptide on TCR-induced tyrosine phosphorylation of PLC-γ_1 were tested (Fig. 6). Cells expressing the *neo* gene alone (Neo) or F505 p56^{lck} were stimulated with anti-TCR MAb F23.1 and SAM IgG for 1 min and lysed in Nonidet P-40-containing buffer. PLC-γ_1 was isolated by immunoprecipitation with a specific rabbit anti-PLC-γ_1 serum. The extent of PLC-γ_1 tyrosine phosphorylation was evaluated by antiphosphotyrosine immunoblotting (Fig. 6A). Unlike Neo cells (lanes 1-4), cells containing F505 p56^{lck} (lanes 5 to 8) demonstrated detectable tyrosine phosphorylation of a 150-kDa polypeptide after TCR stimulation (lanes 6 and 8). This product was not recovered when normal rabbit serum was used as the immunoprecipitating antibody (lanes 9 and 10) and was consistent with PLC-γ_1 (32, 36, 39, 55). As is the case for overall tyrosine protein phosphorylation, no significant tyrosine phosphorylation of PLC-γ_1 was noted prior to TCR stimulation (lanes 1, 3, 5, and 7). Importantly, the increased TCR-induced tyrosine phosphorylation of PLC-γ_1 in F505 p56^{lck}-expressing cells was not secondary to elevated levels of PLC-γ_1, as indicated by anti-PLC-γ_1 immunoblotting of PLC-γ_1 immunoprecipitates (Fig. 6B).

We also explored the impact of mutation of tyrosine 394 or deletion of the SH2 or SH3 domain on the enhancement of TCR-induced PLC-γ_1 tyrosine phosphorylation by F505 p56^{lck} (Fig. 6C to E). Time course studies were performed with representative cells expressing F394F505 Lck (Fig. 6C), ΔSH2F505 Lck (Fig. 6D), and ΔSH3F505 Lck (Fig. 6E). Patterns of PLC-γ_1 tyrosine phosphorylation were compared with those of cells expressing the neomycin resistance gene alone (Neo) or F505 p56^{lck}. First, in agreement with other reports (32, 36, 54), these experiments collectively demonstrated a small and reproducible increase (between 2 and 5 min) in PLC-γ_1 tyrosine phosphorylation after TCR stimulation of Neo cells (Fig. 6 and data not shown). Expression of F505 p56^{lck} resulted in a prominent enhancement (approximately 10-fold) of TCR-induced PLC-γ_1 tyrosine phosphorylation which was maintained over the time period analyzed

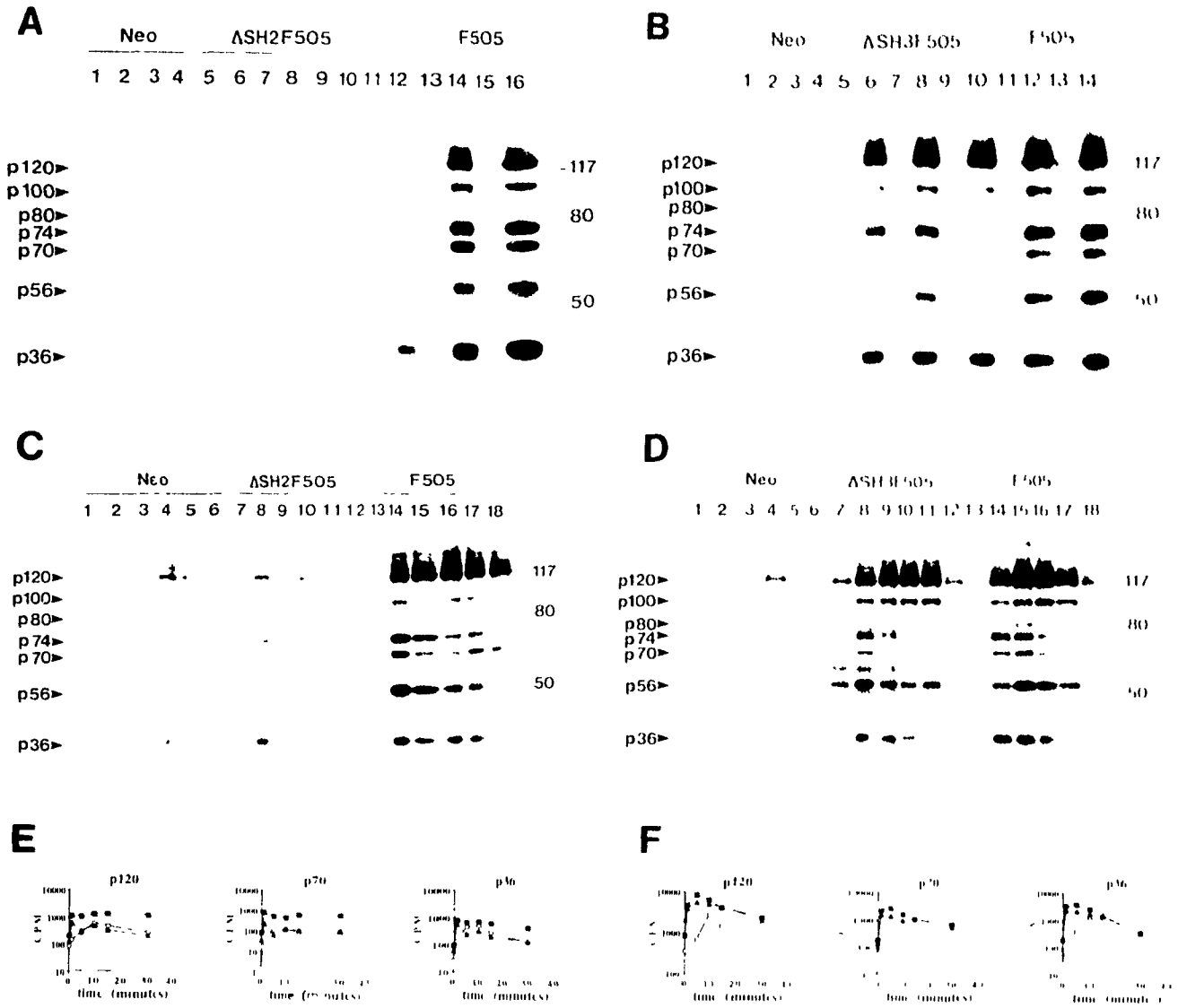

FIG. 5. Effects of SH2 or SH3 domain deletions on the ability of F505 Fck to enhance TCR-induced tyrosine protein phosphorylation. (A) TCR-induced tyrosine protein phosphorylation (antiphosphotyrosine immunoblot). Immunoblotting was same as for Fig. 3 except that cell lines expressing the neomycin resistance marker alone (Neo; lanes 1 to 4), ΔSH2F505 Fck (ΔSH2F505; lanes 5 to 8), or F505 p56^{lck} (F505; lanes 13 to 16) were tested. Moreover, TCR stimulation was performed for 1 min. Lanes 1, 3, 5, 7, 9, 11, 13, and 15 contain RAM IgG alone; lanes 2, 4, 6, 8, 10, 12, 14, and 16 contain MAb F23.1 plus RAM IgG. Lanes 1 and 2, Neo 1; lanes 3 and 4, Neo 2; lanes 5 and 6, ΔSH2F505 7; lanes 7 and 8, ΔSH2F505 7; lanes 9 and 10, ΔSH2F505 9; lanes 11 and 12, ΔSH2F505 20; lanes 13 and 14, F505 7; lanes 15 and 16, F505 9. The positions of the major phosphotyrosine-containing polypeptides (on the left) and of prestained molecular mass markers (on the right) are indicated. Exposure, 6 h. (B) TCR-induced tyrosine protein phosphorylation (antiphosphotyrosine immunoblot). Procedure was same as for panel A except that cell lines expressing the neomycin resistance marker alone (Neo; lanes 1 to 4), ΔSH3F505 Fck (ΔSH3F505; lanes 5 to 10), or F505 p56^{lck} (F505; lanes 11 to 14) were tested. Anti-TCR antibody stimulation was for 1 min. Lanes 1, 3, 5, 7, 9, 11, and 13 contain RAM IgG alone; lanes 2, 4, 6, 8, 10, 12, and 14 contain MAb F23.1 plus RAM IgG. Lanes 1 and 2, Neo 1; lanes 3 and 4, Neo 2; lanes 5 and 6, ΔSH3F505 15; lanes 7 and 8, ΔSH3F505 15; lanes 9 and 10, ΔSH3F505 24; lanes 11 and 12, F505 7; lanes 13 and 14, F505 9. The position of the major phosphotyrosine-containing protein (on the left) and of prestained molecular weight markers (on the right) are indicated. Exposure, 11 h. (C) Effect of expression of ΔSH2F505 Fck polypeptides in a time course experiment. Procedure and lane contents are same as for Fig. 3B; the one time point were examined. Lanes 1 to 6, Neo 2; 7 to 12, ΔSH2F505 3; 13 to 18, F505 9. Exposure, 24 h. (D) Effects of expression of ΔSH3F505 Fck polypeptides in a time course experiment. Procedure and lane contents are same as for Fig. 3B. Lanes 1 to 6, Neo 1; 7 to 12, ΔSH3F505 15; 13 to 18, F505 9. Exposure, 5 h. (E and F) Quantitative analyses of the experiments depicted in panels C and D, respectively. Procedure and abbreviations are same as for Fig. 3C. Symbols: ○, Neo; ▲, ΔSH2F505 3 or ΔSH3F505 15; ■, F505 9.

(up to 10 min). The effects of mutations or deletions in F505 p56^{lck} on PLC-γ_1 tyrosine phosphorylation resembled those noted earlier for overall tyrosine protein phosphorylation (Fig. 3 and 5). Indeed, F394F505 p56^{lck}-expressing BI-141 cells (Fig. 6C) showed an intermediate increase in TCR-induced tyrosine phosphorylation of PLC-γ_1. Moreover, the improvement in PLC-γ_1 tyrosine phosphorylation by F505 Fck was greatly reduced by deletion of the SH2 sequence (Fig. 6D) while it was less affected by deletion of the SH3 region (Fig. 6E).

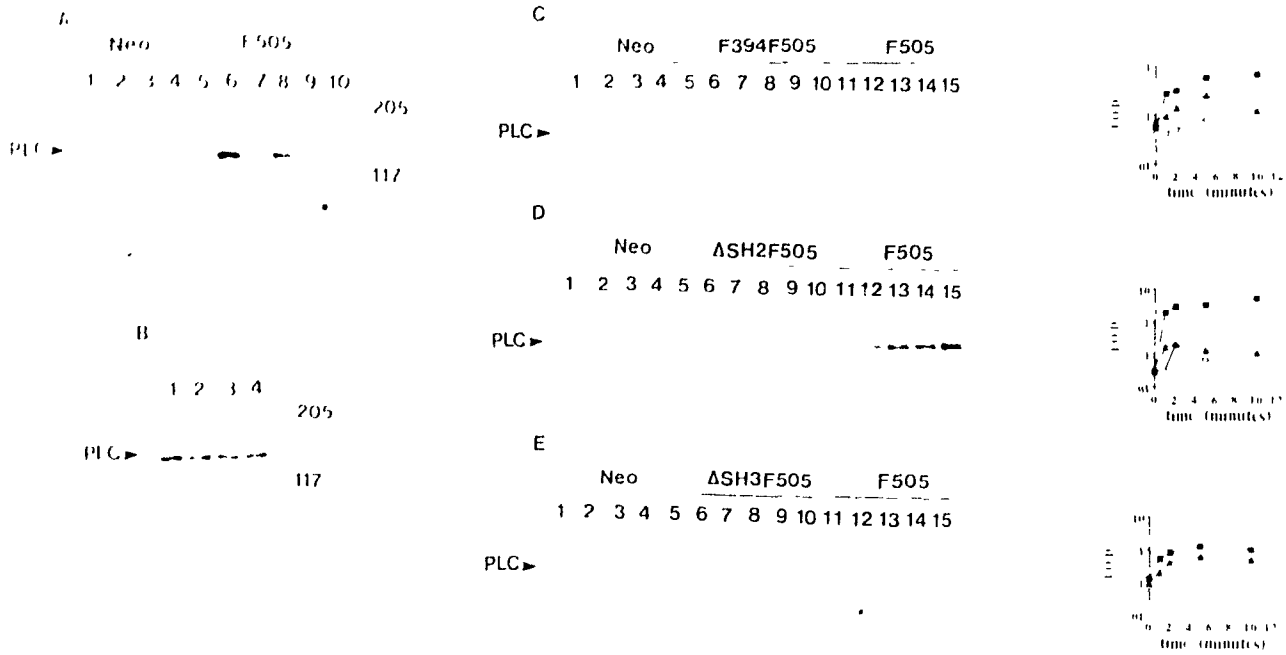

FIG. 6. TCR-induced tyrosine phosphorylation of PLC-γ1. (A) Effects of F505 p56^{lck} expression on the extent of TCR-induced tyrosine phosphorylation of PLC-γ1. Shown is an antiphosphotyrosine immunoblot. Cells were stimulated with anti-TCR MAb F23.1 plus SAM IgG for 1 min at 37°C (lanes 2, 4, 6, 8, and 10) or SAM IgG alone (lanes 1, 3, 5, 7, and 9). Lysates were immunoprecipitated with either a specific polyclonal rabbit anti-PLC-γ1 serum (lanes 1 to 8) or normal rabbit serum (lanes 9 and 10). Lanes 1 and 2, Neo 1-3 and 1-4; lanes 3 and 4, Neo 2-5 and 6; lanes 5 to 10, F505-9. Tyrosine phosphorylated proteins were detected by immunoblotting with antiphosphotyrosine antibodies. The position of PLC-γ1 (PLC) is indicated on the left, while those of prestained molecular mass markers are shown on the right. Exposure, 3 days. (B) Anti-PLC-γ1 immunoblot. Cells were stimulated as for panel A, and PLC-γ1 was recovered by immunoprecipitation. The abundance of PLC-γ1 in the various immunoprecipitates was assessed by immunoblotting with the rabbit anti-PLC-γ1 serum described other. Lanes 1 and 2, Neo 1-3 and 1-4; lanes 3 and 4, F505-9. Lanes 1 and 3 contain SAM IgG alone (1 min); lanes 2 and 4 contain MAb F23.1 plus SAM IgG (1 min). The position of PLC-γ1 (PLC) is indicated on the left, while those of prestained molecular mass markers are shown on the right. Exposure, 3 h. (C) Effects of F394F505 p56^{lck} expression on TCR-induced PLC-γ1 tyrosine phosphorylation in a time course experiment. Lanes 1 to 5, Neo 1-3; lanes 6 to 10, F394F505-8 (▲); 11 to 15, F505-9 (■). Lanes 1 to 5 and 11 to 15 contain SAM IgG alone (1 min); lanes 2 to 5, 7 to 10, and 12 to 15 contain MAb F23.1 plus SAM IgG (lanes 2, 7, and 12, 1 min; lanes 3, 8, and 13, 3 min; lanes 4, 9, and 14, 5 min; lanes 5, 10, and 15, 10 min). Exposure, 3 days. Bands were quantitated by densitometry by using a BioImage (Millipore). A graphic representation of this quantitation is shown on the right. Ordinate, integrated optical density (I.O.D.) (logarithmic scale); abscissa, time (in minutes) (linear scale). (D) Effects of SH2 deletion on the enhancement of TCR-induced PLC-γ1 tyrosine phosphorylation by F505 p56^{lck} in a time course experiment. Lane contents are same as for panel C. Lanes 1 to 5, Neo 1-3; 6 to 10, ΔSH2F505-8 (▲); 11 to 15, F505-9 (■). Exposure, 3 days. (E) Effects of SH3 deletion on the enhancement of TCR-induced PLC-γ1 tyrosine phosphorylation by F505 p56^{lck} in a time course experiment. Lane contents are same as for panel C. Lanes 1 to 5, Neo 1-3; 6 to 10, ΔSH3F505-15 (▲); 11 to 15, F505-9 (■). Exposure, 4 days.

Effects of Lck mutants on antigen responsiveness. We have previously found that expression of F505 p56^{lck}, but not wild-type or kinase-deficient Lck polypeptides, enhanced lymphokine (IL-2) production in response to antigen plus MHC stimulation (1). Consequently, we also analyzed the antigen responsiveness of BI-141 cells expressing V2F505, F394F505, ΔSH2F505, or ΔSH3F505 Lck. Cells were stimulated with various concentrations of bovine insulin in conjunction with the appropriate class II MHC molecules ($A^k_\alpha A^d_\beta$). After 24 h, supernatants were collected and tested for IL-2 content by using the IL-2-sensitive cell line HT-2. Responsiveness of the various cell lines was compared with that of cells expressing the neomycin resistance marker alone (Neo) or F505 p56^{lck}. Each panel of cell lines was analyzed in at least three independent assays. Results of representative assays are shown in Fig. 7. These revealed that mutation of the site of myristylation (Fig. 7A) or autophosphorylation (Fig. 7B) or deletion of the SH2 (Fig. 7C) or SH3 (Fig. 7D) sequence completely abolished the ability of F505 Lck to enhance lymphokine production in response to antigen plus MHC stimulation.

DISCUSSION

Mutation of glycine 2 of p56^{lck} to alanine has previously been shown to prevent myristylation and membrane association of this polypeptide (2). Data presented in this report demonstrate that this amino acid substitution precludes enhancement of TCR-induced tyrosine protein phosphorylation by F505 p56^{lck}. Thus, myristylation and/or membrane association is required for participation of Lck in the TCR signalling pathway. Even though the exact basis for this requirement remains to be clarified, such modifications would certainly favor the interaction of F505 Lck with membrane-bound molecules, possibly the TCR-regulated substrates themselves or molecular intermediates linking p56^{lck} to the TCR signalling pathway (such as the ζ subunit of TCR or the 70-kDa TCR-associated molecule / ZAP-70 [11]). In addition, membrane binding may facilitate further post-translational modifications which improve the efficiency of F505 p56^{lck} at participating in TCR signalling events. Consistently, we have previously shown, with NHH-3H3 cells, that the enzymatic activity of V2F505 p56^{lck} is less than that

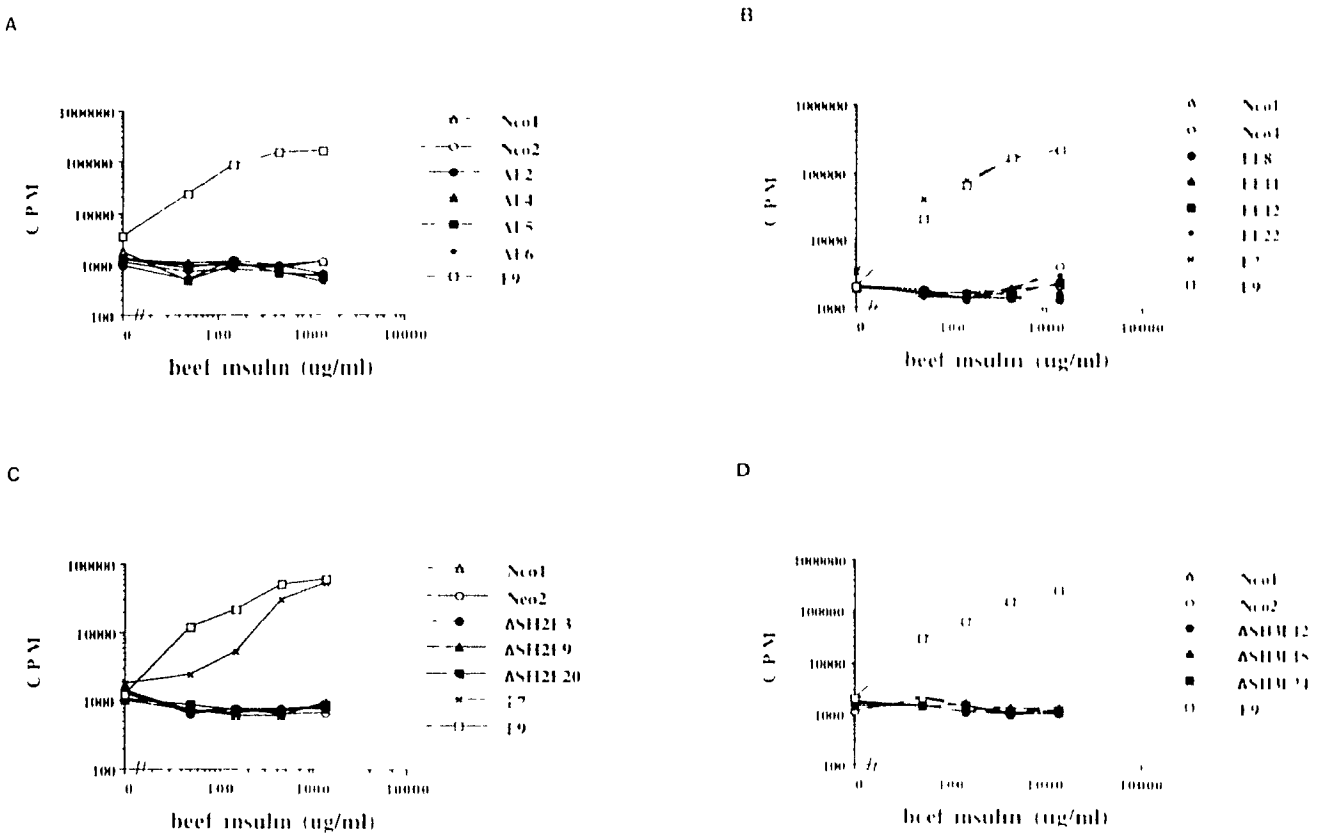

FIG. 7. Antigen stimulation assays. The effects of expression of various Lck mutants on antigen responsiveness of BI-141 cells were assessed by measuring lymphokine production in response to various concentrations of the antigen bovine insulin presented in the context of the appropriate class II MHC molecules ($A^k_1A^k_2$) as described in Materials and Methods. BI-141 clones expressing the neomycin resistance marker alone (Neo) or F505 $p56^{lck}$ (E) were compared with several independent cell lines expressing AE1-505 $p56^{lck}$ (AE; panel A), F394E-505 $p56^{lck}$ (EE; panel B), ΔSEH2F-505 Lck (ΔSEH2F; panel C), or ΔSEH3E-505 Lck (ΔSEH3E; panel D). Ordinates, [^{3}H]thymidine incorporation in counts per minute (CPM) (logarithmic scale); abscissas, concentration of beef insulin (in micrograms per milliliter) (logarithmic scale). Spontaneous IL-2 production is shown at the zero point on the abscissa.

of F505 $p56^{lck}$, possibly because myristylation and/or membrane association is required for adequate in vivo phosphorylation of F505 $p56^{lck}$ at tyrosine 394 (2).

The major site of in vitro autophosphorylation of $p56^{lck}$ tyrosine 394, is involved in positive regulation of the catalytic activity of $p56^{lck}$. Substitution of this residue by phenylalanine interferes with activation of Lck by tyrosine 505 mutation (2) or antibody-mediated aggregation of CD4 (50). In keeping with these observations, we noted that the intensity of the TCR-induced tyrosine phosphorylation signal in BI-141 cells expressing F394E-505 $p56^{lck}$ was intermediate between those of cells expressing F505 $p56^{lck}$ or the neomycin resistance marker alone. Therefore, the decreased enzymatic function of F394E-505 Lck is presumably responsible for its diminished capability to improve TCR signaling. Importantly, these data also indicate that, unlike signal transduction through tyrosine protein kinase receptors (8, 21), $p56^{lck}$-mediated signals in T-cell activation do not require the interaction of the Lck autophosphorylation site with tyrosine phosphorylation substrates. One caveat to this interpretation is that F505 $p56^{lck}$ may undergo tyrosine phosphorylation at sites other than tyrosine 394. However, this possibility seems unlikely, as metabolic labeling and peptide mapping studies have failed to reveal the presence of such sites in F505 $p56^{lck}$ molecules from resting or TCR-stimulated cells (3).

The SH3 domain of $p56^{lck}$ is not required for enhancement of anti-TCR antibody-induced tyrosine protein phosphorylation by F505 Lck (although deletion of this sequence abrogates improvement of antigen-induced IL-2 production, see below). These results are in agreement with those of previous studies conducted with NIH-3T3 cells, which revealed that the catalytic activity and oncogenic potential of ΔSEH3E-505 Lck are equivalent to those of F505 $p56^{lck}$ (48). In contrast to the limited impact of deletion of the SH3 region on TCR signaling, removal of the SH2 domain greatly affected the ability of F505 Lck to augment TCR-induced tyrosine protein phosphorylation. Indeed, expression of ΔSEH2F-505 Lck at best allowed a minor and transient improvement of TCR-induced tyrosine protein phosphorylation.

Earlier studies have shown that the SH2 motif of activated tyrosine protein kinases, such as Src or v-Fps, is critical for the full oncogenic potential of these enzymes, although they appear to be dispensable for the elevated catalytic activity of these products (6, 7, 22, 39). It has been proposed that this function relates to the ability of SH2 domains to associate with phosphotyrosine-containing proteins (5, 31). As the tyrosine protein kinase activity of F505 Lck is seemingly not altered by deletion of the SH2 region (48), our findings are highly consistent with the idea that the SH2 sequence does modulate the interaction of activated Lck molecules with

protein phosphorylation substrates. Moreover, we have found that bacterial fusion proteins containing the SH2 domain of p56^{lck} bind several of the phosphotyrosine-containing proteins from stimulated T cells (9). On the basis of these findings, we postulate that the SH2 domain may stabilize or prolong the interaction of p56^{lck} with protein such as ZAP-70 or TCR-regulated substrates. The SH2 motif may additionally protect these polypeptides from dephosphorylation by tyrosine phosphatases such as CD45.

While searching for potentially relevant substrates of p56^{lck} in T cells, we observed that the TCR-induced tyrosine phosphorylation of PLC-γ1 (but not PLC-γ2) was markedly increased (approximately 10-fold) by expression of E505 p56^{lck}. This response was affected by mutation of tyrosine 394 and deletion of the SH2 domain in manners comparable with those reported above for overall TCR-induced tyrosine protein phosphorylation. These observations provide evidence that the activity of PLC-γ1 can also be regulated by nonreceptor tyrosine protein kinases. As we have been unable to detect association of PLC-γ1 with p56^{lck}, it is conceivable that these polypeptides interact through the intermediate of another molecule. In light of the fact that bacterially produced SH2 sequences from p56^{lck} and PLC-γ1 can bind apparently similar tyrosine phosphorylated proteins from stimulated T cells (9), one of these polypeptides may serve as the bridge between Lck and PLC-γ1.

Despite the differential effects of the tested mutations or deletion on TCR-induced tyrosine protein phosphorylation, all alterations were found to abrogate enhancement of anti-CD4 plus MHC-induced lymphokine production by E505 p56^{lck}. This is especially surprising in the case of F394E505 Lck and YSEH3-505 Lck, which allowed significant enhancement of the anti-TCR antibody-induced signal. Such a discrepancy is perhaps indicative of unsuspected qualitative alterations of the augmented TCR-induced signal as a result of these additional mutations. Indeed, tyrosine 394 or the SH3 region may be required for phosphorylation of substrates poorly represented on antiphosphotyrosine immunoblots of total cell lysates. Alternatively, the use of high-affinity anti-TCR antibodies for T cell triggering may obliterate subtle but meaningful differences in antigen receptor-induced tyrosine phosphorylation. As we have been unable to conclusively study changes in intracellular tyrosine protein phosphorylation after interleukin plus MHC stimulation of BI-141 cells (9), additional efforts will be necessary to test these different possibilities.

In summary, the data presented herein imply that E505 p56^{lck} molecules enhance TCR signalling by a process which requires myristylation and/or membrane association of p56^{lck}. Moreover, this mechanism is dependent on increased p56^{lck} activity, as indicated by the facts that wild-type Lck fails to enhance these signals (1), while F394E505 p56^{lck} allows a partial improvement of TCR signalling. Importantly, the nonmyristylated SH2$^+$ sequence (but not the SH3 sequence) is required for robust and sustained enhancement of TCR-induced tyrosine protein phosphorylation by E505 p56^{lck}. Through its ability to physically associate with tyrosine phosphorylated proteins (9), the SH2$^+$ motif may modulate the interaction of p56^{lck} with the TCR signalling pathway. Interestingly, all the mutations tested, including deletion of the SH3 region, abrogated the enhancement of interleukin-2 (IL-2) production by E505 Lck, thus implying more stringent requirements for enhancement of interleukin responsiveness by E505 Lck. Finally, we observed that expression of E505 p56^{lck} greatly enhanced TCR-induced tyrosine phosphorylation of PLC-γ1, suggesting that PLC-γ1 may be a substrate for p56^{lck} in T lymphocytes.

As the TCR complex does not possess an intrinsic tyrosine protein kinase activity, the mechanism by which this receptor transduces an intracellular tyrosine protein phosphorylation signal during T cell activation has been the object of intense investigations. As discussed above, it is becoming increasingly clear that the lymphocyte-specific tyrosine protein kinase p56^{lck} can participate in this process. Other findings indicate that p59^{fyn}, another Src-related enzyme abundantly expressed in T lymphocytes (13), can also contribute to this signalling pathway (17). It is therefore of great importance that future studies be aimed at defining whether these two products regulate T cell functions through similar, overlapping, or different mechanisms.

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