Title
The role of the SYK protein tyrosine kinase in T cell activation and development

Author
Chu, David Hwa-En

Publication Date
1998

Peer reviewed|Thesis/dissertation

THE ROLE OF THE SYK PROTEIN TYROSINE KINASE
IN T CELL ACTIVATION AND DEVELOPMENT

by

DAVID HWA-EN CHU

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

MICROBIOLOGY AND IMMUNOLOGY

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Date **University Librarian**

Degree Conferred: . . . . . .

In loving memory of my Dad, Dr. Shu-Yuan Chu,
who taught me to be passionate about science, food, and life.

PREFACE

There are so many people who have helped me get this far, I fear that I cannot name them all. First of all, I would also like to thank my collaborators, Hergen Spits, Joe Bolen, and Melissa Elder, who contributed many reagents important to my work. I would also like to thank the members of my thesis committee, David Morgan, Tony DeFranco, and Nigel Killeen. Their guidance, insight, and perspective have been instrumental in the development of my project. I am especially indebted to Nigel Killeen, who not only contributed many valuable resources but has invested a superhuman amount of time, thought, and resources in my project and has served as a second advisor to me.

I would like to acknowledge the people who started me down the path of research, including C.C. and Alice Wang, who introduced me to the world of biochemistry and molecular biology and encouraged me to pursue a career in research. I owe a great deal to Leslie Berg, my undergraduate research advisor, who gave me my first independent project and who has remained been a great advisor, teacher, and friend. Henry Bourne and Harold Varmus, two faculty members in whose labs I had the pleasure of rotating, have been invaluable resources and additional mentors for me, and I appreciate the respect and collegiality they have always shown me.

Members of the Weiss lab, past and present, have made my time in the lab quite enjoyable. For starters, I would like to thank the "old guard" of the Weiss lab, including Dev Desai, who gave me some bench space to start working and who showed me the ropes; Andy Chan, who always made morning coffee entertaining; Bryan Irving, who has always given me useful and energetic advice; and most of all, David Straus, who endured my soul-searching late-night conversations with him. Tomek Sosinowski and I rotated in the lab together, and the last year I have spent working next to him has been round-the-clock entertainment. Ravi Majeti made life in the corner a snug but not stifling experience. I have also been fortunate to have worked with the alumnae of U341J, Terri Kadlecek and

Ginny Shapiro, who have provided both advice and friendship. Marianne Mollenaeur has always had a kind and thoughtful word for me, and for that I will be forever grateful. The newest members of the lab, especially Larry Kane, have provided many enlightening conversations, scientific and otherwise, ranging from thymic development to baseball and the Simpsons. But no one has been more influential and helpful than Nicolai van Oers, whose guidance, friendship, and inexhaustible supply of stories have made my work go more smoothly and the time pass more quickly.

Many people outside of the lab have been important in maintaining my perspective on life. I would like to thank Tai Chen and Scott Alper, my two most recent housemates, have always been around when I wanted to have someone to go with me to a sporting event or to Las Vegas. I am grateful to my first and dearest college roommate, Brian Conrad, for his many interesting and entertaining e-mails. I have had innumerable and invaluable phone conversations with Ryan Schneider and Adam Shaywitz, two of my closest friends who live the furthest from me. Most of all, however, I am indebted to my very best friend, Debbie Yelon, whose companionship, compassion, and generosity have been a guiding force and a vital source of support for me that I will always treasure.

Of course, Art Weiss, my graduate school advisor and mentor, has had a tremendous impact on my development as a scientist and as a person. I will always admire his focus, enthusiasm for science, and tremendous dedication. His lessons and advice for me in both science and medicine will last a lifetime. I look forward to having him as a role model and friend in the years to come.

No greater influence on my life has been provided than that of my family. I am extremely thankful to have my brother Michael as a lifelong companion and friend. My Dad and Mom, Shu-Yuan and Min, have taught me the importance of temperament and patience, of curiosity and enthusiasm, and of discipline and conscientiousness in everything I do. They have always been unfailingly supportive and confident in my abilities, and their love has sustained me through many difficult times. I miss my Dad

dearly, and I hope that I may continue to strive for the intellectual perfection and personal happiness that he has inspired me to achieve.

The coauthor listed in this publication directed and supervised the research which forms the basis for the thesis. Dr. Arthur Weiss was the principal investigator supporting this research. The text of Chapter 2 is a reprint of the material as it appears in the EMBO Journal, referenced below. The other coauthors listed, Drs. Hergen Spits, Jean-François Peyron, R. Bruce Rowley, and Joseph B. Bolen, contributed reagents for these studies. The work presented in Chapter 3 was performed in collaboration with Drs. Nicolai van Oers, Marie Malissen, Jeff Harris, and Melissa Elder, who contributed reagents, provided intellectual advice, and assisted with experiments.

Chu, D.H., Spits, H., Peyron, J.-F., Rowley, R.B., Bolen, J.B., and Weiss, A. (1996). The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. EMBO J *15*, 6251-6261.

ABSTRACT

The role of the Syk protein tyrosine kinase in T cell activation and development

David Hwa-En Chu

The processes of T cell development and activation employ similar immature and mature receptors as well as similar signal transduction pathways to achieve different outcomes. Many signaling molecules are shared between the receptor signaling pathways, including two families of cytoplasmic protein tyrosine kinases (PTKs), the Src family and the Syk family. The two Syk family members expressed in T cells, Syk and ZAP-70, although structurally similar, had previously been demonstrated to have different activation requirements in heterologous systems. I was interested in determining the differences between Syk and ZAP-70 in their regulation of expression and activity in the T cell lineage. Specifically, I have demonstrated that Syk, but not ZAP-70, is capable of transducing a TCR-mediated signal independently of CD45 or Lck, suggesting a coreceptor-independent signaling role for Syk. Using an intracellular staining technique, I have detected elevated Syk expression in pre-T cells and demonstrated a role for Syk in pre-TCR signaling. Furthermore, I have observed an upregulation of Syk expression in a subpopulation of peripheral T cells, suggesting that these cells may have a specialized role in T cells. The overlapping and distinct characteristics of Syk and ZAP-70 in T cell signaling and the potential biological importance of their differences are discussed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 3

CHAPTER 4

CHAPTER 5

APPENDIX A

APPENDIX B

CHAPTER 1

THE SYK FAMILY OF PROTEIN TYROSINE KINASES IN T CELL ACTIVATION AND DEVELOPMENT

Preface

In many processes of vertebrate development and signaling, gene family members provide both overlapping and distinct functions. Because these molecules share many structural similarities, they often share functional similarities as well. Despite overall similarities to other members of their gene family, individual family members often retain certain unique characteristics. For example, different gene family members can have slightly different structures, resulting in altered interactions with regulators or effectors or in altered enzymatic activities and specificities. Alternatively, the regulatory regions of the related genes can diverge, resulting in family members that differ in function due to differential expression, either spatially and/or temporally. The principle of overlapping and distinct functions of gene families is commonly revealed in vertebrate studies, often discovered during experiments with targeted gene disruption. Phenotypes of mutants are often masked because of partial or complete compensatory functions provided by other gene family members, complicating the study of the specific roles of individual family members. In this introductory chapter, I review the processes of T cell development and activation, with emphasis on the role of Syk family protein tyrosine kinases (PTKs) and their potential similarities and differences in function.

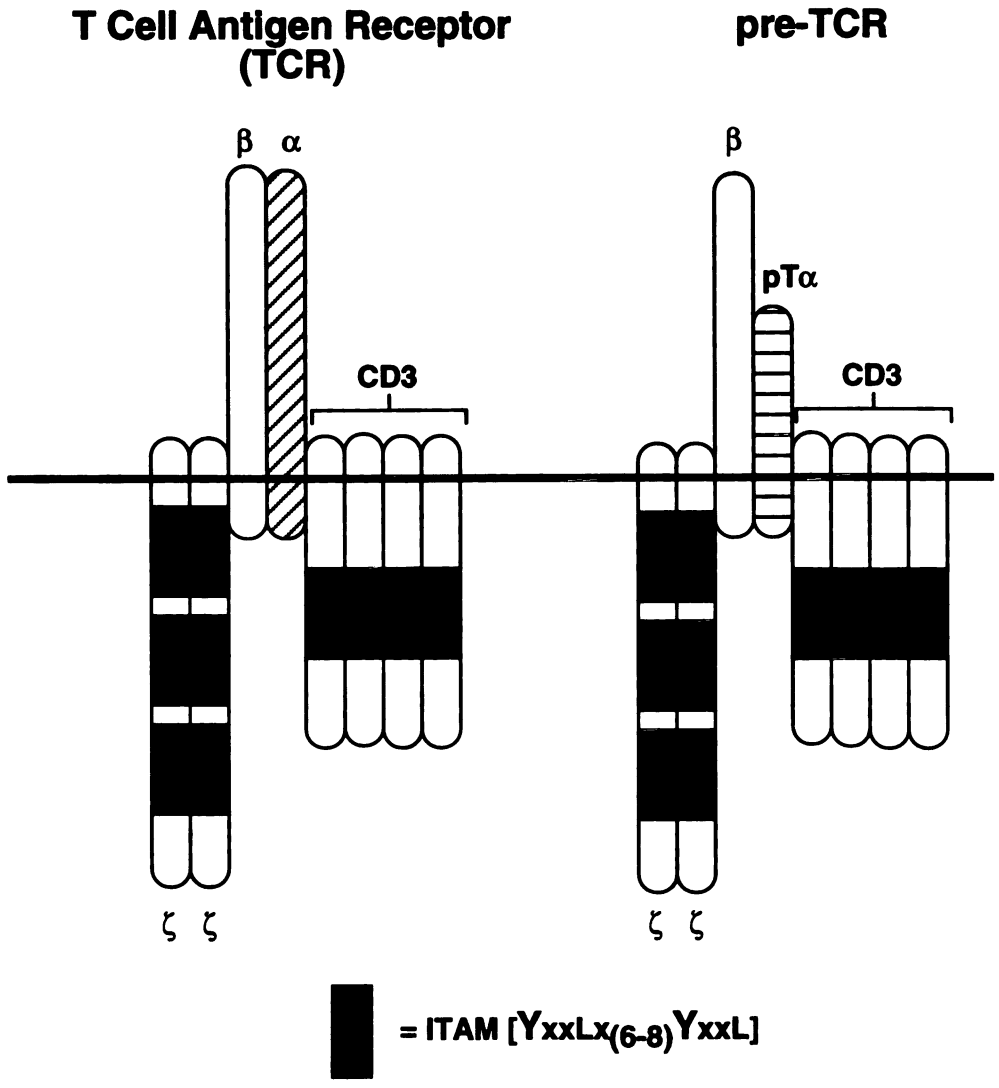
T cell development and activation: common themes in signal transduction

T cell activation and development require signaling through very similar receptors, the T cell antigen receptor (TCR) and the pre-TCR. T cell development requires the pre-TCR as well as the mature TCR, whereas T cell activation uses the TCR exclusively. These two receptor complexes employ many identical modular components as well as similar signal transduction machinery. However, the pre-TCR and TCR use these similar means to produce dramatically different outcomes. Signaling through the pre-TCR promotes T cell maturation early in thymocyte development, whereas signaling through the mature TCR can promote either death or maturation in developing thymocytes and proliferation and differentiation of mature T cells. Critical parameters for the interpretation of information through these receptors include the timing and nature of the signals received as well as alterations in the balance of intracellular signaling molecules propagating these signals.

Because neither the pre-TCR nor TCR complexes has intrinsic enzymatic activity, each relies on recruitment of cytoplasmic signaling molecules for propagation of a receptor signal. Both the TCR and pre-TCR use the CD3 complex, composed of the γ, δ, and ε chains, as well as the ζ chains, as their signal-transducing subunits (Figure 1) [reviewed in (1); see also (2)]. These signaling subunits contain sequence motifs referred to as immunoreceptor tyrosine-based activation motifs (ITAMs), which are repeats of tyrosines and leucines spaced by six to eight amino acids ($YxxLx_{(6-8)}YxxL$). This motif is present once in each of the CD3 chains and three times in the ζ chain. It is also present in the signal-transducing chains of many other lymphoid receptors, such as the B cell antigen receptor (BCR) and the Fc receptors present on macrophages, and mast cells. Once phosphorylated, these ITAMs serve as docking sites for the recruitment of signaling molecules via SH2 domains.

The other subunits within the pre-TCR and TCR complexes define receptor specificity. The α and β chains of the mature TCR are ligand-binding modules composed

Figure 1. Schematic representations of the TCR and pre-TCR. The TCRβ chain pairs with the CD3 chains γ, δ, and ε, and ζ chains as well as the TCRα chain (horizontal shading) in the mature TCR and with the CD3, ζ, and pre-Tα chains (pTα, diagonal shading) in the pre-TCR.



of somatically rearranged VDJ segments for the generation of diverse specificities. The αβ heterodimer recognizes antigenic peptides bound to major histocompatibility molecules (MHC) displayed by antigen-presenting cells. The pre-TCR is quite similar, with the exception that the invariant pre-Tα chain is substituted for the mature TCRα chain (3). A ligand for the pre-TCR has not been identified, and recent evidence suggests that the cell-surface expression of the pre-TCR is sufficient to send a signal leading to differentiation (4). A subset of T cells, the γδ T cells, use somatically rearranged γδ heterodimers for ligand-binding instead of αβ heterodimers but are still complexed with the signal-transducing subunits used by all T cells.

A sequential involvement of PTK families initiates signal transduction

Initial studies in T cell lines and heterologous cell transfection systems have provided a model of signal transduction events following TCR engagement [reviewed in (1)]. Two families of cytoplasmic PTKs are involved in the earliest steps of TCR and pre-TCR signal transduction, the Src family PTKs Lck and Fyn, as well as the Syk family PTKs Syk and ZAP-70 (Figure 2). These two kinase families act in a sequential fashion to transduce receptor signals (Figure 3). Following receptor engagement, Lck, associated with the coreceptors CD4 and CD8, is clustered in proximity to the ITAMs within the TCR complex facilitating Lck-mediated phosphorylation of the tyrosines. These phosphorylated ITAMs then act as docking sites for the Syk family PTKs. Syk and/or ZAP-70 are recruited via their tandem SH2 domains and are then phosphorylated by Src family PTKs on their regulatory loop tyrosines to activate the Syk family PTKs. Lck also appears to bind via its SH2 domain to both Syk and ZAP-70 (5, 6). The activated PTKs are then available to phosphorylate such substrates as Cbl, SLP-76, Vav, PLCγ, and LAT (7-12). These further signal transduction events result in the activation of both calcium/calcineurin- and

Figure 2. Two families of PTKs are involved in TCR and pre-TCR signal transduction. The Src family PTKs Lck and Fyn are composed of a unique domain (Unique) that contains myristoylation and palmitoylation signals and the region of association with CD4 and CD8 coreceptors, an SH2 domain, an SH3 domain, and a kinase domain (Kinase). At the carboxy-terminal end of the kinase domain is the negative regulatory tyrosine (Y) characteristic of Src family PTKs. The Syk family PTKs Syk and ZAP-70 are composed of two SH2 domains at the amino-terminus of the protein (SH2-N and SH2-C) with an intervening interdomain A (IA). Note that a portion of the C-terminal SH2 domain is necessary to complete the N-terminal SH2 domain phosphotyrosine-binding pocket. The above regions of the Syk kinases are depicted as if bound to a phosphorylated ITAM (see text). Following the SH2 domains is an extended interdomain B (IB) and kinase domain (Kinase). Conserved tyrosine phosphorylation sites are indicated (see text). Amino acid numbers refer to those of the human proteins (13, 14).

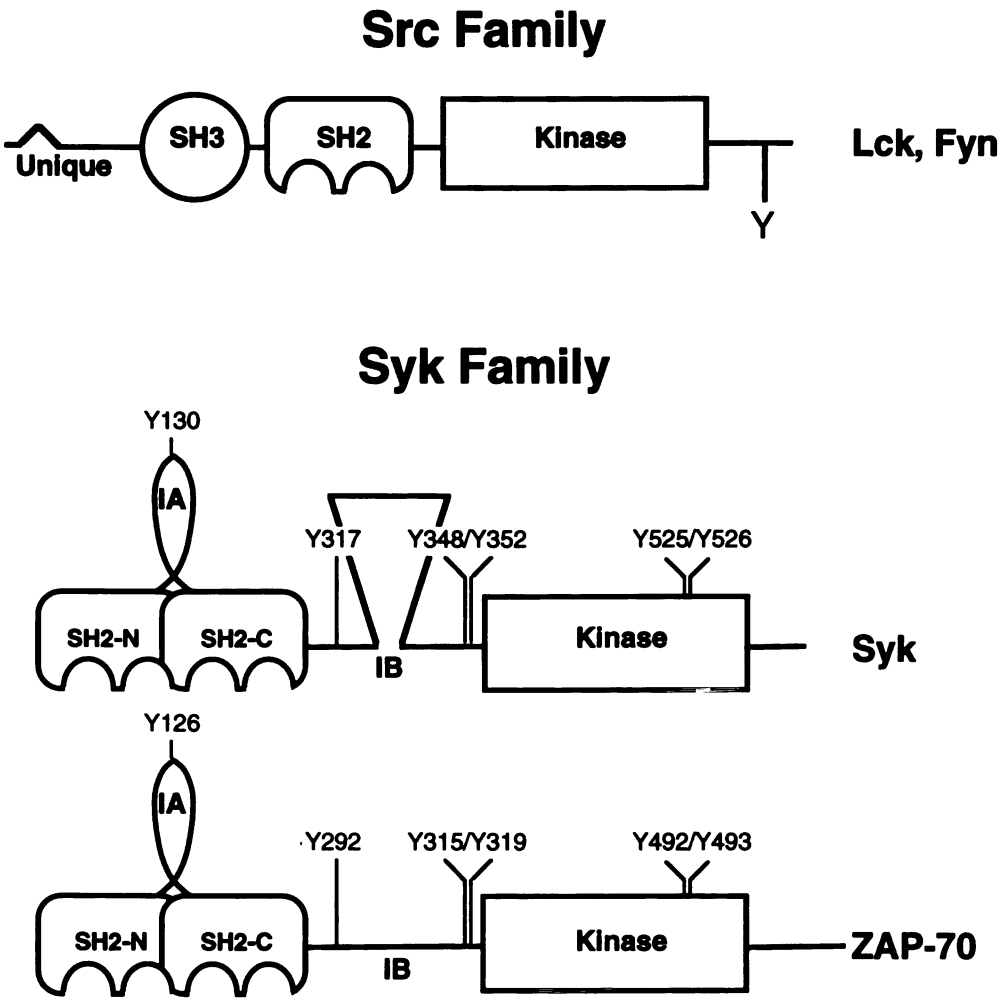
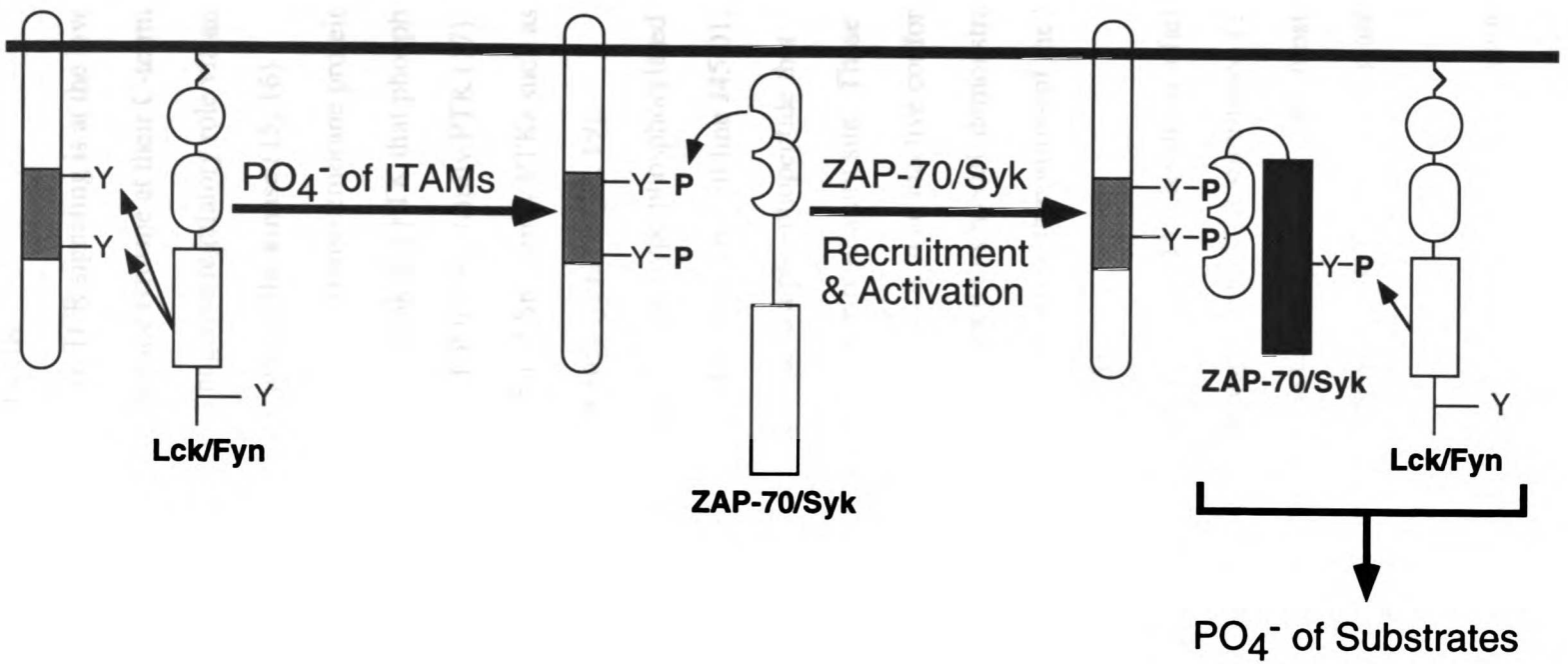


Figure 3. A sequential model of TCR and pre-TCR signal transduction. Following activation by CD45 and colocalization of the Src family PTKs Lck and Fyn to the receptor complex, the Src family PTKs phosphorylate ITAMs within the receptor complex. The phosphorylated tyrosines within the ITAMs serve as docking sites for the Syk family PTKs. Syk and/or ZAP-70 are recruited via their tandem SH2 domains and then are phosphorylated and activated by Src family PTKs. The activated PTKs are then available to phosphorylate downstream substrates.



Ras-mediated signaling pathways which ultimately lead to the differentiation, proliferation, or activation of developing thymocytes or mature T cells.

An earlier level of regulation of these PTKs in TCR signaling is at the level of Src PTK activation. Src family PTKs contain a characteristic tyrosine at their C-terminal ends; phosphorylation of this residue is thought to play a negative regulatory role via an intramolecular association with SH2 and SH3 domains of the kinase (15, 16). Phosphorylation of this site is regulated by two proteins, a transmembrane protein tyrosine phosphatase (PTPase), CD45, and another PTK, Csk. Csk is a PTK that phosphorylates the negative regulatory tyrosine at the C-terminal end of the Src family PTK (17). CD45 counters the activity of Csk and serves as an activator of Src family PTKs such as Lck or Fyn by dephosphorylating the same negative regulatory tyrosine (18, 19).

In T cell lines that lack CD45, both Lck and Fyn are hyperphosphorylated on their negative regulatory tyrosines (16, 20-22). In the CD45-deficient cell line J45.01, the SH2 domain of the hyperphosphorylated Lck is inaccessible to a phosphopeptide that encompasses the carboxy-terminal tyrosine of the negative regulatory site. These results provided the first evidence that Lck in CD45-deficient cells is in an inactive conformation (16). The crystal structures of Src and Hck have confirmed this model, demonstrating that the C-terminal negative regulatory end of the kinase interacts with portions of the SH2 and SH3 domains of the same kinase molecule (16, 23-25).

The inactivity of Src PTKs caused by a deficiency in CD45 results in a failure of CD45-deficient T cell lines to transduce signals in response to TCR stimulation (22, 26-30). Furthermore, in mice that have a targeted disruption of the CD45 gene, most thymocytes are arrested in their development, and those T cells that do develop are unable to be stimulated through their TCR (31, 32).

In vivo experiments have validated this sequential model of PTK activation for signaling by the mature TCR as well as the pre-TCR, with a notable discrepancy. That is, some of the ITAMs of the TCRζ chain appear to be constitutively phosphorylated in *ex*

vivo T cells (33-35). Because of this phosphorylation, Syk family PTKs are found to be associated with the receptor complex in unstimulated cells, although they are not phosphorylated or activated (35-37). The molecular events that are responsible for this observed difference are not clear, but the Src family kinase Lck is required for the *in vivo* constitutive tyrosine phosphorylation of the ζ ITAMs within the receptor complexes (36). Despite this difference in the phosphorylation state of the unstimulated receptor complex, once the receptor is activated, the Syk family PTKs still become tyrosine phosphorylated and function as critical mediators of TCR signaling.

Syk and ZAP-70: variations on a structure

Although both Syk and ZAP-70 can be activated following TCR stimulation, the precise roles that these structurally related PTKs play is not well-defined. Very often, structure-function studies of regions of homology in gene family members support similar functional roles for conserved domains and residues, and this observation appears to be true for the Syk family PTKs. The presumed functions for each domain of these kinases, comparing Syk and ZAP-70 structures and function, will be reviewed.

SH2 domains

Syk and ZAP-70 are both composed of two tandem amino-terminal SH2 domains followed by an extended interdomain region before the kinase domain (Figure 2; see also Chapter 5, Figure 1) (13, 14). Overall, these two PTKs share greater than 50% sequence identity. The SH2 domains of the two kinases are quite similar, with 56% sequence similarity. The most distinctive aspect of the tandem SH2 domains present in this family of PTKs is that they bind to their targets in the TCR/CD3 complex in a cooperative manner (38-41). The molecular basis of this cooperativity for ZAP-70 has been established with the crystallization of its tandem SH2 domains complexed with a phosphorylated ITAM peptide

from the TCRζ chain (39). The N-terminal SH2 domain appears to be incomplete and requires amino acid contributions from the C-terminal SH2 domain to help complete the phosphotyrosine-binding pocket (Figure 2; see also Chapter 5, Figure 1) (38, 39). The critical sequences are conserved in Syk, including many of the residues implicated in interactions either with the phosphorylated ITAM or with the other SH2 domains (Chapter 5, Figure 1) (14, 39). Cooperative binding of the Syk SH2 domains to phosphorylated ITAMs has been demonstrated, and although the structure of the tandem SH2 domains of Syk has not been solved, it is possible that a portion of the C-terminal SH2 domain may contribute to the structure of the N-terminal SH2 domain phosphotyrosine binding pocket (40, 41).

Interdomain A

Between the SH2 domains is an extended sequence referred to as interdomain A. Notably, this sequence is the most highly conserved region between the two PTKs, with greater than 65% of the amino acids identical between Syk and ZAP-70 (Chapter 5, Figure 1). In ZAP-70, this sequence forms a coiled-coil structure that is thought to stabilize the interaction of the C-terminal SH2 domain with the N-terminal SH2 domain (39). One study using anti-peptide antisera specific for interdomain A showed a difference in reactivity depending on whether or not ZAP-70 molecules were bound to phosphorylated ITAMs, suggesting that ITAM binding may induce a conformational change in the structure of this domain (42). Furthermore, these antisera reacted poorly to full-length ZAP-70 compared to the SH2 domains expressed alone, providing evidence that the conformation of interdomain A is also under constraints imposed by regions C-terminal to the SH2 domains (42). A conserved tyrosine residue, Y130 in Syk and Y126 in ZAP-70, may be an important regulator of the stabilizing effect of interdomain A on the tandem SH2 domain structure (Figure 2) (43). This tyrosine has been demonstrated to be an *in vitro* autophosphorylation

site in Syk (43, 44). From the ZAP-70 tandem SH2 domain/ζ peptide crystal structure, this residue appears to be at the tip of the coiled-coil domain, which would be solvent-exposed and accessible (39). Furthermore, mutation of this tyrosine to phenylalanine or glutamic acid affects the activation and receptor-binding of Syk, suggesting that this site of phosphorylation may influence the conformation of the coiled-coil structure, thereby regulating the binding of the SH2 domains to the phosphorylated ITAM (43). Given the conservation and location of this residue, this residue may play a similarly important functional role in ZAP-70.

Structurally, the SH2 domains of Syk and ZAP-70, and the region between them, appear to be conserved. The function of the SH2 domains in these PTKs, namely to allow the kinases to bind to phosphorylated receptor complexes, is conserved as well. Multiple groups have demonstrated that the tandem SH2 domains of Syk and ZAP-70 bind to phosphorylated ITAMs from both TCR- and BCR-associated signaling chains. These SH2 domain affinities have been measured by surface plasmon resonance, indicating that Syk and ZAP-70 SH2 domains have similar affinities for phosphorylated ITAMs, between 10-50 nM (41, 45, 46). Insertion of alanine or non-peptidyl γ-amino butyric acid spacers between the YxxL/I motifs within an ITAM has demonstrated that the correct spatial separation between the YxxL/I repeats is critical for efficient binding of Syk or ZAP-70 SH2 domains (41, 45). This finding is consistent with the crystal structure of the complex which suggests that the extended ζ chain ITAM is a driving force in bringing the SH2 domains together (39).

Interdomain B

Following the SH2 domains and preceding the kinase domain of Syk or ZAP-70 is an extended sequence termed interdomain B that is important in regulating the kinase activity of these proteins. These interdomain regions of Syk and ZAP-70 show about 30%

sequence identity, including a number of tyrosines that appear to be phosphorylated (Figure 2; see also Chapter 5, Figure 1) (47). An obvious difference between Syk and ZAP-70 in this region is the insertion of a 23 amino acid sequence in the Syk interdomain region that is not present in the ZAP-70 molecule (Figure 2; see also Chapter 5, Figure 1). Nevertheless, many of the sites of tyrosine phosphorylation that are present in this interdomain region are conserved and may serve the same functions in both molecules (Figure 2). One of these sites appears to be a positive regulatory site, Y315 in ZAP-70, which corresponds to Y348 in Syk. When phosphorylated, Y315 in ZAP-70 binds to the SH2 domain of Vav, a Rho-family guanine nucleotide exchange factor and one of the tyrosine phosphorylated substrates following TCR stimulation (8). Mutation of this tyrosine to phenylalanine results in decreased Vav association with ZAP-70, as well as the diminished phosphorylation of ZAP-70 and Vav (8). The corresponding tyrosine in Syk, Y348, has been identified as a binding site for Vav in a yeast two-hybrid system and confirmed in cell culture (9). Mutation of this tyrosine also results in the loss of Vav phosphorylation and Vav association with Syk. In Syk, Y348 has been demonstrated to be a site of autophosphorylation (9, 44). Recent work suggests that Y315 of ZAP-70 is also an *in vivo* site of phosphorylation (V. Di Bartolo and O. Acuto, submitted). Mutation of this site results in decreased receptor signaling, confirming the biological importance of this conserved tyrosine.

Another tyrosine within the interdomain region also appears to be an important positive regulatory site for Syk and ZAP-70 function. Mutation of ZAP-70 Y319, which is another *in vivo* autophosphorylation site, results in a dramatic decrease in activation-induced phosphorylation and catalytic activity of the kinase, in phosphorylation of downstream substrates, and ultimately in IL-2 production (V. Di Bartolo and O. Acuto, submitted). In Syk, Y352, which corresponds to Y319 in ZAP-70, has been shown to be a site of autophosphorylation (44). One possible role for this site is the recruitment of the Lck SH2 domain: Lck has been shown to form a stable complex with ZAP-70 and Syk

following TCR stimulation (5, 6). Alternatively, both Y348 and Y352 in Syk have been suggested to be essential for the binding of the C-terminal SH2 domain of PLCγ1 (11). Similarly, mutation of Y319 in ZAP-70 results in a decreased association with PLCγ1 upon treatment of Jurkat T cells with pervanadate (B. Williams and R. Abraham, personal communication). Thus, it appears as if these conserved tyrosines in Syk and ZAP-70 may serve as docking sites for other signal-transducing proteins.

Whereas Y315 and Y319 appear to be positive regulatory sites in ZAP-70, Y292, one of its autophosphorylation sites, appears to be a conserved negative regulatory site. The corresponding site is Y323 in Syk. Mutation of this tyrosine results in an activated form of the ZAP-70 kinase (47-49). Biochemical evidence suggests that Cbl binds to ZAP-70 at this Y292 negative regulatory site (10, 50, 51). Cbl, via a novel N-terminal PTB domain, has been shown to bind to both Syk and ZAP-70 and is thought to act as a negative regulator of receptor signal transduction (10, 50-53). Consistent with this model, binding studies have demonstrated Y323 in Syk to be necessary for the interaction of Syk with Cbl (10). However, the functional effect of mutation of this site in Syk has yet to be addressed. ZAP-70 and Syk may therefore be controlled by a balance between negative regulation via Y292/Y323 and positive regulation via Y315/Y348 and Y319/Y352. Interestingly, deletion of the entire interdomain B in ZAP-70, removing both the positive and negative regulatory sites, results in an activated form of ZAP-70, suggesting that the negative regulatory function is the dominant role of this region (Q. Zhao and A. Weiss, in press?).

Kinase domain

Overall, the kinase domains of Syk and ZAP-70 are very similar, about 60% identical (Chapter 5, Figure 1). Conserved regulatory sites are found within the activation loop of the kinase domain, Y492/Y493 in ZAP-70 and Y525/Y526 in Syk. These are sites for

tyrosine phosphorylation by Lck and for autophosphorylation in the case of Syk (44, 54). In ZAP-70, Y492 has been shown to be a negative regulatory site, whereas Y493 is a positive regulatory site required for ZAP-70 kinase activity (54, 55). Mutation of both Y525 and Y526 to phenylalanine eliminates Syk activity, indicating that these tyrosines are critical for Syk function (40).

These biochemical and structural similarities between Syk and ZAP-70 suggest overall functional similarities, and in fact, Syk and ZAP-70 seem to be interchangeable in numerous situations. Both kinases are able to restore antigen receptor signaling in a Syk-deficient chicken B cell line (49, 56). Furthermore, as will be discussed below, expression of Syk can correct the thymocyte development impairment that is observed in mice rendered deficient in ZAP-70 (57).

Syk and ZAP-70: structural similarities but functional differences

Despite the clear structural resemblance between Syk and ZAP-70, there is growing evidence that these two PTKs are differentially regulated, both in terms of expression and activity. ZAP-70 is expressed in a very restricted manner, present only in T cells and NK cells (13). Syk, however, has a broader pattern of hematopoietic expression, including T and B cells, as well as macrophages, monocytes, mast cells, and platelets (14). Biochemically, although both kinases appear to bind phospho-ITAMs with similar affinities, only Syk kinase activity is enhanced by phospho-ITAM binding (58-60). Syk also appears to have the ability to transphosphorylate other Syk molecules for activation, whereas ZAP-70 requires Lck or Fyn to phosphorylate its activation loop tyrosines (44, 54). Furthermore, there is some evidence from COS cell overexpression studies that suggests that Syk, but not ZAP-70, is capable of phosphorylating ITAMs in the absence of Src kinases (61, 62). Interestingly, biochemical studies have suggested that the full-length Syk and ZAP-70 molecules, in contrast to the SH2 domains alone, have differing abilities to bind to the phosphorylated TCR complex (63). The observation that Syk can bind more

efficiently to phosphorylated ITAMs may reveal an important difference in the ability of these kinases to participate in TCR signaling (63).

The most striking difference in the regulation of kinase activity between Syk and ZAP-70 appears to be the requirement for transactivation by Src family PTKs. Specifically, Syk is less dependent than ZAP-70 on Lck or Fyn for its activation. This feature was first suggested in experiments using CD16/CD7/kinase chimeras (64). These molecules contained heterologous CD16 extracellular and CD7 transmembrane portions linked to either Syk or ZAP-70 as the cytoplasmic portion of the chimera. In COS cells, ZAP-70 chimeras required the co-crosslinking with a Src family PTK to produce a calcium flux, whereas Syk was able to signal after chimera crosslinking alone (64).

The importance of Syk family kinases in mature T cell signaling

Genetic evidence has confirmed that Syk family PTKs are essential for antigen receptor signaling in mature lymphocytes. Notably, several patients have been described who have severe combined immunodeficiency (SCID) caused by a lack of ZAP-70 protein. The decrease in ZAP-70 expression is due to transcriptional loss or various frameshift and insertional mutations that result in a destabilized protein (65-67). Although normal numbers of $CD4^+$ T cells develop in these patients, their peripheral $CD4^+$ T cells are unable to signal in response to stimuli that activate the TCR, such as anti-CD3 antibodies or PHA, as measured by calcium flux, tyrosine phosphoprotein induction, and IL-2 secretion (65-67). However, the T cells are able to respond to pharmacological agents such as phorbol ester and calcium ionophore, reagents that activate the TCR signaling pathway but bypass the proximal receptor-mediated events. In the Jurkat T cell line, a ZAP-70-deficient mutant has also been isolated (68). The signaling phenotype of these cells is similar to that of the T cells seen in the patients of the naturally occurring ZAP-70 deficiency. Again, the loss of ZAP-70 in Jurkat cells does not allow for signal transduction through the TCR, as measured by tyrosine phosphoprotein induction and calcium flux. Several lines of mice

that lack ZAP-70 have been generated, but because of an early block in T cell development that will be described below, there are no mature αβ T cells that can be studied in these mice (69-71).

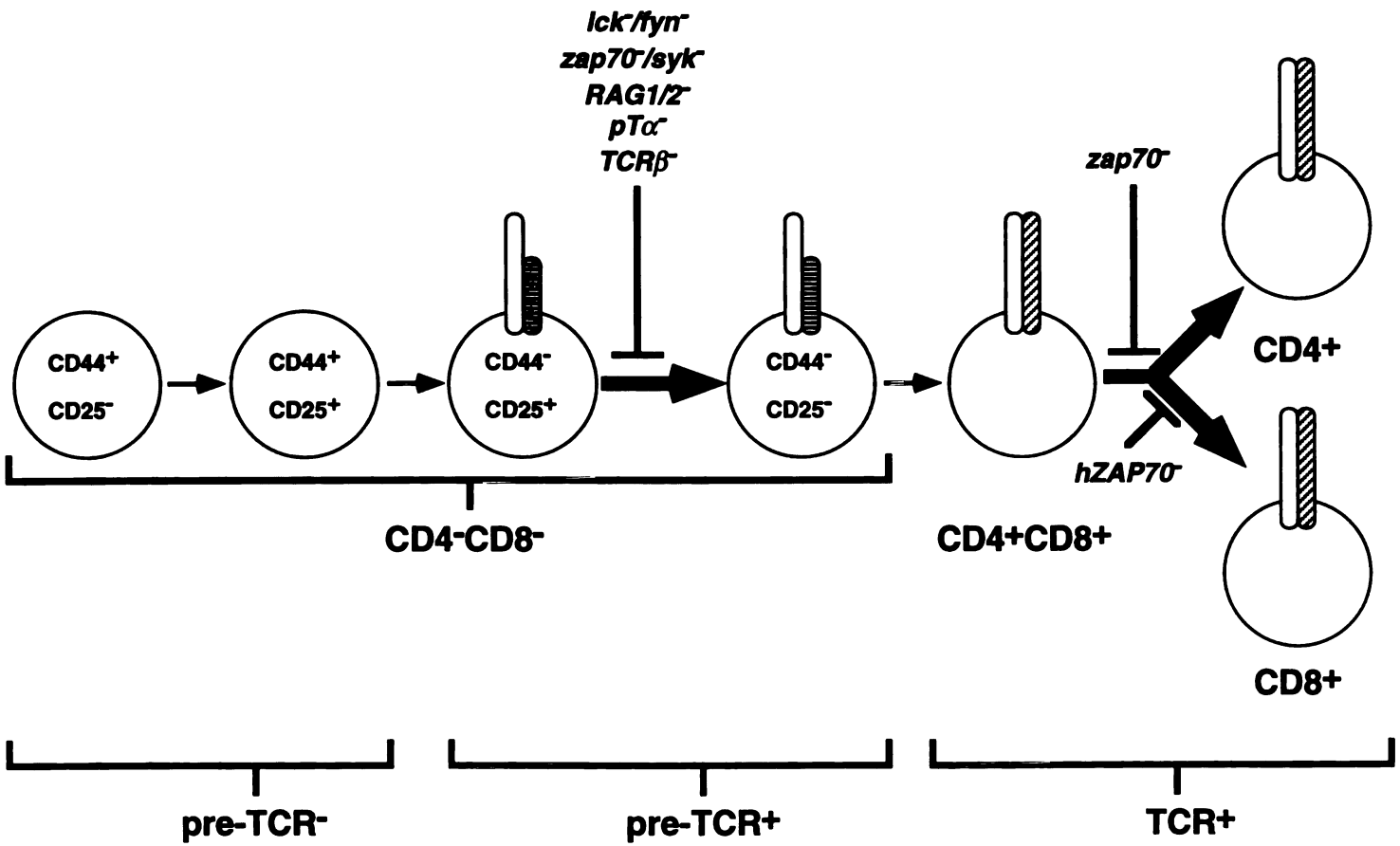
Syk-deficient mice have also been created (72, 73). In these mice, the most striking lymphoid defect is a block in BCR-mediated signaling, as revealed by impaired B cell development and lack of signaling in the B cells that do mature. In contrast, αβ T cell development appears grossly normal in Syk-deficient mice, with no obvious differences in number or composition of T cells in the thymus or periphery (72, 73). Preliminary studies indicate that TCR signaling in mature T cells from these mice is normal, as assessed by their ability to respond to the lectin concanavalin A, a potent activator of TCR signaling (73). Therefore, it appears that Syk is not required for all TCR signaling, although a more specific role for Syk in TCR signaling has not been ruled out.

This conclusion is consistent with studies of wild-type T cells. Analyses of cell lysates have suggested that Syk expression is uniformly low in normal T cells. Most T cells and T cell lines have low or undetectable levels of Syk, as assessed by immunoblotting or *in vitro* kinase assays. Nevertheless, these T cells are able to signal through their TCRs. In Syk-deficient mice, however, there is a selective defect in γδ T cell development which will be discussed in more detail below.

Syk family kinases in early thymocyte development

Another T cell signaling process that employs the Syk family PTKs is the maturation of T cells in the thymus. Once again, differential temporal expression of the Syk family members suggests that these kinases may play unique roles in this process. Thymocyte development proceeds through a number of ordered developmental stages and checkpoints, characterized by the expression of various markers on the cell surface (Figure 4). Two checkpoints that require TCR or pre-TCR signals exist in thymocyte ontogeny, one which

Figure 4. Diagram of thymocyte development. The pre-TCR and TCR checkpoints are indicated by bold-faced arrows. Points of developmental arrest seen in various genetic deficiencies are indicated. *hZAP70* refers to the human *ZAP70* gene; the other genes listed are murine.


lck-/fyn-
zap70-/syk-
RAG1/2-
pTα-
TCRβ-
zap70-
hZAP70-
CD44+
CD25-
CD44+
CD25+
CD44-
CD25+
CD44-
CD25-
CD4+
CD8+
CD4-CD8-
CD4+CD8+
pre-TCR-
pre-TCR+
TCR+

occurs in pre-TCR-expressing thymocytes, and a later checkpoint which involves the mature TCR.

Earliest thymocytes express neither CD4 nor CD8 coreceptors; initially, these thymocytes express no pre-TCR or TCR on their cell surface. These $CD4^-CD8^-$ cells can be further subdivided based on the expression of two other cell surface markers, CD44 (Pgp-1) and CD25 (IL-2 receptor α chain) (Figure 4) (74, 75). Expression of the recombinase-activating genes-1 and -2 (RAG-1 and RAG-2), enzymes that promote the somatic rearrangements of the TCR gene segments of both the α and β chains, initiates the process of β chain rearrangement and subsequent pre-TCR expression on the cell surface. Expression of the pre-TCR occurs specifically at the $CD44^-CD25^+$ stage (75). It is at this point in development that the pre-TCR checkpoint monitors successful β chain rearrangements. A productive rearrangement of the β chain results in its paired cell-surface expression with the pre-Tα chain and upregulation of the CD3 and ζ chains, as well as a subsequent signal through this complex not requiring ligands for pre-Tα or the β chain (4). This pre-TCR signal requires multiple components, a conclusion based on a number of genetic studies in mice; disruption of the genes encoding any of the receptor components or signaling molecules arrests thymocyte development at this $CD44^-CD25^+$ stage. For example, disruption of either the pre-Tα chain or the TCRβ chain arrests development at this stage (76, 77). Similarly, the disruption of the RAG genes abolishes β chain rearrangement and thus arrests thymocyte development in the absence of a pre-TCR (78, 79).

As in mature TCR signaling, multiple PTK family members appear to be crucial for the pre-TCR signal transduction process. Thymocytes from mice doubly deficient for both

Lck and Fyn are arrested at the $CD44^-CD25^+$ stage, indicating that the Src family PTKs are necessary for progression past this point (80, 81). These observations are consistent with the role of this family in initiating signaling by the pre-TCR-associated ITAMs. Disruption of either *lck* or *fyn* individually yields less severe phenotypes. That is, Fyn-deficient mice exhibit no defect in thymocyte development. Development of Lck-deficient thymocytes is only partially blocked at the $CD44^-CD25^+$ stage, although some thymocytes are able to mature to become peripheral T cells (80-84). Thus it appears that once again, related family members can have overlapping biological functions; Lck and Fyn can compensate for each other, albeit incompletely, in this process.

Similarly, the Syk family PTKs are implicated in pre-TCR signaling; in mice doubly deficient for both Syk and ZAP-70, thymocytes do not develop past the pre-TCR checkpoint stage (85). However, mice deficient for only ZAP-70 have a later block in thymocyte development: these mice have normal thymic development past the pre-TCR stage but are unable to progress past the next checkpoint, which will be discussed below (69). On the other hand, mice deficient for Syk do not have any gross thymic developmental abnormalities (72, 73). These results illustrate another case in which related family members are playing overlapping roles in development, since the phenotype of the double knockout is more severe than either single knockout alone.

Syk family PTKs in positive and negative selection

The pre-TCR signal, through a series of signal transduction events that are poorly understood, initiates the somatic rearrangement of the TCRα chain. Once the α chain has successfully rearranged, it is transported to the cell surface together with the β chain to form the mature TCR as the thymocytes develop into $CD4^+CD8^+$ cells. Expression of the mature TCR allows for the second checkpoint in thymic development, the processes of positive and negative selection. These selection events determine the repertoire of mature

$CD4^+$ or $CD8^+$ thymocytes that emigrate from the thymus to populate the peripheral immune system. During negative selection, those thymocytes that express self-reactive TCRs are deleted. In positive selection, thymocytes that express receptors with the potential to recognize self-MHC and foreign peptides are selected to mature.

Genetic evidence again points to important roles for PTK families since the pathways governing TCR signaling at this $CD4^+CD8^+$ stage of thymic development are similar to those employed during TCR signaling in the periphery. Thymocytes from transgenic mice expressing high levels of a dominant-negative form of Lck are unable to undergo positive selection, suggesting that this Src family kinase is important at this stage (86). As mentioned earlier, mice with impaired ZAP-70 kinase function are arrested at the $CD4^+CD8^+$ stage and are unable to undergo positive or negative selection. In this situation, the inability of Syk to compensate for the absence of ZAP-70 is probably a function of low expression rather than functional capability. Indeed, a Syk transgene expressed during all stages of thymic development completely restores thymic development in a mouse deficient for ZAP-70 (57).

To summarize, the differences seen in the roles of Syk and ZAP-70 may be due to differences in expression rather than in biochemical activity at the $CD4^+CD8^+$ checkpoint. Expression of either Syk family kinase is sufficient to allow positive and negative selection to occur. However, the different biochemical activities of Syk and ZAP-70 may affect the strength of the TCR signal during selection; this alteration could in turn lead to a difference in the TCR repertoire of thymocytes that ultimately mature.

Differential Syk expression may explain differences between human and murine ZAP-70 deficiencies

Most of the genetic evidence implicating a role for the Syk family of PTKs in T cell development has come from targeted disruptions of murine genes. The process of human thymocyte development has not been characterized in as much detail, although it is

generally believed that the process is similar. However, recent studies suggest that Syk expression may differ between the two species, a difference that may help to explain some inconsistencies between the human and murine phenotypes of ZAP-70 deficiency (87); see also Chapter 3). As described above, several cases of a rare autosomal recessive SCID have been ascribed to the lack of the ZAP-70 PTK. In these human patients, the characteristics of their peripheral blood and thymus are different from those of ZAP-70-deficient mice. Most notably, a normal number of peripheral T cells develop in the human cases, but they are nonfunctional and are exclusively $CD4^+$, whereas in ZAP-70-deficient mice, no mature peripheral $\alpha\beta$ T cells develop. Thymic biopsies have revealed that the same trend is seen in the precursors of these peripheral cells; in the human ZAP-70-deficient thymus, $CD4^+$ thymocytes are present, but in the mouse deficiency, all thymocytes are arrested at the $CD4^+CD8^+$ stage with no mature thymocytes (69-71, 87). The molecular explanation for this discrepancy is not known, but the development of these human $CD4^+$ thymocytes in the absence of ZAP-70 could reflect differences in the level of Syk expression in subpopulations of thymocytes. In ZAP-70-deficient patients, however, although the $CD4^+CD8^+$ thymocytes are able to signal, the mature peripheral $CD4^+$ T cells are not (87). The inability of mature ZAP-70-deficient $CD4^+$ T cells to signal may reflect a downregulation of Syk expression as these cells develop from thymocytes into peripheral cells. These results raise the point that although the biological function of a gene can be preserved between species, divergence in regulation of that gene can still occur. Recognition of such interspecies differences is important when trying to generalize from an animal model to human disease.

Syk and ZAP-70 may have unique roles in $\gamma\delta$ T cell development

$\gamma\delta$ T cell development is not as well-characterized as $\alpha\beta$ T cell development, although the Syk and ZAP-70 kinases have proven to be important in the developmental processes of

these cells. As discussed earlier, Syk-deficient mice do not have an apparent αβ T cell defect, but they do show a peripheral T cell defect in the development of a specific population of γδ cells, the intraepithelial lymphocytes (IELs) of the intestine (88). Surprisingly, a similar defect in IEL development is also observed in ZAP-70-deficient mice (57, 71), although normal numbers of γδ T cells are present in the spleen and lymph nodes of ZAP-70-deficient mice. Moreover, dendritic epidermal T cells, another subset of γδ cells, are decreased in number and show an abnormal morphology in these animals (71). Thus, in certain γδ T cell subpopulations, Syk and ZAP-70 do not fully compensate for each other in the case of a deficiency in either kinase. It is not clear that the required functions provided by these tyrosine kinases occurs during the same time of development. Therefore, Syk and ZAP-70, although they seem to have overlapping functions in αβ T cell development, may have divergent functions in the development and/or signaling of γδ T cells.

General organization of the thesis

The participation of the Syk family PTKs in T cell activation and development provides an opportunity to investigate the themes of redundancy and specialization in biology. Although these PTKs subserve many overlapping functions, differences in their structure and regulation suggest that they may have certain unique, rather than completely redundant, roles in T cell signaling. I decided to examine in greater detail the differential activity and expression of Syk family PTKs for a better understanding of the complex and intricately regulated mechanisms of T cell signaling. In Chapter 2, I present direct evidence that Syk, but not ZAP-70, is capable of participating in TCR signaling independently of CD45 or Lck, suggesting potentially divergent functions between Syk and ZAP-70 in T cells. In

Chapter 3, I examine the relative importance of Syk in thymocyte development in mice and humans, especially during pre-TCR signaling. In Chapter 4, I suggest that Syk may play a unique role in a subpopulation of peripheral T cells as well. Finally, I conclude by describing future directions based on my observations. The Appendices include information about structure-function studies to define regions critical for the differential kinase regulation of Syk and ZAP-70, as well as preliminary characterization of a "knock-in" mouse created to follow expression of Syk in live cells.

CHAPTER 2

CHARACTERIZATION OF FUNCTIONAL DIFFERENCES BETWEEN SYK AND ZAP-70 DURING T CELL ANTIGEN RECEPTOR SIGNALING

Summary

Members of the Syk family of protein tyrosine kinases (PTKs) are critical mediators of antigen receptor-mediated signal transduction in a variety of hematopoietic cells. They are tandem SH2 domain-containing proteins that are recruited to phosphorylated tyrosines contained within the immunoreceptor tyrosine-based activation motifs (ITAMs) of signal transducing subunits of antigen receptor complexes. Phosphorylation of these tyrosines is accomplished by a separate family of kinases, the Src PTKs, of which Lck and Fyn are expressed in T cells. Src family kinase activity is regulated by the dephosphorylation of a negative regulatory tyrosine at the extreme carboxy-terminal end of all Src family members. Activation of these PTKs is mediated by a hematopoietic cell-specific transmembrane protein tyrosine phosphatase (PTPase), CD45. It had been previously observed that CD45 is required for signal transduction in most T cells and B cells. Surprisingly, however, a T cell line exists that lacks CD45 but is still able to signal through its TCR. During the course of investigation of this cell line, we observed that it expresses higher levels of Syk compared to other T cell lines that have been observed to be CD45-dependent. We demonstrate here that Syk can be introduced into both CD45- and Lck-deficient cell lines, restoring TCR-mediated signal transduction. We further examine the structural requirements for Syk to function in this process. These results provide a clear demonstration that Syk and ZAP-70 have different requirements for activation in T cell lines. They further suggest that Syk may not be completely redundant with ZAP-70 in TCR signaling and suggest it has a specialized and unique role *in vivo*.

The EMBO Journal vol.15 no.22 pp.6251-6261, 1996

The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling

David H.Chu, Hergen Spits[1], Jean-François Peyron[2], R.Bruce Rowley[3], Joseph B.Bolen[4] and Arthur Weiss[5]

Departments of Microbiology and Immunology and of Medicine, and the Howard Hughes Medical Institute, University of California, San Francisco, CA 94143, USA, [1]Department of Immunology, Netherlands Cancer Institute, Plesmanlaan 121, Amsterdam 1060 CX, The Netherlands, [2]INSERM Unit 364 Molecular and Cellular Immunology, Faculté de Médicine Pasteur, 06107 Nice, France, [3]Department of Oncology, Bristol Myers Squibb, Princeton, NJ 08543 and [4]Department of Cellular Signaling, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA 94304, USA

[5]Corresponding author

The protein tyrosine phosphatase CD45 is a critical component of the T cell antigen receptor (TCR) signaling pathway, acting as a positive regulator of Src family protein tyrosine kinases (PTKs) such as Lck. Most CD45-deficient human and murine T cell lines are unable to signal through their TCRs. However, there is a CD45-deficient cell line that can signal through its TCR. We have studied this cell line to identify a TCR signaling pathway that is independent of CD45 regulation. In the course of these experiments, we found that the Syk PTK, but not the ZAP-70 PTK, is able to mediate TCR signaling independently of CD45 and of Lck. For this function, Syk requires functional kinase and SH2 domains, as well as intact phosphorylation sites in the regulatory loop of its kinase domain. Thus, differential expression of Syk is likely to explain the paradoxical phenotypes of different CD45-deficient T cells. Finally, these results suggest differences in activation requirements between two closely related PTK family members, Syk and ZAP-70. The differential activities of these two kinases suggest that they may play distinct, rather than completely redundant, roles in lymphocyte signaling.

Keywords: CD45/Syk/TCR/tyrosine kinase/ZAP-70

Introduction

Stimulation of the T cell antigen receptor (TCR) results in the activation of a series of protein tyrosine kinases (PTKs) and culminates in a variety of distal events which include transcriptional activation of the interleukin-2 (IL-2) gene (reviewed in Weiss and Littman, 1994). According to a sequential model of tyrosine kinase activation, the Src family kinase members Lck or Fyn phosphorylate the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the CD3 and ζ subunits of the TCR complex (Burkhardt *et al.*, 1994; Iwashima *et al.*, 1994). Phosphorylation of these tyrosines creates docking sites for the Syk family member kinases, Syk and ZAP-70. The recruitment and activation of these kinases is important for phosphorylation of downstream substrates.

Genetic evidence from both mice and human T cells underscores the importance of Src PTK family members in TCR signaling. T cell lines and clones lacking Lck are unable to signal through the TCR (Karnitz *et al.*, 1992; Straus and Weiss, 1992). Lck-deficient and Fyn-deficient mice have T cells defective in TCR-mediated activation (Appleby *et al.*, 1992; Molina *et al.*, 1992; Stein *et al.*, 1992; van Oers *et al.*, 1996). Similarly, the importance of ZAP-70 has been demonstrated by genetic studies. Human patients with a ZAP-70 deficiency have a defect in T cell development, and the resulting $CD4^+$ T cells that do develop are unable to signal through the TCR (Arpaia *et al.*, 1994; Chan *et al.*, 1994a; Elder *et al.*, 1994). ZAP-70 deficient mice have a more profound block in T cell development (Negishi *et al.*, 1995).

The role of Syk in T cell signaling is not as clearly defined, although in B cells Syk interacts with the phosphorylated ITAMs present in the Ig-α and Ig-β chains to function in a manner analogous to ZAP-70 in T cells (Law *et al.*, 1993; Saouaf *et al.*, 1994). A potential role for Syk in TCR signaling was suggested by studies in which Syk was found to associate with a CD8/ζ chimera in COS-18 cells (Chan *et al.*, 1994b). In addition, Syk can associate with the phosphorylated ζ chain in murine thymocytes and in the Jurkat T cell line (Chan *et al.*, 1994b; Thome *et al.*, 1995; van Oers *et al.*, 1995). However, Syk does not appear to be required for T cell development: mice deficient in Syk expression are deficient in mature B cells but do not show any gross αβ T cell developmental defect, although γδ T cell development is impaired (Cheng *et al.*, 1995; Turner *et al.*, 1995; Mallick-Wood *et al.*, 1996).

Src family kinases have a characteristic tyrosine at their carboxy-terminal ends; phosphorylation of this residue is thought to play a negative regulatory role via an intramolecular or intermolecular association with the SH2 and SH3 domains of the kinase (Cooper and Howell, 1993). One proposed regulator of the Src family kinases in antigen receptor signaling is the transmembrane protein tyrosine phosphatase, CD45. This phosphatase is believed to act as a positive regulator of Src family kinases by dephosphorylating the negative regulatory tyrosine, thus permitting the kinase to become activated (Weiss and Littman, 1994). CD45 can specifically dephosphorylate both Fyn and Lck *in vitro* at the negative regulatory carboxy-terminal tyrosine (Mustelin *et al.*, 1989, 1992). Furthermore, *in vivo*, the negative regulatory tyrosines of Lck and Fyn are hyperphosphorylated in cell lines that lack CD45 (Ostergaard *et al.*, 1989; Hurley *et al.*, 1993; McFarland *et al.*, 1993; Sieh *et al.*, 1993). In the CD45-deficient cell line J45.01, the SH2 domain of the hyper-

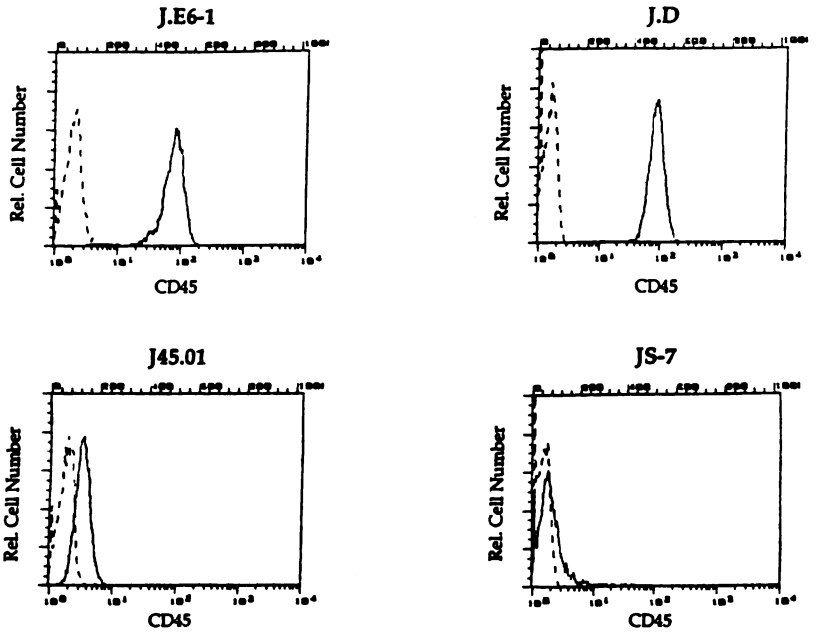


Fig. 1. Cell surface expression of CD45 on wild-type and CD45-deficient Jurkat cells. Cells (5×10^5 cells) were stained with an mAb against CD45 (solid lines), or with an isotype-matched control mAb (dotted lines) and analyzed by flow cytometry. The cell lines stained, either wild-type (J.E6-1 and J.D; upper panels) or their CD45-deficient variants (J45.01 and JS-7, respectively; lower panels), are indicated above each histogram.

phosphorylated Lck is inaccessible to a phosphopeptide that encompasses the carboxy-terminal tyrosine, presumably because it is occupied by the phosphotyrosine of the negative regulatory site. These results suggest that Lck in those CD45-deficient cells is in an inactive conformation (Sieh *et al.*, 1993).

As a positive regulator of the Src family kinases, CD45 is a critical component of early T cell signaling. In most T cell lines deficient in CD45 expression, stimulation of the antigen receptor fails to result in signal transduction (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992; Volarevic *et al.*, 1992; McFarland *et al.*, 1993). Furthermore, in mice that have a targeted disruption of a selected exon of the CD45 gene, most thymocytes are arrested in their development, and those T cells that do develop are unable to be stimulated through their TCR (Kishihara *et al.*, 1993).

Intriguingly, a T cell line exists whose TCR can signal despite the lack of CD45 (Peyron *et al.*, 1991). This CD45-negative variant of the Jurkat cell line has been shown to induce tyrosine phosphoproteins, hydrolyze inositol phosphates and mobilize calcium in response to TCR stimulation. The mechanism by which this cell line, JS-7, is able to signal is unknown. In these studies, we examined the CD45-independent TCR signaling pathway in the JS-7 cell line. It was found that the signaling-competent JS-7 cells express Syk, in contrast to the signaling-incompetent CD45-deficient cell line J45.01 (Koretzky *et al.*, 1991). Most interestingly, we discovered that the Syk PTK is able to mediate TCR signaling in a CD45-independent and Lck-independent manner and that differential expression of Syk is likely to explain the paradoxical signaling phenotypes of different CD45-deficient cells.

Results

The CD45-deficient JS-7 cell line can signal through its TCR

The two CD45-deficient Jurkat T cell lines, J45.01 and JS-7, have been described (Koretzky *et al.*, 1991; Peyron *et al.*, 1991). Both cell lines have almost undetectable levels of CD45 compared with their respective wild-type parental lines, J.E6-1 and J.D, as assessed by cell surface staining and immunoblotting (Figure 1, and Figure 6, lanes 2 and 5). Importantly, the JS-7 cell line is able to signal in response to TCR stimulation despite the absence of CD45 (Peyron *et al.*, 1991). The J45.01 cell line, on the other hand, is not (Koretzky *et al.*, 1991).

To address the reason why the CD45-deficient JS-7 cells are able to respond to TCR stimulation, we first examined the status of Lck in these cells. In the JS-7 cells, Lck is hyperphosphorylated on the negative regulatory tyrosine and its SH2 domain is inaccessible to a phosphopeptide representing the negative regulatory carboxy-terminus of Lck (data not shown). These results suggest that Lck is in an inactive conformation. Furthermore, DNA sequencing of the Lck SH2 domain revealed no mutation (data not shown). We next reasoned that the loss of a positive regulator of Lck such as CD45 may have been compensated for by the absence or decreased expression of the negative regulator of Src family kinases, the Csk PTK

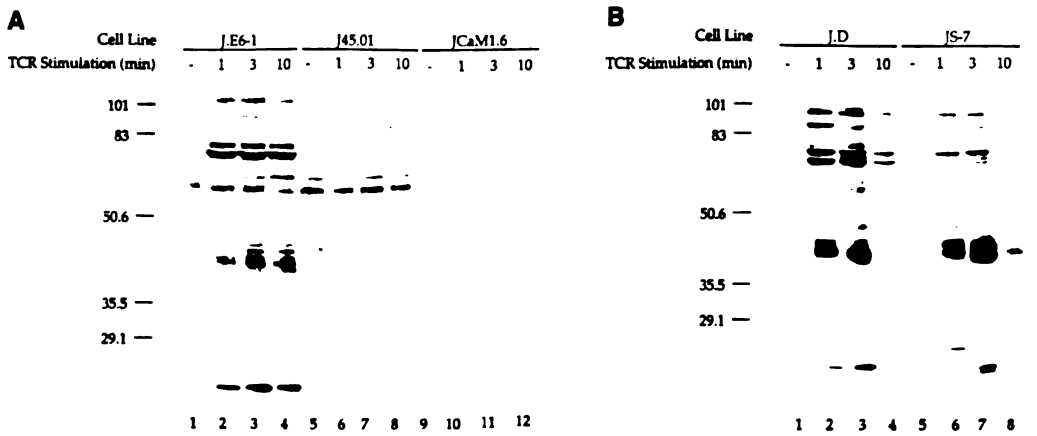


Fig. 2. The CD45-deficient Jurkat line JS-7 can signal through its TCR. (A) Jurkat cells (2×10^6 cells) were left unstimulated (–) or were stimulated for the indicated times with an anti-TCR mAb. Whole cell lysates of J.E6-1 (lanes 1–4), J45.01 (lanes 5–8) and JCaM1.6 (lanes 9–12) were resolved by 12.5% SDS–PAGE and immunoblotted with an anti-phosphotyrosine mAb. (B) J.D (lanes 1–4) and JS-7 (lanes 5–8) Jurkat cells were stimulated and analyzed as described above.

(Chow *et al.*, 1993). However, immunoblotting of whole cell lysates indicated that Csk was expressed at levels comparable with wild-type Jurkat cells in the JS-7 cell line (data not shown).

To examine further the TCR-mediated signals in JS-7 cells, we analyzed one of the earliest steps in the TCR signaling pathway, the induction of tyrosine phosphorylation of cellular proteins. A time course of tyrosine phosphoprotein induction in whole cell lysates from stimulated cells is presented in Figure 2. The anti-phosphotyrosine immunoblot revealed that the pattern and kinetics of tyrosine phosphoprotein induction in the JS-7 cell line are highly similar to that seen in its parental wild-type cell line, J.D (Figure 2B). In marked contrast, the CD45-deficient cell line J45.01 does not show any significant induction of tyrosine phosphoproteins (Figure 2A, lanes 5–8). It should be noted that the wild-type parental cell line of J45.01, J.E6-1, shows a pattern and time course of phosphoprotein induction similar to that seen for J.D and JS-7 cells (Figure 2A, lanes 1–4). The defective signaling phenotype of the J45.01 cell line is similar to that of the Lck-deficient Jurkat cell line, JCaM1.6, consistent with the inactive state of Lck in J45.01 cells (Figure 2A, lanes 9–12; Sieh *et al.*, 1993).

The TCR-ζ chain is phosphorylated upon TCR stimulation in JS-7 cells

We next assessed the status of the earliest substrates phosphorylated following TCR engagement, the ITAMs in the TCR-ζ subunit. Immunoprecipitations of the TCR-ζ chain from unstimulated and stimulated cells revealed that in all three of the signaling-competent lines, J.E6-1, J.D and JS-7, the ζ chain was phosphorylated in response to TCR stimulation (Figure 3). Although the level of ζ phosphorylation in JS-7 appears to be lower in this experiment, this result was not observed consistently (data not shown). Therefore, the induction of TCR-ζ phosphorylation in the CD45-deficient JS-7 cell line appears to be preserved.

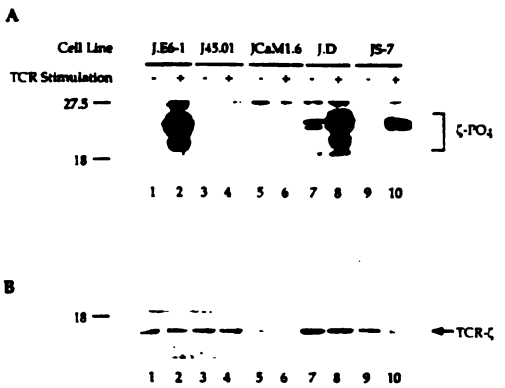


Fig. 3. Phosphorylation of TCR-ζ following TCR stimulation. (A) Jurkat cells (5×10^7 cells) were left unstimulated (lanes 1, 3, 5, 7 and 9) or were stimulated for 2 min with an anti-TCR mAb (lanes 2, 4, 6, 8 and 10). Whole cell lysates of J.E6-1 (lanes 1 and 2), J45.01 (lanes 3 and 4), JCaM1.6 (lanes 5 and 6), J.D (lanes 7 and 8) and JS-7 (lanes 9 and 10) were then immunoprecipitated with an anti-TCR-ζ mAb. The immunoprecipitates were resolved by 12.5% SDS–PAGE and immunoblotted with an anti-phosphotyrosine mAb. (B) The blot in (A) subsequently was stripped and reprobed with an anti-TCR-ζ mAb.

ZAP-70 associates with the phosphorylated ζ chain upon TCR stimulation in JS-7 cells

According to the sequential model of tyrosine kinase activity in the TCR signaling cascade, phosphorylation of the ITAMs in the TCR–CD3 complex leads to the subsequent recruitment of a Syk/ZAP-70 family member. Immunoprecipitations of ZAP-70 were performed using cell lysates prepared from unstimulated and stimulated cells. Anti-phosphotyrosine immunoblotting of ZAP-70 immunoprecipitates revealed that ZAP-70 was inducibly phosphorylated following TCR stimulation in the signaling-competent CD45-deficient JS-7 cell line (Figure 4,

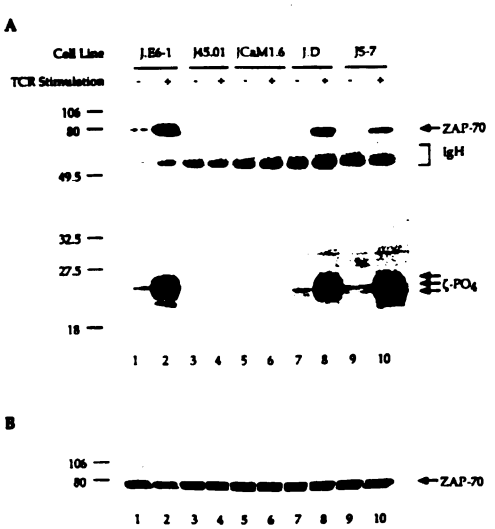


Fig. 4. Phosphorylation of ZAP-70 following TCR stimulation. (A) Jurkat cells were left unstimulated (lanes 1, 3, 5, 7 and 9) or were stimulated (lanes 2, 4, 6, 8 and 10) as in Figure 3. Immunoprecipitations were performed using anti-ZAP-70 antisera and blotted with an anti-phosphotyrosine mAb. (B) The blot was stripped and reprobed with anti-ZAP-70 antisera. Lanes represent: J.E6-1 (lanes 1 and 2), J45.01 (lanes 3 and 4), JCaM1.6 (lanes 5 and 6), J.D (lanes 7 and 8) and JS-7 (lanes 9 and 10).

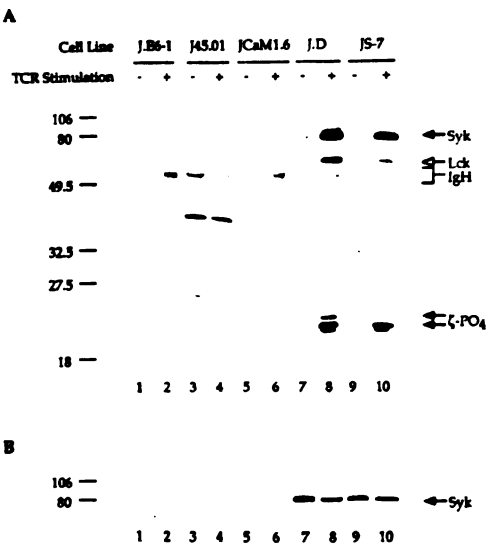


Fig. 5. Phosphorylation of Syk following TCR stimulation. (A) Jurkat cells were left unstimulated (lanes 1, 3, 5, 7 and 9) or were stimulated (lanes 2, 4, 6, 8 and 10) as in Figure 3. Immunoprecipitations were performed using anti-Syk antisera and blotted with an anti-phosphotyrosine mAb. (B) The blot was stripped and reprobed with anti-Syk antisera. Lanes represent: J.E6-1 (lanes 1 and 2), J45.01 (lanes 3 and 4), JCaM1.6 (lanes 5 and 6), J.D (lanes 7 and 8) and JS-7 (lanes 9 and 10).

lane 10), like the response observed with the two wild-type Jurkat cell lines, J.E6-1 and J.D (Figure 4). Furthermore, the phosphorylated ζ chain was associated with phosphorylated ZAP-70 (Figure 4). Therefore, the early TCR signal transduction events appear to be normal in the CD45-deficient JS-7 cell line.

Syk is phosphorylated and associates with the phosphorylated ζ chain upon TCR stimulation only in J.D and JS-7 cells

The Syk PTK, like ZAP-70, has also been implicated in some TCR-mediated signaling processes (Chan *et al.*, 1994b; Couture *et al.*, 1994a; van Oers *et al.*, 1995). To determine whether Syk was involved in the signaling events following TCR stimulation in the JS-7 cell line, we initially examined the phosphorylation status of Syk following TCR stimulation. Immunoprecipitations using an anti-Syk antiserum were performed on unstimulated or stimulated lysates prepared from the five different cell lines. Syk was found to be tyrosine phosphorylated inducibly only in J.D and JS-7 cells (Figure 5A, lanes 7–10). In addition, phosphorylated Syk was also found to associate with the phosphorylated ζ chain in J.D and JS-7 cells (Figure 5A). The phosphorylated bands of ~40 kDa in the J45.01 immunoprecipitates are non-specific and not seen reproducibly (Figure 5A, lanes 3 and 4).

To determine why Syk was not phosphorylated in all of the signaling-competent Jurkat lines, we blotted the anti-Syk immunoprecipitates for Syk expression. Notably, Syk protein was only detected in immunoprecipitates of the cells derived from the J.D Jurkat line, J.D and JS-7 (Figure 5B). In contrast, Syk was not detected in anti-Syk immunoprecipitates from any of the cell lines derived from the E6-1 Jurkat line, J.E6-1, J45.01 or JCaM1.6 (Figure 5B, lanes 1–6). Although no Syk was detected in the anti-Syk immunoblot of wild-type J.E6-1 Jurkat cells, very weak tyrosine phosphorylation of a 72 kDa band can be detected occasionally in anti-Syk immunoprecipitation of stimulated lysates of these cells, indicating that there may be very low levels of Syk expressed (Figure 5B, lanes 1–2 and data not shown). These results are consistent with the recent findings that E6-1-derived Jurkat cells express mutant Syk transcripts containing a nucleotide insertion resulting in a frameshift and premature stop codon (Fargnoli *et al.*, 1995).

Importantly, Syk expression and phosphorylation were detected in the signaling-competent CD45-deficient cell line, JS-7, and not in the signaling-incompetent CD45-deficient cell line, J45.01. Thus, there is a correlation between Syk expression and the TCR signaling capability of CD45-deficient cells. Consistent with this interpretation, another CD45-deficient T cell line that is unable to signal, H45.052, a derivative of the HPB.ALL line, also expresses low to undetectable amounts of Syk protein (Koretzky *et al.*, 1990; Law *et al.*, 1994; and data not shown).

A smaller, 55 kDa phosphorylated band was found to be associated with Syk after stimulation in both the J.D and JS-7 cell lines. At least a portion of this band represents the Lck PTK (data not shown), consistent with previous reports of Syk and ZAP-70 association with Lck (Duplay *et al.*, 1994; Thome *et al.*, 1995).

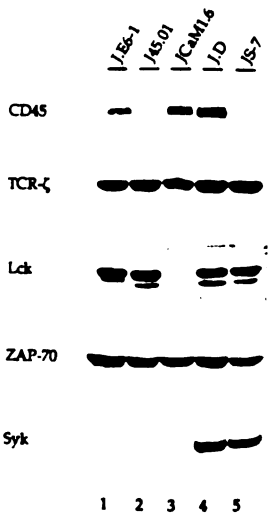


Fig. 6. Expression of proteins involved in early TCR signaling events in various Jurkat cell lines. Cells (2×10^6 cells) were lysed as described. One hundred μg of total cell lysate of J.E6-1 (lane 1), J45.01 (lane 2), JCaM1.6 (lane 3), J.D (lane 4) and JS-7 (lane 5) were resolved by 12.5% SDS–PAGE and immunoblotted with the antibodies indicated: anti-CD45; anti-ζ; anti-Lck; anti-ZAP-70; anti-Syk.

Levels of various proteins involved in proximal TCR signaling

The unexpected difference in levels of Syk expression in the various Jurkat lines we were studying prompted us to compare the relative expression in these cells of a number of the proteins involved in the earliest TCR signaling events, CD45, TCR-ζ, Lck, ZAP-70 and Syk. As mentioned above, neither of the previously described CD45-deficient cell lines, J45.01 and JS-7, expressed CD45 by immunoblotting and only small amounts were expressed by cell surface staining (Figure 1, and Figure 6, lanes 2 and 5). The wild-type parental cell lines, J.E6-1 and J.D, expressed equivalent amounts of CD45 both by immunoblotting and by cell surface staining (Figure 1, and Figure 6, lanes 1 and 4), as did the Lck-deficient derivative of J.E6-1, JCaM1.6 (Figure 6, lane 3; and data not shown). Lck was found to be expressed at equivalent levels in all cell lines except the Lck null mutant, JCaM1.6, which expressed no Lck by immunoblotting (Figure 6). Immunoblots of the ζ chain and ZAP-70 showed that these proteins were expressed at roughly equivalent levels in all five cell lines tested (Figure 6). However, as in the Syk immunoprecipitations, Syk was only expressed in the J.D-derived cell lines, J.D and JS-7 (Figure 6). Wild-type J.E6-1 cells and mutants derived from this Jurkat line (J45.01 and JCaM1.6) expressed low to undetectable levels of the Syk PTK protein (Figure 6).

Expression of Syk can restore TCR signaling in the CD45-deficient cell line J45.01

Based on the above data, one clear difference between the CD45-deficient cell line that can signal, JS-7, and the one that cannot, J45.01, is that the signaling-competent line expresses Syk whereas the signaling-incompetent line does not. We hypothesized, therefore, that the introduction of Syk into the Syk-deficient, CD45-deficient line J45.01 might be able to restore TCR signaling.

To assess this possibility, human Syk was transfected into J45.01 cells. Syk was immunoprecipitated from the transfected cells and its phosphorylation status assessed by anti-phosphotyrosine immunoblotting. Syk was expressed and inducibly phosphorylated upon TCR stimulation only in J45.01 cells transfected with Syk (Figure 7A and B). Furthermore, phosphorylated Syk was found to be associated with phosphorylated TCR-ζ in these transfectants, as was observed for the Syk-expressing Jurkat lines J.D and JS-7 (Figures 7A and 5A). More distal tyrosine phosphorylation events appeared to be restored in the Syk-transfected J45.01 cells as well. The tyrosine phosphorylation pattern in anti-phosphotyrosine immunoprecipitates from stimulated J45.01 cells transfected with Syk appears to be enhanced as compared with J45.01 cells transfected with an empty vector (Figure 7C). Comparison of the anti-phosphotyrosine immunoprecipitates from Syk-transfected J45.01 cells with those from the signaling-competent JS-7 cells reveals a pattern of tyrosine phosphoproteins that is virtually identical. The higher level of phosphoprotein induction in JS-7 cells reflects the result of the low efficiency of transient transfection into J45.01 cells, resulting in a smaller number of J45.01 cells that express Syk compared with the JS-7 cells.

In order to study, in more detail, the restoration of TCR signaling in these Syk-transfected J45.01 cells, we examined a more distal event, activation of an NF-AT reporter construct. This reporter construct consists of three tandem repeats of the NF-AT binding site derived from the IL-2 gene controlling the expression of the luciferase gene (NFAT–Luc). NF-AT-driven transcription is responsive to TCR stimulation (Durand *et al.*, 1988). Rat Syk, epitope-tagged with a portion of the hemagglutinin (HA) protein, was transfected into the J45.01 cell line together with this reporter construct. Figure 8 shows that NF-AT-regulated luciferase activity was restored in a dose-dependent, stimulation-dependent manner when Syk cDNA was transfected into J45.01. In contrast, transfection of comparable amounts of human ZAP-70 cDNA did not reconstitute the response (Figure 8). In this experiment, maximal NF-AT induction in Syk-transfected J45.01 cells in response to TCR stimulation was 15-fold over basal, similar to the 11-fold induction in the parental wild-type J.E6-1 cells, from which J45.01 was selected, transfected with vector alone (data not shown). The slight decrease in NF-AT fold induction at the highest level of expression (40 μg of DNA transfected) was not due to an inhibitory effect on signaling activity, but rather reflects a rise in basal NF-AT activity when Syk is expressed at these levels. Rat Syk was as effective as non-epitope-tagged human Syk in reconstituting these human cell T cell lines (data not shown).

Expression of Syk can restore TCR signaling in the Lck-deficient cell line JCaM1.6

Because the presumed block in TCR signaling in CD45-deficient cells is at the level of Lck activation, we next determined if expression of Syk in an Lck-deficient cell could restore TCR-mediated signaling. As is shown in Figure 9A, a similar dose-dependent, stimulation-

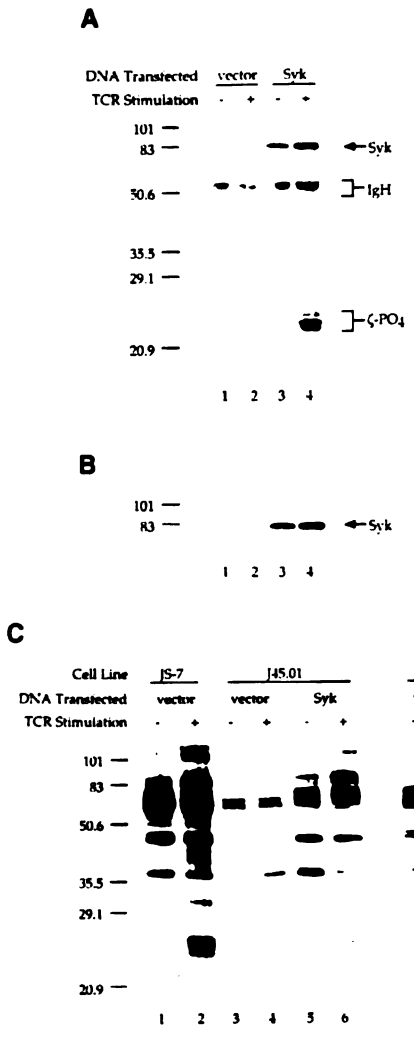


Fig. 7. Induction of tyrosine phosphoproteins in Syk-transfected J45.01 cells. (A) J45.01 cells (2×10^8 cells) were transfected with an empty vector (lanes 1 and 2) or with a Syk expression plasmid (lanes 3 and 4). A total of 10^7 live cells were recovered per transfectant and left unstimulated (lanes 1 and 3) or stimulated with an anti-TCR antibody (lanes 2 and 4). Lysates from these cells were immunoprecipitated with anti-Syk antisera and resolved by 12.5% SDS–PAGE. The immunoprecipitates were then transferred to membranes and probed with an anti-phosphotyrosine mAb. (B) The blot in (A) was stripped and reprobed with anti-Syk antisera. (C) J45.01 cells were transfected with empty vector (lanes 3 and 4) or with a Syk expression plasmid (lanes 5 and 6). Cells were left unstimulated (lanes 1, 3 and 5) or were stimulated with an anti-TCR antibody (lanes 2, 4 and 6). Lysates were immunoprecipitated with an anti-phosphotyrosine mAb covalently coupled to protein A–Sepharose. Immunoprecipitates were then resolved by 12.5% SDS–PAGE and blotted with an anti-phosphotyrosine mAb. JS-7 cells (lanes 1 and 2) were transfected with empty vector as a positive control. In the panel on the right, a shorter exposure of the immunoprecipitates from the JS-7 cells is shown for comparison of the pattern of phosphotyrosine bands seen in the anti-phosphotyrosine immunoprecipitations from transfected J45.01 cells. The results shown are representative of three independent experiments.

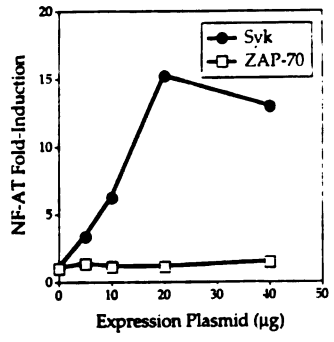


Fig. 8. Syk can reconstitute TCR signaling in J45.01 cells in a dose-dependent, stimulation-dependent manner. J45.01 cells were co-transfected with NFAT–Luc together with either an empty vector or various amounts of epitope-tagged Syk (●) or epitope-tagged ZAP-70 (□). Transfected cells were then either left unstimulated or were stimulated with an anti-TCR mAb and then assayed for luciferase activity. Results are expressed as the fold induction of luciferase activity after stimulation compared with the unstimulated state for each condition. The results shown are representative of four independent experiments.

dependent restoration of NF-AT activity was seen with overexpression of Syk in JCaM1.6 cells, similar to that of J45.01 cells. In the case of JCaM1.6 cells transfected with Syk, maximal TCR-mediated NF-AT induction was 36-fold, compared with 15-fold induction for the vector-transfected parental J.E6-1 cells (data not shown). In this experiment, using both HA-epitope tagged Syk and HA-epitope tagged ZAP-70, equivalent levels of expressed ZAP-70 protein were unable to restore signaling (Figure 9B). This effect was a specific one, as overexpression of Syk in another Jurkat signaling mutant, JCaM2.5, representative of a distinct complementation group (Goldsmith and Weiss, 1987), was not able to reconstitute TCR signaling, as measured by NF-AT activation (data not shown).

Syk requires the SH2 domains, kinase domain and the regulatory domain phosphorylation sites to function in TCR signaling

To address which functional regions of Syk are required for the reconstitution of signaling in these various E6-1-derived Jurkat mutants, and to determine whether this mechanism parallels the well-characterized ZAP-70 pathway in T cells and the Syk pathway in B cells, we transiently expressed various mutants of Syk in the Lck-deficient cell line JCaM1.6.

Three mutants of Syk were used. The first, a kinase-inactive mutant, contains a mutation of a critical lysine to arginine (K395R) in the putative ATP binding site of rat Syk. The second mutant contained changes in the YYKAQ sequence of Syk, present in the putative regulatory loop of the kinase domain (Hubbard *et al.*, 1994). The two tyrosines in this sequence, required for maximal Syk phosphorylation and kinase activity (Couture *et al.*, 1994b), were mutated to phenylalanine (Y519F and Y520F, YYFF mutant). The final mutant is one in which critical arginines within the phosphotyrosine binding pockets of the two SH2 domains were mutated to alanine (R41A and R194A).

These mutants of Syk were transiently co-transfected

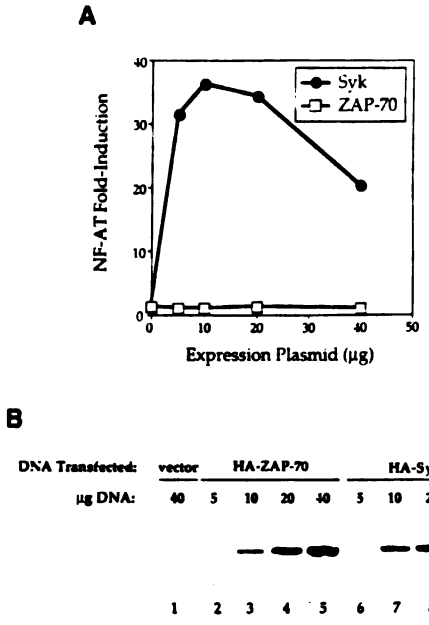


Fig. 9. Syk can reconstitute TCR signaling in JCaM1.6 cells in a dose-dependent, stimulation-dependent manner. (A) JCaM1.6 cells were transfected and analyzed as described for J45.01 transfectants in Figure 8. The results shown are representative of four independent experiments. (B) Aliquots of the cells (5×10^5 cells/lane) transfected in (A) were lysed and immunoblotted with an mAb against the HA epitope. Lane 1 represents JCaM1.6 cells transfected with an empty vector. Lanes 2–5 represent JCaM1.6 cells transfected with 5, 10, 20 and 40 μg of epitope-tagged ZAP-70 DNA, respectively. Lanes 6–9 represent JCaM1.6 cells transfected with 5, 10, 20 and 40 μg of epitope-tagged Syk DNA, respectively.

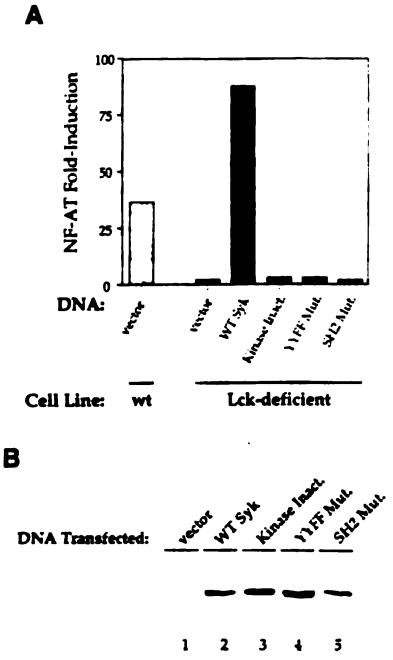


Fig. 10. Syk requires functional SH2 and kinase domains, as well as intact regulatory phosphorylation sites, to restore TCR signaling in JCaM1.6. (A) JCaM1.6 cells were co-transfected with NFAT–Luc and empty vector alone, or with 10 μg of epitope-tagged wild-type Syk (WT Syk), kinase-inactive (Kinase Inact.), regulatory phosphorylation site mutant (YYFF Mut.) or SH2 mutant (SH2 Mut.) plasmid DNA, respectively. The transfectants were then treated and analyzed as described in Figure 8. NF-AT fold induction of wild-type J.E6-1 cells transfected with empty vector alone is shown as a reference (shaded bar). The results shown are representative of four independent experiments. (B) Aliquots of cells (5×10^5 cells/lane) from the transfectants in (A) were lysed and immunoblotted with an anti-Syk antiserum. Lanes represent cells transfected with empty vector (lane 1); wild-type HA–Syk (lane 2); the kinase-inactive mutant (lane 3); the regulatory phosphorylation site mutant (YYFF Mut.; lane 4); and the SH2 mutant (lane 5).

with the NFAT–Luc reporter construct into the Lck-deficient JCaM1.6 cells. Functional TCR signaling was measured by assaying NF-AT-driven transcriptional activity following TCR stimulation. Although wild-type Syk was able to restore signaling, the kinase-inactive mutant of Syk could not, nor could the autophosphorylation mutant of Syk (Figure 10A). Moreover, functional SH2 domains of Syk were required for restoration of TCR signaling, since the Syk SH2 mutant, unable to bind to phosphorylated tyrosine residues and therefore unable to bind to a phosphorylated ITAM, was incapable of restoring NF-AT responsiveness (Figure 10A). As can be seen in Figure 10B, the mutants of Syk were expressed in this transient assay at levels equal to or greater than that of the wild-type kinase. Thus, the SH2 domains, kinase domain and regulatory phosphorylation sites within the kinase domain of Syk are required for the restoration of TCR signaling in JCaM1.6. The same requirements were found for reconstitution of TCR signaling in the J45.01 cell line (data not shown).

Discussion

The results presented here provide evidence that the Syk PTK is able to function in the TCR signaling pathway independently of CD45 and Lck in the Jurkat T cell line. Syk protein expression was found to correlate with the ability of CD45-deficient Jurkat cells to signal through their TCR. Overexpression of Syk was able to restore TCR-mediated signaling in a dose-dependent manner in two Jurkat E6-1-derived mutants, J45.01 and JCaM1.6. This effect was specific since overexpression of equivalent amounts of ZAP-70 was unable to restore TCR signaling function. Thus, our findings demonstrate that the two closely related PTK family members Syk and ZAP-70 differ significantly in their requirements for activation in T cells.

CD45 is an important component of the TCR and B cell antigen receptor signaling pathways (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Justement *et al.*, 1991; Shiroo *et al.*, 1992; Volarevic *et al.*, 1992). It is proposed to be a positive regulator of Lck and Fyn in T cells by acting on the carboxy-terminal negative regulatory tyrosine present in these Src family kinases. In cells expressing CD45, stimulation of the TCR is presumed to induce Lck or Fyn to phosphorylate tyrosines contained

within TCR ITAMs. This phosphorylation, in turn, allows subsequent recruitment of ZAP-70 or Syk to the stimulated receptor complex. However, in the absence of CD45, most investigators have found that Lck and Fyn are hyperphosphorylated at their negative regulatory sites and that the initial events associated with TCR stimulation do not occur (Ostergaard *et al.*, 1989; Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Shiroo *et al.*, 1992; Volarevic *et al.*, 1992; Hurley *et al.*, 1993; McFarland *et al.*, 1993; Sieh *et al.*, 1993). Therefore, the ability of the CD45-deficient JS-7 line to respond to TCR signals represented a paradox. Our studies suggest that the higher level of Syk expression in the CD45-deficient JS-7 Jurkat line can overcome the requirement for both CD45 and Lck. Although Syk transcripts contain a mutation which explains the relative deficiency of Syk in Jurkat clone E6-1-derived mutants (Fargnoli *et al.*, 1995), most mature T cells and lines generally also have low levels of Syk (Chan *et al.*, 1994b), accounting for the similar requirements for CD45 and Lck in TCR signal transduction that have been observed by several other laboratories (Pingel and Thomas, 1989; Koretzky *et al.*, 1990, 1991; Karnitz *et al.*, 1992; Shiroo *et al.*, 1992; Straus and Weiss, 1992; McFarland *et al.*, 1993).

Previous reports have suggested that both Syk and ZAP-70 are recruited to tyrosine-phosphorylated ITAMs in antigen–receptor complexes (reviewed in Weiss and Littman, 1994). The requirement for the SH2 domains of Syk in the reconstitution of both CD45- and Lck-deficient E6-1-derived lines suggests that the phosphorylation of an ITAM is still necessary for Syk to function in these cells, presumably allowing for its recruitment. The kinase responsible for the phosphorylation of ITAMs in the absence of Lck or CD45 is not known. While it is possible that Syk itself is responsible for the ITAM phosphorylation, this seems unlikely since Syk has not been reported to interact with an unphosphorylated ITAM, and the requirement for its SH2 domains would suggest that it only interacts with an ITAM which has already been phosphorylated (Rowley *et al.*, 1995). Moreover, ζ ITAMs are poor substrates for Syk *in vitro* (A.Weiss, unpublished data). A more likely possibility is that Fyn, which appears to be less dependent on the presence of CD45, may partially compensate for the loss of Lck or CD45 (Sieh *et al.*, 1993). Finally, it remains possible that another, as yet unidentified, kinase may be responsible for ITAM phosphorylation. This is an intriguing possibility since the stable association of either Lck or Fyn with the TCR complex has been observed only at low stoichiometry (Samelson *et al.*, 1990; Burgess *et al.*, 1991; Duplay *et al.*, 1994).

Once Syk and ZAP-70 are recruited to the phosphorylated ITAM, their functions appear to be regulated in distinct ways. Phosphorylated but not unphosphorylated ITAM peptides from either FcεRI or Ig-α/β are able to increase the catalytic activity of Syk *in vitro*, independent of an interaction involving a Src family kinase (Rowley *et al.*, 1995; Shiue *et al.*, 1995). This activation may occur by a mechanism of transphosphorylation involving two Syk molecules bound to neighboring ITAMs. Although Syk and ZAP-70 were reported to have similar binding affinities for association with the CD3ε ITAM, the catalytic activity of ZAP-70 is not increased when it binds to an ITAM (Bu *et al.*, 1995; Neumeister *et al.*, 1995). Instead, it appears that an increase in ZAP-70 kinase activity is observed after phosphorylation of tyrosines in the regulatory loop of its kinase domain by Lck (Chan *et al.*, 1995). Whereas substantial data suggest that maximal Syk functional activity is influenced by interactions involving Src family kinases, it is possible that Syk can overcome the deficiency of Lck—or the loss of Lck function due to the absence of CD45—since its catalytic function can be activated directly by ITAM binding.

Our studies suggest that Syk and ZAP-70 are not functionally redundant. Whereas Syk can reconstitute Lck- or CD45-deficient cells, a comparable level of ZAP-70 expression cannot. These results are consistent with the observations of Kolanus *et al.* (1993), who examined the function of chimeric receptors containing Syk or ZAP-70. They found that cross-linking a CD16–Syk chimera alone was sufficient to induce signal transduction events, whereas a CD16–ZAP-70 chimera required cross-linking with a chimera containing a Src family member to induce comparable signaling events. However, Kong *et al.* (1995) suggested that Syk and ZAP-70 are functionally equivalent. It is noteworthy that the latter studies involved the reconstitution of an avian B cell line, not a T cell. The functional difference between Syk and ZAP-70 revealed in our T cell mutants may have been masked in the context of the B cell system, perhaps because the presence of active B cell Src family kinases such as Lyn is sufficient to activate ZAP-70.

Interestingly, ZAP-70 is phosphorylated at wild-type levels in the CD45-deficient JS-7 cells that contain Syk. This is an unexpected finding since in these cells, Lck (the kinase thought to phosphorylate ZAP-70) is in an inactive conformation. It may be that Syk is able to phosphorylate ZAP-70 *in trans*. In support of this model, we have observed that Syk can phosphorylate a kinase-inactive version of ZAP-70 when both proteins are overexpressed in COS-7 cells (data not shown). However, the specific sites of phosphorylation on ZAP-70, and what effect this phosphorylation may have on the kinase activity of ZAP-70, have not been determined. Alternatively, Lck bound to Syk may be able to phosphorylate ZAP-70. Indeed, in JS-7 cells, phosphorylated Syk and Lck are found to be associated. The Lck SH2 domain has been implicated in binding Syk and ZAP-70 (Duplay *et al.*, 1994; Aoki *et al.*, 1995; Thome *et al.*, 1995). Therefore, the phosphorylated negative regulatory tyrosine of the Lck that is bound to Syk may be displaced from its SH2 domain, allowing Lck to phosphorylate other substrates, despite its hyperphosphorylation on the carboxy-terminal tyrosine. Again, this mechanism of Lck activation would have to be specific for Syk and not for ZAP-70.

Couture *et al.* (1994a,b) have proposed that Syk operates upstream of Src family members in TCR signaling by phosphorylating and activating Lck. However, in those studies, signaling was only assessed as the phosphorylation of a 70 kDa band (Couture *et al.*, 1994a). Furthermore, some of those studies were performed using JCaM1.6 cells, which have a mutation in Syk transcripts and therefore are deficient in endogenous Syk expression (Couture *et al.*, 1994a; Fargnoli *et al.*, 1995). Additionally, Syk is not a critical upstream activator of Lck or ZAP-70 in TCR signaling because most mature T cells and lines,

which are able to signal normally through their TCRs, express low levels of Syk. Furthermore, T cells from mice deficient in Syk are able to signal normally following TCR stimulation (Turner *et al.*, 1995). On the other hand, Syk may be sufficient to mediate TCR signaling in the absence of ZAP-70. This is suggested by observations that a cell line made from peripheral T cells of human ZAP-70-deficient patients is able to signal in response to TCR stimulation, and these cells have an increased level of Syk expression as compared with a peripheral T cell line derived from normal patients (Gelfand *et al.*, 1995).

Thus, the findings described here demonstrate the ability of Syk to function independently of CD45 or Lck. Furthermore, differential expression of Syk in various cell lines is likely to explain the variety of signaling phenotypes seen in CD45-deficient cells. Finally the fact that Syk, but not ZAP-70, is able to function in the absence of CD45 or Lck is a clear difference between two closely related family members and indicates that these two kinases have significantly different requirements for activation. The differential activities of these two kinases suggest that they may play distinct, rather than completely redundant, roles in lymphocyte signaling.

Materials and methods

Cells and antibodies

The human leukemic Jurkat T cell lines J.E6-1 (Weiss *et al.*, 1984), J45.01 (Koretzky *et al.*, 1991), JCaM1.6 (Straus and Weiss, 1992), J.D (Peyron *et al.*, 1991) and JS-7 (Peyron *et al.*, 1991) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin and glutamine (Irvine Scientific, Irvine, CA). The J.D cell line is the wild-type parental Jurkat line described by Peyron *et al.* (1991). JS-7 refers to 'Jurkat subclone #7,' a subclone of the CD45-deficient Jurkat clone #25 described in the same paper. Note that J.D is a wild-type Jurkat line that is distinct from the wild-type J.E6-1 Jurkat line (Weiss *et al.*, 1984) that has been described previously. Murine monoclonal antibodies (mAbs) and their specificities include: C305, Jurkat Ti β-chain (Weiss and Stobo, 1984); 6B10.2, ζ (van Oers *et al.*, 1995); 2F3.2, ZAP-70 (Iwashima *et al.*, 1994); 4G10, phosphotyrosine (Upstate Biotechnology, Incorporated, Lake Placid, NY); 1F6, Lck (Burkhardt *et al.*, 1994); 9.4, CD45 (HB 10508, American Type Culture Collection, Rockville, MD); and 12CA5, hemagglutinin epitope (Boehringer-Mannheim, Indianapolis, IN). Rabbit antisera and their specificities are 1373, Syk (van Oers *et al.*, 1995) and 1222, ZAP-70 (Chan *et al.*, 1992). The 4G10 mAb was covalently coupled to protein A–Sepharose CL-4B (Pharmacia LKB, Piscataway, NJ) using dimethylpimelimidate (Harlow and Lane, 1988).

Plasmids

cDNAs encoding wild-type human ZAP-70, wild-type rat Syk or mutants of Syk were subcloned into the mammalian expression vector pEF-BOS (Mizushima and Nagata, 1990) for transient transfections. Mutants of HA epitope-tagged rat Syk (Rowley *et al.*, 1995) were constructed by site-directed mutagenesis using the p-Alter system (Promega, Madison, WI). The kinase-inactive mutant was generated by mutating Lys395 to Arg. The double SH2 mutant has been described (Rowley *et al.*, 1995). For the YYFF mutant, point mutations were introduced to change both of the Tyr residues at positions 518 and 519 to Phe. The *Xba*I fragment of the C-terminal epitope-tagged ZAP-70 cDNA in pSV7d was subcloned into pEF-BOS. For the N-terminal epitope-tagged Syk constructs, the *Sal*I–*Eco*RI fragment containing the HA–Syk sequence in the pMEX vector was subcloned into pEF-BOS. The NF-AT reporter construct (NFAT–Luc) was a generous gift from G.Crabtree.

Cell stimulations, immunoprecipitations, electrophoresis and immunoblots

Jurkat cells were stimulated with anti-TCR antibodies (1:1000 dilution of C305 ascitic fluid) at 37°C for 2 min. Cells were lysed in buffer containing 1% Nonidet P-40, 10 mM Tris (pH 7.8), 150 mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors as previously described (Straus and Weiss, 1992). After normalizing for protein content using the Bio-Rad protein assay, 3.6 mg of total cell lysate were immunoprecipitated per sample. Immunoprecipitations were carried out as previously described (Qian *et al.*, 1993). For immunoprecipitations using ascites, 1 µl of ascitic fluid was used per sample. For those using rabbit heterosera, 4 µl of rabbit antisera were used per sample. For immunoblots of whole cell lysates, 100 µg of protein were loaded per lane. Immunoprecipitates or lysates were resolved by SDS–PAGE and transferred to polyvinylidene difluoride membranes (Immobilon) (Millipore Ltd, Bedford, MA). The membranes were blocked with 5% dry milk powder and 3% bovine serum albumin (BSA) in 10 mM Tris (pH 7.6), 500 mM NaCl and 0.05% Tween-20, or with 5% BSA in phosphate-buffered saline. Blots were then incubated with primary antibody and washed with 10 mM Tris (pH 7.6), 500 mM NaCl and 0.05% Tween-20. After incubation of blots with secondary antibody coupled to horseradish peroxidase or alkaline phosphatase, results were visualized by either enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) or by alkaline phosphatase detection (Zymed, South San Francisco, CA). Blots were stripped according to manufacturer's instructions (Amersham) and reprobed as described above.

Luciferase assays

A total of 10^7 cells were co-transfected with 20 µg of NFAT–Luc reporter plasmid and varying amounts of empty vector, Syk or ZAP-70 plasmids by electroporation at 250 V and 960 µF using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA). Transfected cells were cultured for 24 h in RPMI-1640 supplemented with 10% fetal bovine serum, penicillin, streptomycin and L-glutamine. Cells were then counted and 10^5 live cells were plated per well in 96-well round bottom plates (Corning) in 100 µl. Cells were left unstimulated or stimulated with C305 ascites (1:1000 dilution of ascitic fluid), or phorbol myristate acetate (50 ng/ml) and ionomycin (1.0 µM) for 6 h. Samples were then lysed in a 100 µl volume of 100 mM KPO_4 (pH 7.8), 5.0 mM dithiothreitol and 1% Triton X-100, and this lysate was mixed with 100 µl of assay buffer [200 mM KPO_4 (pH 7.8), 10 mM ATP, 20 mM $MgCl_2$] followed by 100 µl of 1.0 mM luciferin. Luciferase activity, expressed in arbitrary units, was determined in duplicate for each experimental condition. Fold induction was calculated as the ratio of luciferase activity following stimulation divided by the activity in the unstimulated state for each condition.

Acknowledgements

We thank G.Crabtree for the NFAT–Luc reporter plasmid; D.Cantrell for the pEF-BOS plasmid; and Drs A.DeFranco and N.van Oers for helpful discussions and critical reading of the manuscript. This work was supported in part by the Medical Scientist Training Program (D.H.C.) and by National Institutes of Health Grant GM39553 (to A.W.).

References

Aoki,Y., Kim,Y.-T., Stillwell,R., Kim,T.J. and Pillai,S. (1995) The SH2 domains of Src family kinases associate with Syk. *J. Biol. Chem.*, **270**, 15658–15663.

Appleby,M.W., Gross,J.A., Cooke,M.P., Levin,S.D., Qian,X. and Perlmutter,R.M. (1992) Defective T cell receptor signaling in mice lacking the thymic isoform of p59^{fyn}. *Cell*, **70**, 751–763.

Arpaia,E., Shahar,M., Dadi,H., Cohen,A. and Roifman,C.M. (1994) Defective T cell receptor signaling and CD8$^-$ thymic selection in humans lacking ZAP-70 kinase. *Cell*, **76**, 947–958.

Bu,J.-Y., Shaw,A.S. and Chan,A.C. (1995) Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. *Proc. Natl Acad. Sci. USA*, **92**, 5106–5110.

Burgess,K.E., Odysseos,A.D., Zalvan,C., Drucker,B., Anderson,P., Schlossman,S.F. and Rudd,C.E. (1991) Biochemical identification of a direct physical interaction between the CD4:p56lck and Ti(TCR)/CD3 complexes. *Eur. J. Immunol.*, **21**, 1663–1668.

Burkhardt,A.L., Stealey,B., Rowley,R.B., Mahajan,S., Prendergast,M., Fargnoli,J. and Bolen,J.B. (1994) Temporal regulation of non-transmembrane protein tyrosine kinase enzyme activity following T cell antigen receptor engagement. *J. Biol. Chem.*, **269**, 23642–23647.

Chan,A.C., Iwashima,M., Turck,C.W. and Weiss,A. (1992) ZAP-70: a 70kD protein tyrosine kinase that associates with the TCR ζ chain. *Cell*, **71**, 649–662.

Chan.A.C., Kadlecek.T.A., Elder.M.E., Filipovich.A.H., Kuo.W.-L., Iwashima.M., Parslow.T.G. and Weiss.A. (1994a) ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science*, **264**, 1599–1601.

Chan.A.C., van Oers.N.S.C., Tran.A., Turka.L., Law.C.-L., Ryan.J.C., Clark.E.A. and Weiss.A. (1994b) Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in T cell antigen receptor signaling. *J. Immunol.*, **152**, 4758–4766.

Chan.A.C., Dalton.M., Johnson.R., Kong.G.-H., Wang.T., Thoma.R. and Kurosaki.T. (1995) Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *EMBO J.*, **14**, 2499–2508.

Cheng.A.M., Rowley.B., Pao.W., Hayday.A., Bolen.J.B. and Pawson.T. (1995) Syk tyrosine kinase required for mouse viability and B-cell development. *Nature*, **378**, 303–306.

Chow.L.M.L., Fournel.M., Davidson.D. and Veillette.A. (1993) Negative regulation of T-cell receptor signalling by tyrosine protein kinase $p50^{csk}$. *Nature*, **365**, 156–160.

Cooper.J.A. and Howell.B. (1993) The when and how of Src regulation. *Cell*, **73**, 1051–1054.

Couture.C., Baier.G., Altman.A. and Mustelin.T. (1994a) $p56^{lck}$-independent activation and tyrosine phosphorylation of $p72^{syk}$ by T-cell antigen receptor/CD3 stimulation. *Proc. Natl Acad. Sci. USA*, **91**, 5301–5305.

Couture.C. *et al.* (1994b) Activation of $p56^{lck}$ by $p72^{syk}$ through physical association and N-terminal tyrosine phosphorylation. *Mol. Cell. Biol.*, **14**, 5249–5258.

Duplay.P., Thome.M., Hervé.F. and Acuto.O. (1994) $p56^{lck}$ interacts via its src homology 2 domain with the ZAP-70 kinase. *J. Exp. Med.*, **179**, 1163–1172.

Durand.D.B., Shaw.J.-P., Bush.M.R., Replogle.R.E., Gelagaje.R. and Crabtree.G.R. (1988) Characterization of antigen receptor response elements within the interleukin-2 enhancer. *Mol. Cell. Biol.*, **8**, 1715–1724.

Elder.M.E., Lin.D., Clever.J., Cahn.A.C., Hope.T.J., Weiss.A. and Parslow.T.G. (1994) Human severe combined immunodeficiency due to a defect in ZAP-70, a T-cell tyrosine kinase. *Science*, **264**, 1596–1599.

Fargnoli.J., Burkhardt.A.L., Lavert.M., Kut.S.A., van Oers.N.S.C., Weiss.A. and Bolen.J.B. (1995) Syk mutation in Jurkat E6-derived clones results in lack of $p72^{syk}$ expression. *J. Biol. Chem.*, **270**, 26533–26537.

Gelfand.E.W., Weinberg.K., Mazer.B.D., Kadlecek.T.A. and Weiss.A. (1995) Absence of ZAP-70 prevents signaling through the antigen receptor on peripheral blood T cells but not thymocytes. *J. Exp. Med.*, **182**, 1057–1066.

Goldsmith.M.A. and Weiss.A. (1987) Isolation and characterization of a T-lymphocyte somatic mutant with altered signal transduction by the antigen receptor. *Proc. Natl Acad. Sci. USA*, **84**, 6879–6883.

Harlow.E. and Lane.D.P. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hubbard.S.R., Wei.L., Ellis.L. and Hendrickson.W.A. (1994) Crystal structure of the tyrosine kinase domain of the human insulin receptor. *Nature*, **372**, 746–754.

Hurley.T.R., Hyman.R. and Sefton.B.M. (1993) Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the *lck*, *fyn*, and c-*src* tyrosine protein kinases. *Mol. Cell. Biol.*, **13**, 1651–1656.

Iwashima.M., Irving.B.A., van Oers.N.S.C., Chan.A.C. and Weiss.A. (1994) Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science*, **263**, 1136–1139.

Justement.L.B., Campbell.K.S., Chien.N.C. and Cambier.J.C. (1991) Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science*, **252**, 1839–1842.

Karnitz.L., Sutor.S.L., Torigoe.T., Reed.J.C., Bell.M.P., McKean.D.J., Leibson.P.J. and Abraham.R.T. (1992) Effects of $p56^{lck}$ on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. *Mol. Cell. Biol.*, **12**, 4521–4530.

Kishihara.K. *et al.* (1993) Normal B lymphocyte development but impaired T cell maturation in CD45-Exon 6 protein tyrosine phosphatase-deficient mice. *Cell*, **74**, 143–156.

Kolanus.W., Romeo.C. and Seed.B. (1993) T cell activation by clustered tyrosine kinases. *Cell*, **74**, 171–183.

Kong.G.-H., Bu.J.-Y., Kurosaki.T., Shaw.A.S. and Chan.A.C. (1995) Reconstitution of Syk function by the ZAP-70 protein tyrosine kinase. *Immunity*, **2**, 485–492.

Koretzky.G.A., Picus.J., Thomas.M.L. and Weiss.A. (1990) Tyrosine phosphatase CD45 is essential for coupling T cell antigen receptor to the phosphatidylinositol pathway. *Nature*, **346**, 66–68.

Koretzky.G., Picus.J., Schultz.T. and Weiss.A. (1991) Tyrosine phosphatase CD45 is required for both T cell antigen receptor and CD2 mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl Acad. Sci. USA*, **88**, 2037–2041.

Law.D.A., Chan.V.W.-F., Datta.S.K. and DeFranco.A.L. (1993) B cell antigen receptor motifs have redundant signalling capabilities and bind the tyrosine kinases PTK72, Lyn and Fyn. *Curr. Biol.*, **3**, 645–657.

Law.C.-L., Sidorenko.S.P., Chandran.K.A., Draves.K.E., Chan.A.C., Weiss.A., Edelhoff.S., Disteche.C.M. and Clark.E.A. (1994) Molecular cloning of human Syk: a B cell protein tyrosine kinase associated with the sIgM/B cell receptor complex. *J. Biol. Chem.*, **269**, 12310–12319.

Mallick-Wood.C.A., Pao.W., Cheng.A.M., Lewis.J.M., Kulkarni.S., Bolen.J.B., Rowley.B., Tigelaar.R.E., Pawson.T. and Hayday.A.C. (1996) Disruption of epithelial γδ T cell repertoires by mutation of the Syk tyrosine kinase. *Proc. Natl Acad. Sci. USA*, **93**, 9704–9709.

McFarland.E.D.C., Hurley.T.R., Pingel.J.T., Sefton.B.M., Shaw.A. and Thomas.M.L. (1993) Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. *Proc. Natl Acad. Sci. USA*, **90**, 1402–1406.

Mizushima.S. and Nagata.S. (1990) pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res.*, **18**, 5322.

Molina.T.J. *et al.* (1992) Profound block in thymocyte development in mice lacking p56lck. *Nature*, **357**, 161–164.

Mustelin.T., Coggeshall.K.M. and Altman.A. (1989) Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase. *Proc. Natl Acad. Sci. USA*, **86**, 6302–6306.

Mustelin.T., Pessa-Morikawa.T., Autero.M., Gassmann.M., Andersson.L.C., Gahmberg.C.G. and Burn.P. (1992) Regulation of the $p59^{fyn}$ protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. *Eur. J. Immunol.*, **22**, 1173–1178.

Negishi.I., Motoyama.N., Nakayama.K.-I., Nakayama.K., Senju.S., Hatakeyama.S., Zhang.Q., Chan.A.C. and Loh.D.Y. (1995) Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature*, **376**, 435–438.

Neumeister.E.N., Zhu.Y., Richard.S., Terhorst.C., Chan.A.C. and Shaw.A.S. (1995) Binding of ZAP-70 to phosphorylated T-cell receptor ζ and η enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. *Mol. Cell. Biol.*, **15**, 3171–3178.

Ostergaard.H.L., Shackelford.D.A., Hurley.T.R., Johnson.P., Hyman.R., Sefton.B.M. and Trowbridge.I.S. (1989) Expression of CD45 alters phosphorylation of the lck-encoded tyrosine protein kinase in murine lymphoma T-cell lines. *Proc. Natl Acad. Sci. USA*, **86**, 8959–8963.

Peyron.J.-F., Verma.S., Malefyt.R., Sancho.J., Terhorst.C. and Spits.H. (1991) The CD45 protein tyrosine phosphatase is required for the completion of the activation program leading to lymphokine production in the Jurkat human T cell line. *Int. Immunol.*, **3**, 1357–1366.

Pingel.J.T. and Thomas.M.L. (1989) Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell*, **58**, 1055–1065.

Qian.D., Griswold-Prenner.I., Rosner.M.R. and Fitch.F.W. (1993) Multiple components of the T cell antigen receptor complex become tyrosine-phosphorylated upon activation. *J. Biol. Chem.*, **268**, 4488–4493.

Rowley.R.B., Burkhardt.A.L., Chao.H.-G., Matsueda.G.R. and Bolen.J.B. (1995) Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. *J. Biol. Chem.*, **270**, 11590–11594.

Samelson.L.E., Phillips.A.F., Luong.E.T. and Klausner.R.D. (1990) Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc. Natl Acad. Sci. USA*, **87**, 4358–4362.

Saouaf.S.J., Mahajan.S., Rowley.R.B., Kut.S.A., Fargnoli.J., Burkhardt.A.L., Tsukada.S., Witte.O.N. and Bolen.J.B. (1994) Temporal differences in the activation of three classes of non-transmembrane protein tyrosine kinases following B-cell antigen receptor surface engagement. *Proc. Natl Acad. Sci. USA*, **91**, 9524–9528.

Shiroo.M., Goff.L., Biffen.M., Shivnan.E. and Alexander.D. (1992) CD45 tyrosine phosphatase-activated $p59^{fyn}$ couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. *EMBO J.*, **11**, 4887–4897.

Shiue.L., Zoller.M.J. and Brugge.J.S. (1995) Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based

activation motifs of the high affinity receptor for IgE. *J. Biol. Chem.*, **270**, 10498–10502.

Sieh,M., Bolen,J.B. and Weiss,A. (1993) CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. *EMBO J.*, **12**, 315–322.

Stein,P.L., Lee,H.-M., Rich,S. and Soriano,P. (1992) $pp59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. *Cell*, **70**, 741–750.

Straus,D. and Weiss,A. (1992) Genetic evidence for the involvement of the Lck tyrosine kinase in signal transduction through the T cell antigen receptor. *Cell*, **70**, 585–593.

Thome,M., Duplay,P., Guttinger,M. and Acuto,O. (1995) Syk and ZAP-70 mediate recruitment of $p56^{lck}$/CD4 to the activated T cell receptor/CD3/ζ complex. *J. Exp. Med.*, **181**, 1997–2006.

Turner,M., Mee,P.J., Costello,P.S., Williams,O., Price,A.A., Duddy,L.P., Furlong,M.T., Geahlen,R.L. and Tybulewicz,V.L.J. (1995) Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. *Nature*, **378**, 298–302.

van Oers,N.S.C., von Boehmer,H. and Weiss,A. (1995) The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-ζ subunit. *J. Exp. Med.*, **182**, 1585–1590.

van Oers,N.S.C., Killeen,N. and Weiss,A. (1996) Lck regulates the tyrosine phosphorylation of the TCR subunits and ZAP-70 in murine thymocytes. *J. Exp. Med.*, **183**, 1053–1062.

Volarevic,S., Niklinska,B.B., Burns,C.M., Yamada,H., June,C.H., Dumont,F.J. and Ashwell,J.D. (1992) The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca^{2+} oscillations. *J. Exp. Med.*, **176**, 835–844.

Weiss,A. and Littman,D.R. (1994) Signal transduction by lymphocyte antigen receptors. *Cell*, **76**, 263–274.

Weiss,A. and Stobo,J.D. (1984) Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. *J. Exp. Med.*, **160**, 1284–1299.

Weiss,A., Wiskocil,R. and Stobo,J.D. (1984) The role of T3 surface molecules in the activation of human T cells: a two stimulus requirement for IL-2 production reflects events occurring at a pre-translational level. *J. Immunol.*, **133**, 123–128.

Received on January 2, 1996; revised on July 5, 1996

CHAPTER 3

A ROLE FOR THE SYK PROTEIN TYROSINE KINASE IN MURINE AND HUMAN PRE-TCR SIGNALING

Summary

Thymocyte development proceeds through two critical checkpoints that involve signaling events through two different receptors, the T cell antigen receptor (TCR) and the pre-TCR. These receptors employ similar machinery to propagate their signals, including two families of protein tyrosine kinases (PTKs), the Src and Syk families. Genetic and biochemical evidence has shown that the Src family kinases are critical for normal T cell maturation. ZAP-70, a Syk family kinase, has similarly been implicated as a critical component in thymocyte development. However, a unique role for the Syk kinase has not been established in this process. Here, I present evidence that Syk may play an important role in thymocyte maturation during pre-TCR signaling. I have analyzed Syk expression in subpopulations of murine and human thymocytes by intracellular staining and flow cytometry. Syk is expressed at increased levels during the stages in which pre-TCR signaling occurs. Furthermore, I show that Syk is downregulated after the pre-TCR checkpoint has been passed. I therefore believe that Syk may play an important role in thymic development during pre-TCR signal transduction.

Introduction

Development of the αβ T cell receptor (TCR) lineage of T cells proceeds through an ordered series of stages defined by the expression of various cell surface markers (3). Immature thymocytes enter the thymus as $CD4^-CD8^-$ cells that develop into $CD4^+CD8^+$ thymocytes. These cells undergo TCR-dependent positive and negative selection and become $CD4^+$ or $CD8^+$ thymocytes which form the mature αβ T cell repertoire in the periphery.

In mice, $CD4^-CD8^-$ thymocytes can be further subdivided according to the cell surface expression of two other proteins, CD44 (Pgp-1) and CD25 (α chain of the IL-2 receptor) (74, 75). The earliest pro-thymocytes are $CD44^+CD25^+$; these cells go on to downregulate CD25. Next, these thymocytes become $CD44^-CD25^+$, during which time the β chain of the T cell receptor is rearranged and expressed on the cell surface as a heterodimer with the pre-Tα (pTα) chain. The $CD4^-CD8^-$ thymocytes that receive a pre-TCR signal progress through an intermediate $CD4^-CD8^+$ stage prior to becoming $CD4^+CD8^+$ thymocytes (89-93).

Productive β chain rearrangement and expression of the pre-T cell receptor is detected via a signal that is transduced through the pre-TCR complex; the nature of this signaling is poorly understood. However, the signal appears to be independent of a pTα or β chain ligand (4). This critical checkpoint in thymocyte development requires two families of protein tyrosine kinases (PTKs), the Src family of PTKs, including Lck and Fyn in T cells, and the Syk family of PTKs, comprising Syk and ZAP-70. These kinases act in a sequential manner to transmit signals from the pre-TCR and TCR complexes (1).

The TCR and pre-TCR heterodimers are components of multimeric complexes containing non-covalently associated signal transducing CD3 subunits γ, δ, and ε, as well as a ζ chain dimer (1). The signal transducing subunits recruit cytosolic tyrosine kinases for their signaling function. This function is initiated by a Src family kinase that phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAMs), which are present as a single copy within each of the CD3 chains and as three copies within the ζ chain. These phosphorylated tyrosines subsequently serve as docking sites for the tandem SH2 domains of both Syk and ZAP-70. Once recruited to the ITAM, the Syk family kinases are activated by tyrosine phosphorylation of their activation loops and transduce further signals for T cell development, activation, and differentiation.

Genetic studies have demonstrated the importance of both families of tyrosine kinases for the development of murine thymocytes. Mice doubly deficient for Lck and Fyn exhibit a profound block in development at the $CD4^-CD8^-$ stage, the time at which pre-TCR signals are required (80, 81). ZAP-70-deficient mice have thymocytes that are arrested at the $CD4^+CD8^+$ stage (69-71). Syk-deficient mice have no reported αβ T cell defect, although a subset of γδ T cells, contained in intraepithelial lymphocytes (IELs), is impaired in their development (88). However, Syk has been shown to be expressed at higher levels in the thymus and is downregulated in the periphery, suggesting that it might play an unrecognized role at some point during thymocyte development (94).

In humans, ZAP-70 deficient patients have been described (65-67). However, unlike in mice, in which thymocytes are completely arrested at the $CD4^+CD8^+$ stage, human patients with a ZAP-70 deficiency have exclusively $CD4^+$ mature T cells that populate the periphery. Studies with HTLV-1 transformed thymocyte lines from these patients indicate that Syk is increased in expression in those cells when compared to normal thymocytes, suggesting that Syk can compensate for the loss of ZAP-70 function in these patients (87).

Nevertheless, the mature $CD4^+$ cells that populate the periphery are unable to signal, perhaps because they have downregulated Syk expression (87).

Syk has been shown to be able to compensate for ZAP-70 in many situations, including αβ T cell development and signaling (57). However, recent evidence has indicated *in vitro* and in cell lines that Syk and ZAP-70 have different requirements for activation. For instance, Syk has less dependence on Src kinases for its activation (Chapter 2). Thus, Syk may have roles distinct from ZAP-70 in certain *in vivo* situations.

Here, I use an intracellular staining method for detecting Syk expression in subpopulations of cells. I have analyzed the expression of Syk in thymocytes of both mice and humans. These results suggest that Syk is expressed at the highest levels during the pre-TCR signaling stage and downregulated quickly thereafter, suggesting that Syk may play a role in propagation of the pre-TCR signal.

Results

Syk is expressed at higher levels in developmentally arrested thymocytes

Thymocytes in RAG-deficient and Lck/Fyn-doubly deficient mice are arrested at the same $CD44^-CD25^+$ $CD4^-CD8^-$ stage of development [(80, 81); also see below]. During studies of thymocytes of developmentally arrested Lck/Fyn doubly deficient mice, I used immunoblotting to examine the expression of other Src family as well as Syk family PTKs in the thymi of these mice (Figure 1). Strikingly, in these whole thymic preparations, I detected high levels of expression of the Src family kinase Fgr, as well as high levels of expression of the Syk PTK (Figure 1). The same pattern of expression was observed in thymic preparations from RAG-deficient mice (Figure 1). It is possible that the high levels of expression of these kinases reflects the increased proportion of dendritic cells, macrophages, and stromal cells in the lysates, due to the decreased number of thymocytes in the developmentally arrested thymi. However, I also considered the possibility that the high level of Syk might reflect the level of expression of Syk in the developmentally arrested thymocytes. I hypothesized that Syk might play a specific role at the $CD4^-CD8^-$ stage of development, when pre-TCR signaling occurs.

Syk expression can be detected by intracellular staining in murine cells

To analyze the expression of the Syk PTK in thymocyte development with greater sensitivity, I generated a monoclonal antibody (mAb), 5F5.2, against the murine protein. This antibody is specific for a peptide sequence in interdomain B of murine Syk, amino acids 306-333. Immunoblot analysis of whole cell lysates using [^{125}I]-iodinated 5F5.2 antibody revealed that it reacts primarily with a 72-kD protein present in large amounts in murine spleen and lymph nodes and almost undetectable in the thymus (Figure 2A). This band was not detected in 3T3 cells, a murine fibroblast line (Figure 2A). The reactivity against the 72-kD band is specific, as it was competed away with peptide against which the

Figure 1. Syk expression in thymi from different lines of mice. Whole cell lysates from the thymi of the indicated lines of mice were normalized for protein content and resolved by SDS-PAGE. Gels were transferred to PVDF membranes and probed with the indicated antibodies. The lanes were loaded as follows: lane 1, wild-type thymus (N. Thymus); lane 2, *lck*$^{-}$*/fyn*$^{-}$ thymus (Lck/Fyn$^{-/-}$); lane 3, *RAG*$^{-}$ thymus (Rag$^{-/-}$); lane 4, wild-type spleen (N. Spleen).

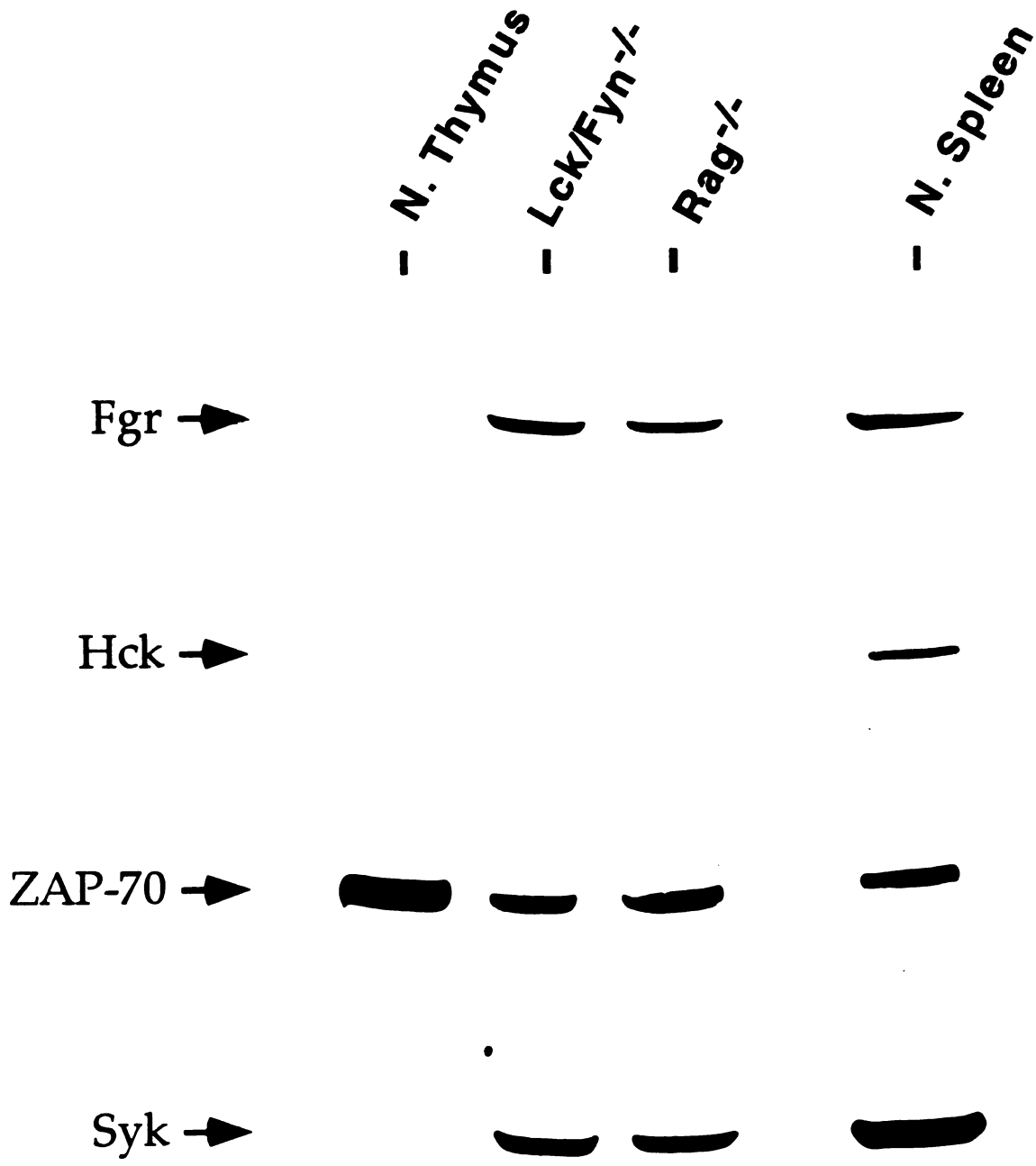
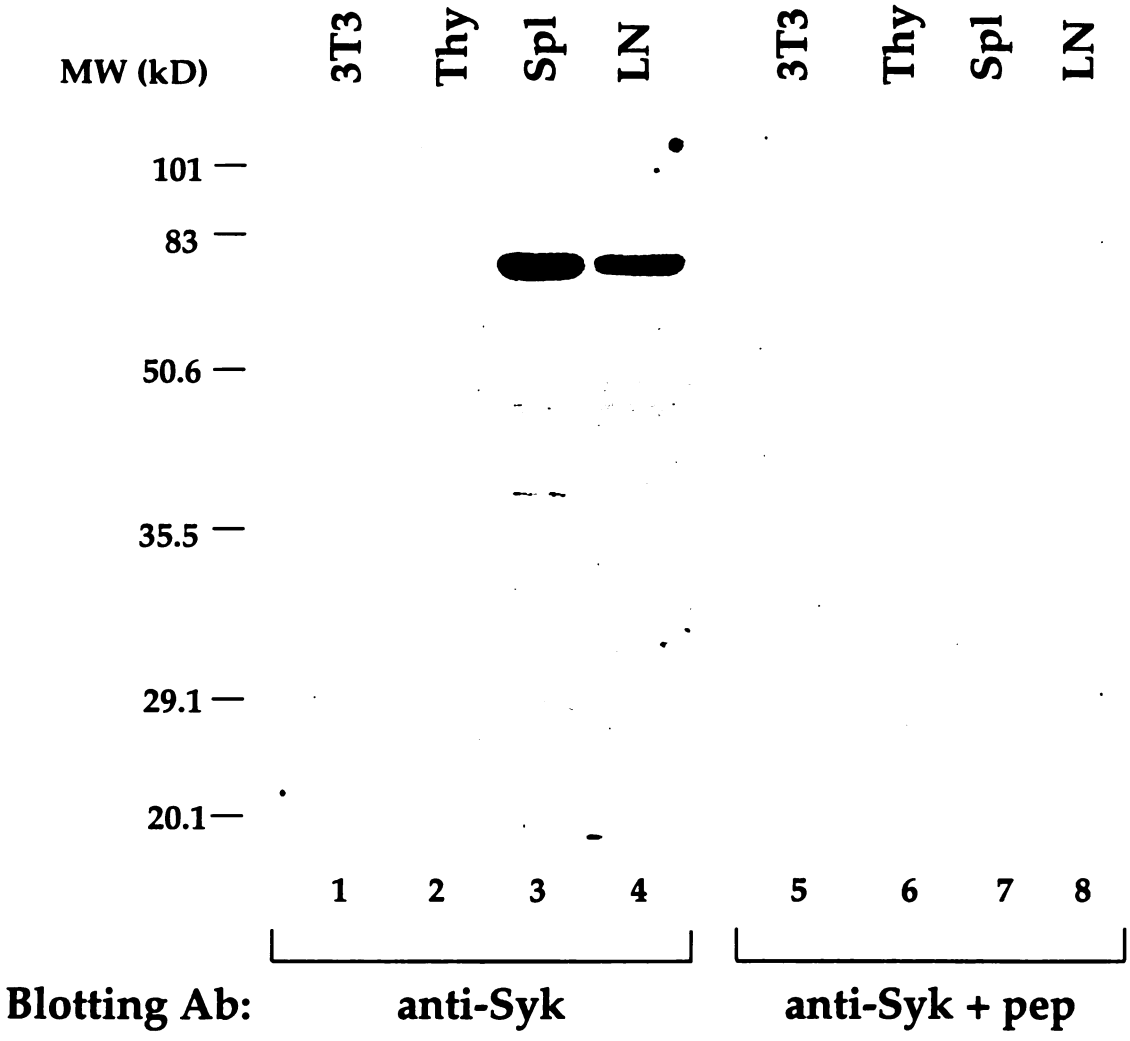
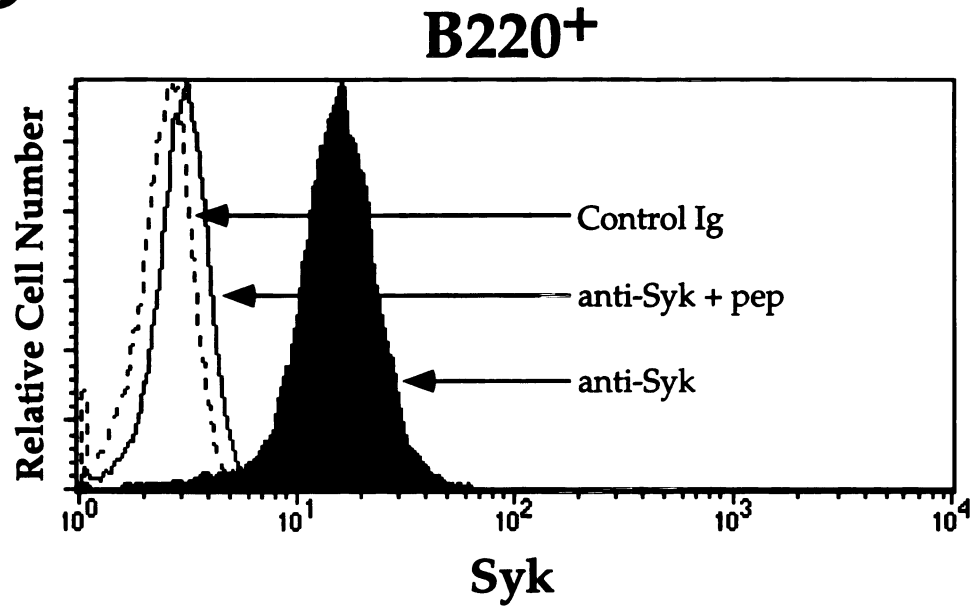


Figure 2. Characterization of the 5F5.2 anti-Syk monoclonal antibody. (A) Blotting specificity of 5F5.2. Equivalent amounts of whole cell lysate (100 μg protein/lane) were loaded in each lane and resolved by 10% SDS-PAGE. After transfer to PVDF membranes, blots were probed with [^{125}I]-iodinated 5F5.2 antibody (left panel, anti-Syk) or [^{125}I]-iodinated 5F5.2 antibody preincubated with competitor peptide (right panel, anti-Syk + pep). Results were visualized by autoradiography. Lanes were loaded as follows: lanes 1 and 5, NIH 3T3 (3T3); lanes 2 and 6, wild-type thymus (Thymus); lanes 3 and 7, wild-type spleen (Spleen); lanes 4 and 8, wild-type lymph nodes (LN). (B) Intracellular staining for murine Syk. Splenocytes from a wild-type C57Bl/6 mouse were stained with anti-B220 PE and 5F5.2 FITC (anti-Syk); 5F5.2 FITC + competitor peptide (anti-Syk + pep); or an isotype-matched staining control, IgG1 FITC (Control Ig). B220^{+} cells were gated and analyzed for Syk staining. The results shown are representative of four independent experiments.



B



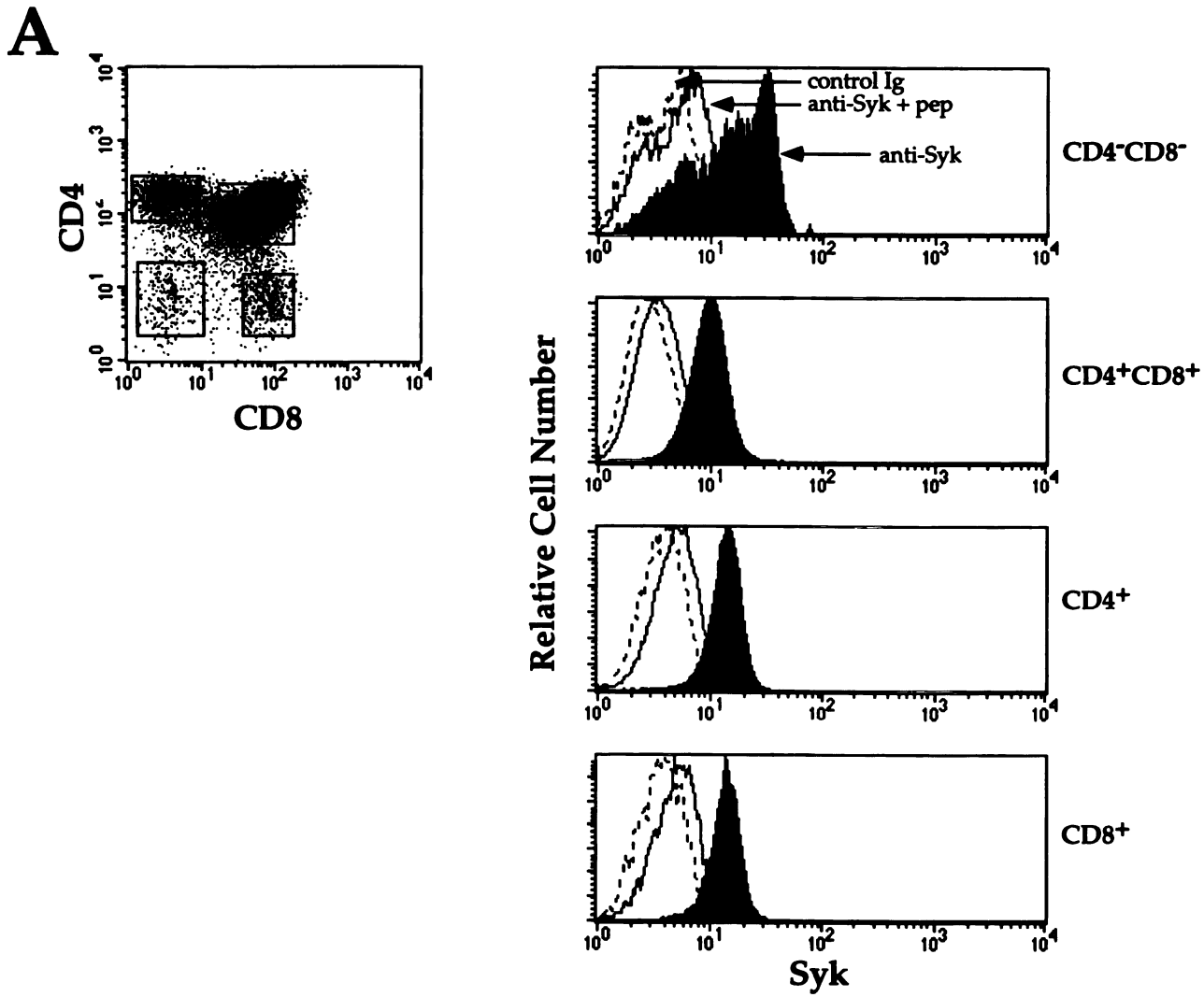
antibody was generated (Figure 2A). Using this antibody, I am also able to detect Syk expression by immunoblotting in a murine B cell line, Bal-17, and a murine macrophage line, RAW 264.7 (data not shown). Furthermore, the 5F5.2 antibody is specific for murine Syk, as it did not detect Syk in human or avian cells (data not shown).

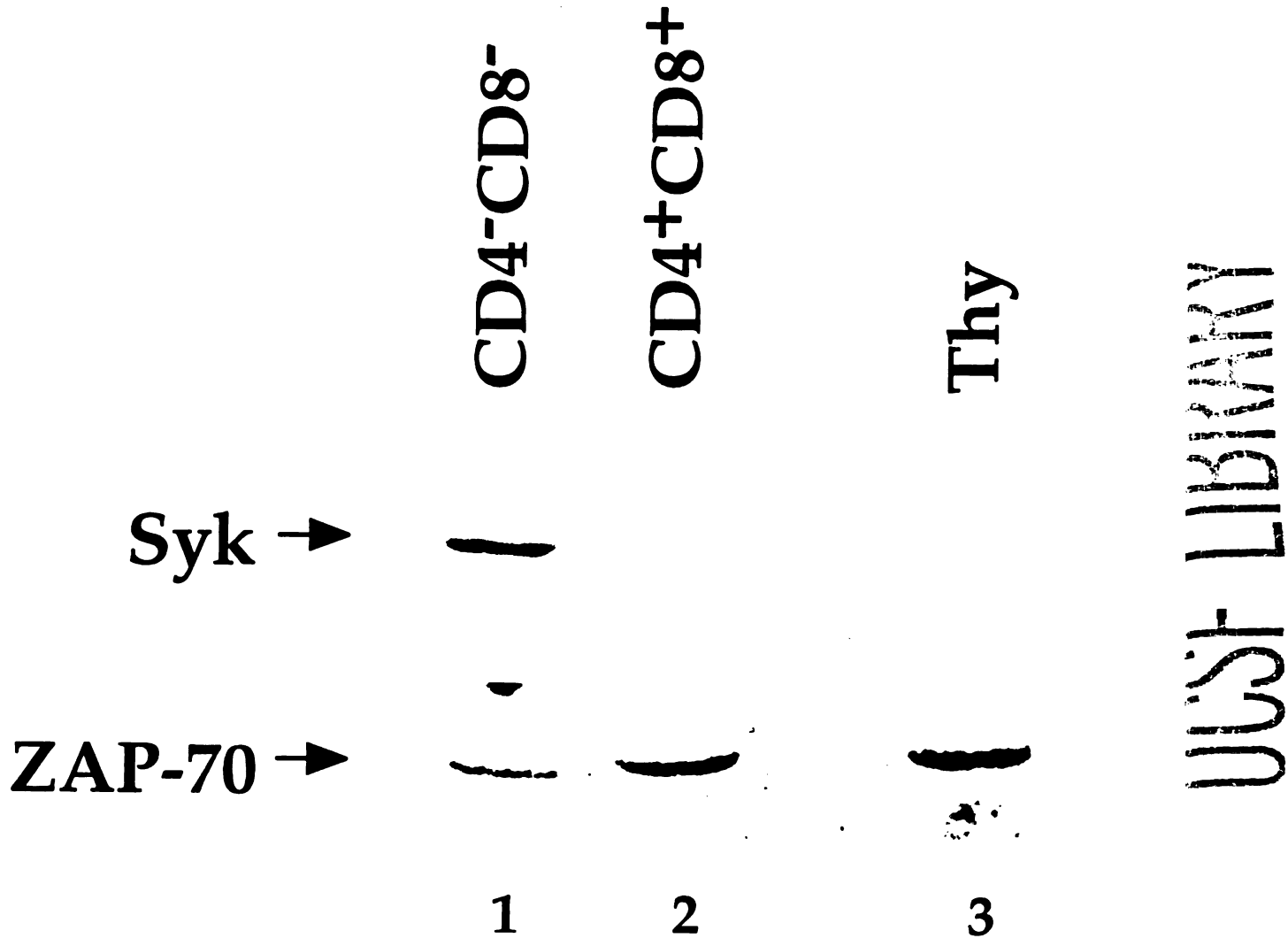
Next, I was interested in determining if the 5F5.2 mAb was capable of staining Syk intracellularly. This technique has been used extensively for the detection of cytokine expression. Briefly, cells were stained for surface markers and were subsequently fixed, permeabilized with saponin, and stained intracellularly with the anti-Syk antibody. Cells were then analyzed by flow cytometry. Syk expression could easily be detected in $B220^+$ splenic B cells (Figure 2B), which express high levels of Syk. Consistent with the immunoblotting results, intracellular staining for Syk revealed high levels in macrophages and low levels in peripheral T cells (data not shown). The staining observed with this mAb was specific, as I have shown with several controls. First, mAb staining could be competed away with the Syk-derived peptide against which the antibody was generated. Furthermore, an isotype-matched antibody control overlapped with the negative peak of the peptide competition control (Figure 2B). Similar to the reactivity observed in immunoblotting, the anti-Syk reagent did not recognize Syk from human or avian cells when used for intracellular staining (data not shown). Finally, no reactivity was seen when non-permeabilized cells were stained with the anti-Syk reagent, indicating that the antigen recognized by the antibody is an intracellular protein (data not shown).

Syk is expressed at the $CD4^-CD8^-$ stage in murine thymocytes

Based on experiments using anti-murine Syk polyclonal antibodies, it has been reported that in the T cell lineage, Syk is expressed at highest levels during thymic development (94). To investigate this observation in more detail, I stained for intracellular levels of Syk in murine thymocytes. I co-stained thymocytes from wild-type mice for CD4, CD8, and Syk and used flow cytometry to define the subsets of cells expressing Syk (Figure 3A).

Figure 3. Syk is expressed in murine $CD4^-CD8^-$ thymocytes. (A) Intracellular staining for Syk in murine thymocytes. Thymic tissue from a wild-type C57Bl/6 mouse was isolated and stained on the cell surface using anti-CD4 APC, anti-CD8 PE, anti-B220 TC, and anti-Mac-1 TC antibodies. TC-positive cells were excluded by fluorescent gating. Syk expression in the different CD4/CD8 subpopulations is depicted in the histograms to the right of the dot plot. The histograms represent the following intracellular stains: anti-Syk 5F5.2 FITC, filled histogram (anti-Syk); 5F5.2 FITC + competitor peptide, open histogram, solid line (anti-Syk + pep); isotype-matched IgG1 FITC staining control, open histogram, dashed line (control Ig). The results shown are representative of five independent experiments. (B) Immunoblotting for Syk in sorted thymocytes. Thymocytes from wild-type C57Bl/6 mice were isolated and stained for CD4 and CD8 expression and sorted by flow cytometry. The sorted populations were 99% pure. Equivalent amounts of whole cell lysate (75 μg/lane) were resolved by 8% SDS-PAGE, transferred to membranes, and probed with the anti-Syk (5F5.2) mAb followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody. Bands were visualized by enhanced chemiluminescence (ECL). The blots were subsequently stripped according to manufacturer's instructions and reprobed with anti-ZAP-70 antisera followed by an HRP-conjugated Protein A secondary reagent. Bands were again visualized by ECL. Lanes were loaded as follows: lane 1, sorted $CD4^-CD8^-$ thymocytes ($CD4^-CD8^-$); lane 2, sorted $CD4^+CD8^+$ thymocytes ($CD4^+CD8^+$); lane 3, whole thymus (Thymus). The results shown are representative of three independent experiments.


A
CD4
CD8
control Ig
anti-Syk + pep
anti-Syk
CD4-CD8-
CD4+CD8+
CD4+
CD8+
Relative Cell Number
Syk



Interestingly, Syk was expressed at variable levels in $CD4^-CD8^-$ thymocytes, with a subpopulation of $CD4^-CD8^-$ cells expressing relatively high levels of Syk (Figure 3A). These cells were not contaminating B cells or macrophages, since we excluded those $B220^+$ or Mac-1^+ populations by fluorescent gating. Furthermore, the majority of cells that expressed high levels of Syk were not γδ cells. Although γδ T cells do express significant levels of Syk in the thymus, after gating on $CD3^-$ populations, as well as after directly excluding γδ TCR-expressing cells, I still detected a Syk-expressing population in the $CD4^-CD8^-$ cells (data not shown).

In $CD4^+CD8^+$ cells, Syk levels were substantially reduced (Figure 3A). These levels remain low in the progression to more mature $CD4^+$ and $CD8^+$ thymocytes as well as later as these cells emerge as mature T cells in the periphery (Figure 3A).

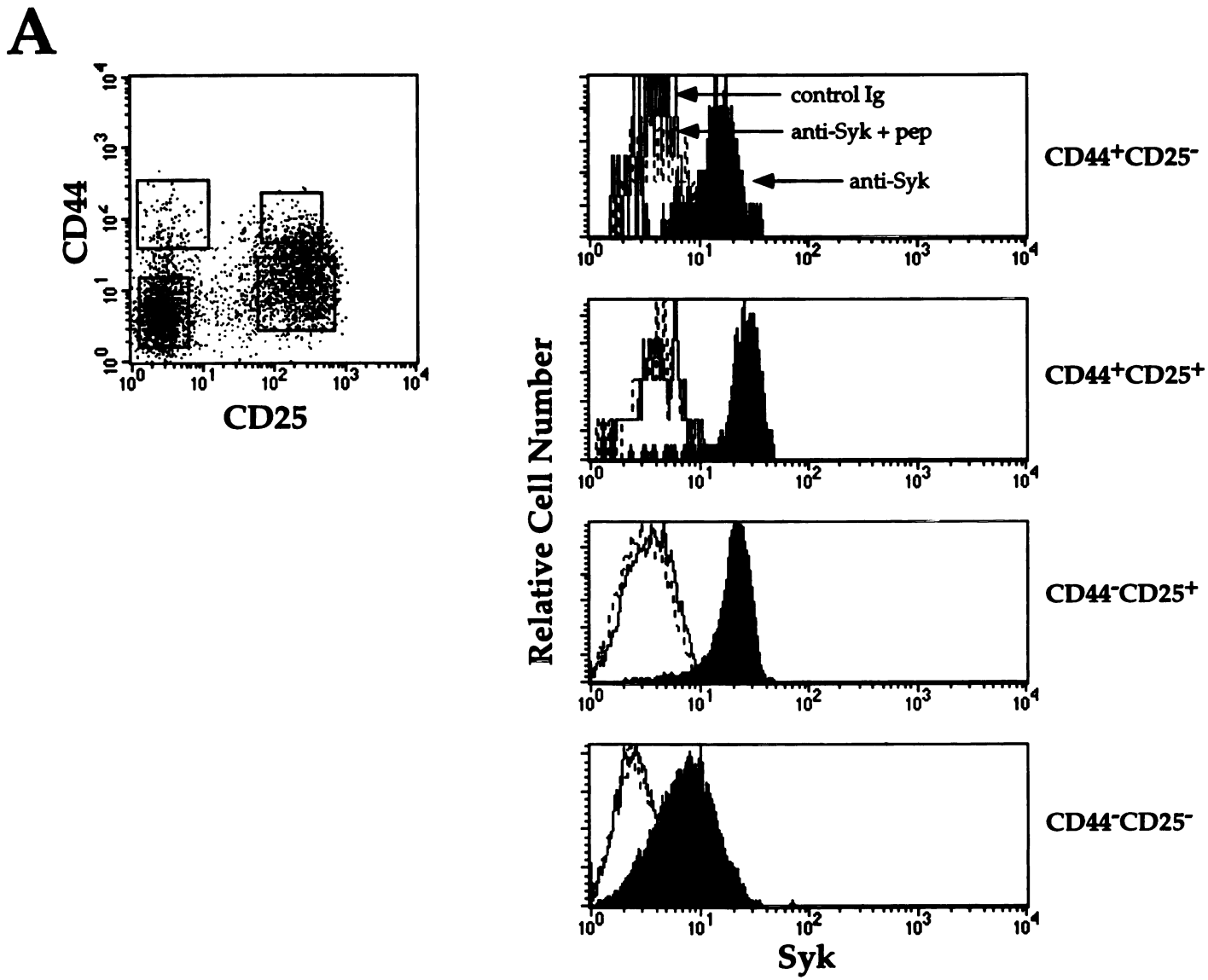
The above results contrast with previously published data using polyclonal antibodies in which immunoblotting experiments suggested that Syk was expressed at highest levels in the $CD4^+CD8^+$ population (94). To address this issue further, we sorted $CD4^-CD8^-$ thymocytes and $CD4^+CD8^+$ thymocytes for immunoblotting whole cell lysates with this new anti-Syk mAb and anti-ZAP-70 antisera for comparison. Whole cell lysates were generated from FACS-sorted cells which were 99% pure. (Figure 3B). The results I obtained with these antibodies are consistent with the intracellular staining results: Syk levels were highest in the $CD4^-CD8^-$ population and decreased in the $CD4^+CD8^+$ population (Figure 3B). In contrast, ZAP-70 was expressed throughout thymic development [Figure 3B; see also (94)]. The reasons for the discrepancies in Syk expression between previous results and my current results are not clear. It is possible that in the previous experiments, contaminating Syk-expressing cells such as B cells were present in the enriched populations or, more likely, that the Syk antibodies that had been previously used cross-reacted with a protein of similar size, perhaps ZAP-70.

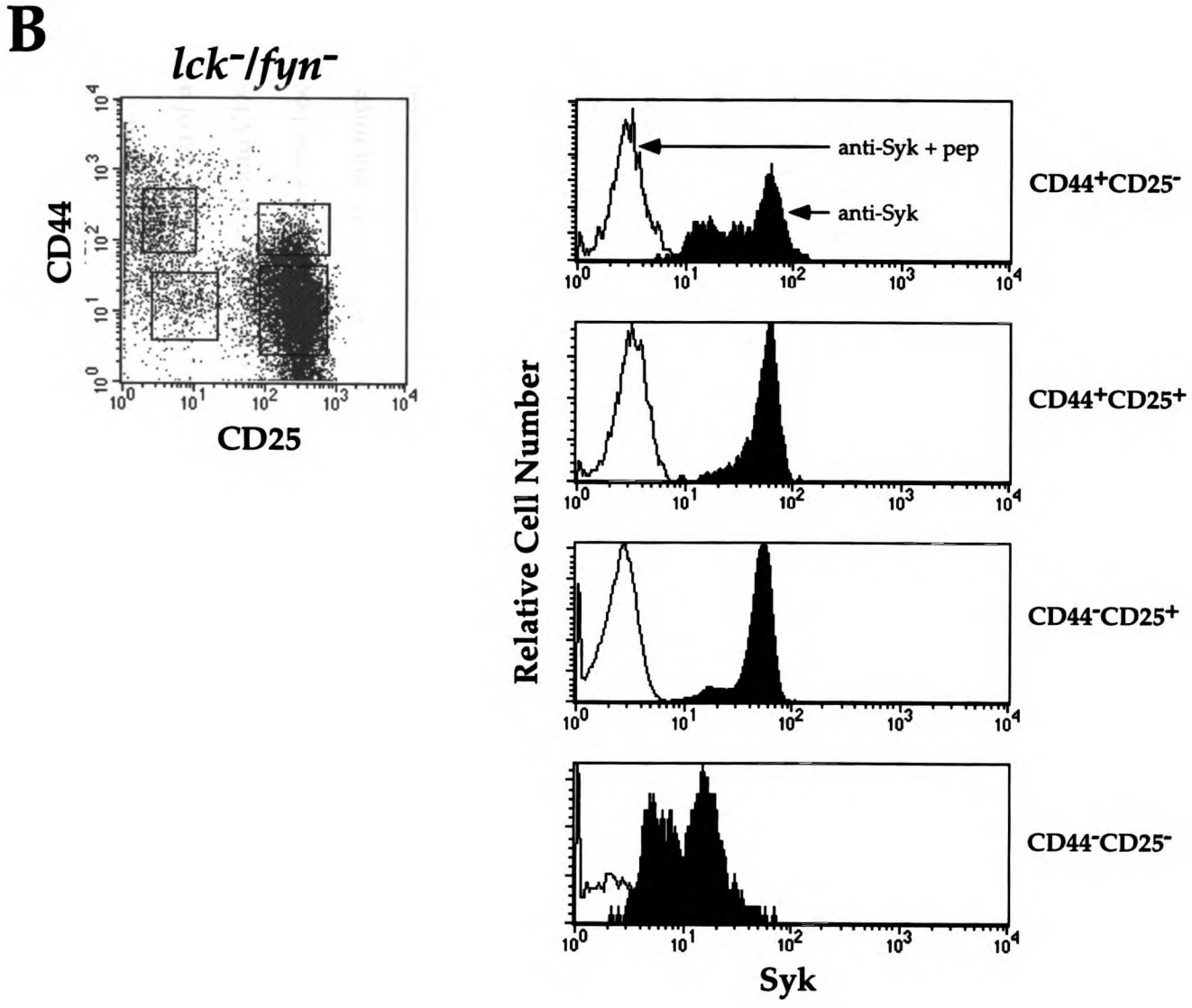
Syk is expressed at the pre-TCR signaling stage in mice

In mice, $CD4^-CD8^-$ thymocytes can be further subdivided into subpopulations based on expression of the CD44 and CD25 cell surface proteins (74, 75). The expression of Syk within the $CD4^-CD8^-$ population was analyzed. Using four-color analysis, wild-type thymocytes expressing CD4, CD8, CD3, B220, and Mac-1 were excluded. The remaining thymocytes were analyzed for CD44, CD25, and Syk expression (Figure 4A). From these staining results, it can be seen that Syk expression is elevated from the $CD44^+CD25^-$ through the $CD44^-CD25^+$ stage (Figure 4A). After the $CD44^-CD25^+$ stage, Syk levels appear to be downregulated, with a notable decrease in Syk expression by the $CD44^-CD25^-$ stage, and uniformly low by the time the thymocytes have matured to $CD4^+CD8^+$ cells (Figure 3A and Figure 4A). Interestingly, the $CD44^-CD25^+$ stage of thymocyte development corresponds to the stage at which thymocytes receive signals through the pre-TCR complex (75). Thus, it appears that Syk expression is elevated until the stage at which the pre-TCR signal is propagated and then its expression is rapidly downregulated.

As shown in Figure 1, whole-cell lysates of thymocytes from Lck/Fyn-deficient mice and RAG-deficient mice appeared to express higher levels of Syk than wild-type mice. Since these mice are arrested in thymic development at the $CD44^-CD25^+$ stage (80, 81), I reasoned that the increased Syk expression seen in whole cell lysates results from the fact that the majority of thymocytes are arrested at the point when Syk is most highly expressed. I verified this hypothesis by staining for CD44 and CD25 in thymic populations from Lck/Fyn-deficient and RAG-deficient mice (Figure 4B and data not shown). As in the wild-type thymus, Syk was expressed most highly at the $CD44^-CD25^+$ stage and decreased in expression by the $CD44^-CD25^-$ stage. Note that the differences in maximal staining intensity of Syk staining between wild-type and mutant mice are the result of using different flow cytometers (see legend to Figure 4).

Figure 4. Syk is downregulated in murine thymocytes after the pre-TCR checkpoint. (A) Syk expression in wild-type $CD4^-CD8^-$ thymocytes. Thymocytes from a wild-type C57Bl/6 mouse were cell-surface stained with biotinylated anti-CD44, anti-CD25 PE, anti-CD3 TC, anti-CD4 TC, anti-CD8 TC, anti-B220 TC, and anti-Mac-1 TC followed by streptavidin-APC. TC-positive cells were excluded by fluorescent gating. Syk expression in the different CD44/CD25 subpopulations is depicted in the histograms to the right of the dot plot. (B) Syk expression in *lck*$^-$*/fyn*$^-$ thymocytes. Thymocytes from *lck*$^-$*/fyn*$^-$ mice were isolated and stained with anti-CD44 TC and anti-CD25 PE. Syk expression in the CD44/CD25 subpopulations, as measured by intracellular staining, is depicted in the histograms to the right of the dot plot. In (A) and (B), the histograms represent the following intracellular stains: Syk 5F5.2 FITC, filled histogram (anti-Syk); Syk FITC + competitor peptide, open histogram, solid line (anti-Syk + pep); isotype-matched IgG1 FITC staining control, open histogram, dashed line (control Ig). Note that the differences in the maximal intensity of Syk staining in (A) and (B) are the result of using different flow cytometers. Four-color analysis in (A) was performed on a FACStar Plus, whereas three-color analysis in (B) was performed on a FACScan. The results shown are representative of three independent experiments.


A
CD44
CD25
control Ig
anti-Syk + pep
anti-Syk
CD44+CD25-
CD44+CD25+
CD44-CD25+
CD44-CD25-
Relative Cell Number
Syk



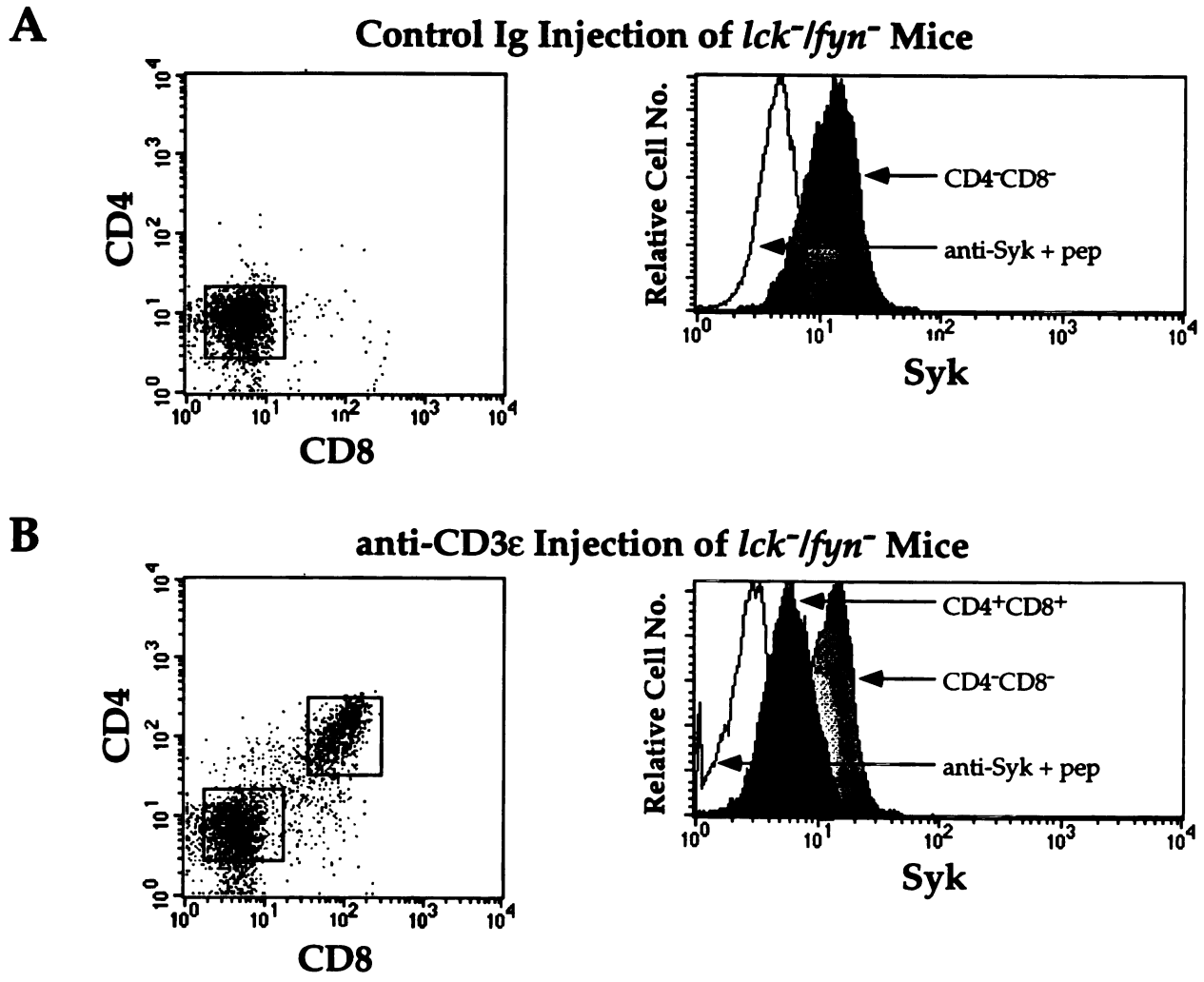
Syk expression is downregulated from the CD4$^-$CD8$^-$ to CD4$^+$CD8$^+$ transition in murine thymocytes

I next sought to determine if I could induce a pre-TCR signal to downregulate Syk during the transition from CD4$^-$CD8$^-$ thymocytes to CD4$^+$CD8$^+$ thymocytes. To do so, I used *RAG*$^-$ and *lck*$^-$*/fyn* mice. As mentioned above, the majority of thymocytes from these mice are arrested at the pre-TCR checkpoint during CD4$^-$CD8$^-$ thymocyte development. Previous experiments in RAG-1-deficient mice have demonstrated that injection of anti-CD3 antibodies can induce the arrested CD44$^-$CD25$^+$ thymocytes to mature into CD4$^+$CD8$^+$ thymocytes, presumably by crosslinking low levels of CD3 chains that have reached the surface in the absence of the pre-TCR chains (95). Because Lck/Fyn-deficient mice are arrested at an identical stage in development, I wished to determine if injection of anti-CD3 antibodies would have a similar effect.

Surprisingly, the CD4$^-$CD8$^-$ cells from *lck*$^-$*/fyn*$^-$ mice could be induced to develop, similar to the results observed in *RAG*$^-$ mice (Figure 5 and data not shown). These results show that the developmental arrest in these thymocytes can be overcome by the stimulation of the CD3 complex. In the Lck/Fyn-deficient thymocytes, the TCR β chain was detected by intracellular staining even prior to antibody treatment (data not shown), suggesting that a functional pre-TCR complex can be generated in a subpopulation of these thymocytes, which may allow for further development of these cells. Induction of the CD4$^+$CD8$^+$ cells following anti-CD3 stimulation also resulted in TCR α chain rearrangements, as assessed by RT-PCR (data not shown), suggesting that the signal through the pre-TCR is generating *bona fide* CD4$^+$CD8$^+$ thymocytes. This effect might reflect the decreased dependence of Syk, which is expressed in these cells, on Lck/Fyn function.

To examine the expression of Syk during this transition, we stained for Syk intracellularly in the CD4$^-$CD8$^-$ and CD4$^+$CD8$^+$ cells after *in vivo* anti-CD3 treatment of both *lck*$^-$*/fyn* and *RAG*$^-$ mice (Figure 5B and data not shown). Consistent with the results

Figure 5. *In vivo* induction of maturation of $CD4^-CD8^-$ thymocytes from *lck*$^-$*/fyn*$^-$ mice leads to downregulation of Syk expression. (A) Syk expression in *lck*$^-$*/fyn*$^-$ thymocytes. *lck*$^-$*/fyn*$^-$ mice were injected with 250 μg normal hamster Ig intraperitoneally in PBS. After seven days, thymocytes from these mice were isolated and stained for CD4, CD8, and Syk. (B) Syk is downregulated upon *in vivo* treatment with anti-CD3ε antibodies. *lck*$^-$*/fyn*$^-$ mice were injected with 250 μg anti-CD3ε antibody (2C11) IP in PBS. After seven days, thymocytes from these mice were processed as in (A). In both (A) and (B), the histograms to the right of the dot plot represent Syk expression in the indicated gated populations: anti-Syk FITC, $CD4^-CD8^-$ thymocytes, shaded histogram; anti-Syk FITC, $CD4^+CD8^+$ thymocytes, black histogram. In addition, 5F5.2 FITC + competitor peptide staining was used as a negative control for staining (anti-Syk + pep, open histogram). The results shown are representative of six independent experiments.

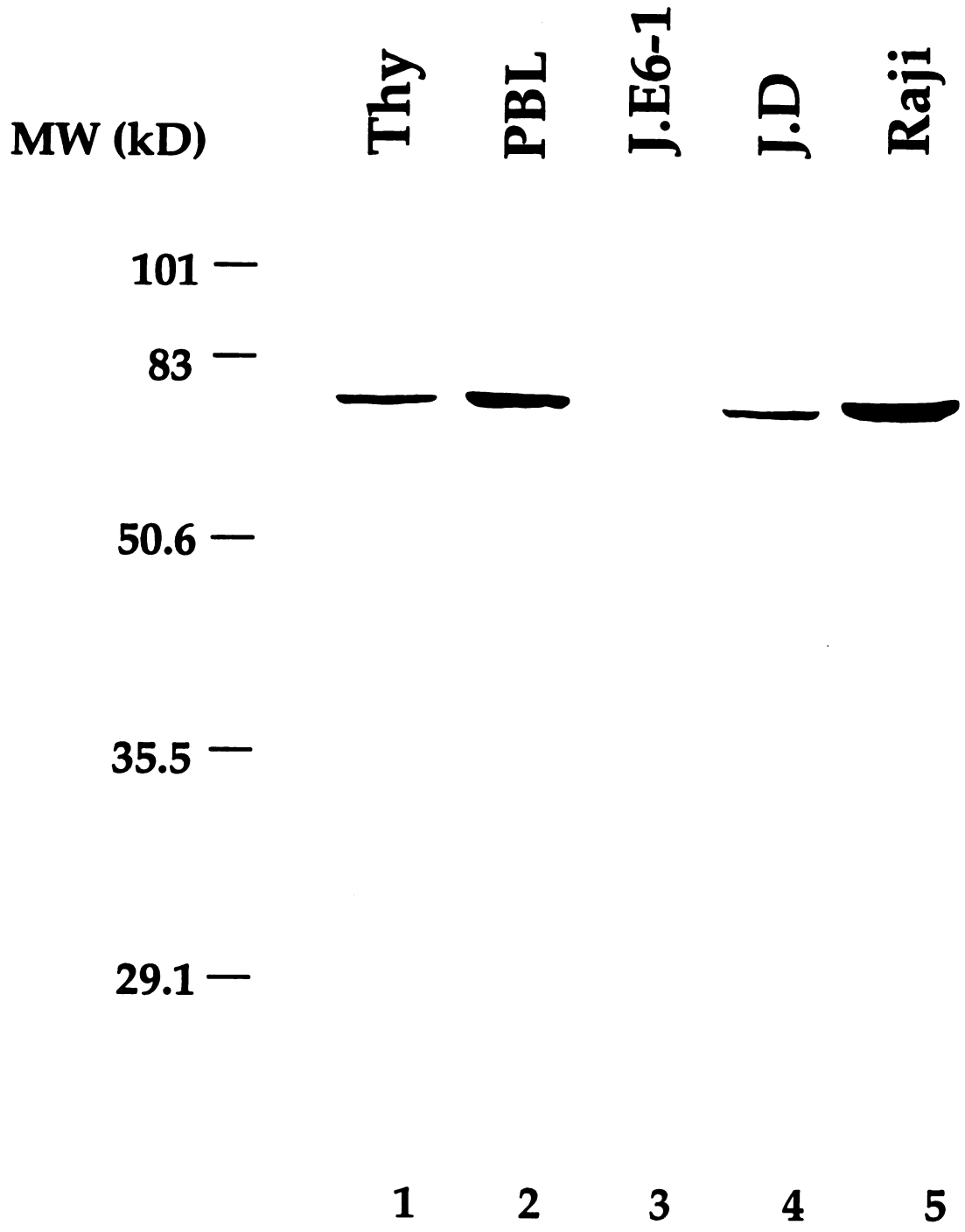


observed in wild-type mice, the induced $CD4^+CD8^+$ thymocytes showed decreased levels of Syk expression compared with the $CD4^-CD8^-$ thymocytes (Figure 5B and data not shown). Thus, Syk appears to be actively downregulated following a pre-TCR signal during the $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocyte transition. The elevated levels of Syk at the $CD44^-CD25^+$ stage may allow anti-CD3 treatment to overcome the developmental block in *lck⁻/fyn⁻* and *RAG⁻* thymocytes. This result is especially noteworthy since Syk activation is less dependent on Src kinases than is ZAP-70 activation (Chapter 2).

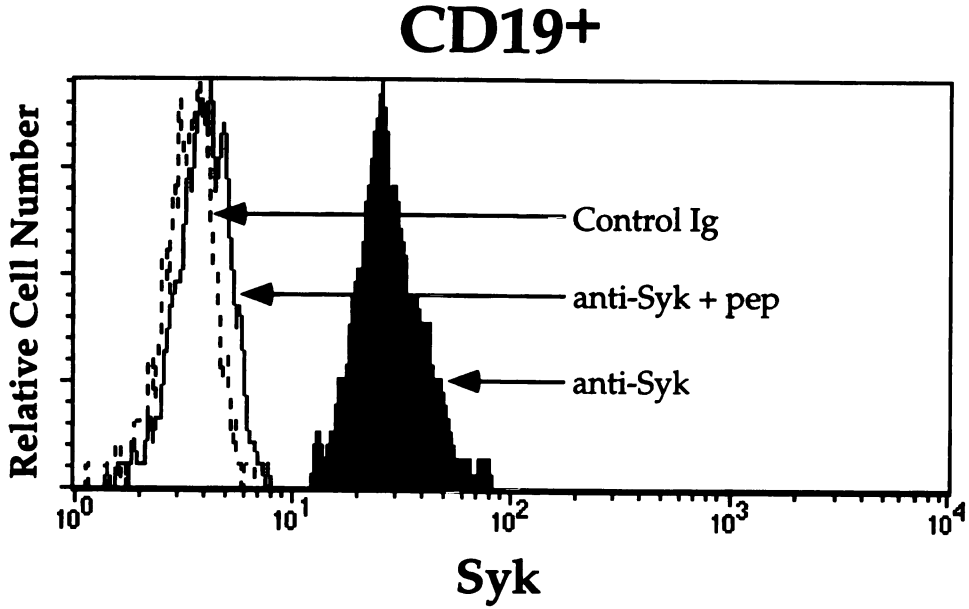
Syk is expressed in human thymocytes

Having determined that Syk is downregulated during the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition in murine thymocytes, I was next interested in determining the pattern of Syk expression in the human thymus. I believed that regulation of Syk family members might differ between humans and mice, as suggested by the differences in T cell development resulting from ZAP-70-deficiency in mice and humans (65-67, 69-71). Patients with severe combined immunodeficiency due to ZAP-70 deficiency have nonfunctional peripheral $CD4^+$ T cells, whereas in mice, loss of ZAP-70 results in a complete arrest at the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition. I reasoned that one possible explanation for the ability of human but not murine thymocytes to mature was a differential regulation of Syk expression in humans. To examine more closely the expression of Syk in human thymocytes, I used an anti-Syk mAb that has previously been used for immunoprecipitation and immunoblotting of human Syk (96). When used for immunoblotting, this antibody detects Syk in lysates of human thymocytes as well as peripheral blood lymphocytes (PBLs) (Figure 6A). To assess if this antibody was also capable of staining Syk intracellularly, I co-stained B cells from human PBLs with antibodies against surface CD19 and intracellular Syk. Syk could be stained in this population of cells, and the specificity of staining once again was demonstrated by the ability of specific peptide to compete with the staining (Figure 6B).

Figure 6. The 4D10.1 anti-human Syk monoclonal antibody can detect intracellular Syk. (A) Blotting specificity for human tissues. Whole cell lysates were made from the indicated tissues or cell lines and quantitated for protein content. Equivalent amounts of lysate (100 μg) were loaded in each lane resolved by 10% SDS-PAGE. Gels were transferred to PVDF membranes and probed with an anti-human Syk mAb (4D10.1) followed by an HRP-conjugated goat anti-mouse secondary antibody. Bands were visualized by ECL. The lanes were loaded as follows: lane 1, whole thymus (Thy); lane 2, peripheral blood leukocytes (PBL); lane 3, Jurkat, J.E6-1 clone (Syk-non-expressing, J.E6-1); lane 4, Jurkat, J.D clone (Syk-expressing, J.D); and lane 5, Raji B cell line (Raji). The results shown are representative of five independent experiments. (B) Intracellular staining of human Syk. Human PBLs were isolated by Ficoll/Hypaque separation. Cells were washed and stained on the cell surface using CD19 TC and intracellularly using 4D10.1 FITC (anti-Syk); 4D10.1 FITC + competitor peptide (anti-Syk + pep); and isotype-matched IgG1 FITC staining control (Control Ig). $CD19^+$ cells were gated and analyzed for Syk staining. The results shown are representative of fifteen independent experiments.



B



To analyze the expression of Syk during human thymic development, thymocytes from human thymi were stained for surface CD4 and CD8 expression, as well as intracellularly with the anti-Syk mAb 4D10.1. As can be seen in Figure 7, in the normal human thymus, similar to the mouse thymus, an elevated level of Syk expression was present in a subpopulation of $CD4^-CD8^-$ cells; in this case, a smaller subset express high levels of Syk. In contrast to the case in the murine thymus, Syk levels were not decreased as much in the $CD4^+CD8^+$ cells. Instead, Syk levels remained somewhat elevated until the transition to either the $CD4^+$ or $CD8^+$ stage, where a majority of these thymocytes showed decreased Syk expression. Strikingly, and in dramatic contrast to the murine thymus, in the $CD4^+$ compartment, 5-10% of the thymocytes expressed substantially elevated levels of Syk (Figure 7). To confirm that the Syk^{hi} cells that I observed in the $CD4^-CD8^-$ and $CD4^+$ populations were thymocytes and not other contaminating cell types, I examined forward and side scatter gating, indicating that the Syk^{hi} cells were small lymphocytes (data not shown). Exclusion of $CD19^+$ and Mac-1^+ (CD11b) cells by fluorescence gating indicated that the $CD4^-CD8^-$ and the $CD4^+$ cells with elevated Syk expression were not B cells, macrophages, or monocytes (data not shown).

Syk is expressed coordinately with the pre-TCR in human thymocytes

I hypothesized that this subpopulation of Syk^{hi} $CD4^+$ thymocytes might represent a subset of cells that become precursors for the human $CD4^+$ peripheral T cells that develop in ZAP-70-deficient patients. Alternatively, I considered the possibility that the $CD4^+$ thymocytes were an immature thymic population that is an intermediate between $CD4^-CD8^-$ and $CD4^+CD8^+$, as has been described in studies of human thymocyte development (97). Unlike the murine system, in which thymocytes develop from $CD4^-CD8^-$ cells to $CD4^+CD8^+$ via a $CD8^+$ intermediate, human thymocyte development proceeds through a $CD4^+$ intermediate during the transition from $CD4^-CD8^-$ to $CD4^+CD8^+$ thymocytes.

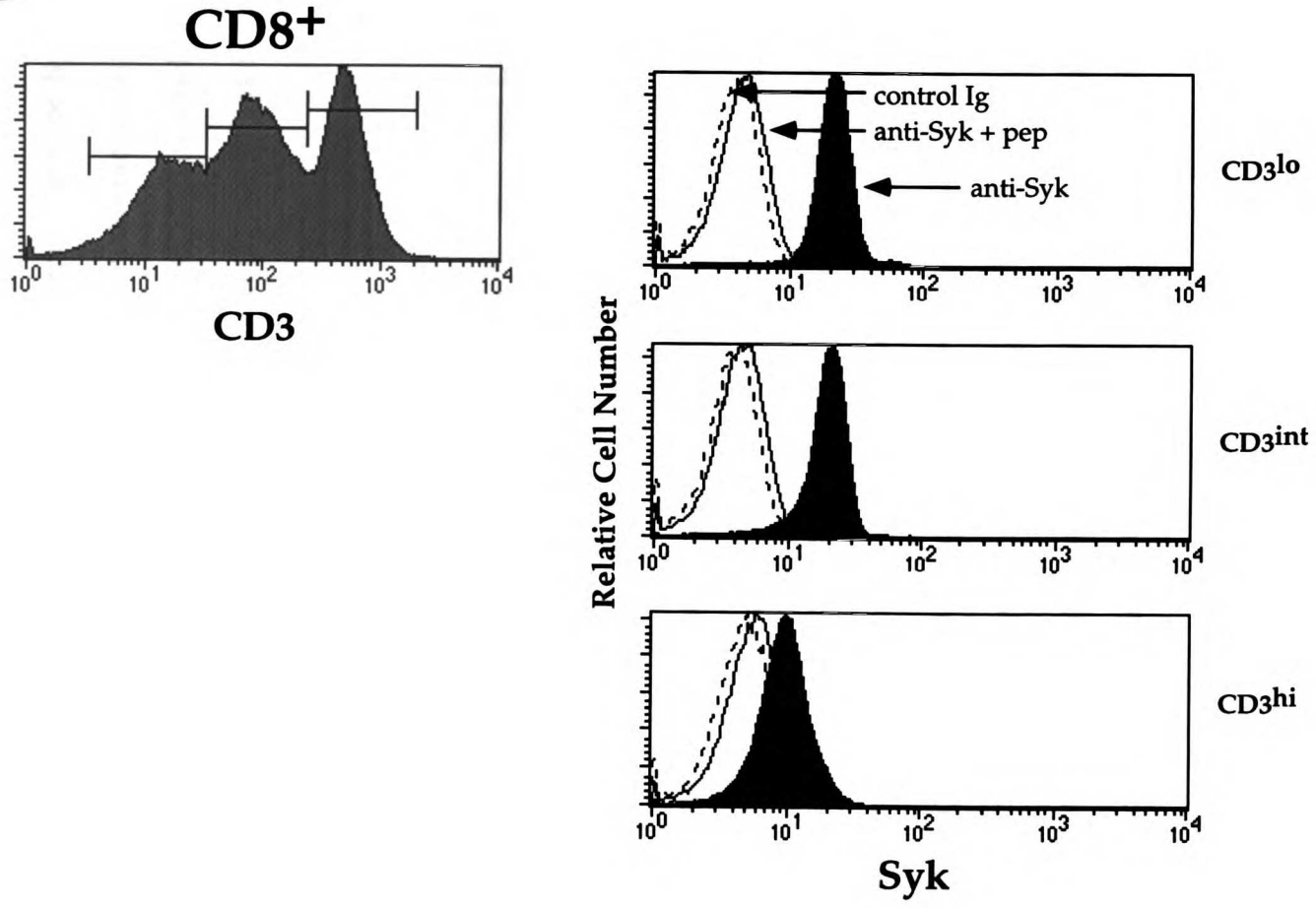
Figure 7. Human thymocytes express elevated levels of Syk in a subpopulation of $CD4^-$ $CD8^-$ and $CD4^+$ cells. Human thymocytes were stained on the cell surface with anti-CD4 TC and anti-CD8 PE antibodies. Syk expression in the CD4/CD8 subpopulations, as measured by intracellular staining, is depicted in the histograms to the right of the dot plot: 4D10.1 Syk FITC, filled histogram (anti-Syk); Syk FITC + competitor peptide, open histogram, solid line (anti-Syk + pep); isotype-matched IgG1 FITC staining control, open histogram, dashed line (control Ig). The results shown are representative of twelve independent experiments.

To assess if the small subpopulation of $CD4^+$ cells expressing elevated levels of Syk was immature or mature, I examined their CD3 expression. Mature $CD4^+$ thymocytes express high levels of CD3, whereas the immature $CD4^+$ intermediates express low levels of CD3. CD4 vs. CD3 staining indicated that, in fact, the Syk-expressing cells expressed low levels of CD3 and therefore represent the $CD4^+$ developmental intermediates between $CD4^-CD8^-$ and $CD4^+CD8^+$ populations (Figure 8A). A comparable population of Syk^{hi} cells in $CD8^+$ thymocytes was not detected (Figure 8B). The expression of Syk in the $CD4^+CD3^{lo}$ population corroborates the idea that Syk is expressed in immature cells and decreases in expression as thymocytes mature.

Unlike murine thymocytes, in which the pre-TCR is expressed during a discrete period of $CD4^-CD8^-$ thymocyte development, only a small number of human $CD4^-CD8^-$ thymocytes express the pre-TCR. Instead, it appears that it is during the $CD4^+$ intermediate stage that the human pTα chain and the pre-TCR are most highly expressed (98). Thus, the $CD4^+$ intermediate stage in humans appears to correspond functionally to the $CD44^-$ $CD25^+$ stage of murine $CD4^-CD8^-$ cells. It appears the elevation of Syk expression during pre-TCR signaling has been preserved between mice and humans, and that Syk is downregulated following the pre-TCR signal. Note also that $CD3^{int}$ cells, contained within the $CD4^+CD8^+$ stage of development, again have higher levels of Syk than the more mature subset. This higher level of Syk expression in $CD4^+CD8^+$ human thymocytes may be important for the ability of $CD4^+$ cells to develop in ZAP-70-deficient patients.

Figure 8. Syk^{hi} human $CD4^+$ thymocytes are immature. (A) Syk is elevated in a subpopulation of $CD4^+CD3^{lo}$ cells. Human thymocytes were stained on the cell surface with anti-CD4 TC and anti-CD3 PE antibodies. Syk expression in the CD4/CD8 subpopulations, as measured by intracellular staining, is depicted in the histograms to the right of the dot plot: 4D10.1 Syk FITC, filled histogram (anti-Syk); Syk FITC + competitor peptide, open histogram, solid line (anti-Syk + pep); isotype-matched IgG1 FITC staining control, open histogram, dashed line (control Ig). (B) Syk is not elevated in $CD8^+$ cells. Human thymocytes were stained on the cell surface with anti-CD8 TC and anti-CD3 PE antibodies. Syk expression is depicted as in (A). The results shown are representative of five independent experiments.

B


CD8+
CD3
control Ig
anti-Syk + pep
anti-Syk
CD3lo
CD3int
CD3hi
Relative Cell Number
Syk

Discussion

I have isolated a new monoclonal anti-Syk antibody and used cytoplasmic staining and flow cytometry for detecting the Syk PTK in subpopulations of cells. Using this method, I have analyzed the expression of Syk during thymic development. In mice, Syk expression was elevated prior to pre-TCR signaling and was then quickly downregulated. In humans, Syk expression was upregulated in a small subpopulation of $CD4^+$ cells which correspond to pre-TCR-expressing cells. These results highlight a potential role for Syk in pre-TCR signaling.

Syk expression in $CD4^-CD8^-$ thymocytes

Syk was found to be highly expressed in $CD4^-CD8^-$ thymocytes up to the $CD44^-CD25^+$ stage. I have confirmed the elevated expression using my anti-Syk mAb both by intracellular staining and by immunoblot analysis of sorted thymocyte subpopulations. The reasons for the contrasting results between our current results and the previously published report (94) that Syk is expressed at the $CD4^+CD8^+$ stage are not clear, but one possibility is that the polyclonal rabbit heterosera previously used for Syk detection may have exhibited low-level cross-reactivity with another protein of similar size to give misleading results. Another less likely possibility is that the previously sorted preparations contained contaminating B cells or other Syk-expressing populations. The thymocytes purified in this study were sorted by flow cytometry, which may have increased the purity of these samples compared with the previously used strategy of magnetic bead depletion.

A role for Syk in pre-TCR signaling

The expression of Syk in $CD4^-CD8^-$ thymocytes raises the possibility that Syk may be involved in pre-TCR signaling. A role for Syk in pre-TCR signaling has also been suggested by prior genetic data (85). Although ZAP-70 deficient murine thymocytes progress to the $CD4^+CD8^+$ stage, thymocytes from Syk/ZAP-70 doubly-deficient mice are arrested at the $CD44^-CD25^+$ pre-TCR checkpoint in $CD4^-CD8^-$ thymocytes (69-71, 85). Thus, Syk appears to be able to compensate partially for a lack of ZAP-70 in thymocyte development, during pre-TCR signaling. As I show here, Syk is in fact expressed at its highest levels just before the $CD4^-CD8^-$ to $CD4^+CD8^+$ transition, at the $CD44^-CD25^+$ pre-TCR checkpoint.

The expression of Syk at the pre-TCR signaling stage has apparently been preserved between mice and humans. In humans, as mentioned earlier, only a small number of $CD4^-CD8^-$ cells express the pre-Tα chain (98). Instead, the $CD4^+$ intermediate thymocytes express the highest proportion of pre-TCR-expressing cells. Mature TCR β chains are first detected at the $CD4^+$ intermediate stage, suggesting that the pre-TCR is expressed at this stage and that pre-TCR signaling occurs during this stage as well (98). Syk expression in human thymocytes reflects the pattern of expression of the pTα chain. It may be that in humans, the small number of Syk^{hi} $CD4^-CD8^-$ cells are the precursors of the eventual $CD4^+$ pre-TCR-expressing thymocytes. The relationship between Syk and pre-TCR expression could be more directly confirmed by staining for the coexpression of these molecules on the same cell. Similarly, the relationship between the Syk^{hi} $CD4^-CD8^-$ and Syk^{hi} $CD4^+$ cells may be determined by performing lineage analysis with long-lasting membrane-intercalating fluorescent dyes or thymic reconstitutions using the $CD4^-CD8^-$ cells. The conservation of Syk expression and the pre-TCR in both mice and humans suggests that Syk and the pre-TCR may in fact be functionally linked.

Downregulation of Syk after pre-TCR signaling

In mice, Syk is downregulated as $CD4^-CD8^-$ cells become $CD4^+CD8^+$ cells. Thus, only low amounts of Syk remain at the $CD4^+CD8^+$ stage, when positive and negative selection occur. Therefore, in ZAP-70-deficient $CD4^+CD8^+$ thymocytes, neither Syk nor ZAP-70 is expressed, so positive and negative selection cannot occur. However, in ZAP-70-deficient mice in which a Syk transgene is constitutively expressed throughout thymic development, complete thymic development is restored, indicating that Syk can compensate for ZAP-70 when present (57).

In humans, the downregulation of Syk expression is not as complete following pre-TCR signaling (Figure 6). The fact that $CD4^+CD8^+$ human thymocytes still express significant levels of Syk may explain the differences between the severity of the developmental phenotype of ZAP-70-deficient mice and humans. Because the relative amounts of Syk between human CD4-CD8- and CD4+CD8+ thymocytes appear to differ less than the comparable murine thymocytes, Syk may be able to compensate for the loss of ZAP-70 more effectively in human than in murine thymocytes, thereby allowing some human $CD4^+$ thymocytes to develop despite the lack of ZAP-70. This subpopulation of $CD4^+$ thymocytes would then become the $CD4^+$ peripheral T cells observed in ZAP-70-deficient patients. However, because Syk expression is downregulated between the $CD4^+CD8^+$ and the mature $CD4^+$ stage of thymic development (Figure 8), the $CD4^+$ T cells that develop in ZAP-70-deficient patients remain unable to signal (65-67, 87). The fact that Syk expression appears to decrease again after human CD4+CD8+ TCR signaling, not just after pre-TCR signaling earlier in thymic development, suggests that the processes of positive and negative selection may also affect Syk expression in human thymocyte development.

The signals that cause Syk to be downregulated have not been identified. It is possible that the signal is a direct result of pre-TCR signaling, but it may be that another

signal, perhaps delivered via a cytokine receptor, is responsible. Analysis of the *syk* promoter may indicate what types of transcriptional regulation control Syk expression.

Syk and coreceptor-independent signaling

The proposed function of Syk during pre-TCR signaling is consistent with the idea that Syk, when compared to ZAP-70, has a decreased dependence on Lck/Fyn function (62, 64, 99). One *in vivo* example of a situation in which Lck plays less of a role in TCR signaling is during coreceptor-independent signaling. In such situations, the TCR can signal in the absence of CD4 or CD8 coreceptors and therefore in the absence of the Lck molecules that are associated with the cytoplasmic tails of those coreceptors. Pre-TCR signaling is by definition coreceptor-independent, because it occurs in the absence of expression of either CD4 or CD8. Thus, during pre-TCR signaling, because CD4 and CD8 are not expressed, Lck is presumably not recruited to the CD3 signaling complex as efficiently.

Based on the observed differences in regulation of expression and kinase activity of Syk and ZAP-70, I propose the following model. Syk and ZAP-70 both function during thymocyte development, but they are most critical during different stages. Their individual characteristics are appropriate for the delivery of two different types of signals through the thymocyte receptor complex. Consistent with this model, the expression of these kinases correlates with the time in development when they are proposed to be most useful. The pre-TCR appears to be less discriminating than the mature TCR in terms of signal initiation: a truncated pre-TCR lacking extracellular domains can restore thymic development in a RAG-deficient background, suggesting that no specific ligand-binding is necessary for stimulation of the pre-TCR (4). On the other hand, the TCR/coreceptor complex is responsible for discriminating ligand affinities over a wide concentration range. Syk is less dependent on upstream activators than ZAP-70: it is capable of autophosphorylation on its activation loop tyrosines, and it is therefore less dependent on CD45 or Lck (40, 61, 99).

ZAP-70, in contrast, relies on transphosphorylation by Lck and Fyn for activation (54). Thus, Syk can more readily act as a signaling molecule during the pre-TCR signaling process and is expressed at the pre-TCR, but not the TCR, signaling stage. Conversely, ZAP-70 is likely to be more important than Syk during the processes of positive and negative selection at the $CD4^+CD8^+$ stage, when differences in the strength of the TCR signal are important to transmit and therefore tighter regulation of signaling is required. The fact that human $CD4^+CD8^+$ thymocytes appear to express relatively more Syk than their murine counterparts suggests that positive selection in the human may be less coreceptor dependent. Although Syk-deficient mice have no severe defect in thymocyte development (72, 73), the efficiency of pre-TCR signaling and thymocyte development, as well as potential effects on repertoire development, have not been studied in these mice. These results therefore suggest a role for Syk in pre-TCR, but not TCR, signaling during thymocyte development. Our observations further demonstrate a sensitive and powerful application of the combination of intracellular staining and flow cytometry for the study of signaling proteins in situations in which cells must be analyzed at high purity or limiting cell number.

Experimental Procedures

Generation of 5F5.2

The anti-murine Syk mAb 5F5.2 was generated by standard protocols (100). Briefly, Balb/C mice were immunized intraperitoneally with TiterMax adjuvant (CytRx, Norcross, GA) and KLH-conjugated anti-Syk peptide (100 μg) corresponding to amino acids 306-333 in murine Syk. Mice were boosted with 100 μg KLH-Syk peptide 3 more times over 2 months in the absence of adjuvant. A final boost of 100 μg peptide was given intravenously via tail vein injection and spleens harvested three days later. Fusions were performed with the Ag8.653 murine hybridoma fusion partner using polyethlyene glycol, grown in selective medium (HAT), and screened by ELISA. Positive clones were then subcloned by limiting dilution and rescreened for stable, high-level secretion.

Mice, tissues, and antibodies

Mice were maintained at the UC San Francisco Animal Care Facility. Wild-type C57Bl/6 mice were obtained from Jackson Labs. *lck*$^{-}$*/fyn*$^{-}$ mice (81) and *RAG1*$^{-}$ mice (generous gifts of Dr. Nigel Killeen) have been described. Human thymic tissue was obtained from pediatric cardiac surgery patients between 1 week and 8 years of age.

The following antibodies with the following specificities were used: rabbit heterosera, 1598, anti-ZAP-70; anti-Fgr; anti-Hck (anti-Fgr and anti-Hck were gifts of Dr. Clifford Lowell); monoclonal antibodies, 1F6, anti-Lck; 5F5.2, anti-murine Syk; 4D10.1, anti-human Syk (96). Antibodies against cell-surface markers were obtained from Pharmingen (San Diego, CA): anti-CD44, anti-mCD4; from Becton-Dickinson (San Jose, CA): anti-hCD3, anti-hCD4, anti-hCD8; and from Caltag Laboratories (Burlingame, CA): anti-mCD4, anti-mCD8, anti-hCD19 anti-mCD25, anti-mB220. Streptavidin conjugates

were obtained from Pharmingen (streptavidin-allophycocyanin, APC) and from Caltag (streptavidin-tricolor, TC).

Antibody iodination and FITC conjugation

5F5.2 antibody was dialyzed against iodination buffer and reacted with IodoBeads (Pierce, Rockford, IL) and 1 mCi carrier-free [^{125}I]-sodium iodide according to manufacturer's instructions. Reactions were stopped upon removal of the IodoBead reagent. Free radioisotope was removed using a dextran matrix desalting column (D-Salt Columns, Pierce).

Conjugation of affinity purified 5F5.2 and 4D10.1 mAbs to fluorescein isothiocyanate (FITC, Molecular Probes, Eugene, OR) was performed as described (101).

Cell lysates and immunoblotting

Cell were lysed in 1% NP-40 lysis buffer containing 10 mM Tris, pH 7.6; 150 mM NaCl; and protease and phosphatase inhibitors as previously described. Protein content of lysates was determined using the Bio-Rad protein assay reagent (BioRad, Hercules, CA); values were determined using a spectrophotometer. Lysates were resolved by SDS-PAGE and transferred to polyvinylidine difluoride membranes (PVDF, Immobilon-P, Amersham, Arlington Heights, IL). Blots were blocked with nonfat powdered milk and bovine serum albumin and incubated with the appropriate primary antibody. Following washes in Tris-buffered saline + 0.05% Tween-20, blots were incubated with horseradish-peroxidase conjugated secondary antibodies. Bands were visualized using the enhanced chemiluminescence system and autoradiographic film (ECL, Amersham).

Intracellular staining and flow cytometry

Tissues were strained through a wire mesh to generate a single-cell suspension. Cells were washed multiple times with phosphate buffered saline (PBS), resuspended in staining buffer, and stained with the appropriate conjugated antibodies in the presence of normal mouse serum (10 μg/ml) or Fc receptor blocking antibody, 2.4G3. Cells were then fixed in 4% paraformaldedhyde in PBS and washed. Antibody for intracellular staining was added in staining buffer + 0.1% saponin in the presence of normal mouse serum or Fc receptor blocking antibody. Cells were washed twice in permeabilization buffer and analyzed by flow-cyometry. Four-color analysis was performed on a FACStar Plus (Becton-Dickinson). All other samples were collected on a FACScan (Becton-Dickinson). Results were analyzed using CellQuest software (Becton-Dickinson).

Gene rearrangement assays

These methods have been previously described (102). Briefly, RT-PCR was performed on thymic preparations from the mice of interest using sets of primers specific for certain V, D, and J regions. The products of these rearrangements were quantitated and compared to those found in a wild-type murine thymus.

anti-CD3 injections

250 μg of purified anti-CD3ε antibodies (2C11) were injected intraperitoneally into *lck*$^{-}$*/fyn* or *RAG-1*$^{-}$ mice in PBS. After seven days, thymi were removed from the mice and stained for Syk expression as outlined above. Results were analyzed by flow cytometry on a FACScan.

CHAPTER 4

EXPRESSION OF SYK IN PERIPHERAL T CELLS

Summary

Peripheral T cells recognize antigen through the T cell antigen receptor (TCR). Two families of protein tyrosine kinases (PTKs), the Src family and Syk family, are involved in transducing the signals received through the TCR. The Src family PTKs Lck and Fyn phosphorylate tyrosines within the signal-transducing subunits of the TCR. These phosphorylated tyrosines then recruit the Syk family PTKs Syk and ZAP-70 to the receptor complex. Src family members have been shown to be critical for TCR-mediated signal transduction. Genetic evidence indicates that ZAP-70 is required for signal transduction, but the role of Syk is less clear. Syk has been demonstrated to be critical for signal transduction through the B cell antigen receptor, but a unique role for Syk in TCR signaling has not been defined. I have demonstrated that Syk is selectively upregulated in early thymocytes (Chapter 3). I now provide evidence that Syk is also upregulated in a subpopulation of mature peripheral T cells. These T cells appear to include both αβ and γδ cells. The cells that have upregulated Syk express some cell-surface markers associated with T cell memory but not with recent activation.

Introduction

T cell activation in response to antigen results from a signal transduction cascade that begins with stimulation of the T cell antigen receptor (TCR). The earliest events during TCR signaling involve two families of protein tyrosine kinases (PTKs), the Src family, of which Lck and Fyn are most important in T cells; and the Syk family, comprising Syk and ZAP-70. Lck and Fyn are capable of phosphorylating tyrosine-containing motifs within the TCR complex, known as immunoreceptor tyrosine-based activation motifs (ITAMs). The phosphorylated tyrosines then serve as a docking site for the SH2 domains of Syk and/or ZAP-70. Once recruited to the TCR complex, Syk and/or ZAP-70 are subsequently activated and, together with the Src kinases, phosphorylate other substrates in the signaling pathway, ultimately resulting in proliferation, cytokine production, or other effector activities.

There is ample genetic evidence that Src family kinases are important in TCR signal transduction. T cell lines and clones lacking Lck are unable to transduce signals through their TCR (103, 104). Loss of either Fyn or Lck through targeted disruption of murine genes results in a situation in which the receptor is less responsive to stimulation (36, 82, 84, 105). Furthermore, in T cells from Lck-deficient mice, the ITAMs within the TCR complex are no longer phosphorylated, supporting the model that Src kinases are responsible for phosphorylating ITAMs (36).

Syk family kinases are also critical to signal transduction in mature T cells. Experiments with dominant negative versions of ZAP-70 block TCR signal transduction (106). Furthermore, a ZAP-70-deficient Jurkat T cell line is unable to signal through its receptor (68). Peripheral T cells from ZAP-70-deficient patients are also unable to respond to stimuli against the TCR (65-67). Interestingly, Syk deficiency results in only a selective defect in certain subpopulations of peripheral T cells. Syk is apparently required for the development of γδ intraepithelial lymphocytes, but not for splenic γδ T cells. In the

absence of Syk, splenic γδ cells, as well as αβ T cells, have been reported to develop normally (72, 73). Furthermore, TCR activation of αβ T cells from Syk-deficient mice is apparently normal, at least in terms of responses to concanavalin A, a lectin that is a polyclonal activator of T cells (73). In addition, analysis of whole cell lysates of peripheral tissues and mature sorted T cells has revealed a very low level of Syk expression (14). Collectively, these results suggest that ZAP-70, but not Syk, is required for the signaling of the majority of peripheral αβ T cells.

Other than in some γδ T cells, Syk function would appear to be redundant to ZAP-70 in TCR signaling. Despite this preliminary evidence that Syk function is redundant in most T cells, some recent evidence has suggested that Syk may in fact have a more specialized role than was previously appreciated. I have shown that Syk can function in the absence of CD45 or Lck in Jurkat T cells (Chapter 2). Other groups have confirmed these observations and demonstrated that Syk, but not ZAP-70, can enhance TCR signals in an antigen-specific hybridoma line (62). Thus, I was interested in finding a correlate for these observations from T cell lines in peripheral T cells *in vivo*, that is, a subpopulation of T cells that would preferentially use or express Syk over ZAP-70.

Here I use a sensitive intracellular staining assay for detection of Syk expression by flow cytometry to show that Syk is indeed expressed in a small proportion of the total number of T cells in the periphery. These Syk-expressing cells also co-express some of the markers that have been associated with memory T cells, and do not express markers associated with recently activated T cells. Thus, these cells may be an important subpopulation of T cells that possesses a specific coreceptor-independent ability to signal, or they may be a subpopulation of memory cells.

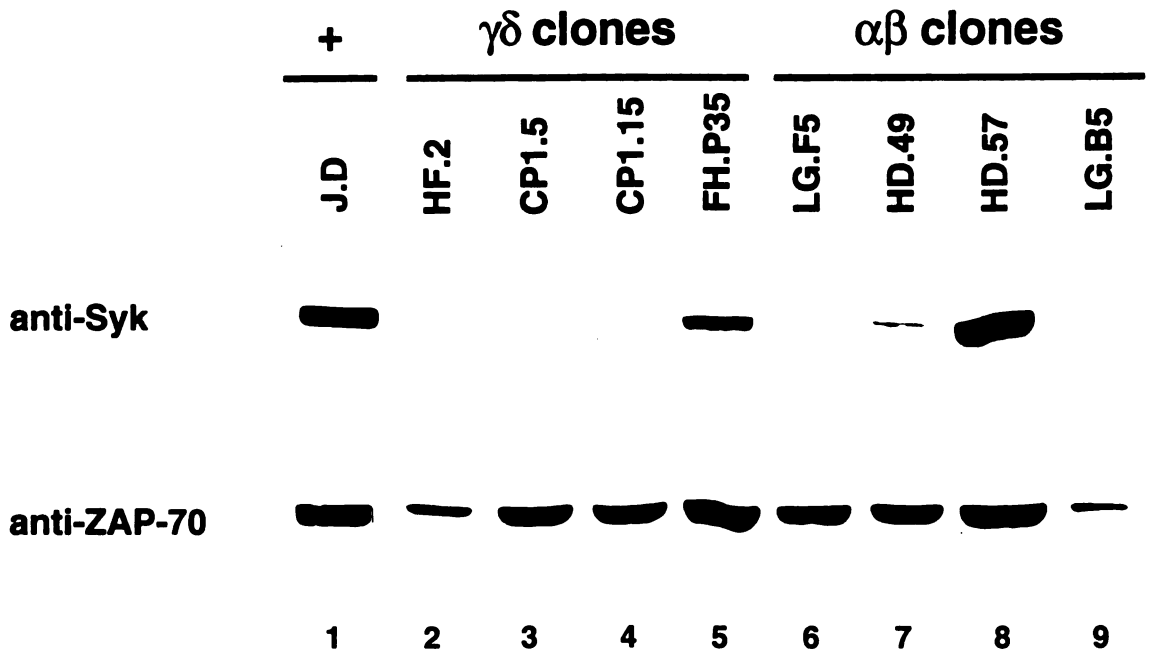
Results

I have described the results of examining the expression of the Syk PTK in small subpopulations of thymocytes by intracellular staining and flow cytometry (Chapter 3). Based on these results and the observations that Syk can play a distinct role from ZAP-70 in T cell lines (Chapter 2), I was interested in determining whether a differential expression occurred within mature peripheral T cell populations.

Syk is variably expressed in human T cell clones

It has been reported that in Syk-deficient mice, certain γδ T cell populations are absent, suggesting that Syk may be an important kinase in the normal function of these populations (88). To study the expression of Syk in γδ T cells, I initially analyzed a panel of human γδ and αβ T cell clones (Figure 1) (107), expecting to see high levels of Syk expressed in the γδ clones and low levels of Syk in the αβ clones. Surprisingly, however, there was heterogeneous expression of Syk in both γδ and αβ T cell clones, based on the analysis of Syk expression in whole cell lysates. Syk is variably expressed in different γδ clones, as well as in αβ clones. Thus, the overall low expression of Syk in peripheral T cells that others have reported (14) may represent the average of the majority of cells that express low levels and a small subpopulation of cells that express high levels of Syk. The selective expression of Syk in these subpopulations may confer special signaling characteristics upon these cells.

Figure 1. Syk is differentially expressed in αβ and γδ T cell clones. Whole cell lysates of human T cell clones were made in 1% NP-40 lysis buffer. 100 μg of lysate was loaded per lane and resolved by SDS-PAGE. The gels were transferred to PVDF membranes and probed using an anti-Syk antibody (4D10.1) or an anti-ZAP-70 antibody (2F3.2). Lane 1, Syk- and ZAP-70-expressing Jurkat line, J.D (99); lanes 2-5, γδ clones; lanes 6-9, αβ clones.



Syk is upregulated in a subpopulation of $CD4^+$ and $CD8^+$ splenic T cells

To further analyze Syk expression in the periphery, I stained splenocytes from wild-type mice. I performed CD4 vs. CD8 cell-surface staining, accompanied by Syk intracellular staining, to determine if T cell subpopulations expressed higher levels of Syk. Interestingly, I observed that in 1-6% of $CD4^+$ or $CD8^+$ cells, Syk expression was elevated (Figure 2). This elevated level of Syk staining was comparable to that previously detected in B cells and $CD4^-CD8^-$ thymoctes (Chapter 3). The same percentage of $CD3^+$ cells expressed elevated levels of Syk (4% of total $CD3^+$ cells, data not shown), consistent with these cells being T cells. Importantly, the staining of the anti-Syk antibody could be competed away with a Syk-derived peptide against which the staining antibody was raised (Figure 2). Therefore, the staining detected by the anti-Syk mAb represented true Syk expression and was not an artifact due to nonspecific binding. Most T cells appeared to express very low levels of Syk.

Syk^{hi} T cells are primarily $\alpha\beta$ T cells

In order to identify what type of TCR these Syk^{hi} T cells expressed, I next stained splenocytes with antibodies that distinguish $\alpha\beta$ from $\gamma\delta$ T cells. As shown in Figure 3, the majority of Syk^{hi} cells are $\alpha\beta$ cells. Of the total $\alpha\beta$ cells stained, approximately 4% expressed elevated levels of Syk, consistent with the finding that Syk^{hi} cells express CD4 and CD8. Of note, $\gamma\delta$ cells, in addition to being a rather small proportion of the cells in the periphery, were also heterogeneous in Syk expression, with only a small subset of splenic $\gamma\delta$ T cells expressing high levels of Syk (6% Syk^{hi}). The variable Syk expression I observed in both $\alpha\beta$ and $\gamma\delta$ T cells is consistent with the variable expression of Syk we have previously obtained from immunoblotting of T cell clones (Figure 1).

Figure 2. Syk is upregulated in a subset of $CD4^+$ and $CD8^+$ murine splenocytes. Splenocytes from a wild-type mouse were stained with anti-CD4 TC, anti-CD8 PE, and anti-Syk FITC. The filled histograms represent Syk expression within the $CD4^+$ and $CD8^+$ gates. The open histograms represent the staining with anti-Syk FITC in the presence of competitor peptide. The percentages indicate the proportion of $CD4^+$ or $CD8^+$ cells that express elevated levels of Syk. These results shown are representative of eight independent experiments. The percentage of Syk^{hi} $CD4^+$ cells ranged from 2.5-7.7%, with an average value of 6.1%, whereas Syk^{hi} $CD8^+$ cells ranged from 0.6-3.4%, with an average value of 2.6%.

CD4
CD8
Relative Cell No.
Syk
4.4%
CD4+
Relative Cell No.
Syk
2.7%
CD8+

Figure 3. Syk is upregulated in a subset of αβ TCR$^+$ and γδ TCR$^+$ splenocytes. Splenocytes were stained with anti-αβ TC, anti-γδ PE, and anti-Syk FITC. The filled histograms represent Syk expression in αβ- or γδ- gated populations. The open histograms represent the staining with anti-Syk FITC in the presence of competitor peptide (solid line) or with an isotype matched staining control (dashed line). The percentages indicate the proportion of αβ TCR$^+$ or γδ TCR$^+$ cells that express elevated levels of Syk. The results shown are representative of three independent experiments. The values of Syk^{hi} αβ$^+$ cells ranged from 3.9-4.7%, with a mean of 4.3%. The values of Syk^{hi} γδ$^+$ cells ranged from 6.1-7%, with an average value of 6.3%.

Syk^{hi} T cells express a subset of memory markers, but not markers of recent activation

I was interested in further characterizing this subpopulation of Syk^{hi} T cells. I first determined whether these cells were resting or recently activated cells. Two markers of early T cell activation are the cell-surface proteins CD25 and CD69 (108). I therefore stained the CD4^{+} and CD8^{+} Syk^{hi} cells for these two activation markers (Figure 4). I determined that the neither of these activation markers distinguished Syk^{hi} from Syk^{lo} cells (Table 1). Thus, elevated Syk expression does not appear to correlate with recent activation.

Next, I determined whether these resting cells expressed cell-surface markers characteristic of memory cells. Some of the best-characterized memory markers are CD44, L-selectin, and CD45RB. I therefore assessed how Syk expression correlated with memory marker expression in CD4^{+} and CD8^{+} T cell populations. I found that, like memory T cells, Syk^{hi} cells were almost uniformly CD44^{hi} (91%, Figure 5 and Table 1). Also consistent with the idea that Syk^{hi} cells represent a memory T cell population is the fact that a significant portion of these cells had downregulated CD62L (L-selectin; 66%, Figure 5 and Table 1). However, the Syk^{hi} cells were uniformly CD45RB^{hi} (91%, Figure 5 and Table 1); most memory cells have been reported to downregulate CD45RB. I therefore believe that the correlation of Syk expression and memory cell marker expression is not complete; it is possible that Syk^{hi} cells may be a subset of memory cells or a subpopulation that has not been well-characterized using these markers.

Syk^{hi} T cells are primarily found in the spleen

I next wished to determine the tissues in which these Syk^{hi} T cells were present. Thus, I stained for Syk expression in CD4^{+} or CD8^{+} splenocytes, lymph node cells, and peripheral blood lymphocytes (PBLs), the major sites of T cell recirculation (Figure 6). I observed that the elevated expression of Syk in CD4^{+} or CD8^{+} cells occurred primarily in the spleen.

Figure 4. Syk^{hi} T cells are not recently activated. Splenocytes were stained with anti-CD4 TC, anti-CD8 TC, anti-Syk FITC, and anti-CD25 PE, or with anti-CD4 PE, anti-CD8 PE, anti-Syk FITC, and anti-CD69 biotin followed by streptavidin-TC. Stained splenocytes were gated on $CD4^+$ or $CD8^+$ (i.e. TC-positive in 3A and PE-positive in 3B) cells; the dot plots represent the distribution of Syk expression of these cells with regard to (A) CD25 or (B) CD69 expression. The results shown are representative of five independent experiments.

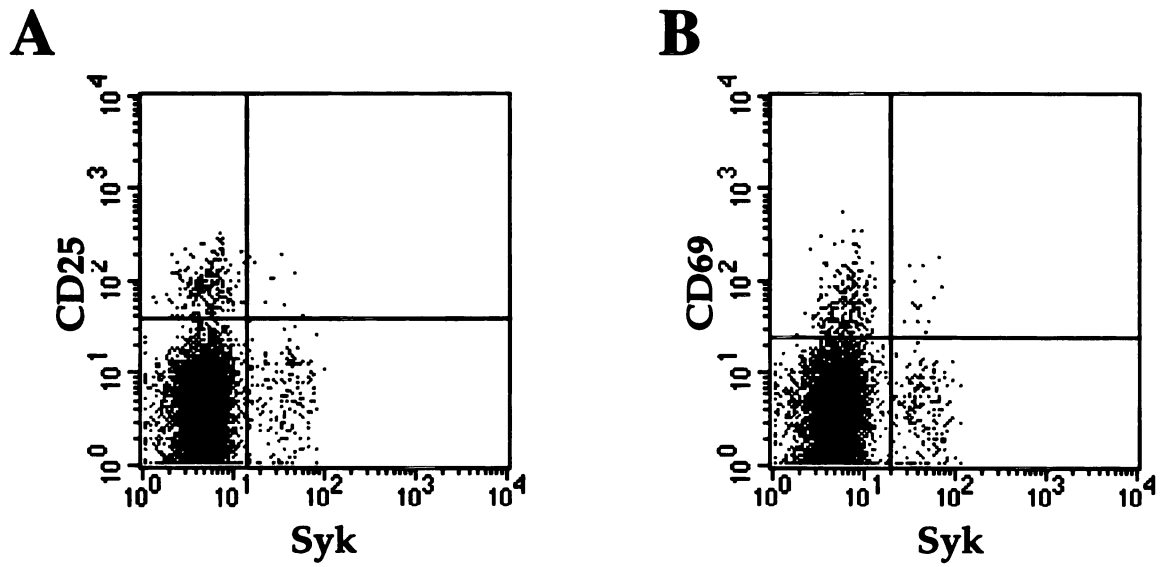

A
CD25
Syk
B
CD69
Syk

Figure 5. Correlation of Syk upregulation with T cell memory markers. Splenocytes were stained with anti-CD4 PE, anti-CD8 PE, and anti-Syk FITC, together with one of the following: anti-CD44 TC, anti-CD62L biotin, or anti-CD45RB biotin. Samples stained with biotinylated antibodies were stained with a streptavidin-TC secondary reagent. Stained splenocytes were gated on $CD4^+$ or $CD8^+$ (PE-positive) cells; the dot plots represent the distribution of Syk expression of these cells with regard to (A) CD44, (B) CD62L, and (C) CD45RB expression. The results shown are representative of four independent experiments.

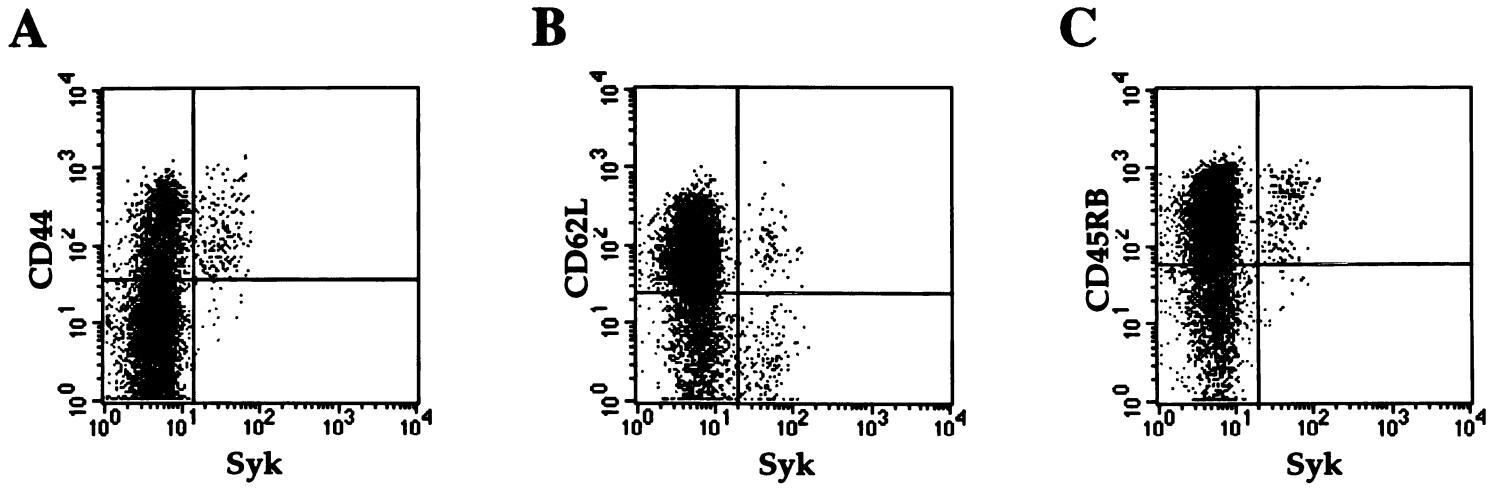

A
CD44
Syk
B
CD62L
Syk
C
CD45RB
Syk

Table 1. Expression of activation and memory markers in T cells. The percentages of T cells that are either Syk^{hi} or Syk^{lo} that exhibit the cell-suface phenotype indicated were calculated from dot plots like those in Figures 4 and 5. The values indicate the correlation of the activation markers CD25 or CD69, or of the memory markers CD44, CD62L, and CD45RB, with the indicated phenotype of Syk expression. "Syk^{hi} % of marker^{+}" refers to the percentage of total T cells that have the indicated cell-surface phenotype that are Syk^{hi}.

Table 1. Correlation of Syk expression with memory marker and activation marker expression

Marker	% of Syk^{lo} T Cells	% of Syk^{hi} T cells	Syk^{hi} % of $marker^{+}$
$CD44^{hi}$	28	91	10.5
$CD62L^{lo}$	19	66	19.0
$CD45RB^{lo}$	24	9	0.8
$CD25^{hi}$	14	7	7.3
$CD69^{hi}$	4	5	6.0

Lymph node T cells did not express high levels of Syk, nor did $CD4^+$ or $CD8^+$ cells from peripheral blood (Figure 6).

A subset of human T cells also express elevated levels of Syk

I was also curious if a similar Syk^{hi} peripheral T cell population is present in humans as well as mice. To do so, I used a different monoclonal antibody, specific for human Syk, for intracellular staining (Chapter 3). As mentioned above, I have observed that in the mouse, the majority of the Syk^{hi} cells were found in the spleen and fewer in lymph nodes or peripheral blood (Figure 6). I also detected a small number of peripheral T cells that express elevated levels of Syk in human $CD8^+$ PBLs (1-2%, Figure 7). In other cases, the proportion of $CD4^+$ PBLs that shows elevated levels of Syk expression is greater (up to 2%, data not shown). The small number of Syk^{hi} cells in human peripheral blood is consistent with the tissue distribution of Syk^{hi} cells in the mouse (Figure 6). It is possible that an examination of human splenocytes would yield a higher proportion of Syk^{hi} T cells, consistent with the results from the mouse. Nevertheless, the fact that these Syk^{hi} T cells are present in both mice and humans suggests that the upregulation of Syk in a specific subpopulation of cells may be an important event during the immune response.

Figure 6. Syk^{hi} T cells are found in the spleen more often than in lymph nodes or peripheral blood. Single-cell suspensions were made from the indicated tissues and stained with anti-CD4 TC, anti-CD8 PE, and anti-Syk FITC. (A) Syk expression of $CD4^+$ and $CD8^+$ cells in spleen. (B) Syk expression of $CD4^+$ and $CD8^+$ cells in lymph nodes (LN). (C) Syk expression of $CD4^+$ and $CD8^+$ cells in peripheral blood lymphocytes (PBL). The filled histograms represent Syk expression of the cells within the $CD4^+$ and $CD8^+$ gates. The open histograms represent the staining with anti-Syk FITC in the presence of competitor peptide. The percentages indicate the proportion of $CD4^+$ or $CD8^+$ cells that express elevated levels of Syk. The results shown are representative of three independent experiments. The average values of Syk^{hi} $CD4^+$ or $CD8^+$ cells in the lymph nodes or peripheral blood never exceeded 1%. Note that the lower percentage of Syk^{hi} splenocytes, particularly $CD8^+$ splenocytes, from (A) compared to Figure 2 may be due to the use of a different batch of anti-Syk staining antibody.

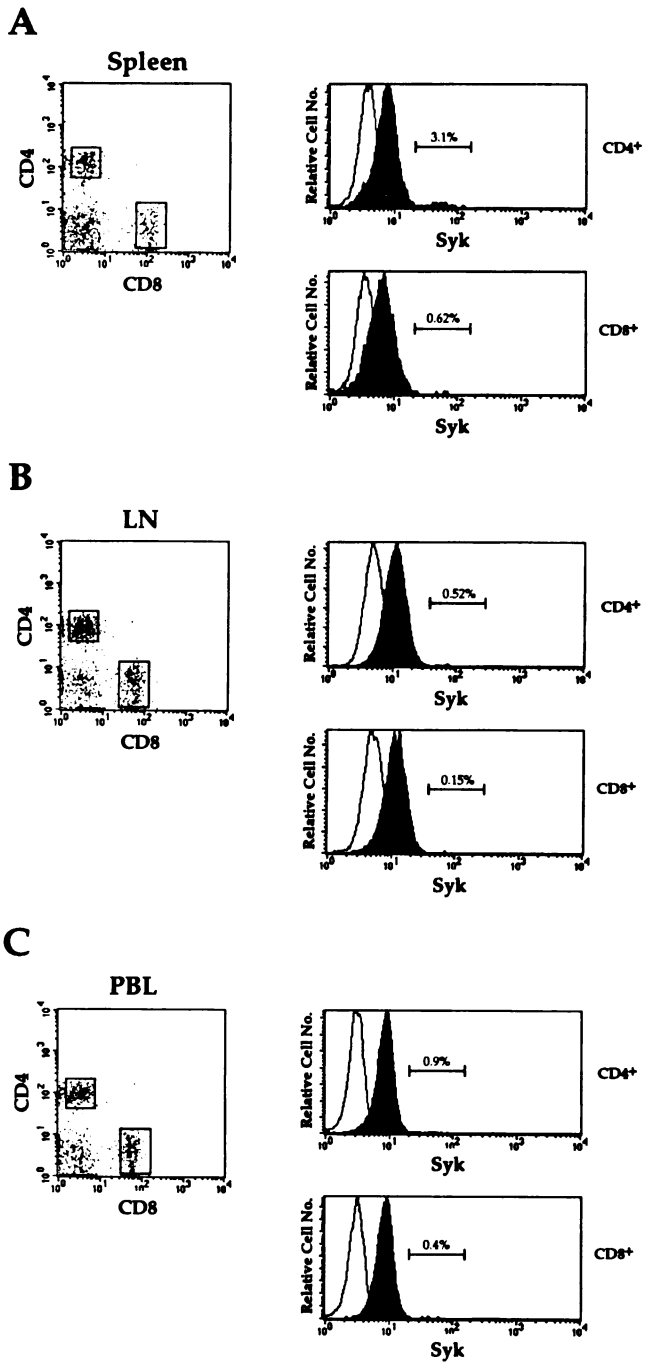


Figure 7. Syk is upregulated in a subpopulation of human $CD4^+$ and $CD8^+$ PBLs. Human PBLs were isolated by Ficoll-Hypaque gradients and stained with anti-CD4 TC, anti-CD8 PE, and anti-Syk FITC. The filled histograms represent Syk expression in the gated $CD4^+$ or $CD8^+$ cells. The open histograms represent the staining with anti-Syk FITC in the presence of competitor peptide. The results shown are representative of eight independent experiments. The percentage of Syk^{hi} $CD4^+$ cells ranged from 0.3-2.0%, with an average value of 1.5%. The percentage of Syk^{hi} $CD8^+$ cells ranged from 0.5-3.1%, with an average value of 1.8%.

PBLs. Hum
TC. anti-CD
he gated CD
TC in the
t indepenc
th an averag
with an

Discussion

I have presented evidence that Syk is expressed at higher levels in a subset of T cells in the periphery of both mice and humans. Analysis of Syk expression by intracellular staining and flow cytometry has allowed us to correlate the expression of Syk with certain markers of memory phenotype in murine cells, suggesting that Syk may be involved in T cell memory, either in its induction or its maintenance.

Most previous analyses of mature T cell lines and bulk populations of peripheral T cells have suggested that Syk is expressed at low levels in mature T cells (14). However, I have determined that Syk is variably expressed in a panel of human αβ and γδ T cell clones (Figure 1). Moreover, using a technique of detecting Syk by intracellular staining, I have now been able to demonstrate that Syk is in fact expressed at high levels in small subpopulations of peripheral T cells, a finding that contrasts with the reports of low levels of Syk detected in most T cell populations by immunoblotting (14). My current analysis further emphasizes the value of the intracelluar staining technique, as differences in Syk expression can now be analyzed in bulk populations at the single cell level.

Identification of $CD4^+$ and $CD8^+$ Syk-expressing splenic T cells

The expression of Syk appears to occur in a small proportion of T cells that express CD3 and either CD4 or CD8. Because of the small numbers of cells involved, it may be argued that the Syk-expressing cells are an artifact of non-specific staining. However, a peptide against which the anti-Syk antibody was raised is able to compete away the staining. Furthermore, not all antibodies react with the Syk^{hi} population. For example, some antibodies, such as the L-selectin antibody, are bound by this subset of cells preferentially at low levels, not just at the highest levels which might be expected if the staining represented nonspecific staining. In CD4 vs. CD8 stains, there are no double positive cells present. Of note, staining is performed in the presence of an antibody that blocks Fc

receptor binding as well as a 500-fold excess of normal mouse immunoglobulin. That some mature T cell do express high levels of Syk protein is also supported by the immunoblot analysis of human T cell clones (Figure 1).

Proposed Syk function in memory and coreceptor-independent T cells

Studies of Syk function in T cell lines suggest that Syk, but not ZAP-70, can be activated in the absence of Lck or CD45 (99). This suggests that Syk may have a role in coreceptor-independent signaling. Since Lck is associated with CD4 and CD8 coreceptors, in situations where T cells respond to TCR stimulation in the absence of coreceptor, Syk might play a more important role than ZAP-70, which requires Lck to phosphorylate and activate it. Multiple groups have suggested that the characteristics of a secondary immune response are not due solely to an increase in precursor frequency, but appear to correlate with a qualitative difference in the requirement for TCR stimulation in memory cells (108-112). It has also been observed that memory phenotype cells have a decreased requirement for coreceptor and for costimulation (111, 112). Moreover, it has been suggested that TCR signaling function is qualitatively different in naïve and memory cells, independent of any accessory molecule contribution (112). These results could be accounted for by a selective increase in Syk expression in the memory T cell.

Of particular interest is the fact that these Syk-expressing cells appear to be $CD3^+$ and present in both $CD4^+$ and $CD8^+$ populations. Studies on the cell-surface phenotype of these cells indicate that they represent a subset of cells which are $CD44^{hi}$, $CD62L^{int}$, and $CD45RB^{hi}$. Thus, these cells exhibit some, but not all, of the cell surface phenotypes associated with memory cells, usually described as $CD44^{hi}$, $CD62^{lo}$, and $CD45RB^{lo}$ (Table 1). Furthermore, the Syk^{hi} cells do not appear to be recently activated cells, since they are $CD69^{lo}$ and $CD25^{lo}$. Whether these cells may correspond to some intermediate stage of memory cell or a subset of memory precursors remains to be determined. These cells are

not exclusively γδ cells, although γδ cells also express heterogeneous levels of Syk (Figure 2).

Syk^{hi} cells may represent small numbers of cells that have been exposed to some environmental factor and have mounted an immune response against this antigen. In that case, a small number of memory cells would remain in mice that have had environmental exposure to various antigenic stimuli. These cells may reflect such a population. Because these cells are not the direct effect of controlled immunization, it is difficult to verify or predict that they are in fact present in every mouse that is analyzed. Although we have attempted to detect an upregulation of Syk expression after immunization with well-characterized antigens such as pigeon cytochrome c or influenza virus (113, 114), we have not detected dramatic increases in Syk expression in these systems (data not shown). In these cases, however, the low frequency of antigen-specific memory cells may still be below the limits of our capability to detect such populations. Therefore, the role of these Syk^{hi} cells is unclear at present and a connection with immunological memory still remains to be more firmly established. Current studies are aimed at determining the requirement for Syk in this process in a Syk-deficient background.

An alternative model would suggest that these cells may not be related to a memory population but may instead correlate more with a coreceptor-independent T cell population. Coreceptor independence has been described both for CD4 cells and for CD8 cells (115-117). The expression of the cell-surface markers for memory and activation have not been reported for such cells. The correlation of Syk expression and these cell populations could be studied by inducing the development of these cell populations and studying them for Syk expression by intracellular staining.

Enrichment of Syk^{hi} T cells in the spleen

Interestingly, the frequency of these Syk^{hi} cells appears to be higher in the spleen, and lower in lymph nodes or in PBLs. The precise reasons for this distribution are not clear. Memory cells have been reported to be enriched at sites of infection, presumably by a CD44/hyaluronate interaction that allows for the memory cells to extravasate to the site of infection (118-125). Phenotypic differences between splenic, lymph node, and effector site T cells have also been described (120, 124, 126), demonstrating that different populations of memory and effector cells are concentrated in different lympoid organs. In one report, immunization with P815 cells intraperitoneally resulted in an initial proliferation of T cells in the spleen, but not in the lymph node or peritoneum (124). These T cells expressed high levels of CD44, intermediate levels of L-selectin, and low levels of CD25. This surface phenotype corresponds to that of the cells detected in the spleen that express elevated levels of Syk. It is possible that the Syk^{hi} cells represent a subset of the sensitized T cells prior to their circulation and proliferation, as has been suggested during the transition from the naïve to memory cell (125).

Thus, despite the uncertainty of the precise identity of this Syk-expressing subpopulation of T cells, the existence of this subpopulation raises interesting questions with regard to the specific biological role of Syk in these cells and whether they are distinct from other T cells that express only ZAP-70. Furthermore, the selective upregulation of Syk also suggests that the study of the regulation of the *syk* gene may reveal a previously unappreciated complexity of transcriptional control in T cells. These findings serve to highlight the potential for unique and nonredundant functions for gene family members as well as to provide possible molecular explanations for classic immunological phenomena.

Experimental Procedures

Mice and antibodies

C57Bl/6 were obtained from Jackson Research Laboratories (Bar Harbor, ME) and maintained at the UC San Francisco Animal Care Facility. The following antibodies were used: anti-murine Syk FITC, 5F5.2 (Chapter 3); anti-CD4 TC, anti-CD8 PE, biotinylated anti-CD44, biotinylated anti-CD62L (Caltag Laboratories); biotinylated anti-CD25, biotinylated anti-αβ, biotinylated anti-γδ (Pharmingen, San Diego, CA); anti-human Syk FITC, 4D10.1 (Chapter 3).

Cells and tissues

Lymph nodes and spleens from mice were removed and made into single-cell suspensions and washed in phosphate buffered saline (PBS). Peripheral blood was obtained from both mice and humans and fractionated using Ficoll/Hypaque (Histopaque 1077, Sigma Research Chemicals, St. Louis, MO, for human PBLs; Lympholyte M, Accurate Chemicals, Westbury, NY, for murine PBLs).

Cell lysates and immunoblotting

Whole cell lysates were generated in 1% NP-40 lysis buffer as described (Chapter 3). Lysates were resolved by SDS-PAGE, transferred to polyvinylidine difluoride membranes (PVDF, Immobilon P, Amersham, Arlington Heights, IL), and probed with the appropriate antibodies. Results were visualized by enhanced chemiluminescence (ECL, Amersham).

Intracelluar staining and flow cytometry

Intracellular staining was performed as previously described (Chapter 3). Briefly, cells were stained on the cell-surface with appropriate primary and/or secondary antibodies and washed in staining buffer containing 1% FCS and 0.1% sodium azide in PBS. Following

fixation in 4% paraformaldehyde in PBS, cells were stained with staining buffer containing 0.1% saponin and antibodies against intracellular antigens. All staining was performed in the presence of 50 μg/ml normal mouse immunoglobulin and 2.4G3 anti-Fc receptor blocking antibody. Competitor peptide (murine Syk-derived, amino acids 306-333) was added at 10 μg/ml for negative control staining. Stained cells were analyzed by flow cytometry on a FACScan (Becton-Dickinson) using CellQuest software (Becton-Dickinson).

CHAPTER 5

CONCLUDING REMARKS

Summary

I have observed and characterized roles for the Syk protein tyrosine kinase (PTK) in T cell signaling and development that had been previously unappreciated. Growing evidence suggests that Syk and ZAP-70 may play some unique and non-overlapping roles in T cell biology. I now suggest future areas of investigation that will help to clarify the biological significance of the differences in activities of the Syk and ZAP-70 PTKs and their importance in immune function.

Participation of Syk in TCR signaling

Based on the studies reported here, it is apparent that Syk is able to function independently of CD45 or Lck, and presumably independently of Src family kinase activation. However, the mechanism by which the TCR signaling cascade is initiated in the absence of CD45 or Lck is not clear. There have been some indications that Syk is capable of phosphorylating immunoreceptor tyrosine-based activation motifs (ITAMs) within antigen receptor complexes, but these results have come from cotransfection and overexpression experiments in heterologous COS cell systems, which may not accurately reflect the physiological situation in a T cell (61, 62). One way to assess the ability of Syk to phosphorylate ITAMs in T cells would be to express different forms of Syk in the Lck-deficient JCaM1.6 Jurkat line. In a stable line expressing Syk in the absence of Lck, it would be possible to assess the ability of Syk to restore ITAM phosphorylation. This biochemical analysis was not possible in the transient transfection studies reported here because of the insensitivity of the biochemical analysis. Next, generating a stable JCaM1.6 line expressing the kinase inactive mutant of Syk would allow the determination of whether Syk kinase activity is required for the phosphorylation of the ITAMs. If, as in expression of kinase inactive ZAP-70, kinase inactive Syk protects ITAMs from dephosphorylation (106), one might expect to increase the amount of phosphorylation of ITAMs mediated by a different kinase. This experiment would clarify whether Syk or some other kinase is responsible for the phosphorylation of ITAMs in the absence of Lck.

Further applications of intracellular staining and flow cytometry

The Syk family of protein tyrosine kinases (PTKs) is an interesting one to study both in terms of understanding the events in TCR signaling and in studying differences in closely related family members. Analysis by intracellular staining and flow cytometry allows for the detection of a protein within small subpopulations of cells with great purity and sensitivity. This methodology can be expanded for use in examining the expression and

coexpression of many different signaling molecules when small subpopulations need to be examined. Thymic and peripheral cell subpopulations are prime examples, as I have demonstrated. Studies of the coexpression of other signaling molecules would be an interesting subject to investigate in peripheral populations. These can be used to look at the expression of ZAP-70 and related kinases to document any changes in expression in the T cell lineage. Furthermore, these studies can be extended to examine the expression of other families of tyrosine kinases or signaling molecules of interest, to ascertain if any are controlled in a development-specific or activation-specific manner.

The effect of Syk expression on T cell repertoire development

I have suggested that Syk plays a role in pre-T cell receptor signaling (see Chapter 3). The fact that Syk expression can restore development in ZAP-70-deficient mice (57) raises the question of why Syk expression is downregulated prior to positive and negative selection. Perhaps if Syk is capable of increasing sensitivity of signaling of the TCR, the thresholds for TCR signals mediating selection events could be perturbed in a manner independent of avidity or affinity of the extracellular interactions between the TCR and antigen presenting cells (127). Looking at shifts in repertoire or at TCR transgenic models of selection in the presence of Syk overexpression would help address this issue. The CD4 knockout mouse would be an interesting model to use, since CD4-lineage cells do develop in these mice (128). Whether Syk is upregulated in those mice, and what effects that has on the affinity of the receptors that are selected, are intriguing questions to examine.

Syk and its role in peripheral T cell signaling--memory and coreceptor independence

The speculation that Syk may be involved in situations in which TCR signals are not as stringently controlled arises from the differences in activation requirements between Syk and ZAP-70. Because Syk is less dependent on Src family kinases for activation, Syk may

have increased importance in situations when less coreceptor-associated Lck is available. This situation may occur in coreceptor-independent signaling or in memory cells, which have been shown to be less dependent on costimulation or coreceptor function for activation (108-112). The fact that Syk may be involved in memory is further supported by our evidence that Syk is upregulated in a subpopulation of cells which express some markers characteristic of memory cells (see Chapter 4). This correlation with a memory cell population is suggestive that Syk may be involved, but a true test of the requirement for Syk in memory induction would have to be done in a Syk-deficient background. Because Syk-deficient mice die perinatally, the assessment of this function is not trivial. However, it is possible to reconstitute irradiated or immunocompromised hosts with fetal liver cells from Syk-deficient mice. This will allow for the restoration of the T cell immune system with Syk-deficient cells. These mice can then be assessed for memory responses after immunization. The immunogens used can be alloantigens, to assess the general memory response, or more specific antigens such as hen egg lysozyme, which have been well-characterized and for which transgenic TCR models exist. Because B cells fail to develop in Syk-deficient animals, it is possible that mixed fetal liver reconstitutions will be required to provide B cell function. Ideally, such a B cell donor would be T cell-deficient, such as a CD3- or ZAP-70-deficient mouse, so that any T cell memory responses would result solely from the Syk-deficient T cells.

The importance of Syk in T cell signaling need not correspond strictly to memory responses. It is possible that Syk may play a more general role in coreceptor-independent signaling. Thus, it is of interest to study if coreceptor-independent function can be correlated with the induction of Syk expression. CD8-independent clones can be assayed by performing CTL assays in the presence of anti-CD8 blocking antibodies and comparing the CTL activity compared to the absence of anti-CD8 antibodies (116). Alternatively, coreceptor-independent clones can be generated by culturing bulk populations of cells in the presence of anti-CD8 antibodies (115). Mice can be primed with an allogeneic stimulus,

such as P815 cells in C57Bl/6 mice, in order to increase the frequency of coreceptor-independent clones, and spleens harvested and cultured from these animals. These coreceptor-independent clones can then be assayed for Syk expression. Correlation of increased levels of Syk in coreceptor-independent but not coreceptor-dependent T cell clones would suggest that Syk expression may be important for the differences in receptor signaling.

Identification of Syk-expressing cells *in vivo*

The value of intracellular staining in conjunction with flow cytometry for the assessment of expression of proteins has been demonstrated in studies of expression of Syk. One particular shortcoming of this assay, however, is the requirement for fixation of these cells prior to intracellular staining. Ideally, one would like to have an assay whereby Syk expression could be assayed on viable cells, so that they could be sorted and studied for their biochemical properties. One method for distinguishing Syk-expressing cells is the generation of a mouse that expresses a reporter gene under transcriptional control of the endogenous *syk* locus (a "knock-in" mouse). I have attempted to construct such a mouse (see Appendix B) but have been unsuccessful in expressing our particular reporter of interest, a human CD2 cDNA targeted to the initiation codon of the *syk* gene. One consequence of this strategy is that the targeted *syk* allele is disrupted upon introduction of the reporter gene. If *syk* were haploinsufficient, this approach would not work; however, $syk^{+/-}$ mice do not have any reported abnormalities (72, 73). Similarly, if *syk* were monoallelically expressed, the strategy to study reporter-containing heterozygotes would not work, since the reporter would have to be silenced to maintain viability. Again, there is currently no evidence that this is the case, although it has not been studied in detail. One attractive alternative strategy for overcoming these complications is to use internal ribosomal entry sequences (IRES) to create a bicistronic message that includes the

endogenous transcript as well as that of the reporter, so that the message from the endogenous locus will remain unaffected (129-131).

In any event, construction of a mouse with a reporter gene to monitor Syk expression will be useful for the physical sorting of Syk-expressing cells and will allow for further biochemical studies on the Syk-expressing versus Syk-nonexpressing populations. Comparison of the sensitivity of the TCR to antigen stimulation and evaluation of coreceptor and costimulatory dependence between Syk-expressing and Syk-nonexpressing cells will be important for the establishment of a connection between Syk expression and memory or coreceptor independent function (112).

Evaluation of the functional redundancy of Syk PTK family members

The use of knock-in technology has also been reported for studying the effects of related family members or isoforms of particular genes in place of other family members (132-135). To do so, gene targeting by homologous recombination has been used to introduce the alternate gene in the endogenous locus of the target gene of interest. This technology could also be applied to study the functional differences between Syk and ZAP-70. Although a Syk transgene has been demonstrated to reconstitute thymic development in ZAP-70-deficient mice, the promoter used is active only in the thymus, so further characterization of peripheral cells is not possible (57). Furthermore, that experiment does not address the potentially more interesting question of when it is that ZAP-70, a PTK with more restricted activity and expression, is unable to compensate for Syk. To develop a system for studying the situations in which Syk and ZAP-70 are not interchangeable, one could construct a mouse that expressed ZAP-70 under control of the endogenous Syk regulatory elements. It would then be possible to assess the ability of ZAP-70, which has more stringent regulation by Src family kinases, to restore development and signaling in hematopoietic lineages that normally depend on Syk. These lineages include platelets, B

cells, mast cells, and macrophages, cells that require a Syk family PTK to be recruited to ITAMs for proper receptor signaling (72, 73, 136-139).

The role of Syk PTKs in γδ T cells

Syk family PTKs have been shown to be critical not only in αβ T cell development, but in γδ T cell development as well (57, 71, 88). γδ T cell development is not as well-characterized as αβ T cell development, although the Syk and ZAP-70 kinases have proven to be important in the developmental process of these cells. As discussed earlier, adult Syk-deficient mice do not have an apparent αβ T cell defect, but they do show a peripheral T cell defect in the development of a specific population of γδ cells, the intraepithelial lymphocytes (IELs) of the intestine (88). Surprisingly, a similar defect in IEL development is also observed in ZAP-70-deficient mice (57, 71). Although normal numbers of γδ T cells are present in the spleen and lymph nodes of ZAP-70-deficient mice, dendritic epidermal T cells, another subset of γδ cells, are decreased in number and show an abnormal morphology in these animals (57, 71). Thus, in certain γδ T cell subpopulations, Syk and ZAP-70 do not fully compensate for each other in the case of a deficiency in either kinase. It is not clear that the required functions provided by these tyrosine kinases occur during the same time of development. Therefore, Syk and ZAP-70, although they seem to have overlapping functions in αβ T cell development, may in fact have divergent functions in the development and/or signaling of γδ T cells. Analysis of Syk expression in the ontogeny of the γδ T cell lineage, using the methods that I have described for studying Syk in the αβ T cell lineage, may provide some insights with regard to these processes.

Transcriptional control of the *syk* locus

The biological role of Syk in T cells *in vivo* is only beginning to be understood. These studies have generated a number of questions that will lead to heightened appreciation for this kinase in T cell biology. First, what is its role in development? Is it a signal amplifier that allows a weak pre-TCR signal to be transmitted that indicates that further progression is possible? Our studies would suggest this to be the case and that this function is only required during a specific developmental period (see Chapter 3). After the pre-TCR signal, it appears that Syk is rapidly decreased in expression via a signal that has not been characterized. The downregulation of Syk would be consistent with the need for tighter regulation of mature TCR signaling at the $CD4^+CD8^+$ selection checkpoint. How is *syk* expression regulated? It may be controlled at the transcriptional level, in which case the promoter region of the *syk* locus could be interesting to study. Indeed, expression of *syk* message appears to correlate with the expression of Syk protein in many cell lines and tissues (14). Thus, it may be that some early elements of transcription, specific for pro- and pre-T cells, are the ones that are responsible for the expression of Syk at this stage. Alternatively, there may be a transcriptional silencer that becomes activated following pre-TCR signal, which then remains active throughout the rest of development and for the most part in the periphery.

It is also unclear what controls the expression of Syk in a subset of T cells in the periphery. It is possible that a small subset of these cells is constitutively expressing Syk from the time it exits the thymus. On the other hand, it may be that a second event that causes a subsequent upregulation of Syk expression. Such an inductive signal could be a cytokine that is expressed after T cell activation or a signal directly from the TCR itself. Determining the timecourse of induction of Syk expression would be of interest in trying to sort out this problem. Examination of the *syk* promoter region may reveal the presence of binding sites for transcriptional activators or repressors that are expressed in a similar

temporal/activation-specific manner. These transcriptional regulators may indicate what is controlling the expression of this kinase in such a tightly regulated manner. Transcriptional reporter assays based on the *syk* gene could aid in this analysis. Whether the thymic expression of Syk is controlled by the same elements that control its peripheral reexpression, or whether there are "proximal" and "distal" *syk* promoter elements, as there are in *lck*, remains to be clarified.

Consequences of loss of regulation of murine and human Syk expression

Not only does the transcriptional regulation of *syk* present an interesting problem for the study of normal gene regulation, but it may have important consequences in disease states as well. Based on our model of Syk activity, one would imagine that if Syk were misexpressed in the periphery, an autoimmune state might develop. Because Syk can increase the sensitivity of a TCR to stimulation, T cells whose receptors are slightly cross-reactive to self-antigen may now become activated. It would be interesting to determine if such a state could be induced experimentally. For example, mice carrying a *syk* transgene selectively expressed in peripheral T cells, but not during thymic selection, could provide such a system. One would predict that, in these Syk-misexpressing mice, autoimmune disease would be observed. By coupling such Syk expression with a TCR transgenic mouse specific for certain organ-specific antigens such as myelin basic protein, it might be possible to increase the incidence or speed the progression of a disease state.

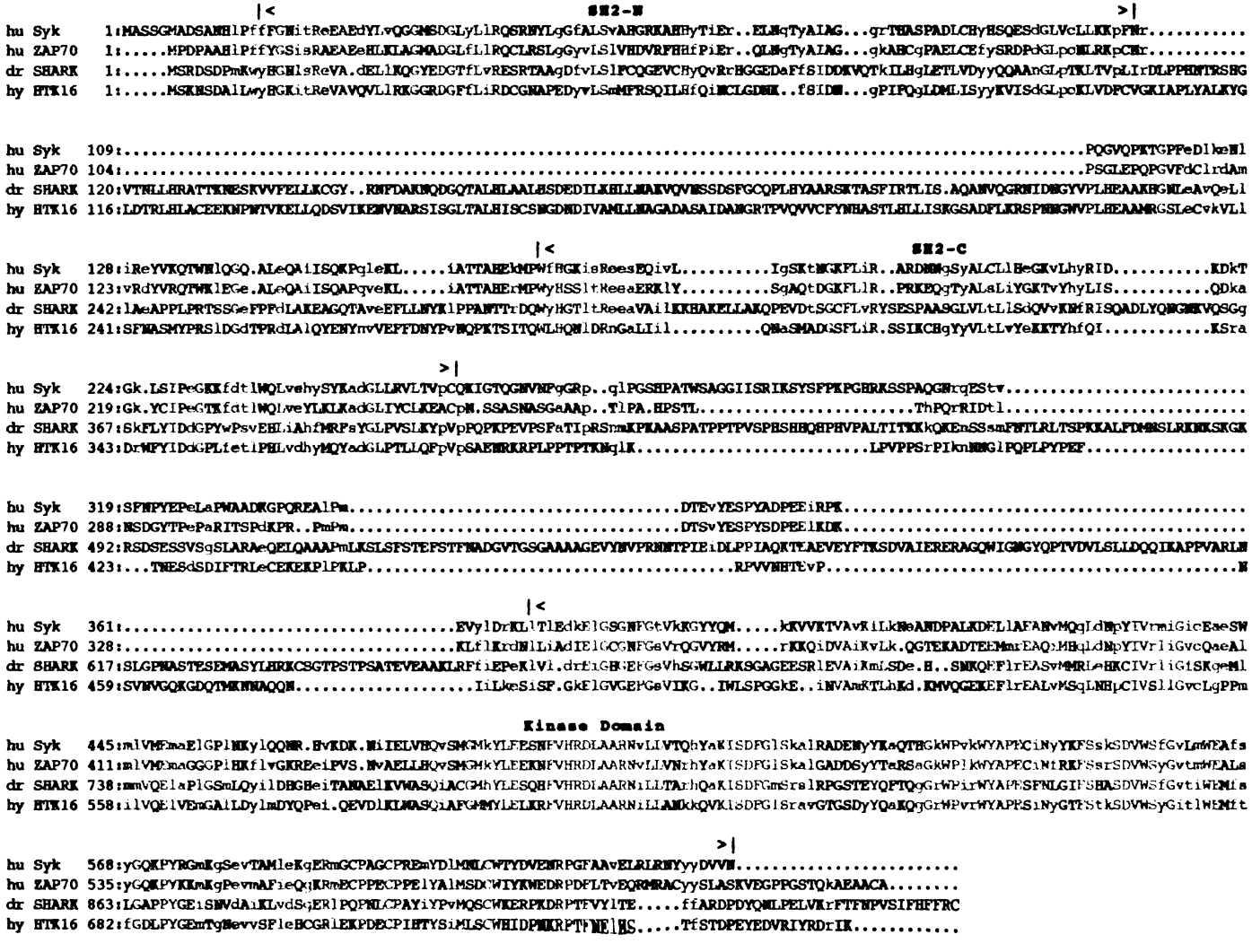
The expression and potential misexpression of Syk may also be important in human disease. Correlation of the upregulation of Syk-expressing T cells in autoimmune diseases such as diabetes or rheumatoid arthritis would provide an important molecular mechanism for these diseases and provide a new target for pharmacological intervention. If misexpression of Syk is a contributory factor for autoimmunity, screening for elevated levels of Syk in patients with a genetic predisposition to certain rheumatological diseases may become an important diagnostic tool.

Identification of new members of the Syk gene family

Because I have seen a functional difference between Syk and ZAP-70, the question arises about the presence of other family members and what their role is in signaling. In fact, this family of kinases has been conserved throughout evolution; other Syk family kinases have been identified in more primitive organisms, *Hydra vulgaris* and *Drosophila melanogaster*. These other tyrosine kinases, HTK16 in *Hydra* and SHARK in *Drosophila*, both contain tandem SH2 domains in a similar configuration to Syk and ZAP-70 (140, 141). Although the sequences of the individual SH2 domains are more similar to the Src and Abl families of tyrosine kinases, Syk family kinases are the only cytoplasmic tyrosine kinases that have two consecutive SH2 domains at the N-terminus of the protein. Both HTK and SHARK also contain the extended interdomain B between the C-terminal SH2 domain and kinase domain found in Syk and ZAP-70 (Figure 1). Furthermore, the kinase domains of HTK and SHARK are highly similar to those of Syk and ZAP-70 (Figure 1) (140, 141). Based on these shared structural features, it seems reasonable to consider HTK and SHARK as part of the Syk gene family.

One distinction between the *Hydra* and *Drosophila* proteins and the mammalian Syk family kinases is that both HTK16 and SHARK contain five ankyrin repeats in interdomain A, between the SH2 domains, that are not present in their vertebrate counterparts (140, 141). Thus, HTK and SHARK appear to be more related to each other than to Syk or ZAP-70. The secondary structure of the ankyrin repeat domains has not been determined for either HTK16 or SHARK, although the structure of ankyrin repeats from other proteins suggests that they may form helix-turn-helix motifs (142, 143). It would be interesting to determine if the SH2 domains in HTK16 or SHARK exhibit the same cooperative binding to their targets as those of Syk and ZAP-70 and if the ankyrin-repeat-containing inter-SH2 domain can stabilize this interaction. Following the ankyrin repeats is another portion of interdomain B that appears to be more conserved between all the Syk family members,

Figure 1. Sequence alignment of Syk family kinases. Human Syk and ZAP-70, *Drosophila melanogaster* SHARK, and *Hydra vulgaris* HTK16 are compared. Identical amino acids are indicated by red capital letters. Conserved or similar amino acids are indicated by lower case letters.


|<
SH2-N
>|
|<
SH2-C
>|
Kinase Domain
>|

suggesting a potential conservation of function (Figure 1). Conservation of these characteristics would support the idea of a common evolutionary origin of the Syk family of PTKs. Recently, another HTK-related gene was cloned from Hydra. Interestingly, this kinase does not contain the ankyrin repeats found in HTK and SHARK, suggesting that this kinase may be the true *Hydra* homolog of Syk and ZAP-70 (R. Steele, personal communication).

The biological functions of HTK16 and SHARK have not been determined; however, it is interesting to note that Src family PTKs have been identified in both *Hydra* and *Drosophila* as well, suggesting that the signaling pathway involving the Src and Syk family PTKs may also have been conserved during evolution (144-146). One test of the conservation of signaling function of the new family members would be to assess the ability of these PTKs to reconstitute Syk-deficient or ZAP-70 deficient cell lines. Study of these conserved kinases in genetically tractable systems such as *Drosophila* would be useful for identifying interacting genes that could be potential regulators or effectors of this family of PTKs.

Final thoughts

I have characterized biochemical differences between Syk and ZAP-70 that have suggested differing *in vivo* roles for these two related PTKs. To study these differences in a physiological context, I have developed new reagents and demonstrated a powerful application of intracellular staining and flow cytometry as a means for detecting expression of proteins in small subpopulations of cells. These results have provided intriguing evidence with regard to the potential nonredundant functions of Syk compared to ZAP-70. These issues of overlapping and distinct functions are commonly encountered in biology, and the T cell system of activation and development provides a useful model for the study of such issues. The knowledge gained from studying the Syk PTK in T cells has raised interesting and complex questions about immune regulation, memory induction, and

APPENDIX A

STRUCTURE-FUNCTION ANALYSIS OF THE DIFFERENCES BETWEEN SYK AND ZAP-70

Summary

Syk and ZAP-70 are two protein tyrosine kinases that participate in early events of T cell antigen receptor (TCR) signal transduction. I have previously shown that Syk, but not ZAP-70, can function independently of CD45 or Lck in Jurkat T cells. I extended these results by demonstrating that the qualitative differences in Syk and ZAP-70 kinase activation reside in the kinase domain, although additional modification of kinase activity by other domains has not been ruled out.

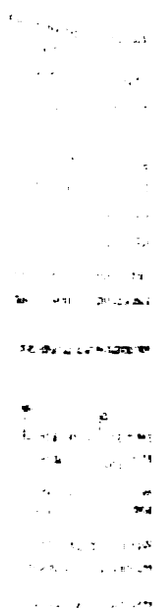
Introduction

Syk and ZAP-70 are two protein tyrosine kinases (PTKs) of the same gene family that participate in early events of T cell antigen receptor (TCR) signal transduction. My recent results have demonstrated that although both PTKs are similar in structure, the function of Syk and ZAP-70 can be differentially regulated (Chapter 2). In Jurkat cells, I demonstrated that Syk can function independently of CD45 or Lck in TCR signaling. Furthermore, I determined that the Syk SH2 and kinase domains, as well as activation loop tyrosines, are required for that function.

Despite these differences, in other systems, Syk and ZAP-70 have been shown to act similarly. For example, both Syk and ZAP-70 have been shown to reconstitute the Syk-deficient avian B cell line DT-40, in terms of antigen receptor-induced tyrosine phosphoproteins as well as IL-2 and NF-AT signaling restoration (49, 56).

In an attempt to dissect the functional domains that are responsible for the differences between Syk and ZAP-70 function, I constructed chimeric kinases using Syk and ZAP-70, replacing the SH2 domains, kinase domain, or interdomain B from one kinase with that of the other. I used the reconstitution of the mutant lymphocyte cell lines described above as our functional readouts for the reconstitution of signaling. Specifically, I used reconstitution of the Syk-deficient DT-40 cells as an assay to confirm the integrity of the chimeras, as well as the J45.01 and JCaM1.6 Jurkat mutants to assay each chimera for the ability to behave like Syk.

I have determined that the kinase domains are primarily responsible for the differences in kinase regulation between Syk and ZAP-70, both functionally and in terms of kinase activity.



Results and Discussion

I have previously shown that Syk, but not ZAP-70, is capable of reconstituting signaling in the Jurkat mutant lines J45.01 and JCaM1.6. To determine the domains responsible for these differences between Syk and ZAP-70, I constructed chimeric kinases in which the three major domains of the kinases were interchanged (Figure 1).

My initial strategy was to create identical restriction enzyme sites within the two kinases to facilitate the swapping of domains, as well as to provide modularity to the different domains for more detailed mutagenesis. This was achieved by introducing short spacers of alanines and/or glycines into the junctions of the predicted domains (Figure 1)(14). Using PCR mutagenesis, I created these linker-containing constructs, which I termed SSS and ZZZ. These were subcloned into the pEF-BOS expression vector and transiently transfected into Syk-deficient DT-40 chicken B cells. Both SSS and ZZZ restored BCR-mediated NF-AT induction, consistent with the level of induction seen with the wild-type versions of each enzyme (Figure 2, Figure 3, and data not shown). Thus, the function of the kinases in DT-40 cells did not appear to be perturbed substantially by insertion of these junctions.

Next, to test whether these constructs behaved like their wild-type counterparts in a T cell context, I transfected the new SSS and ZZZ constructs into JCaM1.6 and J45.01 cells. I expected SSS, but not ZZZ, to reconstitute TCR signaling in these mutant cell lines. The qualitative trend observed between wild-type Syk and ZAP-70 remained consistent in the SSS vs. ZZZ constructs, namely that SSS was active in the absence of CD45 or Lck, but ZZZ was not (Figure 4 and data not shown). To my surprise, however, the SSS construct constitutively activated the NF-AT reporter, even in the absence of receptor stimulation (Figure 4). These results have also been observed in other chimeras between Syk and ZAP-70 whose junctions are in interdomain B [S. Hunter and A. Schreiber, submitted; (147)]. I therefore believe that interdomain B is sensitive to

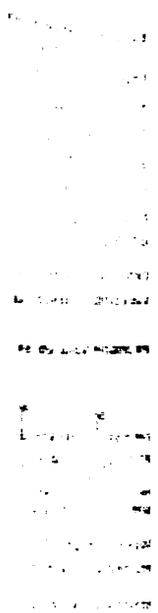


Figure 1. Schematic representation of the Syk/ZAP-70 chimeric kinases. The boundaries of the three major domains of the kinases, the tandem SH2 domains (SH2); interdomain B (IB); and the kinase domain (Kinase), are indicated. The insertion points for the alanine and glycine spacers in ZZZ and SSS are depicted. For the chimeric kinases, the shaded domains are derived from Syk, whereas the open domains are derived from ZAP-70.

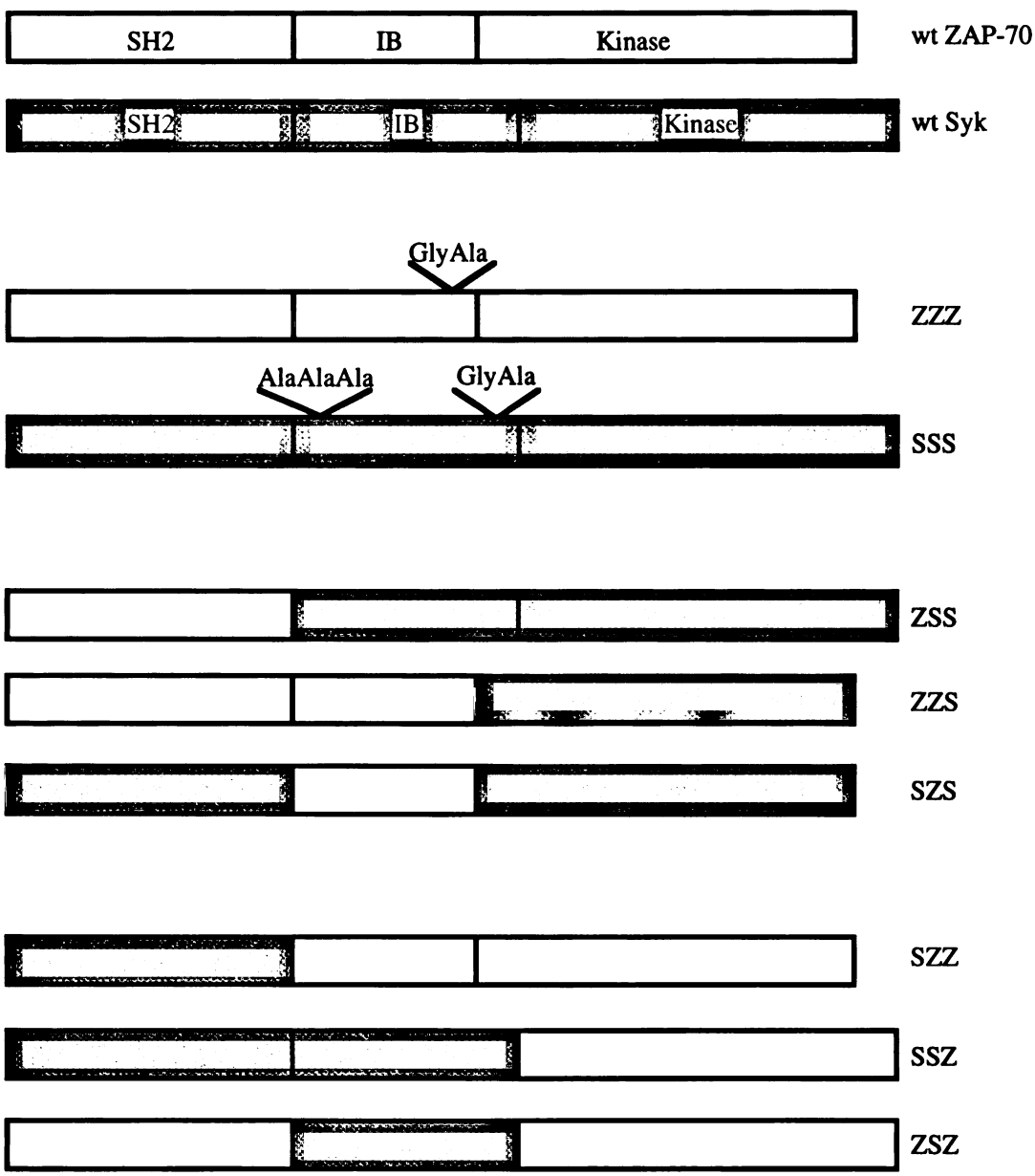
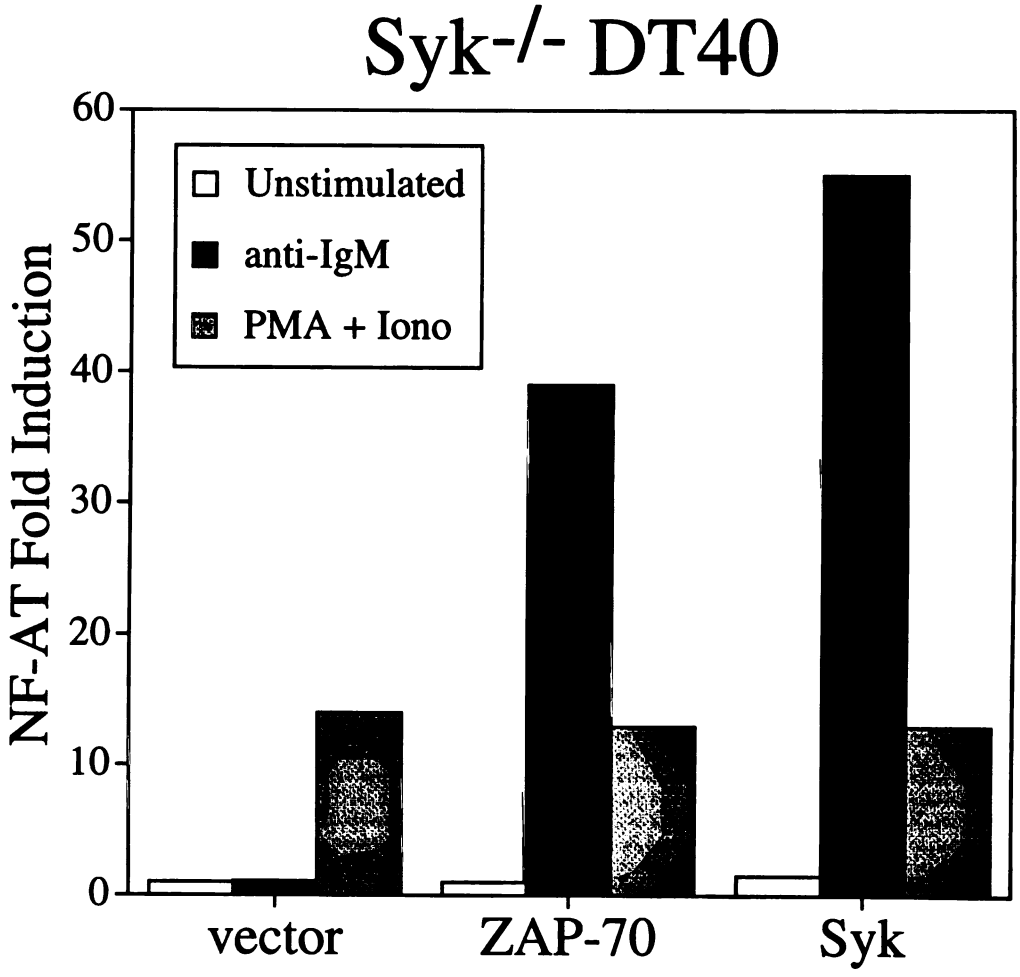

SH2
IB
Kinase
wt ZAP-70
SH2
IB
Kinase
wt Syk
GlyAla
ZZZ
AlaAlaAla
GlyAla
SSS
ZSS
ZZS
SZS
SZZ
SSZ
ZSZ

Figure 2. Syk and ZAP-70 can both reconstitute BCR signaling in Syk-deficient DT40 B cells. Syk-deficient DT40 B cells were transfected with 20 μg of the indicated plasmid and 10 μg of the NF-AT-Luc reporter. After 20 hr, cells were left unstimulated (open histogram) or stimulated with an anti-BCR antibody (anti-IgM, black histogram) or with PMA and ionomycin (PMA + Iono, shaded histogram) for 6 hr. Luciferase activity was then assessed and expressed as the ratio of the activity from the stimulation of interest to the unstimulated vector control (NF-AT Fold Induction).


Syk-/- DT40
NF-AT Fold Induction
60
50
40
30
20
10
0
Unstimulated
anti-IgM
PMA + Iono
vector
ZAP-70
Syk

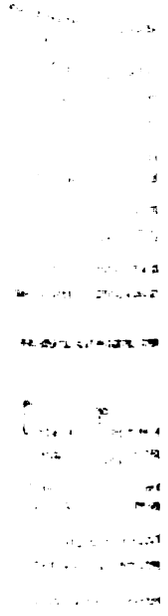
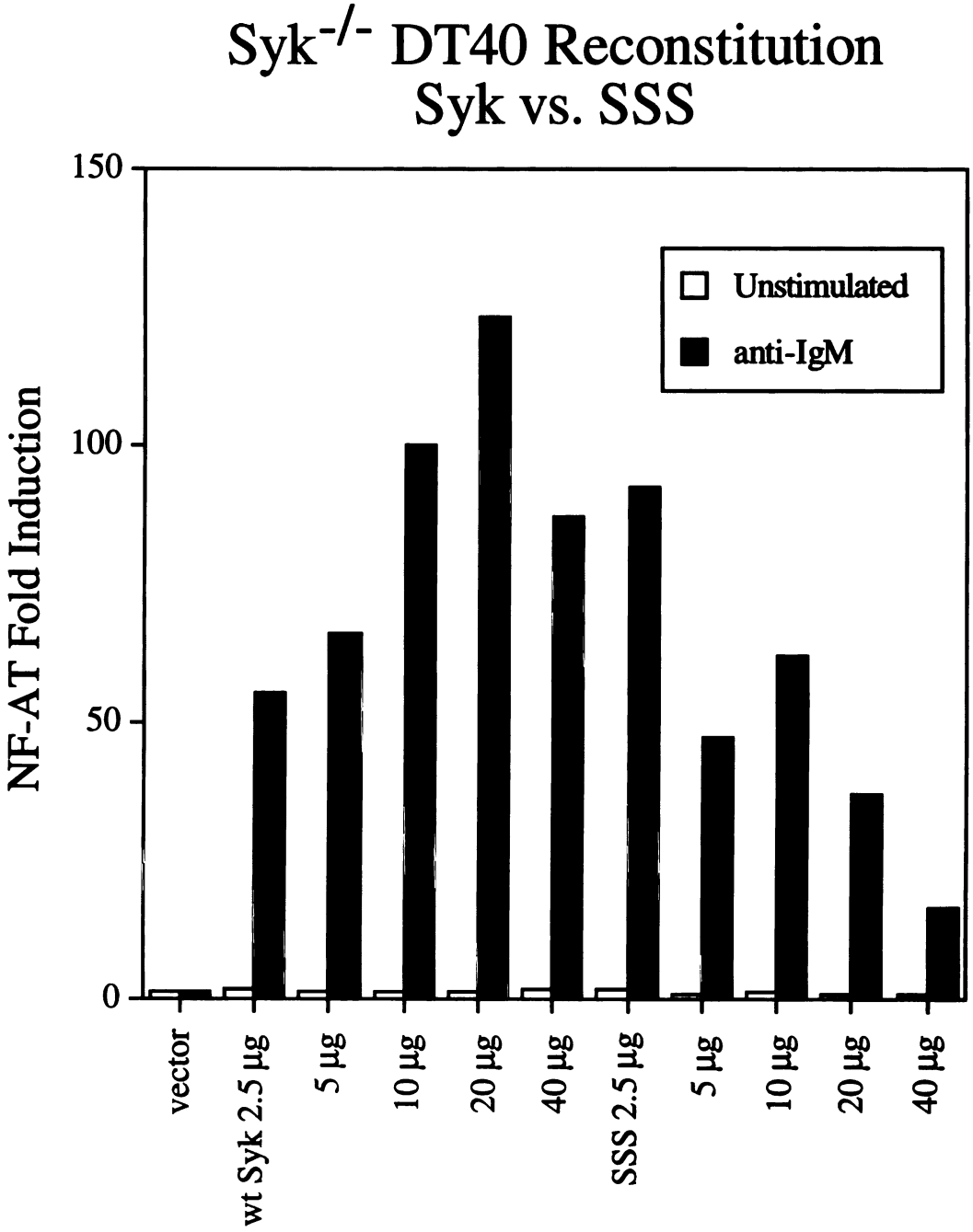


Figure 3. Wild-type Syk and SSS can both reconstitute BCR-inducible NF-AT responses in Syk-deficient DT40 B cells. Syk-deficient DT40 B cells were transfected with the indicated amount of plasmid, along with 10 μg of the NF-AT-Luc reporter. Empty vector plasmid was added so that the total amount of DNA transfected was the same for every sample. After 20 hr, cells were left unstimulated (open histogram) or stimulated with an anti-BCR antibody (anti-IgM, shaded histogram) for 6 hr. Luciferase activity was then assessed and expressed as the ratio of the activity from the stimulation of interest to the unstimulated vector control (NF-AT Fold Induction).


Syk-/- DT40 Reconstitution
Syk vs. SSS
NF-AT Fold Induction
150
100
50
0
Unstimulated
anti-IgM
vector
wt Syk 2.5 µg
5 µg
10 µg
20 µg
40 µg
SSS 2.5 µg
5 µg
10 µg
20 µg
40 µg

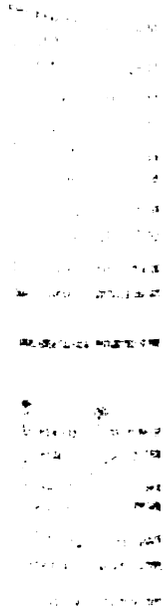
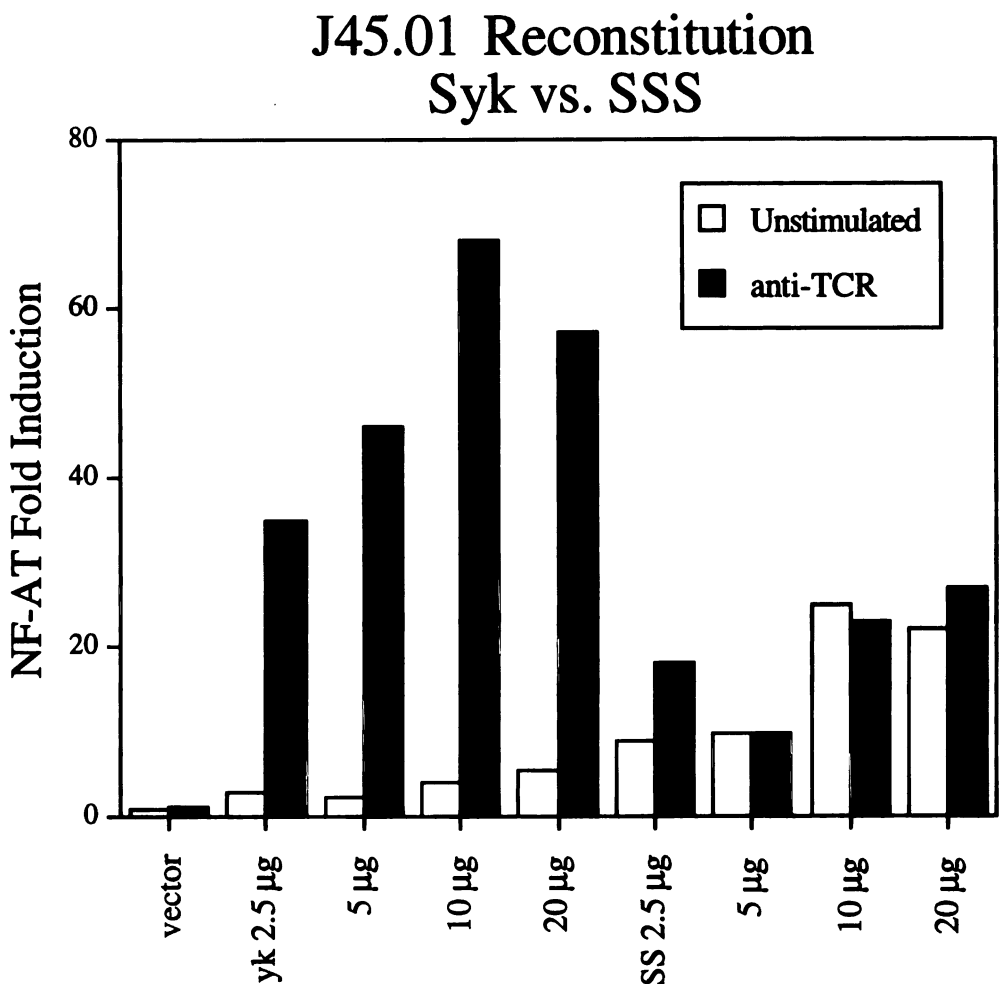
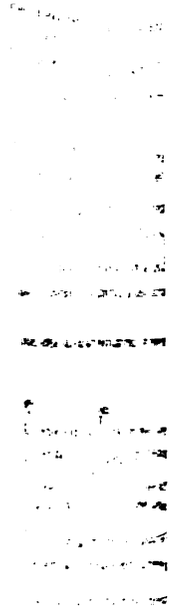


Figure 4. The SSS chimera is constitutively active in CD45-deficient J45.01 Jurkat T cells. J45.01 Jurkat T cells cells were transfected with the indicated amount of plasmid, along with 10 μg of the NF-AT-Luc reporter. Empty vector plasmid was added so that the total amount of DNA transfected was the same for every sample. After 20 hr, cells were left unstimulated (open histogram) or stimulated with an anti-TCR antibody (anti-TCR, shaded histogram) for 6 hr. Luciferase activity was then assessed and expressed as the ratio of the activity from the stimulation of interest to the unstimulated vector control (NF-AT Fold Induction).


J45.01 Reconstitution
Syk vs. SSS
NF-AT Fold Induction
80
60
40
20
0
Unstimulated
anti-TCR
vector
wt Syk 2.5 µg
5 µg
10 µg
20 µg
SSS 2.5 µg
5 µg
10 µg
20 µg



conformational changes that can activate the kinase activity of Syk. The introduction of extra amino acids into ZAP-70, however, did not render the ZZZ construct independent of CD45 or Lck (data not shown).

Because of the lack of inducibility of the first set of constructs, I chose to use a different strategy of creating the chimeras, one in which only endogenous sequences of Syk or ZAP-70 were used, without introduction of additional amino acids (Figure 1). Once again, the kinases were aligned and overlap PCR used to generate the chimeras (S. Hunter and A. Schreiber, submitted). The coding regions of these constructs were verified by sequencing.

Initially, I transiently transfected these constructs into heterologous COS cells to assess the *in vitro* kinase activity of the chimeric kinases. It appears that kinase activity of the chimeras corresponds to the kinase domain, namely that those constructs with the Syk kinase domain are more active than those that contain the ZAP-70 kinase domain (data not shown; also S. Hunter and A. Schreiber, submitted). These results are in agreement with the *in vitro* assays performed by other groups (148). Blotting for protein expression with an antibody against the myc epitope in these constructs confirmed that the constructs were expressed at comparable levels (data not shown).

I next transfected the constructs into Syk-deficient DT-40 cells to test the functional integrity of all of these chimeras. Because both Syk and ZAP-70 are capable of reconstituting these cells (Figure 2) (49, 56), these transfections tested the ability of the chimeras to behave like either Syk or ZAP-70. All of the kinases are able to reconstitute the Syk-deficient DT-40 cells, as measured by NF-AT reporter assays (Figure 5). Of note, those constructs that contained the Syk kinase domain restored greater NF-AT inducibility than those that contained the ZAP-70 kinase domain, consistent with the trend seen between Syk and ZAP-70. In fact, SSZ and ZSZ only restored low levels of BCR-mediated signaling, although the NF-AT response was higher than that of cells transfected with empty vector. However, levels of protein expression in these transfectants were not

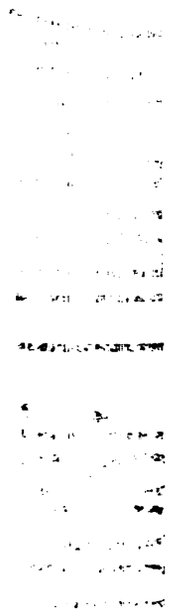
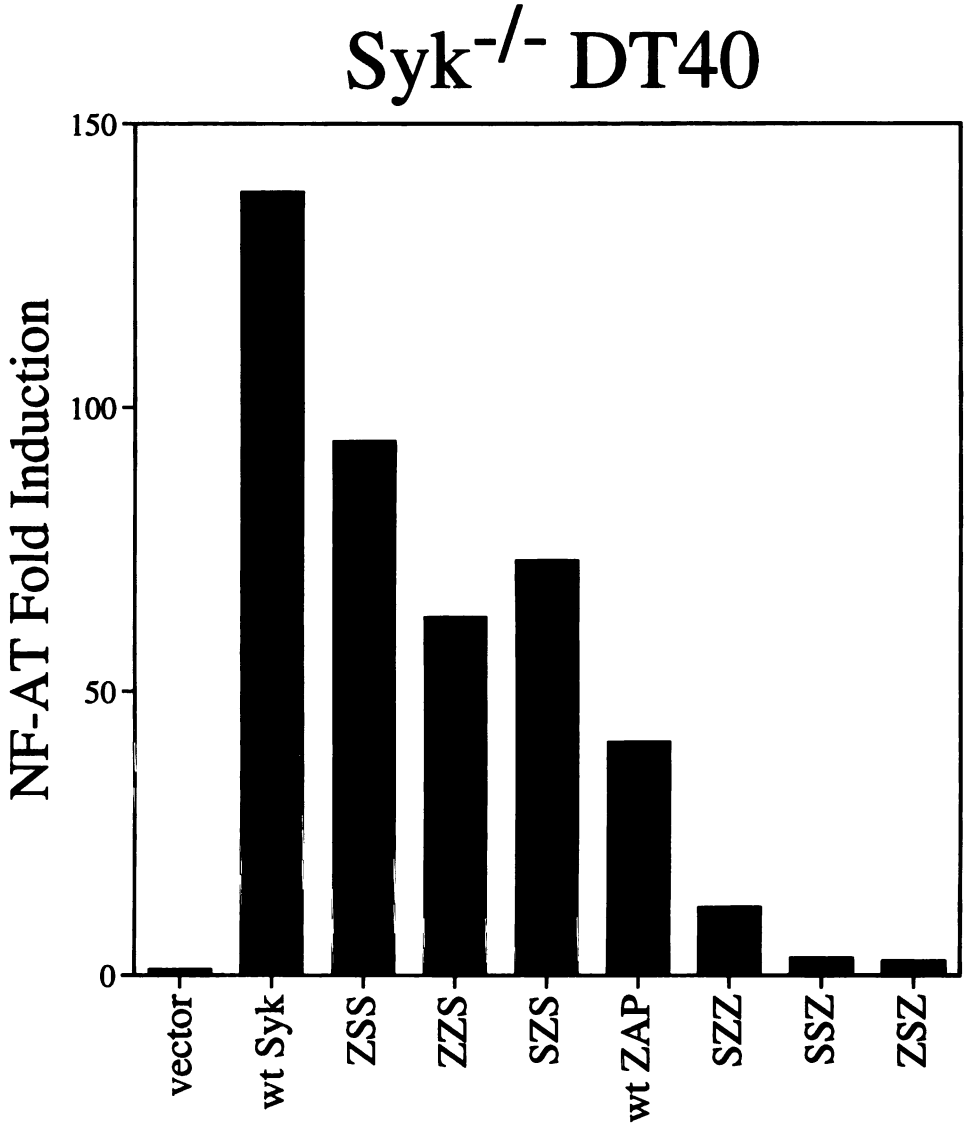
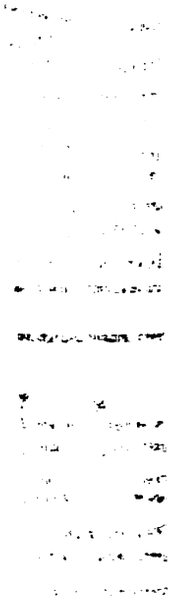


Figure 5. The Syk/ZAP-70 chimeric kinases can all reconstitute Syk-deficient DT40 B cells. Syk-deficient DT40 B cells were transfected with 20 μg of the indicated plasmid, along with 10 μg of the NF-AT-Luc reporter. After 20 hr, cells were left unstimulated or stimulated with an anti-BCR antibody (shaded histogram) for 6 hr. Luciferase activity was then assessed and expressed as the ratio of the activity from the stimulation of interest to the unstimulated vector control (NF-AT Fold Induction).


Syk-/- DT40
NF-AT Fold Induction
150
100
50
0
vector
wt Syk
ZSS
ZZS
SZS
wt ZAP
SZZ
SSZ
ZSZ



assessed, so it is possible that these particular constructs were expressed at lower levels.

To assess the regulation requirements of these chimeric kinases, I next transfected the chimeras into J45.01 and JCaM1.6 along with the NF-AT reporter construct. My results suggest that the ability of the chimeras to behave like Syk, that is, to reconstitute NF-AT signaling in J45.01 and JCaM1.6, is dependent on the presence of the Syk kinase domain (Figure 6 and data not shown). Any of the chimeras with the Syk kinase domain, with the exception of ZSS, were able to reconstitute the Jurkat mutants, suggesting that the Syk kinase domain is the critical region for reconstitution. Again, protein expression was not assessed, but I presume the constructs can be expressed, based on the results from the COS cell experiments.

Based on the results from these chimeras, I conclude that the ability of Syk, but not ZAP-70, to function independently of Lck or CD45, can be accounted for by the differences in the kinase domains of Syk and ZAP-70. These results are consistent with reports that Syk is capable of autophosphorylation, but that ZAP-70 requires Lck to phosphorylate its activation loop tyrosines to activate it.

On the other hand, quantitative differences in kinase activity between activated Syk and ZAP-70 are more difficult to dissect. Syk has been reported to be at least 100-fold as active an enzyme as ZAP-70 (148); however, these assays were performed using GST fusion proteins. Because Syk can autophosphorylate, it can activate itself in the absence of Src family kinases. However, ZAP-70 cannot. Therefore, the in vitro assay is not a fair comparison of the efficiency of fully activated kinases. For such an assessment, one would need to phosphorylate ZAP with Lck and compare that to the level of Syk phosphorylation, and then perform the enzymatic assays. However, even interpretation of these results is complicated. Syk, but not ZAP-70, kinase activity is increased upon binding phosphorylated ITAMs (58, 59). In addition, a portion of the Syk interdomain B and tyrosines within the SH2 domains of Syk, are apparently required for more efficient phospho-ITAM binding (43, 63). ZAP-70 does not have the sequence in interdomain B

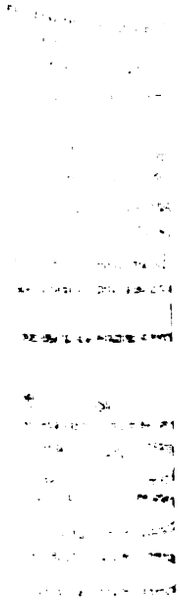
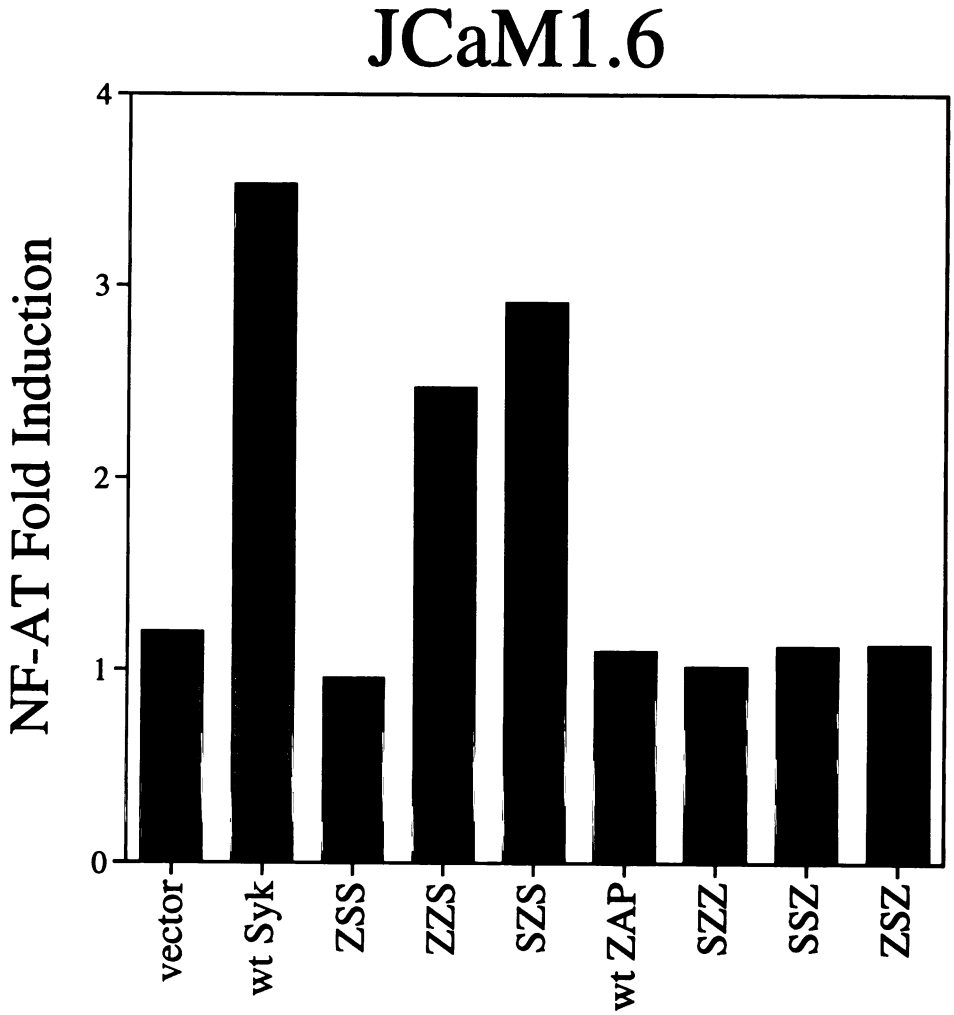
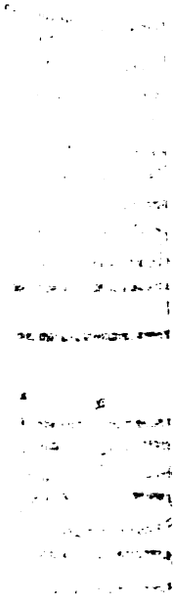


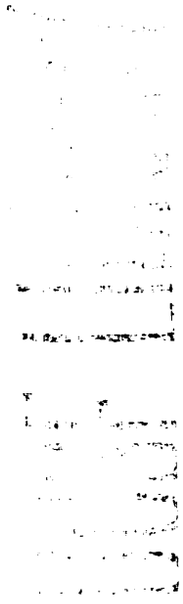
Figure 6. Only chimeras containing the Syk kinase domain are capable of reconstituting TCR-induced NF-AT induction in JCaM1.6 cells. Lck-deficient JCaM1.6 Jurkat T cells were transfected with 20 μg of the indicated plasmid, along with 10 μg of the NF-AT-Luc reporter. After 20 hr, cells were left unstimulated or stimulated with an anti-TCR antibody (shaded histogram) for 6 hr. Luciferase activity was then assessed and expressed as the ratio of the activity from the stimulation of interest to the unstimulated vector control (NF-AT Fold Induction).


JCaM1.6
NF-AT Fold Induction
0
1
2
3
4
vector
wt Syk
ZSS
ZZS
SZS
wt ZAP
SZZ
SSZ
ZSZ



that promotes binding to phospho-ITAMs. The effect of mutation of the tyrosine within theSH2 domains has not been determined. Nevertheless, these factors could influence the ability of Syk to bind to phosphorylated ITAMs and affect *in vivo* kinase activity which would not be detected by performing *in vitro* assays on the isolated kinase.

In conclusion, our results suggest that the kinase domain is primarily responsible for the ability of Syk, but not ZAP-70, to function relatively independently of Src family PTKs. These results are in agreement with the results of other groups (62, 68, 147). Other observations by us and others suggest that the actual regulation of the kinase activity of these proteins is much more intricate than this simplistic view. A more specific understanding of the complex interactions of the various domains within Syk and ZAP-70 awaits the crystallization of the full-length kinase molecules.



Experimental Procedures

Cells and antibodies

J45.01 and JCaM1.6 cells were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. Syk-deficient DT40 cells were maintained in 5% RPMI-1640 medium supplemented with 1% chicken serum (Sigma, St. Louis, MO), penicillin, streptomycin, and glutamine. Murine monoclonal antibodies and their specificities include: C305, Jurkat Ti β-chain; M4, anti-chicken IgM receptor.

Plasmids and constructs

Constructs were created by overlap PCR using the following oligos:

For ZZZ: 5'-atc atc agc cag gcc cc-3' (5' primer, sense strand, starting at nt 633)

5'-ggg agt gtg gga gcg gcc gcc cct gag gcg ttg c-3' (antisense, Not I site)

5'-aac gcc tca ggg gcg gcc gct ccc aca ctc cca gc-3' (sense, Not I site)

5'-atc gcg ctt cag ggc gcc gaa gag ctt ctt gtc ctt g-3' (antisense, Nar I site)

5'-caa gaa gct ctt cgg cgc cct gaa gcg cga taa cct c-3' (sense, Nar I site)

5'-aag gcc atg acc tcc gg-3' (3' primer, antisense strand, starting at nt 1861)

For SSS: 5'-gag tct gat ggc ctg gtc-3' (5' primer, sense strand, starting at nt 430)

5'-acg gcc tcc aaa cgc ggc cgc att aac att tcc ctg tgt gc-3' (antisense, Not I site)

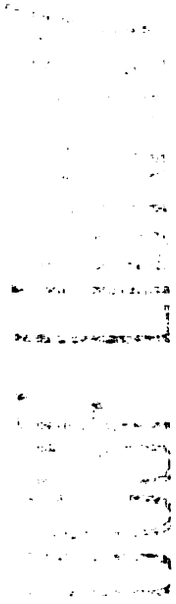
5'-gga aat ggt aat gcg gcc gcg ttt gga ggc cgt cca caa c (sense, Not I site)

5'-gct ttc ggt cca ggg cgc cgt aaa cct cct tgg gcc tg (antisense, Nar I site)

5'-cca agg agg ttt acg gcg ccc tgg acc gaa agc tgc tg (sense, Nar I site)

5'-ttc atc agc acg cag tgc-3' (3' primer, antisense strand, starting at nt 1706)

Syk was engineered to include an extra Not I and an extra Nar I site by adding AlaAlaAla and GlyAla, respectively, creating the SSS construct. The sites of these extra amino acids were chosen to be ten amino acids into interdomain B from either end.

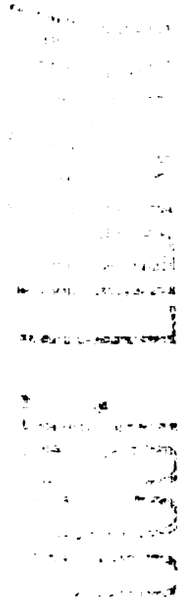


ZAP-70 was also altered to include an extra Nar I and an extra Nar I site. Coincidentally, ZAP-70 already includes a sequence of AlaAlaAla at the desired position, so the coding sequence was altered,without the introduction of new amino acids. The GlyAla spacer was inserted into the part corresponding to the region in Syk, creating the ZZZ construct. All constructs were sequenced to ensure that the coding regions were correct.

The ZSS, ZZS, SZS, SZZ, SSZ, and ZSZ constructs will be described elsewhere (S. Hunter and A. Schreiber, submitted).

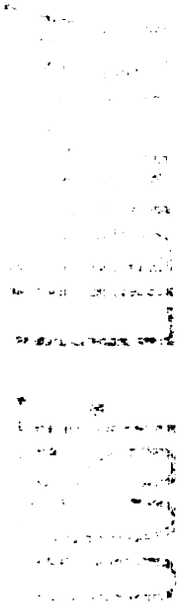
Transfections and luciferase assays

Transfections and luciferase assays were performed as previously described (Chapter 2).



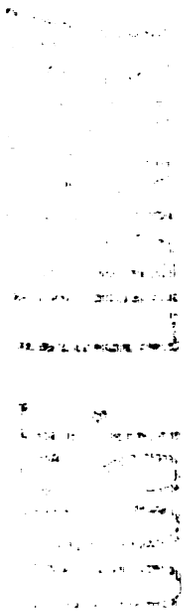
APPENDIX B

GENERATION OF A "KNOCK-IN" MOUSE TO MONITOR SYK EXPRESSION



Summary

In mice, the ability to disrupt genes by homologous recombination has been an invaluable tool for the study of gene function. I chose to use this technology to study the importance of the *syk* gene in T cells. I designed a targeting construct for the expression of a reporter gene under control of the murine *syk* locus. Preliminary studies indicate that although the construct has targeted appropriately, expression of the reporter gene cannot be detected. Successful generation of a mouse with this reporter would allow us to sort for small populations of Syk-expressing cells for the study of their biochemical properties.

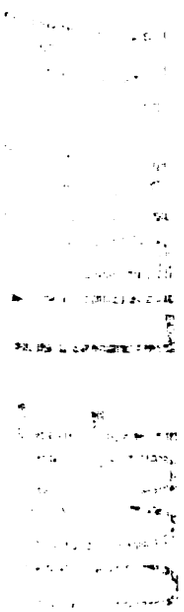


Introduction

Gene targeting by homologous recombination has been a useful tool in many genetic systems, including mice, allowing for the selective disruption of specific genes by introduction of a heterologous segment of DNA into the genomic locus of the gene of interest. Refinements of this technology have included the concomitant expression of a reporter gene or replacement gene in the targeted locus, which remain under the transcriptional control of the endogenous gene. Such "knock-in" studies have made it possible to trace cells that express a particular gene, as well as to express family members or other genes in a specific expression pattern (132, 134, 135, 149, 150).

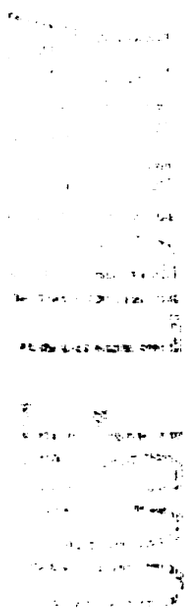
Using intracellular antibody staining, I have previously described Syk expression in a subpopulation of cells of the T cell lineage (see Chapter 3). The Syk-expressing T cells that I have detected are $CD44^{hi}$ and $CD62L^{lo}$, markers characteristic of memory cells. Memory T cells have been demonstrated to be more sensitive to antigen stimulation and to have a decreased requirement for coreceptor and costimulatory function to become activated (108-110, 112). I was therefore interested in isolating these Syk-expressing T cells and comparing their T cell receptor (TCR) signaling characteristics with those of naïve T cells that do not express Syk. However, I am unable to isolate Syk-expressing T cells using the intracellular staining technique for detecting Syk expression. Although this technique is extremely sensitive, it suffers from the disadvantage that cells must be fixed prior to permeabilization and staining, making it impossible to perform functional assays on the stained cells.

To overcome this complication, I chose to construct a "knock-in" mouse using a cell-surface protein, human CD2 (hCD2), as a reporter for Syk expression. The hCD2 gene has been used previously in transgenic mice under control of the CD4 promoter. The primary ligand for hCD2 is human LFA-3; a murine homolog for LFA-3 has not been identified, and hCD2 has not been demonstrated to bind to murine CD48, the ligand for



murine CD2 (151, 152). Therefore, I do not believe that hCD2 will interfere with murine thymocyte or T cell function. Although high-level overexpression of a hCD2 transgene has been demonstrated to affect murine thymic development (153), I believe that expression of our construct will not approach those levels.

Here, I describe the construction of a targeting vector and characterization of a "knock-in" mouse in which a hCD2 reporter has been introduced into the endogenous *syk* locus. Despite targeting of the reporter to the correct genomic locus, expression of the reporter was not detected in peripheral blood B cells.



Results

Construction of the hCD2 targeting construct

The reporter construct was designed such that a reporter gene, human CD2 (hCD2), would be expressed under regulatory control of the endogenous *syk* promoter. To do so, I introduced a hCD2 cDNA cassette followed by a neomycin resistance gene. The *neo* gene was flanked by two loxP sites (floxed) to allow for excision of the *neo* cassette by expression of the Cre recombinase (Figure 1). The excision of the *neo* gene prevents its transcriptional regulatory elements from interfering with endogenous *syk* promoter function. I targeted the first exon of the murine *syk* gene, which contains the ATG start codon. The resulting targeting construct contains 3.6 kB of homologous genomic sequence 5' and 2.0 kB of homologous genomic 3' flanking the reporter (Figure 1).

To verify that the loxP sites were functional and that the *neo* cassette could be excised *in vitro*, I introduced our targeting construct into BNN' bacteria, which express the Cre recombinase, to assess if the neo cassette would be removed in these bacteria (Figure 2). I detected a 1.2 kB decrease in insert size, the size of the *neo* cassette, in the Cre-expressing BNN' strain; I did not detect this decrease in the DH5α strain, which does not express Cre. I therefore concluded that the loxP sites contained in my targeting construct were functional signals for the Cre recombinase.

Generation of hCD2neo founder mice

Next, the targeting construct was electroporated into embryonic stem (ES) cells. These ES cells were selected for G418 resistance and screened for correct recombination. Of 300 clones screened, 13 clones were found to contain the correctly targeted insert by Southern blotting, a targeting efficiency of 4% (data not shown). Of these, three clones were expanded, 1.2C1, 1.2F1, and 3.1F1. Secondary screening of the expanded clones with

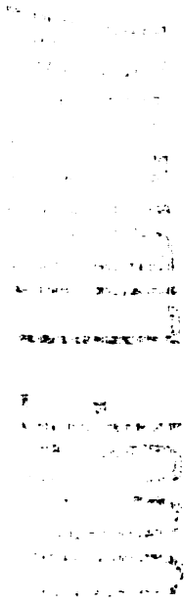
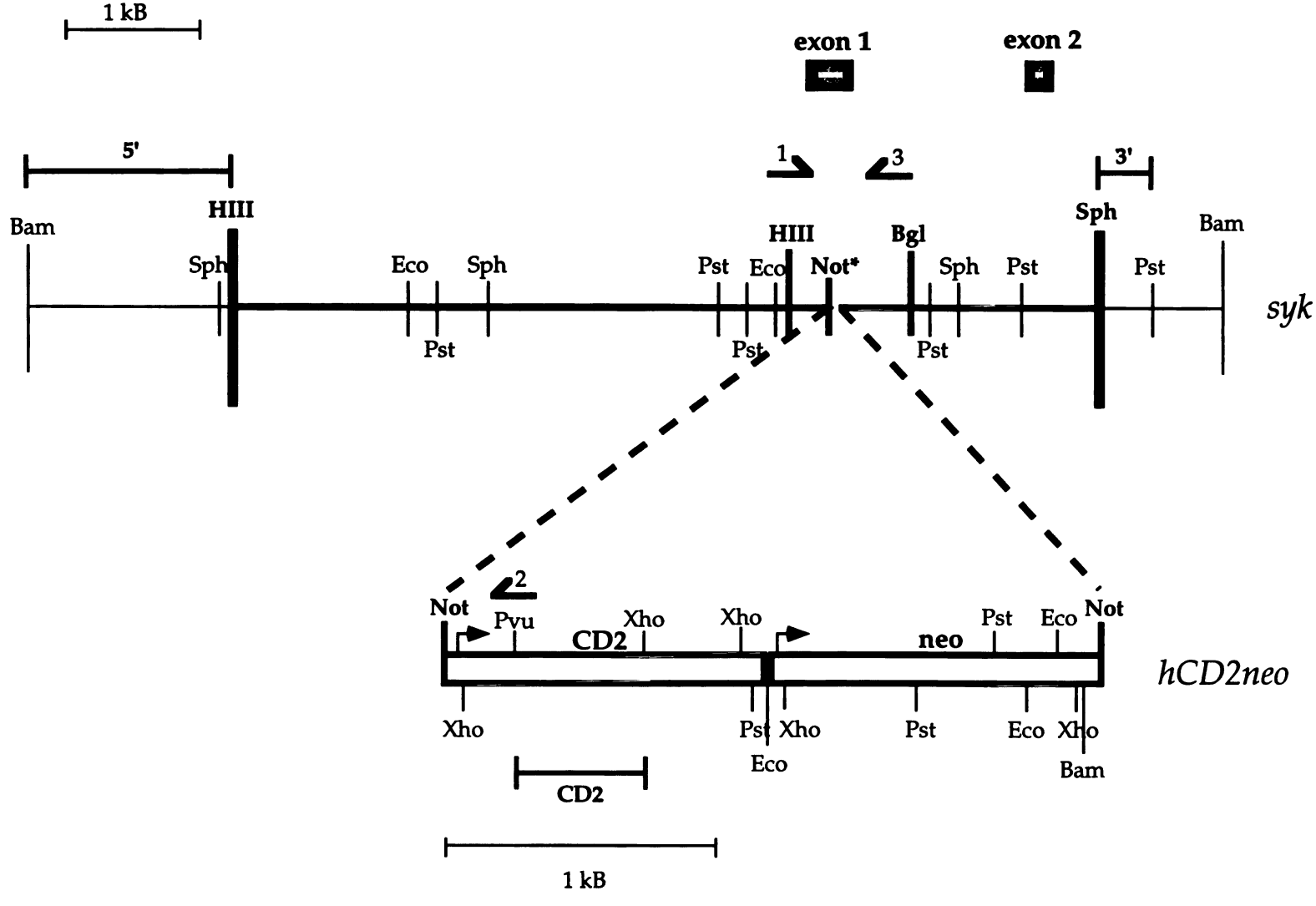


Figure 1. Schematic diagram of the hCD2neo targeting construct. The 8 kB Bam HI fragment of the syk locus is depicted. Exons 1 and 2 are denoted by shaded boxes. The heavy lines indicate the boundaries of the targeting construct, a 5' Hind III site and a 3' Sph I site. Relevant restriction enzyme sites are indicated. The genomic fragments used for Southern blot probes are labeled 5', 3', and CD2. PCR primers are numbered 1, 2, and 3, with arrows indicating their homologous genomic sequence. The Not I* site was created by site-directed mutagenesis for insertion of the hCD2neo cassette. The diamonds flanking the *neo* cassette denote the loxP sites. The scales for the genomic fragment and the hCD2neo cassette are shown. The restriction enzyme abbreviations used are as follows: Bam, Bam HI; Bgl, Bgl II; Eco, Eco RI; HIII, Hind III; Not, Not I; Sph, Sph I; Pst, Pst I; Pvu, Pvu II; Xho, Xho I.


1 kB
exon 1
exon 2
5'
3'
1
3
Bam
HIII
Sph
Eco
Sph
Pst
Eco
Not*
HIII
Bgl
Sph
Pst
Sph
Pst
Bam
Pst
Pst
Pst
syk
2
Not
Pvu
Xho
Xho
CD2
neo
Pst
Eco
Not
hCD2neo
Xho
Pst
Xho
Pst
Eco
Xho
Eco
Bam
CD2
1 kB

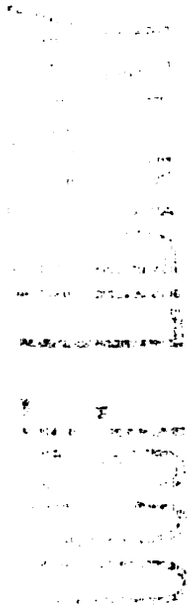
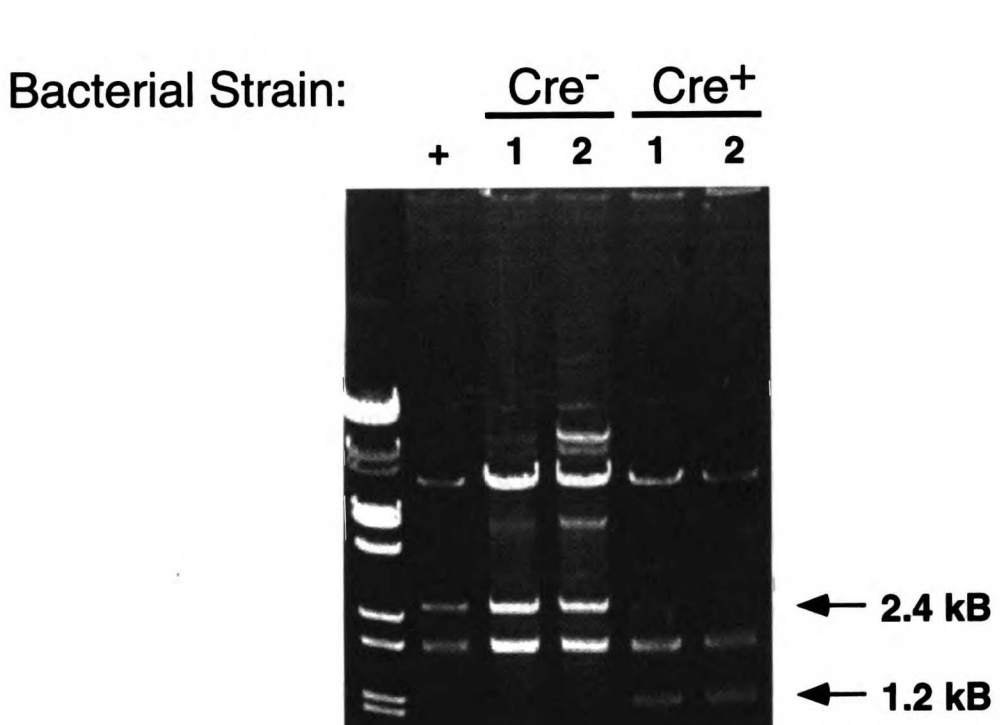


Figure 2. Cre can excise the loxP-flanked *neo* cassette in the hCD2neo targeting construct. DH5α (Cre^-) or BNN' (Cre^+) bacteria were transformed with the Bluescript-hCD2neo targeting construct. Two separate bacterial colonies were cultured for each bacterial strain (1 and 2). Plasmid DNA was isolated from these bacteria and a Not I restriction enzyme digest was performed. DNA fragments were resolved on an ethidium bromide-stained 0.8% agarose gel. The 2.4 kB fragment contains both the hCD2 reporter and the neo cassette. The 1.2 kB fragment represents the hCD2 reporter alone. "+" denotes a digest of the original Bluescript-hCD2neo plasmid that was used for the bacterial transformations.


Bacterial Strain:
Cre-
Cre+
+
1
2
1
2
2.4 kB
1.2 kB
Not I Digest

probes specific for the regions 5', 3', and internal to the targeted region confirmed that correct targeting had occurred in these three ES cell clones (Figure 3)

The three positive clones were microinjected into C57Bl/6 blastocysts and the blastocyts implanted into pseudopregnant females. Male chimeric offspring were assessed by coat color and bred to C57Bl/6 females. F1 offspring were assessed for transmission of ES cell-derived DNA by assessing coat color of the animals. Of the three clones injected, only one clone, 3.1F1, produced founders that transmitted the hCD2neo allele. This transmission was verified by Southern blotting of tail DNA from the offspring of the chimeric founder (data not shown).

Removal of the neo cassette from hCD2neo

I next wished to remove the neomycin resistance gene from the *syk* locus, because the *neo* regulatory elements could potentially interfere with the fidelity of *syk* regulation. To remove the *neo* cassette, I employed two strategies. First, I bred the hCD2neo heterozygous mice to transgenic mice expressing the bacterial Cre recombinase under control of the β-actin promoter (generously provided by Dr. G. Martin), which is expressed ubiquitously. Offspring of this mating were screened by Southern blot for the transmission of the hCD2 reporter and excision of the *neo* cassette (Figure 4).

As an alternative to breeding the mice to Cre mice for removing the neo cassette, I also transiently transfected Cre into 3.1F1 ES cells. These clones were screened for loss of G418 resistance. Excision of the *neo* gene was confirmed by Southern blotting (data not shown). Of the 100 clones screened for loss of G418 resistance, 10 clones were found to contain the hCD2 reporter without the *neo* cassette (data not shown).

The hCD2 reporter does not express in mice

To assess the expression of the hCD2 reporter in Syk-expressing cells, I decided to evaluate the expression of the reporter in B cells from peripheral blood. Mice of different

Figure 3. Screening ES cell clones for targeted integration of the hCD2neo reporter construct. Genomic DNA was harvested from ES cell clones and digested with the indicated enzymes. The digests were resolved on 0.8% agarose gels and transferred to nylon membranes. The probes used for Sourthern blotting are diagrammed in Figure 1. For the assessment of 5' integration, the targeted allele gives the 5.5 kB band, whereas the endogenous *syk* allele gives an 8.0 kB band. The band of intermediate size is a result of partial digestion of the DNA. For the assessment of 3' integration, the targeted allele gives a 2.9 kB band. The 8.0 kB band visualized with the CD2 probe is diagnostic for the presence of the hCD2 reporter. The clones analyzed were as follows: "1," 1.2C1; "2," 1.2F2; "3," 3.1F1; "-" JM1 wild-type ES cells.

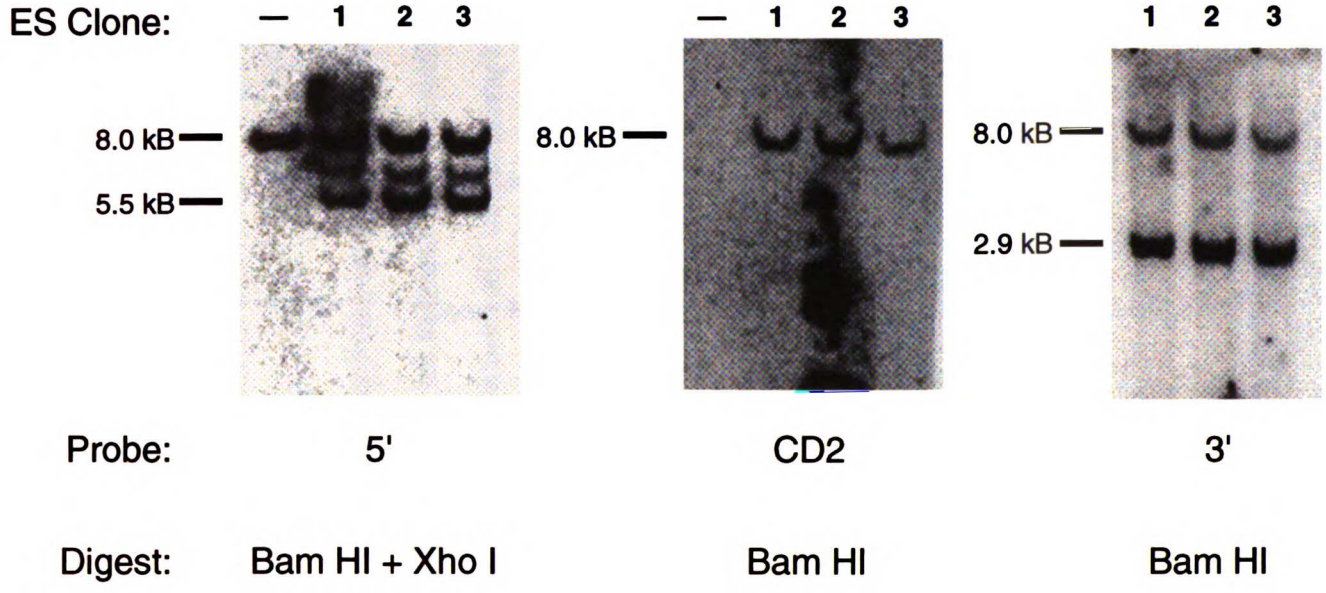

ES Clone:
—
1
2
3
8.0 kB
5.5 kB
8.0 kB
8.0 kB
2.9 kB
Probe:
5'
CD2
3'
Digest:
Bam HI + Xho I
Bam HI
Bam HI

Figure 4. Southern blot analysis of the genotypes of hCD2neo reporter mice. Genomic DNA was obtained from the tails of mice of the indicated genotype after Proteinase K digestion and ethanol precipitation. The resulting DNA was digested with the indicated restriction enzymes, resolved on 0.8% agarose gels, and transferred to nylon membranes. The probes used for Southern blotting are diagrammed in Figure 1. The diagnostic bands are indicated by arrows. The lanes are labeled with the genotypes of the mice, where "+" denotes a wild-type allele of the *syk* gene.

5' Integration
Digest:
Bam HI + Xho I
Probe:
5'
wt
CD2neo
SykCD2neo; +
SykCD2; +
SykCD2; SykCD2
3' Integration
Bam HI
3'
wt
CD2neo
SykCD2neo; +
SykCD2; +
SykCD2; SykCD2
CD2 Integration
Bam HI
CD2
CD2neo
CD2
SykCD2neo; +
SykCD2; +
SykCD2; SykCD2
CD2neo vs. CD2
Bam HI + Hind III
CD2
CD2neo
CD2
SykCD2neo; +
SykCD2; +
SykCD2; SykCD2

genotypes, $syk^{+/+}$, $syk^{+/hCD2neo}$, and $syk^{+/hCD2}$ were bled from the tail vein. These peripheral blood lymphocytes were stained for B220 and hCD2 cell-surface expression. Co-staining of hCD2 was not observed in $B220^{+}$ B cells, although anti-CD2 staining of the Jurkat human T cell line was clearly positive (Figure 5). This result indicates that the hCD2 reporter is not being expressed in peripheral B cells, a population of lymphocytes that expresses high levels of Syk.

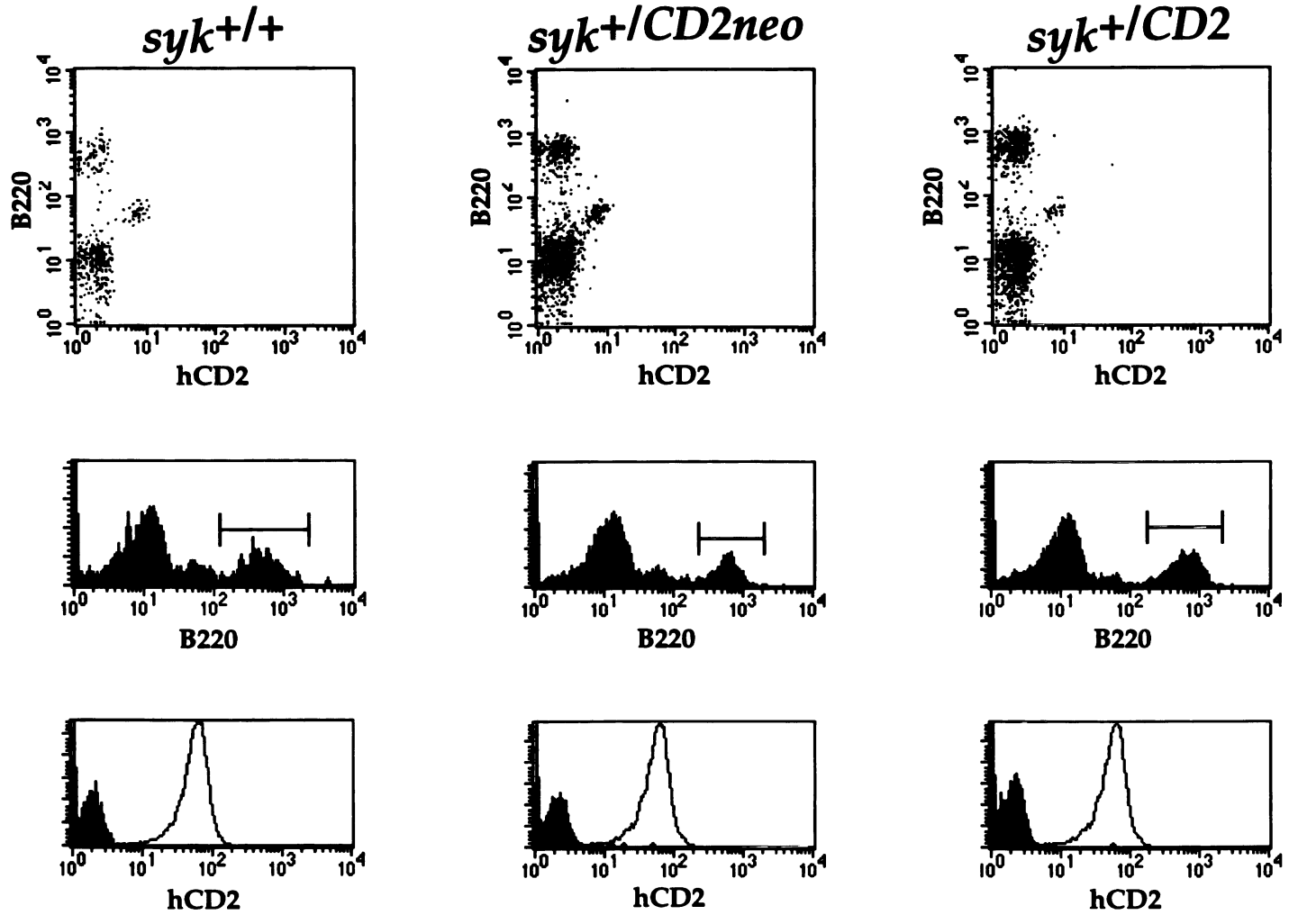
syk may be disrupted in hCD2neo, but not hCD2, mice

Because I did not detect any expression of the hCD2 reporter, I next wanted to assess if the allele disrupted endogenous expression of *syk*. Disruption of the endogenous *syk* locus would provide further confirmation that the reporter was targeted correctly. I predicted that, if the mice were functional knockouts of *syk*, I would observe the perinatal lethality that has previously been described for *syk* knockout mice (72, 73). I therefore set up matings of heterozygous $syk^{+/hCD2neo}$ x $syk^{+/hCD2neo}$ and $syk^{+/hCD2}$ x $syk^{+/hCD2}$ mice and analyzed the segregation of the different *syk* alleles.

F1 offspring of these matings were genotyped by PCR and confirmed by Southern blotting (Figure 4 and data not shown). For Southern blotting, the following parameters were assessed: 1) 5' integration; 2) 3' integration; 3) presence of the hCD2 reporter; 4) presence of the neo cassette. I did not use a probe for the actual *neo* cassette but instead probed for a 1.2 kB decrease in the size of the genomic fragment containing the hCD2 reporter. A summary of the genotyping results is provided in Table 1.

For the $syk^{+/hCD2neo}$ heterozygous cross, I obtained 32% wild-type and 68% $syk^{+/CD2neo}$ mice, but no mice homozygous for the reporter allele. These percentages would be expected if the reporter allele were homozygous lethal, consistent with idea that endogenous *syk* expression is being disrupted due to the introduction of the reporter construct. I therefore conclude that the targeting construct is disrupting the endogenous *syk* gene and that the lack of reporter expression is not due to inappropriate targeting.

Figure 5. The hCD2 reporter mice do not express hCD2 in Syk-expressing cells. B cells were obtained from peripheral blood via tail vein bleed. Red blood cells were lysed and the remaining cells were stained with anti-hCD2 FITC and anti-B220 PE antibodies. The stained cells were then analyzed on a FACScan. The dot plots depict the expression of B220 and hCD2 in peripheral blood lymphocytes. $B220^+$ cells were gated and assessed for hCD2 expression (shaded histogram). hCD2 expression of the human Jurkat T cell line is also depicted (open histogram).


syk+/+
syk+/CD2neo
syk+/CD2
B220
hCD2
B220
hCD2
B220
hCD2
B220
hCD2
B220
hCD2

Somewhat surprisingly, the $syk^{+/CD2}$ x $syk^{+/CD2}$ cross yielded two $syk^{CD2/CD2}$ mice, or 11% of the offspring analyzed (Table 1). The results of these crosses yielded percentages of offspring approaching those expected of typical Mendelian inheritance, suggesting that the introduction of the hCD2 reporter alone may not completely disrupt endogenous *syk* function. Because the larger insertion, including the *neo* cassette, apparently disrupts expression of the *syk* gene, whereas the hCD2 cDNA alone does not appear to do so completely, it may be that a cryptic splice site is present in the hCD2 cDNA that allows for a chimeric *hCD2/syk* message and protein to be expressed. This fusion protein may be able to function at least in part to compensate for the loss of wild-type Syk protein. Indeed, cryptic splice sites have been reported in other transgenic mice (154). RT-PCR sequencing of transcripts from the syk^{CD2} locus would help to clarify these issues.

Table 1. Genotypes of F1 offspring from syk^{CD2neo} or syk^{CD2} heterozygote crosses. Mice of the indicated genotypes were crossed and their progeny genotyped by PCR and Southern blot. The numbers of animals of the indicated genotypes are shown, where "+" refers to the wild-type *syk* allele.

Table 1. Genotypes of F1 offspring from syk^{CD2neo} or syk^{CD2} heterozygote crosses

	Number of animals		
Mating	+/+	+/CD2neo or +/CD2	CD2neo/CD2neo or CD2/CD2
$syk^{+/CD2neo}$ x $syk^{+/CD2neo}$	7	15	0
$syk^{+/CD2}$ x $syk^{+/CD2}$	5	11	2

Discussion

I have attempted to create a "knock-in" mouse expressing a human CD2 reporter under transcriptional control of the endogenous *syk* locus. This mouse was designed to allow me to isolate live Syk-expressing cells by sorting using the reporter protein. I have demonstrated that the targeting construct I created has targeted to the correct genomic locus, but that the reporter is not expressed in B cells from peripheral blood, cells that would be expected to express high levels of Syk.

Potential explanations for the lack of expression of the CD2 reporter include incorrect targeting of the reporter cassette, lack of expression of reporter message, mutation in the reporter sequence, and failure of CD2 to be expressed at the cell surface. I believe that the construct is targeted correctly, as Southern blot analysis of genomic DNA from the reporter mice have been performed using both flanking and internal probes. Furthermore, mice homozygous for the reporter allele do not mature, consistent with the notion that the targeted construct has disrupted the endogenous *syk* locus.

It remains to be determined if hCD2 message is being expressed in mice containing the reporter allele, which can be assessed by Northern blot or RT-PCR analysis of hCD2 transcripts. At the same time, it would be informative to examine the expression of endogenous *syk* message, to determine if these "knock-in" mice are functional *syk* knockouts. If no message is expressed, it is possible, although unlikely, that a mutation in the *syk* promoter has occurred in the genomic sequence used for the targeting construct. Sequencing of the genomic DNA of the reporter mice would address that possibility. Alternatively, it may be that introduction of the hCD2 cDNA is interfering with proper transcriptional regulation of the genomic locus. It is also conceivable that the hybrid *hCD2/syk* gene is not transcribed as efficiently as the endogenous *syk* gene. Introduction of a splice site and/or a polyadenylation sequence into the hCD2 reporter may enhance the

abundance of the hCD2 message. The hCD2 reporter used in this study was a cDNA which relies on the endogenous *syk* polyadenylation sequence.

If hCD2 transcripts are detected, it would be more likely that the hCD2 reporter carries a spontaneous mutation that does not allow for the detection of CD2 protein. It is possible that mutations in the coding sequence of the hCD2 reporter encode truncated proteins or missense mutations that do not allow for the anti-CD2 antibody used to detect the protein. Sequencing of transripts by RT-PCR would clarify that issue. Alternatively, one could use different antibodies against hCD2, or one could try to detect the protein by immunoblotting.

Another potential explanation for the apparent lack of hCD2 expression may in fact be the sensitivity of the anti-CD2 detection reagent. This explanation does not seem likely, since the number of molecules of Syk expressed in B cells, which we assume would be similar to the number of hCD2 molecules expressed, is above the limits of detection by FACS staining (57). Nevertheless, the sensitivity of hCD2 detection could be increased by using a secondary detection reagent to amplify the anti-hCD2 staining instead of using the directly conjugated reagent used in this analysis. Furthermore, the lack of hCD2 expression should also be assayed in B cells from other tissues, such as lymph nodes or spleen.

Despite the fact that this particular reporter for Syk expression does not appear to be functional, it is still of great interest to generate a Syk reporter mouse. Because I have not yet found other markers that can uniquely define the Syk^{hi} T cell population, a comparable reporter mouse will be invaluable in allowing us to isolate and define the cellular and biochemical properties of this very intriguing subpopulation of T cells.

Experimental Procedures

Generation of targeting construct

Genomic clones of an 8 kB Bam HI fragment of the *syk* locus was generously provided by C. Schmedt and A. Tarakhovsky. Fragments were subcloned into the Bluescript vector (Stratagene, La Jolla, CA). The construct was made in a Bluescript vector with a modified multiple cloning site, composed of the following restriction enzyme sites: Bam HI, Sph I, Bgl II, Hind III. Constructs were confirmed by minprep digests and sequencing using the SP6 and T7 sequences present in the vector backbone.

A Hind III to Bgl II fragment containing the first exon of *syk* was subcloned into the pSP73 vector (Promega, Madison, WI). The ATG initiation codon of the *syk* gene was mutated to a Not I site using the MORPH mutagenesis system (5 Prime -> 3 Prime, Inc., Boulder, CO) using an oligonucleotide of the following sequence:

5'-ctgaaggggtgcagacgcggccgcaagtgctgtggacagcg-3'.

The hCD2neo cassette was subcloned into this new construct as a Not I fragment.

Following completion of this step, I constructed the entire targeting construct from the 3' end. I used a 1.4 kB Bgl II to Sph I fragment for the 3' section of the targeting construct. I then added the Hind III to Bgl II fragment containing the mutated ATG and hCD2neo cassette. Finally, I ligated a 3.6 kB Hind III genomic fragment as the 5' piece of the targeting construct. The resulting targeting construct contains 3.6 kB of homologous 5' flanking DNA and 2.0 kB of homologous 3' flanking DNA surrounding the hCD2neo insert.

Electroporation of ES cells

JM1 embryonic stem cells (ES cells, generously provided by Dr. N. Killeen) at a concentration of 2 x 10^7 cells/0.8 ml phosphate buffered saline (PBS) were electroporated with 20 μg of DNA containing the targeting construct at 250 V, 500 μF in 0.4 cm

electroporation cuvettes using a GenePulser (BioRad, Hercules, CA). Cells were plated in varying dilutions on 10 cm plates containing monlayers of irradiated mouse embyronic fibroblast feeder cells. 1 day after transfection, ES cells were cultured in the presence of 250 μg/ml G418 in DME-H21 (Gibco, Gaithersburg, MD) supplemented with 10% characterized fetal calf serum (HyClone, Logan, UT), non-essential amino acids, 2-mercaptoethanol, vitamins, penicillin, streptomycin, and glutamine (Gibco). Media was changed daily. After 14 days, visible single colonies were picked, transferred to flat-bottom 96-well plates, and trypsinized. Clones were fed daily until confluent. Cells were then trypsinized and frozen, as well as grown up in the absence of selection until cells once again reached confluence. Cells were then digested with Proteinase K at 60°C overnight. Genomic DNA was then generated from these clones and ethanol precipitated.

Of the 300 clones tested, 13 clones were positive upon screening for correct integration, as assessed by Southern blotting for the 3' region. Three of these clones, 1.2C1, 1.2F2, and 3.1F1, were expanded and rescreened to confirm the correct homologous recombination using 5' and 3' genomic probes, and a probe from the hCD2 cDNA, to verify that the hCD2neo cassette had integrated appropriately.

Southern blotting

ES cells were digested overnight in ES cell lysis buffer (10 mM Tris, pH 7.4, 10 mM EDTA, 10 mM NaCl, 0.5% SDS + 1 mg/ml Proteinase K) and tails were digested overnight in tail extraction buffer (0.05 mM Tris, pH 8.0, 3 mM EDTA, 0.1 M NaCl, 0.5% SDS + 1 mg/ml Proteinase K) at 60°C. DNA was then ethanol precipitated and resuspended for digestion in TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH8.0). Genomic digests were performed on ES cell DNA or tail DNA at 37°C in the presence of RNAse, BSA, and spermidine, and the digests were resolved on 0.8% agarose/TAE gels. Gels were depurinated, alkali denatured, and neutralized as described, and then transferred to

MagnaCharge nylon membranes overnight (MSI, Westboro, MA). Following UV crosslinking, [^{32}P]-labeled probes (RediPrime, Amersham, Arlington Heights, IL) were incubated with the blots. Results were visualized by autoradiography (BioMax MS film, Kodak, Rochester, NY).

Cell staining

Peripheral blood was obtained from tail vein bleeding into heparinized PBS. Red blood cells were lysed, and the cells were washed. Antibodies to cell surface receptors were added and the samples incubated on ice 10'. Cells were then washed and analyzed on a FACScan (Becton Dickinson, San Jose, CA).

PCR genotyping of mice

The following oligonucleotide primers were used:

SykCD2-1 (5'-tacacttcccagaactctg-3') is the 5' primer including sequence from exon 1;

SykCD2-2 (5'-cccattttatatcgtcaatatc-3') is a 3' primer from sequence within hCD2;

SykCD2-3 is a 3' primer that corresponds to sequence 3' of the CD2neo insertion site.

When these three primers are used in combination for screening, the wild-type *syk* allele produces a 100 bp band, while the targeted allele produces a 210 bp band.

BIBLIOGRAPHY

1. Weiss, A., and Littman, D. R. (1994). Signal transduction by lymphocyte antigen receptors. Cell *76*, 263-274.

2. van Oers, N. S. C., von Boehmer, H., and Weiss, A. (1995). The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-ζ subunit. J. Exp. Med. *182*, 1585-1590.

3. Malissen, B., and Malissen, M. (1996). Functions of TCR and pre-TCR subunits: lessons from gene ablation. Curr. Opin. Immunol. *8*, 383-393.

4. Irving, B. A., Alt, F. W., and Killeen, N. (1998). Thymocyte development in the absence of pre-T cell receptor extracellular immunoglobulin domains. Science *280*, 905-908.

5. Thome, M., Duplay, P., Guttinger, M., and Acuto, O. (1995). Syk and ZAP-70 mediate recruitment of $p56^{lck}$/CD4 to the activated T cell receptor/CD3/ζ complex. J Exp Med *181*, 1997-2006.

6. Duplay, P., Thome, M., Herve, F., and Acuto, O. (1994). $p56^{lck}$ interacts via its src homology 2 domain with the ZAP-70 kinase. J Exp Med *179*, 1163-72.

7. Wardenburg, J. B., Fu, C., Jackman, J. K., Flotow, H., Wilkinson, S. E., Williams, D. H., Johnson, R., Kong, G., Chan, A. C., and Findell, P. R. (1996). Phosphorylation of SLP-76 by the ZAP-70 protein-tyrosine kinase is required for T-cell receptor function. J Biol Chem *271*, 19641-4.

8. Wu, J., Zhao, Q., Kurosaki, T., and Weiss, A. (1997). The Vav binding site (Y315) in ZAP-70 is critical for antigen receptor-mediated signal transduction. J Exp Med *185*, 1877-82.

9. Deckert, M., Tartare-Deckert, S., Couture, C., Mustelin, T., and Altman, A. (1996). Functional and physical interactions of Syk family kinases with the Vav proto-oncogene product. Immunity *5*, 591-604.

10. Deckert, M., Elly, C., Altman, A., and Liu, Y. C. (1998). Coordinated regulation of the tyrosine phosphorylation of Cbl by Fyn and Syk tyrosine kinases. J Biol Chem *273*, 8867-74.

11. Law, C. L., Chandran, K. A., Sidorenko, S. P., and Clark, E. A. (1996). Phospholipase Cγ1 interacts with conserved phosphotyrosyl residues in the linker region of Syk and is a substrate for Syk. Mol Cell Biol *16*, 1305-15.

12. Zhang, W., Sloan-Lancaster, J., Kitchen, J., Trible, R. P., and Samelson, L. E. (1998). LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. Cell *92*, 83-92.

13. Chan, A. C., Iwashima, M., Turck, C. W., and Weiss, A. (1992). ZAP-70: A 70kD protein tyrosine kinase that associates with the TCR ζ chain. Cell *71*, 649-662.

14. Law, C.-L., Sidorenko, S. P., Chandran, K. A., Draves, K. E., Chan, A. C., Weiss, A., Edelhoff, S., Disteche, C. M., and Clark, E. A. (1994). Molecular cloning of human Syk: A B cell protein tyrosine kinase associated with the surface immunoglobulin M/B cell receptor complex. J. Biol. Chem. *269*, 12310-12319.

15. Cooper, J. A., and Howell, B. (1993). The when and how of Src regulation. Cell *73*, 1051-1054.

16. Sieh, M., Bolen, J. B., and Weiss, A. (1993). CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck. EMBO J. *12*, 315-322.

17. Chow, L. M. L., Fournel, M., Davidson, D., and Veillette, A. (1993). Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50^{csk}. Nature *365*, 156-160.

18. Mustelin, T., Coggeshall, K. M., and Altman, A. (1989). Rapid activation of the T-cell tyrosine protein kinase pp56lck by the CD45 phosphotyrosine phosphatase. Proc. Natl. Acad. Sci. USA *86*, 6302-6306.

19. Mustelin, T., Pessa-Morikawa, T., Autero, M., Gassmann, M., Andersson, L. C., Gahmberg, C. G., and Burn, P. (1992). Regulation of the p59*fyn* protein tyrosine kinase by the CD45 phosphotyrosine phosphatase. Eur. J. Immunol. *22*, 1173-1178.

20. Ostergaard, H. L., Shackelford, D. A., Hurley, T. R., Johnson, P., Hyman, R., Sefton, B. M., and Trowbridge, I. S. (1989). Expression of CD45 alters phosphorylation of the lck--encoded tyrosine protein kinase in murine lymphoma T-cell lines. Proc. Natl. Acad. Sci. USA *86*, 8959-8963.

21. Hurley, T. R., Hyman, R., and Sefton, B. M. (1993). Differential effects of expression of the CD45 tyrosine protein phosphatase on the tyrosine phosphorylation of the *lck, fyn,* and c-*src* tyrosine protein kinases. Mol. Cell. Biol. *13*, 1651-1656.

22. McFarland, E. D. C., Hurley, T. R., Pingel, J. T., Sefton, B. M., Shaw, A., and Thomas, M. L. (1993). Correlation between Src family member regulation by the protein-tyrosine-phosphatase CD45 and transmembrane signaling through the T-cell receptor. Proc. Natl. Acad. Sci. USA *90*, 1402-1406.

23. Xu, W., Harrison, S. C., and Eck, M. J. (1997). Three-dimensional structure of the tyrosine kinase c-Src. Nature *385*, 595-602.

24. Sicheri, F., Moarefi, I., and Kuriyan, J. (1997). Crystal structure of the Src family tyrosine kinase Hck. Nature *385*, 602-609.

25. Moarefi, I., LaFevre-Bernt, M., Sicheri, F., Huse, M., Lee, C.-H., Kuriyan, J., and Miller, W. T. (1997). Activation of the Src-family tyrosine kinase Hck by SH3 domain displacement. Nature *385*, 650-653.

26. Pingel, J. T., and Thomas, M. L. (1989). Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. Cell *58*, 1055-1065.

27. Koretzky, G.A., Picus, J., Thomas, M. L., and Weiss, A. (1990). Tyrosine phosphatase CD45 is essential for coupling T cell antigen receptor to the phosphatidylinositol pathway. Nature *346*, 66-68.

28. Koretzky, G., Picus, J., Schultz, T., and Weiss, A. (1991). Tyrosine phosphatase CD45 is required for both T cell antigen receptor and CD2 mediated activation of a protein tyrosine kinase and interleukin 2 production. Proc. Natl. Acad. Sci. USA *88*, 2037-2041.

29. Shiroo, M., Goff, L., Biffen, M., Shivnan, E., and Alexander, D. (1992). CD45 tyrosine phosphatase-activated $p59^{fyn}$ couples the T cell antigen receptor to pathways of diacylglycerol production, protein kinase C activation and calcium influx. EMBO J. *11*, 4887-4897.

30. Volarevic, S., Niklinska, B. B., Burns, C. M., Yamada, H., June, C. H., Dumont, F. J., and Ashwell, J. D. (1992). The CD45 tyrosine phosphatase regulates phosphotyrosine homeostasis and its loss reveals a novel pattern of late T cell receptor-induced Ca^{2+} oscillations. J. Exp. Med. *176*, 835-844.

31. Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J., and Mak, T. W. (1993). Normal B lymphocyte development but impaired T cell maturation in CD45-Exon6 protein tyrosine phosphatase-deficient mice. Cell *74*, 143-156.

32. Byth, K. F., Conroy, L. A., Howlett, S., Smith, A. J., May, J., Alexander, D. R., and Holmes, N. (1996). CD45-null transgenic mice reveal a positive regulatory role for CD45 in early thymocyte development, in the selection of $CD4^+CD8^+$ thymocytes, and B cell maturation. J. Exp. Med. *183*, 1707-1718.

33. Samelson, L. E., Davidson, W. F., Morese, H. C., III, and Klausner, R. D. (1986). Abnormal tyrosine phosphorylation on T-cell receptor in lymphoproliferative disorders. Nature *324*, 674-676.

34. van Oers, N. S. C., Tao, W., Watts, J. D., Johnson, P., Aebersold, R., and Teh, H.-S. (1993). Constitutive tyrosine phosphorylation of the T cell receptor (TCR) ζ subunit: Regulation of TCR-associated protein kinase activity by TCR ζ. Mol. Cell. Bio. *13*, 5771-5780.

35. van Oers, N. S., Killeen, N., and Weiss, A. (1994). ZAP-70 is constitutively associated with tyrosine-phosphorylated TCR ζ in murine thymocytes and lymph node T cells. Immunity *1*, 675-85.

36. van Oers, N. S., Killeen, N., and Weiss, A. (1996). Lck regulates the tyrosine phosphorylation of the T cell receptor subunits and ZAP-70 in murine thymocytes. J Exp Med *183*, 1053-62.

37. Madrenas, J., Wange, R. L., Wang, J. L., Isakov, N., Samelson, L. E., and Germain, R. N. (1995). ζ phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. Science *267*, 515-8.

38. Iwashima, M., Irving, B. A., van Oers, N. S., Chan, A. C., and Weiss, A. (1994). Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. Science *263*, 1136-9.

39. Hatada, M. H., Lu, X., Laird, E. R., Green, J., Morgenstern, J. P., Lou, M., Marr, C. S., Phillips, T. B., Ram, M. K., Theriault, K., and et al. (1995). Molecular basis for interaction of the protein tyrosine kinase ZAP-70 with the T-cell receptor. Nature *377*, 32-8.

40. Kurosaki, T., Johnson, S. A., Pao, L., Sada, K., Yamamura, H., and Cambier, J. C. (1995). Role of the Syk autophosphorylation site and SH2 domains in B cell antigen receptor signaling. J. Exp. Med. *182*, 1815-1823.

41. Ottinger, E. A., Botfield, M. C., and Shoelson, S. E. (1998). Tandem SH2 domains confer high specificity in tyrosine kinase signaling. J Biol Chem *273*, 729-35.

42. Grazioli, L., Germain, V., Weiss, A., and Acuto, O. (1998). Anti-peptide antibodies detect conformational changes of the inter-SH2 domain of ZAP-70 due to binding to the ζ chain and to intramolecular interactions. J Biol Chem *273*, 8916-21.

43. Keshvara, L. M., Isaacson, C., Harrison, M. L., and Geahlen, R. L. (1997). Syk activation and dissociation from the B-cell antigen receptor is mediated by phosphorylation of tyrosine 130. J Biol Chem *272*, 10377-81.

44. Furlong, M. T., Mahrenholz, A. M., Kim, K. H., Ashendel, C. L., Harrison, M. L., and Geahlen, R. L. (1997). Identification of the major sites of autophosphorylation of the murine protein-tyrosine kinase Syk. Biochim Biophys Acta *1355*, 177-90.

45. Vely, F., Nunes, J. A., Malissen, B., and Hedgecock, C. J. (1997). Analysis of immunoreceptor tyrosine-based activation motif (ITAM) binding to ZAP-70 by surface plasmon resonance. Eur J Immunol *27*, 3010-4.

46. Bu, J. Y., Shaw, A. S., and Chan, A. C. (1995). Analysis of the interaction of ZAP-70 and syk protein-tyrosine kinases with the T-cell antigen receptor by plasmon resonance. Proc Natl Acad Sci U S A *92*, 5106-10.

47. Watts, J. D., Affolter, M., Krebs, D. L., Wange, R. L., Samelson, L. E., and Aebersold, R. (1994). Identification by electrospray ionization mass spectrometry of the sites of tyrosine phosphorylation induced in activated Jurkat T cells on the protein tyrosine kinase ZAP-70. J Biol Chem *269*, 29520-9.

48. Kong, G., Dalton, M., Wardenburg, J. B., Straus, D., Kurosaki, T., and Chan, A. C. (1996). Distinct tyrosine phosphorylation sites in ZAP-70 mediate activation and negative regulation of antigen receptor function. Mol Cell Biol *16*, 5026-35.

49. Zhao, Q., and Weiss, A. (1996). Enhancement of lymphocyte responsiveness by a gain-of-function mutation of ZAP-70. Mol Cell Biol *16*, 6765-74.

50. Lupher, M. L., Jr., Reedquist, K. A., Miyake, S., Langdon, W. Y., and Band, H. (1996). A novel phosphotyrosine-binding domain in the N-terminal transforming region of Cbl interacts directly and selectively with ZAP-70 in T cells. J Biol Chem *271*, 24063-8.

51. Lupher, M. L., Jr., Songyang, Z., Shoelson, S. E., Cantley, L. C., and Band, H. (1997). The Cbl phosphotyrosine-binding domain selects a D(N/D)XpY motif and binds to the Tyr292 negative regulatory phosphorylation site of ZAP-70. J Biol Chem *272*, 33140-4.

52. Ota, Y., Beitz, L. O., Scharenberg, A. M., Donovan, J. A., Kinet, J. P., and Samelson, L. E. (1996). Characterization of Cbl tyrosine phosphorylation and a Cbl-Syk complex in RBL-2H3 cells. J Exp Med *184*, 1713-23.

53. Ota, Y., and Samelson, L. E. (1997). The product of the proto-oncogene c-cbl: a negative regulator of the Syk tyrosine kinase. Science *276*, 418-20.

54. Chan, A. C., Dalton, M., Johnson, R., Kong, G. H., Wang, T., Thoma, R., and Kurosaki, T. (1995). Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. EMBO J *14*, 2499-508.

55. Wange, R. L., Guitian, R., Isakov, N., Watts, J. D., Aebersold, R., and Samelson, L. E. (1995). Activating and inhibitory mutations in adjacent tyrosines in the kinase domain of ZAP-70. J Biol Chem *270*, 18730-3.

56. Kong, G. H., Bu, J. Y., Kurosaki, T., Shaw, A. S., and Chan, A. C. (1995). Reconstitution of Syk function by the ZAP-70 protein tyrosine kinase. Immunity *2*, 485-92.

57. Gong, Q., White, L., Johnson, R., White, M., Negishi, I., Thomas, M., and Chan, A. C. (1997). Restoration of thymocyte development and function in zap-$70^{-/-}$ mice by the Syk protein tyrosine kinase. Immunity *7*, 369-77.

58. Rowley, R. B., Burkhardt, A. L., Chao, H. G., Matsueda, G. R., and Bolen, J. B. (1995). Syk protein-tyrosine kinase is regulated by tyrosine-phosphorylated Igα/Igβ immunoreceptor tyrosine activation motif binding and autophosphorylation. J Biol Chem *270*, 11590-4.

59. Shiue, L., Zoller, M. J., and Brugge, J. S. (1995). Syk is activated by phosphotyrosine-containing peptides representing the tyrosine-based activation motifs of the high affinity receptor for IgE. J. Biol. Chem. *270*, 10498-10502.

60. Neumeister, E. N., Zhu, Y., Richard, S., Terhorst, C., Chan, A. C., and Shaw, A. S. (1995). Binding of ZAP-70 to phosphorylated T-cell receptor ζ and η enhances its autophosphorylation and generates specific binding sites for SH2 domain-containing proteins. Mol. Cell. Biol. *15*, 3171-3178.

61. Zoller, K. E., MacNeil, I. A., and Brugge, J. S. (1997). Protein tyrosine kinases Syk and ZAP-70 display distinct requirements for Src family kinases in immune response receptor signal transduction. J Immunol *158*, 1650-9.

62. Latour, S., Fournel, M., and Veillette, A. (1997). Regulation of T-cell antigen receptor signalling by Syk tyrosine protein kinase. Mol Cell Biol *17*, 4434-41.

63. Latour, S., Zhang, J., Siraganian, R. P., and Veillette, A. (1998). A unique insert in the linker domain of Syk is necessary for its function in immunoreceptor signalling. EMBO J. *17*, 2584-2595.

64. Kolanus, W., Romeo, C., and Seed, B. (1993). T cell activation by clustered tyrosine kinases. Cell *74*, 171-183.

65. Arpaia, E., Shahar, M., Dadi, H., Cohen, A., and Roifman, C. M. (1994). Defective T cell receptor signaling and $CD8^+$ thymic selection in humans lacking zap-70 kinase. Cell *76*, 947-58.

66. Chan, A. C., Kadlecek, T. A., Elder, M. E., Filipovich, A. H., Kuo, W. L., Iwashima, M., Parslow, T. G., and Weiss, A. (1994). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. Science *264*, 1599-601.

67. Elder, M. E., Lin, D., Clever, J., Chan, A. C., Hope, T. J., Weiss, A., and Parslow, T. G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. Science *264*, 1596-9.

68. Williams, B. L., Schreiber, K. L., Zhang, W., Wange, R. L., Samelson, L. E., Leibson, P. J., and Abraham, R. T. (1998). Genetic evidence for differential coupling of Syk family kinases to the T-cell receptor: reconstitution studies in a ZAP-70-deficient Jurkat T-cell line. Mol Cell Biol *18*, 1388-99.

69. Negishi, I., Motoyama, N., Nakayama, K., Nakayama, K., Senju, S., Hatakeyama, S., Zhang, Q., Chan, A. C., and Loh, D. Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. Nature *376*, 435-438.

70. Wiest, D. L., Ashe, J. M., Howcroft, T. K., Lee, H. M., Kemper, D. M., Negishi, I., Singer, D. S., Singer, A., and Abe, R. (1997). A spontaneously arising mutation in the DLAARN motif of murine ZAP-70 abrogates kinase activity and arrests thymocyte development. Immunity *6*, 663-71.

71. Kadlecek, T. A., van Oers, N. S. C., Lefrancois, L., Olson, S., Finlay, D., Chu, D. H., Connolly, K., Killeen, N., and Weiss, A. (1998). Differential requirements for ZAP-70 in TCR signaling and T cell development. J. Immunol., in press.

72. Cheng, A. M., Rowley, B., Pao, W., Hayday, A., Bolen, J. B., and Pawson, T. (1995). Syk tyrosine kinase required for mouse viability and B-cell development. Nature *378*, 303-6.

73. Turner, M., Mee, P. J., Costello, P. S., Williams, O., Price, A. A., Duddy, L. P., Furlong, M. T., Geahlen, R. L., and Tybulewicz, V. L. (1995). Perinatal lethality and blocked B-cell development in mice lacking the tyrosine kinase Syk. Nature *378*, 298-302.

74. Godfrey, D. I., Kennedy, J., Suda, T., and Zlotnik, A. (1993). A developmental pathway involving four phenotypically and functionally distinct subsets of $CD3^-CD4^-CD8^-$ triple-negative adult mouse thymocytes defined by CD44 and CD25 expression. J. Immunol. *150*, 4244-4252.

75. Godfrey, D. I., Kennedy, J., Mombaerts, P., Tonegawa, S., and Zlotnik, A. (1994). Onset of TCR-β rearrangement and role of TCR-β expression during $CD3^-CD4^-CD8^-$ thymocyte differentiation. J. Immunol. *152*, 4783-4792.

76. Mombaerts, P., Clarke, A. R., Rudnicki, M. A., Iacomini, J., Itohara, S., Lafaille, J. J., Wang, L., Ichikaway, Y., Jaenish, R., Hooper, M. L., and Tonegawa, S. (1992). Mutations in the T-cell antigen receptor genes α and β block thymocyte development at different stages. Nature *360*, 225-231.

77. Fehling, H. J., Krotkova, A., Saint-Ruf, C., and Von Boehmer, H. (1995). Crucial role of the pre-T-cell receptor α gene in development of αβ but not γδ T cells. Nature *375*, 795-798.

78. Mombaerts, P., Iacomini, J., Johnson, R. S., Herrup, K., Tonegawa, S., and Papaioannou, V. E. (1992). RAG-1-deficient mice have no mature B and T lymphocytes. Cell *68*, 869-877.

79. Shinkai, Y., Rathbun, G., K.-P., L., Oltz, E. M., Stewart, V., Mendelsohn, M., Charron, J., Datta, M., Young, F., Stall, A. M., and Alt, F. W. (1992). RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. Cell *68*, 855-867.

80. Groves, T., Smiley, P., Cooke, M. P., Forbush, K. F., Perlmutter, R. M., and Guidos, C. J. (1996). Fyn can partially substitute for Lck in T lymphocyte development. Immunity *5*, 417-428.

81. van Oers, N. S., Lowin-Kropf, B., Finlay, D., Connolly, K., and Weiss, A. (1996). αβ T cell development is abolished in mice lacking both Lck and Fyn protein tyrosine kinases. Immunity *5*, 429-36.

82. Appleby, M. W., Gross, J. A., Cooke, M. P., Levin, S. D., Qian, X., and Perlmutter, R. M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59*fyn*. Cell *70*, 751-763.

83. Cooke, M. P., Abraham, K. M., Forbush, K. A., and Perlmutter, R. M. (1991). Regulation of T cell receptor signaling by a *src* family protein-tyrosine kinase (p59fyn). Cell *65*, 281-292.

84. Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K.-U., Veillette, A., Davidson, D., and Mak, T. W. (1992). Profound block in thymocyte development in mice lacking $p56^{lck}$. Nature *357*, 161-164.

85. Cheng, A. M., Negishi, I., Anderson, S. J., Chan, A. C., Bolen, J., Loh, D. Y., and Pawson, T. (1997). The Syk and ZAP-70 SH2-containing tyrosine kinases are implicated in pre-T cell receptor signaling. Proc Natl Acad Sci U S A *94*, 9797-801.

86. Wildin, R. S., Wang, H. U., Forbush, K. A., and Perlmutter, R. M. (1995). Functional dissection of the murine *lck* distal promoter. J. Immunol. *155*, 1286-1295.

87. Gelfand, E. W., Weinberg, K., Mazer, B. D., Kadlecek, T. A., and Weiss, A. (1995). Absence of ZAP-70 prevents signaling through the antigen receptor on peripheral blood T cells but not on thymocytes. J Exp Med *182*, 1057-65.

88. Mallick-Wood, C. A., Pao, W., Cheng, A. M., Lewis, J. M., Kulkarni, S., Bolen, J. B., Rowley, B., Tigelaar, R. E., Pawson, T., and Hayday, A. C. (1996). Disruption of epithelial γδ T cell repertoires by mutation of the Syk tyrosine kinase. Proc Natl Acad Sci U S A *93*, 9704-9.

89. Crispe, I. N., and Bevan, M. J. (1987). Expression and functional significance of the J11d marker on mouse thymocytes. J. Immunol. *138*, 2013-2018.

90. Shortman, K., Wilson, A., Egerton, M., Pearse, M., and Scollay, R. (1988). Immature $CD4^{-}CD8^{+}$ murine thymocytes. Cell. Immunol. *113*, 462-479.

91. MacDonald, H. R., Budd, R. C., and Howe, R. C. (1988). A $CD3^{-}$ subset of $CD4^{-}$ $CD8^{+}$ thymocytes: a rapidly cycling intermediate in the generation of $CD4^{+}8^{+}$ cells. Eur. J. Immunol. *18*, 519-523.

92. Paterson, D. J., and Williams, A. F. (1987). An intermediate cell in thymocyte differentiation that expresses CD8 but not CD4 antigen. J. Exp. Med. *166*, 1603-1608.

93. Miyazaki, T. (1997). Two distinct steps during thymocyte maturation from CD4-CD8- to CD4+CD8+ distinguished in the early growth response (Egr)-1 transgenic mice with a recombinase activating gene-deficient background. J. Exp. Med. *186*, 877-885.

94. Chan, A. C., van Oers, N. S., Tran, A., Turka, L., Law, C. L., Ryan, J. C., Clark, E. A., and Weiss, A. (1994). Differential expression of ZAP-70 and Syk protein tyrosine kinases, and the role of this family of protein tyrosine kinases in TCR signaling. J Immunol *152*, 4758-66.

95. Levelt, C. N., Mombaerts, P., Iglesias, A., Tonegawa, S., and Eichmann, K. (1993). Restoration of early thymocyte differentiation in T-cell receptor β-chain-deficient mutant mice by transmembrane signaling through CD3ε. Proc. Natl. Acad. Sci. USA *90*, 11401-11405.

96. van Oers, N. S., von Boehmer, H., and Weiss, A. (1995). The pre-T cell receptor (TCR) complex is functionally coupled to the TCR-zeta subunit. J Exp Med *182*, 1585-90.

97. Kraft, D. L., Weissman, I. L., and Waller, E. K. (1993). Differentiation of $CD3^-4^-8^-$ human fetal thymoctyes *in vivo*: characterization of a $CD3^-4^+8^-$ intermediate. J. Exp. Med. *178*, 265-277.

98. Ramiro, A. R., Trigueros, C., C., M., San Millan, J. L., and Toribio, M. L. (1996). Regulation of pre-T cell receptor (pTα-TCRβ) gene expression during human thymic development. J. Exp. Med. *184*, 519-530.

99. Chu, D. H., Spits, H., Peyron, J. F., Rowley, R. B., Bolen, J. B., and Weiss, A. (1996). The Syk protein tyrosine kinase can function independently of CD45 or Lck in T cell antigen receptor signaling. EMBO J *15*, 6251-61.

100. Harlow, E., and Lane, D. P. (1988). Antibodies: a laboratory manual (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory), 522-523.

101. Coligan, J. E., Kruisbeek, A. M., Margulies, D. H., Shevach, E. M., and Strober, W. (1994). Current Protocols in Immunology, R. Coico, ed. (New York: John Wiley & Sons, Inc.), pp. 5.3.5.

102. Malissen, M., Gillet, A., Ardouin, L., Bouvier, G., Trucy, J., Ferrier, P., Vivier, E., and Malissen, B. (1995). Altered T cell development in mice with a targeted mutation of the CD3-ε gene. EMBO J. *14*, 4641-4653.

103. Karnitz, L., Sutor, S. L., Torigoe, T., Reed, J. C., Bell, M. P., McKean, D. J., Leibson, P. J., and Abraham, R. T. (1992). Effects of $p56^{lck}$ on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line. Molecular and Cellular Biology *12*, 4521-4530.

104. Straus, D., and Weiss, A. (1992). Genetic evidence for the involvement of the Lck tyrosine kinase in signal transduction throught the T cell antigen receptor. Cell *70*, 585-593.

105. Stein, P. L., Lee, H.-M., Rich, S., and Soriano, P. (1992). $pp59^{fyn}$ mutant mice display differential signaling in thymocytes and peripheral T cells. Cell *70*, 741-750.

106. Qian, D., Mollenauer, M. N., and Weiss, A. (1996). Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. J Exp Med *183*, 611-20.

107. Morita, C. T., Verma, S., Aparicio, P., Martinez, C., Spits, H., and Brenner, M. B. (1991). Functionally distinct subsets of human γδ T cells. Eur. J. Immunol. *21*, 2999-3007.

108. Pihlgren, M., Dubois, P. M., Tomkowiak, M., Sjögren, T., and Marvel, J. (1996). Resting memory $CD8^+$ T cells are hyperreactive to antigenic challenge in vitro. J. Exp. Med. *184*, 2141-2151.

109. Byrne, J. A., Butler, J. L., and Cooper, M. D. (1988). Differential activation requirements for virgin and memory T cells. J. Immunol. *141*, 3249-3257.

110. Sanders, M. E., Makgoba, M. W., June, C. H., Young, H. A., and Shaw, S. (1989). Enhanced responsiveness of human memory T cells to CD2 and CD3 receptor-mediated activation. Eur. J. Immunol. *19*, 803-808.

111. Croft, M., Bradley, L. M., and Swain, S. L. (1994). Naive versus memory CD4 T cell response to antigen. Memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. J. Immunol. *152*, 2675-2685.

112. Curtsinger, J. M., Lins, D. C., and Mescher, M. F. (1998). $CD8^+$ memory T cells ($CD44^{high}$, Ly-$6C^+$) are more sensitive than naive cells ($CD44^{low}$, Ly-$6C^-$) to TCR/CD8 signaling in response to antigen. J. Immunol. *160*, 3236-3243.

113. McHeyzer-Wiliams, M. G., and Davis, M. M. (1995). Antigen-specific development of primary and memory T cells in vivo. Science *268*, 106-111.

114. Flynn, K. J., Belz, G. T., Altman, J. D., Ahmed, R., Woodland, D. L., and Doherty, P. C. (1998). Virus-specific CD8+ T cells in primary and secondary influenza pneumonia. Immunity *8*, 683-691.

115. McCarthy, S. A., Kaldjian, E., and Singer, A. (1988). Induction of anti-CD8 resistant cytotoxic T lymphocytes by anti-CD8 antibodies. Functional evidence for T cell signaling induced by multi-valent cross-linking of CD8 on precursor cells. J. Immunol. *141*, 3737-3746.

116. MacDonald, H. R., Thiernesse, N., and Cerottini, J.-C. (1981). Inhibition of T cell-mediated cytolysis by monoclonal antibodies directed against Lyt-2: heterogeneity of inhibition at the clonal level. J. Immunol. *126*, 1671-1675.

117. Fowell, D. J., Magram, J., Turck, C. W., Killeen, N., and Locksley, R. M. (1997). Impaired Th2 subset development in the absence of CD4. Immunity *6*, 559-569.

118. Lawrence, M. B., and Springer, T. A. (1991). Leukocytes roll on a selectin at phsyiological flow rates: distinction from and prerequisite for adhesion through integrins. Cell *65*, 859-873.

119. Butcher, E. C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to sepcificity and diversity. Cell *67*, 1033-1036.

120. Baumgarth, N., Egerton, M., and Kelso, A. (1997). Activated T cells from draining lymph nodes and an effector site differ in their responses to TCR stimulation. J. Immunol. *159*, 1182-1191.

121. DeGrendele, H. C., Estess, P., and Siegelman, M. H. (1997). Requirement for CD44 in activated T cell extravasation into an inflammatory site. Science *278*, 672-675.

122. Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J. D., Zajac, A. J., Miller, J. D., Slansky, J., and Ahmed, R. (1998). Counting antigen-sepcific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity *8*, 177-187.

123. Butz, E. A., and Bevan, M. J. (1998). Massive expansion of antigen-specific $CD8^+$ cells during an acute virus infection. Immunity *8*, 167-175.

124. Kedl, R. M., and Mescher, M. F. (1997). Migration and activation of antigen-specific $CD8^+$ T cells upon in vivo stimulation with allogeneic tumor. J. Immunol. *159*, 650-663.

125. Mackay, C. R., Marston, W. L., and Dudler, L. (1990). Naive and memory T cells show distinct pathways of lymphocyte recirculation. J. Exp. Med. *171*, 801-817.

126. Binns, R. M., Licence, S. T., and Pabst, R. (1992). Homing of blood, splenic and lung emigrant lymphoblasts: comparison with the behaviour of lymphocytes from these sources. Int. Immunol. *4*, 1011-1019.

127. Jameson, S. C., Hogquist, K. A., and Bevan, M. J. (1995). Positive selection of thymocytes. Annu. Rev. Immunol. *13*, 93-126.

128. Killeen, N., and Littman, D. R. (1993). Helper T-cell development in the absence of CD4-$p56^{lck}$ association. Nature *364*, 729-732.

129. Mountford, P., Zevnik, B., Duwel, A., Nichols, J., Li, M., Dani, C., Robertson, M., Chambers, I., and Smith, A. (1994). Dicistronic targeting constructs: reporters and modifiers of mammalian gene expression. Proc. Natl. Acad. Sci. USA *91*, 4303-4307.

130. Li, M., Sendtner, M., and Smith, A. (1995). Essential function of LIF receptor in motor neurons. Nature *378*, 724-727.

131. Collignon, J., Varlet, I., and Robertson, E. J. (1996). Relationship between asymmetric *nodal* expression and the direction of embryonic turning. Nature *381*, 155-158.

132. Zou, Y. R., Muller, W., Gu, H., and Rajewsky, K. (1994). Cre-loxP-mediated gene replacement: a mouse strain producing humanized antibodies. Curr. Biol. *4*, 1099-1103.

133. Stacey, A., Schnieke, A., Mcwhir, J., Cooper, J., Colman, A., and Melton, D. W. (1994). Use of double-replacement gene targeting to replace the murine α-lactalbumin gene with its human counterpart in embryonic stem cells and mice. Mol. Cell. Biol. *14*, 1009-1016.

134. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B., and Joyner, A. L. (1995). Rescule of the *En-1* mutant phenotype by replacement of *En-1* with *En-2*. Science *269*, 679-682.

135. Wang, Y., Schnegelsberg, P. N., Dausman, J., and Jaenisch, R. (1996). Functional redundancy of the muscle-specific transcription factors Myf5 and myogenin. Nature *379*, 823-825.

136. Costello, P. S., Turner, M., Walters, A. E., Cunningham, C. N., Bauer, P. H., Downward, J., and Tybulewicz, V. L. (1996). Critical role for the tyrosine kinase Syk in signalling through the high affinity IgE receptor of mast cells. Oncogene *13*, 2595-605.

137. Poole, A., Gibbins, J. M., Turner, M., van Vugt, M. J., van de Winkel, J. G., Saito, T., Tybulewicz, V. L., and Watson, S. P. (1997). The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. EMBO J *16*, 2333-41.

138. Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., and DeFranco, A. L. (1997). A critical role for Syk in signal transduction and phagocytosis mediated by Fcγ receptors on macrophages. J Exp Med *186*, 1027-39.

139. Kiefer, F., Brummel, J., Al-Alawi, N., Latour, S., Cheng, A., Veillette, A., Grinstein, S., and Pawson, T. (1998). The Syk protein tyrosine kinase is essential for Fcγ receptor signaling in macrophages and neutrophils. Mol. Cell. Biol. *18*, 4209-4220.

140. Chan, T. A., Chu, C. A., Rauen, K. A., Kroiher, M., Tatarewicz, S. M., and Steele, R. E. (1994). Identification of a gene encoding a novel protein-tyrosine kinase containing SH2 domains and ankyrin-like repeats. Oncogene *9*, 1253-9.

141. Ferrante Jr., A. W., Reinke, R., and Stanley, E. R. (1995). Shark, a Src homology 2, ankyrin repeat, tyrosine kinase, is expressed on the apical surfaces of ectodermal epithelia. Proc Natl Acad Sci U S A *92*, 1911-1915.

142. Batchelor, A. H., Piper, D. E., de la Brousse, F. C., McKnight, S. L., and Wolberger, C. (1998). The structure of GABPα/β: an ETS domain-ankyrin repeat heterodimer bound to DNA. Science *279*, 1037-41.

143. Gorina, S., and Pavletich, N. P. (1996). Structure of the p53 tumor suppressor bound to the ankyrin and SH3 domains of 53BP2. Science *274*, 1001-5.

144. Simon, M. A., Drees, B., Kornberg, T., and Bishop, J. M. (1985). The nuclotide sequence and the tissue-specific expression of Drosophila c-src. Cell *42*, 831-840.

145. Gregory, R. J., Kammermeyer, K. L., Vincent, W. S., and Wadsworth, S. G. (1987). Primary sequence and developmental expression of a novel Drosophila melanogaster src gene. Mol. Cell. Biol. *7*, 2119-2127.

146. Bosch, T. C. G., Unger, T. F., Fisher, D. A., and Steele, R. E. (1989). Structure and expression of *STK*, a *src*-related gene in the simple metazoan *Hydra attenuata*. Mol. Cell. Biol. *9*, 4141-4151.

147. Wong, J., Straus, D., and Chan, A. C. (1998). Genetic evidence of a role for Lck in T-cell receptor function independent or downstream of ZAP-70/Syk protein tyrosine kinases. Mol Cell Biol *18*, 2855-66.

148. Latour, S., Chow, L. M. L., and Veillette, A. (1996). Differential intrinsic enzymatic activity of Syk and Zap-70 protein-tyrosine kinases. J Biol Chem *271*, 22782-90.

149. Tajbakhsh, S., and Buckingham, M. E. (1994). Mouse limb muscle is determined in the absence of the earliest myogenic factor *myf-5*. Proc. Natl. Acad. Sci. USA *91*, 747-751.

150. Mansour, S. L., Thomas, K. R., Deng, C. X., and Capecchi, M. R. (1990). Introduction of a *lacZ* reporter gene into the mouse *int-2* locus by homologous recombination. Proc. Natl. Acad. Sci. USA *87*, 7688-7692.

151. Kato, K., Koyanagi, M., Okada, H., Takanashi, T., Wong, Y. W., Williams, A. F., Okumura, K., and Yagita, H. (1992). CD48 is a counter-receptor for mouse CD2 and is involved in T cell activation. J. Exp. Med. *176*, 1241-1249.

152. Kato, K., Tamura, N., Okumura, K., and Yagita, H. (1993). Identification of the T cell surface signal-transducing glycoprotein sgp-60 as CD48, a counter-receptor for mouse CD2. Eur. J. Immunol. *23*, 1412-1415.

153. Melton, E., Sarner, N., Torkar, M., Van der Merwe, P. A., Russell, J. Q., Budd, R. C., Mamalaki, C., Tolaini, M., Kioussis, D., and Zamoyska, R. (1996). Transgene-encoded human CD2 acts in a dominant negative fashion to modify thymocyte selection signals in mice. Eur. J. Immunol. *26*, 2952-2963.

154. Meyers, E. N., Lewandoski, M., and Martin, G. R. (1998). An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. Nature Genet. *18*, 136-141.