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SRC-LIKE ADAPTOR PROTEIN NEGATIVELY REGULATES T CELL RECEPTOR SIGNALING AND THYMOCYTE DEVELOPMENT

by

Tomasz Sosinowski

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

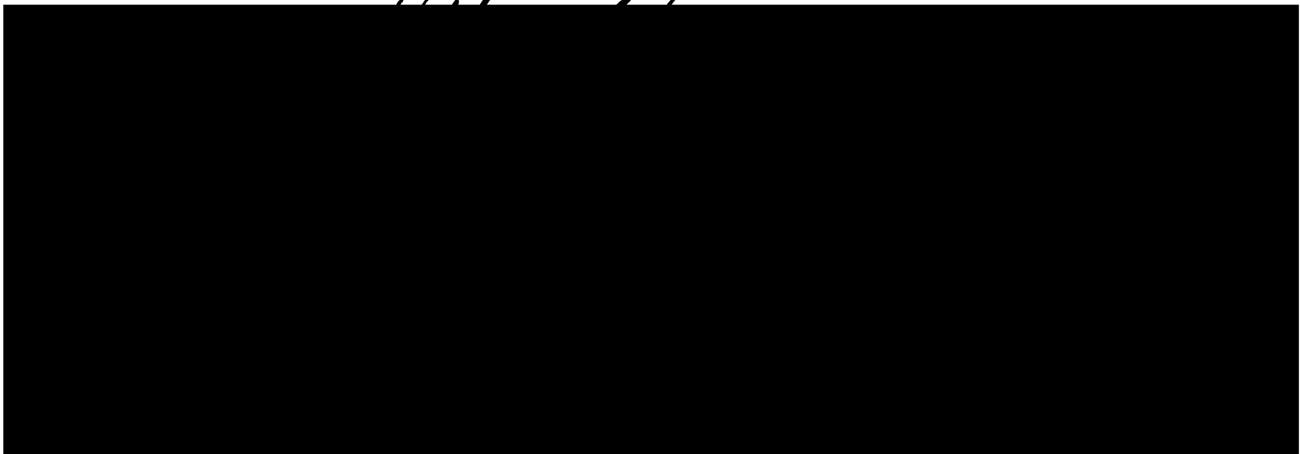
Microbiology and Immunology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



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Dedicated to my mother Cecylia Sosinowska

who always encouraged me to pursue my dreams,

and to my father Jerzy Sosinowski,

who taught me to be passionate about my work

PREFACE

I feel very privileged to be able to pursue my Graduate Studies in the Department of Microbiology and Immunology at the University of California in San Francisco. The path to graduation was neither easy nor short, but I cherished every moment of it. It is because of the people I have met here, that this part of my life was both fun and unforgettable. During my work at UCSF I have received help from many people and I would like to thank them all.

First, I would like to express my appreciation and gratitude to my graduate advisor, Arthur Weiss, for his guidance, support, and patience. He will always be my scientific inspiration, for I admire his knowledge, his enthusiasm and his integrity.

Second, I would like to thank the members of my thesis committee, Jason Cyster, Anthony DeFranco, and Nigel Killeen. Their guidance and support was instrumental in the successful culmination of my research project. I am especially thankful to Nigel Killeen for his help in generating SLAP deficient mice, and for the time he spent on insightful discussions about thymic development.

I would like to acknowledge my undergraduate research advisor Dennis Thiele, who was instrumental in my decision to pursue a scientific carrier. Not only did he personally recruit me to his lab, but he also gave me the foundations of how to "do" science. Importantly, he encouraged me to pursue graduate education, and he highly recommended the University of California in San Francisco for my graduate studies.

During my long graduate career I met many people in the lab and I am afraid I will not be able to thank them all. I would like to thank Peter Stein, who supervised my

rotation and who initiated my first research project in the lab; Nicolai van Oers, who introduce me to "mouse" work; Bente Lowin-Kropf, who always found the time to discuss science and to help in solving day-to-day problems; the members of the "cooking club": Alicia Fry, Jen Liou, Ginny Shapiro, Louise Howe, Jun Wu, Debbie Yablonsky, Ravi Majeti, and Zheng Xu for all the social evenings and wonderful food from all over the world; my roommates in U312, Bryan Irving, Jun Wu, David Chu, Jeanne Baker, Larry Kane, and Jeff Critchfield, for creating the enlightening scientific, social, and political conversations; Marianne Mollenaeur who made the lab feel like home. Special thanks go to Jen Liou, who gave me encourangment during tough times, was very patient reminding me of important deadlines and events, and is a great friend and a little sister I never had. Finally, I would like to thank Joseph Lin for his expert advice on dealing with computers, and for many outdoors trips we took together.

I would like to thank my family, and especially my parents who always believed in me. They both taught me to be curious about life and to pursue my dreams. I also thank my brothers, who were always there when I needed their support.

Dr. Arthur Weiss was the principal investigator supporting this research. Dr. Nigel Killeen was involved in generating SLAP-deficient mice and the analysis of their phenotype. Dr. Akhilesh Pandey and Dr. Vishva Dixit supplied the reagents for the experiments presented in Chapter II, and Dr. Hua Gu donated c-Cbl-deficient mice, which were used for the research presented in Chapter IV. The data of Chapter II was published in the Journal of Experimental Medicine, and the data presented in Chapter III

was accepted for publication in the journal Immunity, both of which are referenced below.

Sosinowski, T., Pandey, A., Dixit, V. M., and Weiss, A. (2000). Src-like adaptor protein (SLAP) is a negative regulator of T cell receptor signaling. J Exp Med *191*, 463-74.

Sosinowski, T., Killeen, N., and Weiss, A. (2001). The Src-Like Adaptor Protein Downregulates the T Cell Receptor on CD4+CD8+ Thymocytes and Regulates Positive Selection. Immunity: *15*(3).

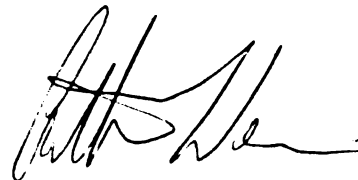
ABSTRACT

SRC-LIKE ADAPTOR PROTEIN NEGATIVELY REGULATES T CELL RECEPTOR SIGNALING AND THYMOCYTE DEVELOPMENT

TOMASZ SOSINOWSKI

Initiation of T-cell antigen receptor (TCR) signaling is dependent on Lck, a Src-family kinase. A molecule structurally resembling Src family protein tyrosine kinases was recently cloned. Like Lck, this Src-like adaptor protein (SLAP) contains SH3 and SH2 domains, two protein-protein interaction modules. Unlike Lck, however, SLAP does not contain a tyrosine kinase domain. Because of these structural features, I hypothesized that SLAP might negatively regulate TCR signaling by interfering with Lck functions. To test this hypothesis I studied the effects of SLAP overexpression on TCR signaling in Jurkat T cells, and the effects of SLAP depletion on thymocyte development in SLAP deficient mice. In addition, I characterized SLAP expression in various tissues and at different stages during T cell development, its subcellular localization, and its biochemical properties. Here I show that SLAP is expressed predominantly in lymphoid tissues, and in particular in T cell compartments. Overexpression of SLAP in Jurkat T cells can specifically inhibit TCR signaling leading to NFAT-, AP-1-, and IL-2-dependent transcription. SLAP colocalizes with endosomes in Jurkat and in HeLa cells,

and is insoluble in mild detergents. In stimulated Jurkat cells, SLAP associates with a molecular signaling complex containing CD3ζ, ZAP-70, SLP-76, Vav, and LAT. SLAP expression is developmentally regulated; it is low in $CD4^-CD8^-$ thymocytes, it peaks in the $CD4^+CD8^+$ subset, and it decreases to low levels in more mature cells. Disruption of the *SLAP* gene leads to a marked upregulation of TCR and CD5 expression at the $CD4^+CD8^+$ stage. The absence of SLAP was also developmentally significant as it enhanced positive selection in mice expressing the DO11.10 transgenic TCR. Moreover, SLAP deletion at least partially rescued the development of ZAP-70 deficient thymocytes. These results demonstrate that SLAP participates in a novel mechanism of TCR downregulation at the $CD4^+CD8^+$ stage, and regulates positive selection. A further genetic analysis suggests that SLAP and c-Cbl, a known negative regulator of TCR signaling, are in the same pathway leading to TCR downregulation at the $CD4^+CD8^+$ stage. In summary, the data presented in this thesis supports the hypothesis that SLAP plays negative regulatory role in TCR signaling and thymocyte development.



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CHAPTER I

STRUCTURAL FEATURES AND A POTENTIAL ROLE OF SLAP IN T CELL RECEPTOR SIGNALING AND THYMOCYTE DEVELOPMENT

Preface

T cells play an essential part in the vertebrate immune system by both, organizing and performing the effector functions in the adaptive response. By doing so, they not only efficiently eliminate foreign invaders from the host organism, but also participate in the undesired events of transplant rejection or the generation of autoimmunity. Therefore, an understanding of the mechanisms underlying T cell activation could be of a tremendous benefit in clinical applications.

A T cell becomes activated following the crosslinking of T cell receptors (TCRs) by the ligands expressed on antigen presenting cells. Crosslinked TCRs generate signals at the plasma membrane that ultimately result in the activation of genes in the nucleus. Transmission of the signal from cell surface to the nucleus involves a complex network of interdependent pathways relying mainly on biochemical modifications of proteins and lipids. Full comprehension of TCR signaling requires not only identification of the components, but also understanding their functions, interactions and regulation. In this introduction I present a short summary of the TCR signal transduction, and its role in development of T cells in the thymus. I concentrate especially on the role and structure of Lck and other members of the Src-family kinases. Finally, I introduce a Src-Like Adaptor Protein (SLAP), the major topic of my thesis.

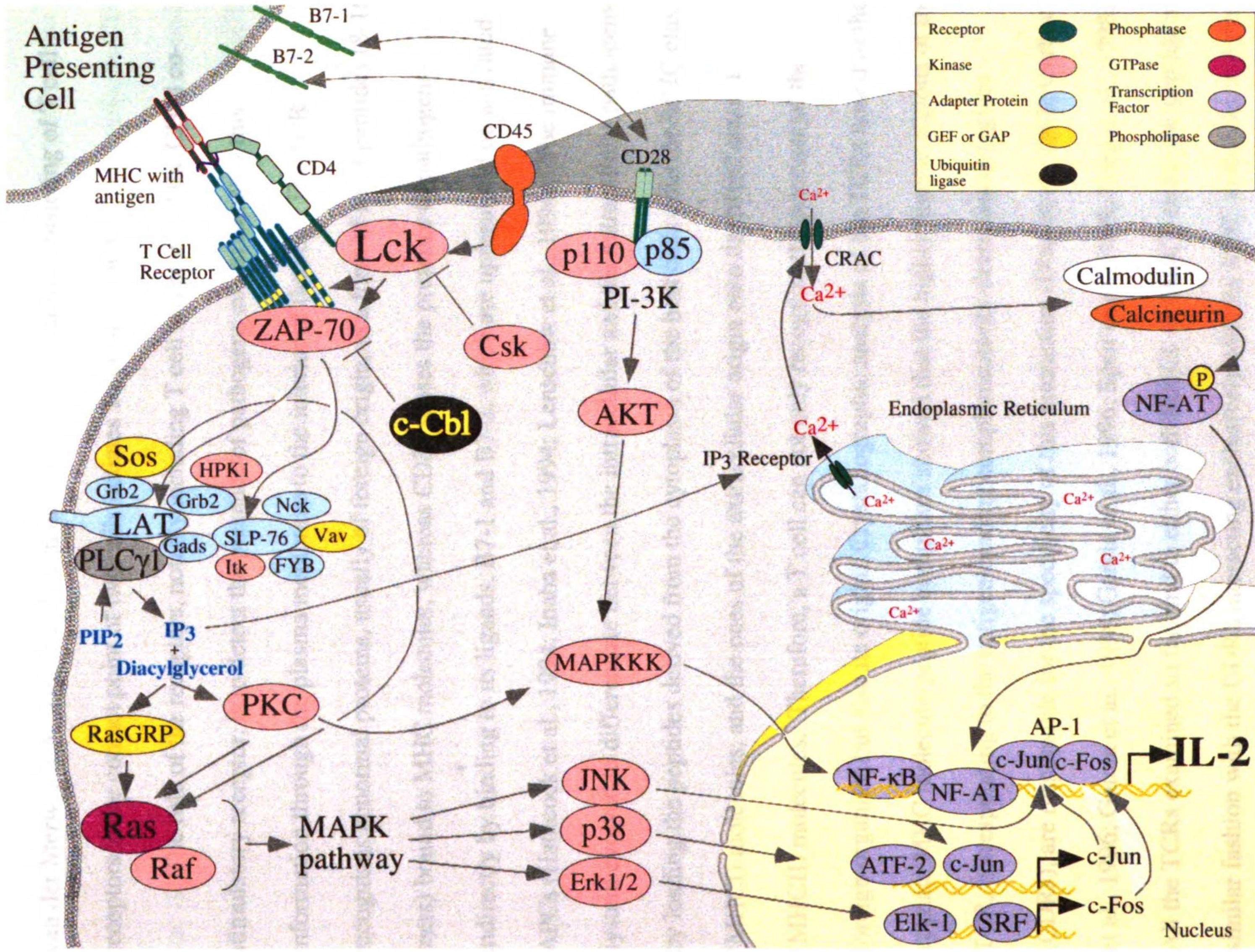
Immunological challenges trigger T cell receptor signaling

T cells respond to immunological challenges by activating T cell receptor (TCR)-dependent signaling pathway. TCR signaling is characterized by phosphorylation of multiple proteins on tyrosine residues and increase in intracellular Ca^{2+} concentration, two events that lead to transcription of novel genes ultimately driving cellular proliferation and acquisition of the effector functions. Induction of the *Interleukin-2* (*Il-2*) gene transcription is one of the earliest responses and is considered to be a hallmark of T cell activation. A simplified cartoon representation of TCR signaling (Figure 1) shows some of the proteins participating in the generation and propagation of the signals leading to transcriptional activation of the *Il-2* gene.

Receptors and coreceptors: complex interactions at the cell surface

Mature T cells residing in the immunologically unchallenged host are small, circulating cells arrested at the G_0 stage of the cell cycle. They become "activated" upon interaction with the antigen presenting cells (APCs), or target cells, expressing on their surface pathogen-derived antigenic peptides in the context of a major histocompatibility complex (MHC) molecules. Experiments performed more than a decade ago documented the formation of long-lived conjugates between T cells and the relevant APCs or target cells, but only recently has the nature and the complexity of this interaction begun to be appreciated. Results from several laboratories have shown that the T cell forms a highly organized and dynamic interface with an APC (for review see Anton van der Merwe et al., 2000; Delon and Germain, 2000). This structure, designated as immunological synapse or supra molecular activation cluster (SMAC), integrates interactions of many pairs of receptors and their ligands including T cell receptor (TCR)/MHC molecule-

Figure 1. Cartoon representation of the T cell receptor (TCR)- mediated signal transduction. The receptors, colored in green, are essential for the initiation of the signal. The TCR recognizes and binds to an agonist peptide bound to a MHC molecule expressed on the antigen presenting cell (APC). The CD4 or CD8 (not shown) co-receptors stabilize the APC-T cell interactions by binding to the non-polymorphic regions of MHC molecule. At the same time, the coreceptors bring a Src-family kinase, Lck, to the TCR complex. Lck initiates the cascade of protein tyrosine phosphorylation, which leads to, among other things, assembly of the signaling structures known as "signalosomes". The formation of a hypothetical signalosome is nucleated by tyrosine phosphorylation of a transmembrane adaptor protein LAT. Interactions among many proteins in these signaling complexes activate several enzymes (e.g. PLCγ1, Sos, RasGRP) which generate two second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Next, the signaling leads to activation of the Ras, PKC, and Ca^{2+} pathways. Ultimately, the three pathways synergize in the nucleus to initiate the Il-2 gene transcription. This figure is modified form Lin and Weiss "T cell receptor signalling" Journal of Cell Science 114, 243-244 (2001).



Antigen Presenting Cell
B7-1
B7-2
MHC with antigen
CD4
T Cell Receptor
CD45
CD28
Lck
ZAP-70
Csk
c-Cbl
p110
p85
PI-3K
AKT
Sos
HPK1
Grb2
Grb2
Nck
LAT
Gads
SLP-76
Vav
PLCγ1
Itk
FYB
PIP2
IP3
+
Diacylglycerol
PKC
RasGRP
Ras
Raf
MAPK pathway
MAPKKK
JNK
p38
Erk1/2
IP3 Receptor
Ca2+
CRAC
Calmodulin
Calcineurin
P
NF-AT
Endoplasmic Reticulum
AP-1
NF-κB
NF-AT
c-Jun
c-Fos
IL-2
ATF-2
c-Jun
c-Jun
Elk-1
SRF
c-Fos
Nucleus
Receptor
Kinase
Adapter Protein
GEF or GAP
Ubiquitin ligase
Phosphatase
GTPase
Transcription Factor
Phospholipase

peptide complex, CD4/8:MHC, CD28:B7.1/B7.2, LFA-1:ICAM-1, CD2:CD48/58, and other surface molecules with no known ligands, like CD45 or CD43. Even though the role of the immunological synapse in TCR signaling remains controversial (Davis and van der Merwe, 2001; Delon and Germain, 2001), it is clear that clustering of T cell receptors at the contact point of two cells initiates T cell activation.

The role of the receptors, notably including T cell receptor and the CD28 co-stimulatory receptor, is to detect the presence of pathogens and to transmit this information through the plasma membrane to the interior of the cell. The TCR recognizes abnormal proteins, usually of foreign origin, in a form of short peptides (8-16-mers) bound to MHC molecules, whereas CD28 senses the presence of pathogens indirectly by binding to its ligands, B7-1 and B7-2, which are upregulated on activated APCs (Hathcock et al., 1994; Inaba et al., 1994; Lenschow et al., 1994). The immune system evolved to differentiate between the intracellular and the extracellular pathogens by loading the peptides derived from the cytoplasm of the host cells onto the MHC class I (MHCI) molecules, and the ones of the extracellular origin onto the MHC class II (MHCII) molecules. Therefore, a T cell can not only recognize the presence of the foreign organism but also its origin. Crystallographic analysis of the TCRs bound to the cognate MHC molecule-peptide complexes showed that the highly diverse regions of the TCR, corresponding to the antigen-binding complementarity-determining regions (CDRs), are responsible for the specificity of this interaction (Ding et al., 1998; Garboczi et al., 1996; Garcia et al., 1998; Garcia et al., 1996; Speir et al., 1998; Teng et al., 1998). All the TCRs examined so far bind to either both MHCI- or MHCII molecule-peptide in a similar fashion with the CDR2 interacting exclusively with the MHC molecule, and the

CDR1 and CDR3 making contacts with the atoms of both MHC molecule and peptide (Hennecke and Wiley, 2001).

The similar pattern of TCR binding to both MHCI and MHCII molecule-peptide complexes implies that TCR by itself is unable to differentiate between these two types of antigen presenting molecules. This recognition is accomplished by the co-receptors CD4 and CD8 expressed on T cells, since CD4 binds exclusively to the MHCII molecule and CD8 bind only to the MHCI molecules. The interactions between co-receptors and MHC molecules are of low affinities, with extremely fast kinetics (e.g. for CD8 and MHCI molecule, dissociation rate constant, $k_{off} > 18\ s^{-1}$) compared to TCR/MHC molecule-peptide binding ($k_{off} = 0.01\text{-}0.1\ s^{-1}$) (Davis et al., 1998; Garcia et al., 1996; Kern et al., 1999; Wyer et al., 1999). The low affinity and transient nature of the co-receptor/MHC interactions suggest that the co-receptors cannot bind stably to MHCs on their own (therefore confirming that they are true co-receptors), and that both the TCR and the co-receptor bind to the same MHC molecule (Gao and Jakobsen, 2000; Janeway, 1992). In fact, this cooperative binding is synergistic and results in several fold increase of the overall affinity (Garcia et al., 1996; Luescher et al., 1995). In addition to contributing to the overall TCR/MHC-peptide binding, the co-receptors contribute to the efficiency of the TCR signaling on the inside of the T cell by bringing a protein tyrosine kinase Lck to the TCR complex, which itself is devoid of intrinsic catalytic activities.

Lck and ZAP-70 kinases activate adaptor proteins to form signaling scaffolds

Lck, a member of Src-family protein tyrosine kinases, has been shown to be responsible for initiating TCR signaling by phosphorylating tyrosines of the

immunoreceptor tyrosine-based activation motifs (ITAMs) present within CD3 and ζ chains of the TCR (Weiss and Littman, 1994). In turn, tyrosine phosphorylated ITAMs serve as docking sites for SH2 domains of the two members of another family of tyrosine kinases, ZAP-70 and Syk. Recruitment of both types of kinases to the TCR complex leads to their activation via transphosphorylation by Lck, or by autophosphorylation in the case of Syk. Subsequently, these kinases transduce TCR signals downstream by phosphorylating a number of targets on tyrosine residues.

Many of the targets phosphorylated by Lck/ZAP-70 kinases belong to a group of adaptors proteins (Figure 1). By definition, these proteins do not possess any catalytic or transcriptional activities, but instead contain a variety of protein modules enabling them to function as molecular scaffolds (for review see Tomlinson et al., 2000). The key representative of this group is a hematopoietic specific transmembrane protein termed linker for activated T cells (LAT). LAT becomes tyrosine phosphorylated on many tyrosine residues and serves as a nucleation point for assembly and interactions of many signaling proteins. Because of the large number of important signaling molecules present in the complex, and its placement at the intersection of several signaling pathways, this assembly has been designated a "signalosome" (Tomlinson et al., 2000). Two important outcomes of the functional signalosome are activation of phospholipase C gamma 1 (PLCγ1) and a mammalian homologue of the *Drosophila* Son of Sevenless (Sos), which subsequently lead to Ca^{2+} flux (Ca^{2+} pathway), and activation protein kinase C (PKC) and Ras.

Ca^{2+} and Ras pathways converge in the nucleus to induce transcription of IL-2

The Ca^{2+} pathway is initiated when the activated PLCγ1 cleaves a membrane lipid, phosphatidylinositol 4,5 bisphosphate (PIP_2), into two second messengers: a soluble inositol 1,4,5-trisphosphate (IP_3) and a membrane bound diacylglycerol (DAG). IP_3, by binding to the IP_3 receptors present on the endoplasmic reticulum (ER), causes release of calcium from the intracellular stores into the cytoplasm. This depletion of Ca^{2+} from the ER triggers opening of the calcium-release-activated calcium (CRAC) channels in the plasma membrane, resulting in sustained influx of the extracellular Ca^{2+}. High levels of Ca^{2+} trigger calmodulin to activate the protein phosphatase calcineurin, which by dephosphorylating serine residues, allows a nuclear factor of activated T cells (NF-AT) to translocate into the nucleus and bind to the promoter region of the *IL-2* gene. DAG, the other product of PIP_2 cleavage by PLCγ1, activates PKC and guanine nucleotide exchange factor for Ras.

Ras is a 21-Kda protein that has been shown to activate the MAPK pathway in many cell types, including T cells. Two forms of Ras exist at equilibrium: an active guanosine triphosphate (GTP)-bound, and an inactive guanosine diphosphate (GDP)-bound state. Ras has a built-in device for self-deactivation since it displays a GTPase activity. There are two potential ways to regulate Ras activity. In resting cells, the slow rate of endogenous Ras GTPase activity can be enhanced by GTPase-activating proteins (GAPs), thus shifting the equilibrium toward the GDP- bound state. In activated cells, stimulation could suppress GAP activity and/or enhance the function of guanine nucleotide-exchange factors (GEFs) which increase the rate of GDP-for-GTP exchange, thereby shifting the equilibrium toward the GTP-bound state. Historically, the DAG-dependent activation of Ras was assigned to downstream functions of the family of PKC

proteins. However, the proposed PKC-dependent inhibition of Ras-GAPs has not been established (Downward et al., 1990; Izquierdo et al., 1992). More recent genetic data indicates that DAG activates Ras by localizing Ras guanyl nucleotide-releasing protein (Ras-GRP) to the plasma membrane (Dower et al., 2000). Another GEF that participates in activation of Ras in T cells is Sos, but unlike Ras-GRP, Sos is recruited to the plasma membrane by a transmembrane protein LAT. Specifically, targeting of Sos to the plasma membrane is accomplished by the interactions between tyrosine phosphorylated LAT and an SH2 domain of Sos-bound adaptor protein Grb 2 (Buday et al., 1994; Finco et al., 1998; Lowenstein et al., 1992; Olivier et al., 1993; Simon et al., 1993; Zhang et al., 1998). That targeting of Sos to the membrane is sufficient for Ras activation was shown by fusing the v-Src myristylation sequence to the N-terminus of Sos (Holsinger et al., 1995). In summary, the current model of Ras activation in T cells proposes that Ras is activated by two guanine nucleotide-exchange factors: Ras-GRP and Sos (Dower et al., 2000; Roose and Weiss, 2000). This model does not exclude the role of PKCs in activation of Ras.

Activated Ras initiates a cascade of protein serine/threonine phosphorylation *via* the mitogen-activated protein kinase (MAPK) pathway (Chang and Karin, 2001). The Ras-dependent stimulation branches into three MAPK pathways, c-Jun N-terminal kinase (JNK), p38, and extracellular signal-regulated kinase 1/2 (Erk1/2). These kinases can directly phosphorylate transcription factors, including AP-1 and NF-AT, and together with signals generated by the Ca^{2+} flux, they can initiate *IL-2* gene transcription. Ras and Ca^{2+}pathways are both necessary and sufficient for T cell activation, since stimulation of T cells with pharmacological agents which directly induce Ras activation (PMA) and

Ca^{2+} flux (ionomycin) leads to upregulation of *IL-2* gene transcription. Moreover, a direct evidence for the role of Ras and Ca^{2+} pathways in T cell activation came from work of Woodrow et al., who demonstrated that expression of constitutively active Ras and constitutively active calcineurin in Jurkat T cells was sufficient to induce transcriptional activity of the NF-AT element (Woodrow et al., 1993).

Summary of the TCR signaling

As outlined above, activation of T cells is a complex process involving several categories of proteins. The receptors detect immunological challenges and initiate the response by activating a class of protein tyrosine kinases of Src- and ZAP-70/Syk-families. These two types of kinases start a cascade of phosphorylation that leads to modification of adaptor proteins. The adaptor proteins connect interacting proteins resulting in an assembly of signaling scaffolds. They in turn allow for activation of enzymes and accessory proteins, such as phospholipases and GAPs/GEFs. Coordinate action of these proteins lead to generation of second messengers and activation of Ca^{2+} and Ras pathways. The ultimate result of all these activities is activation of transcription factors and thus induction of novel genes, including transcription of *IL-2*. The complexity of the TCR signaling pathways poses a challenge for researchers, but at the same time, it presents us with an opportunity to discover ways to control and modify the immune responses.

Thymocyte development

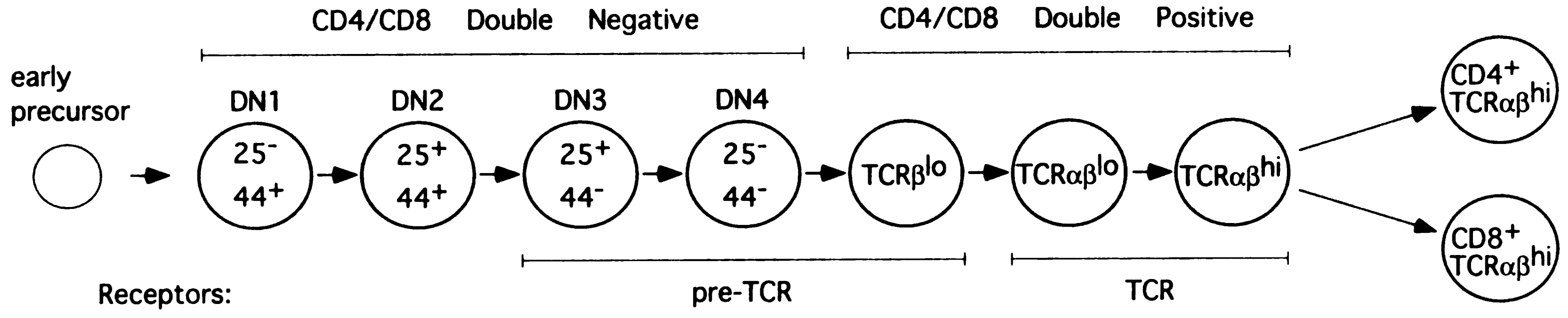
The function and the means

The function of thymic development is to generate a pool of mature T cells capable of recognizing and responding to a diverse set of pathogen-derived peptides bound to MHC molecules. A large repertoire of thymocytes with unique antigen receptors is generated by random rearrangements at the *TCR*α and β gene loci. While creating an immense TCR diversity, this process generates many thymocytes with either non-functional or self-reactive receptors. To solve this problem, all developing thymocytes undergo a process of thymic selection: the non-functional and potentially autoreactive cells are eliminated during negative selection, whereas the potentially useful ones receive survival signals during positive selection. The selected clones mature in the thymus and ultimately seed the periphery (for review see (Sebzda et al., 1999; Wiest et al., 1999).

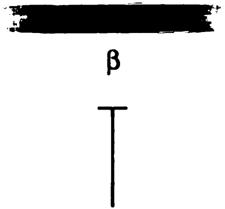
Major stages of thymic development

As illustrated in Figure 2, three major populations of developing thymocytes can be defined on the basis of their ordered expression of CD4 and/or CD8 coreceptors. These populations are: (1) an early double negative (DN) lacking both coreceptors; (2) an intermediate double positive (DP) expressing both; and, (3) the most mature single positive (SP) marked by either CD4 or CD8. The DN population is frequently subdivided into four stages marked by the differential expression of CD25 and CD44. Resolution of the DN population based on the expression of these markers is especially useful for studying the progression of gene rearrangement at the *TCR*β locus. Rearrangement of *TCR*α gene takes place at the $CD4^{+}CD8^{+}$ stage.

Figure 2. Diagram representing the major stages of thymic development. The timing of the TCR α and β gene rearrangements, and of the subsequent selection processes, is shown. Points in the development at which the indicated positive and negative regulators play essential roles are also shown. Modified from Sebzda et al., Annu. Rev. Immunol.,17:829-874 (1999).



CD4/CD8 Double Negative
CD4/CD8 Double Positive
early precursor
DN1
25⁻ 44⁺
DN2
25⁺ 44⁺
DN3
25⁺ 44⁻
DN4
25⁻ 44⁻
TCRβlo
TCRαβlo
TCRαβhi
CD4+ TCRαβhi
CD8+ TCRαβhi
Receptors:
pre-TCR
TCR
Gene rearrangements:
TCRβ
TCRα
Thymic selections:
β
negative
positive
Positive contributors:
TCRβ
CD3ε
CD3γ
pTα
Lck/Fyn
ZAP-70/Syk
SLP-76
LAT
ZAP-70
Lck
TCRα
Negative contributors:
Csk
Csk
c-Cbl
CD5



Pre-TCR signaling: confirming successful rearrangement at the TCRβ locus

The initial gene rearrangement takes place at the *TCRβ* locus among CD4-CD8-CD25+CD44- thymocytes (DN3 stage). The protein product of the rearranged *TCRβ* gene pairs with an invariant pre-TCRα protein to form an immature T cell receptor known as the pre-TCR. The pre-TCR travels to the cell surface to initiate a signal that prevents further rearrangements at the *TCRβ* locus. In addition, this signal is essential for cell survival, expansion, and for further development. Because the major difference between surviving and dying cells is a successful rearrangement of *TCRβ* gene, this process is known as β selection.

Mature TCR signaling: fitting the thymocyte repertoire to the host

Rearrangements of the *TCRα* locus take place at the DP stage. When successful, this process yields TCRα protein that pairs with TCRβ chain to form the unique antigen recognition unit of the mature receptor. Since the mature T cell receptor has a defined specificity for the recognition of a peptide/MHC ligand, a more stringent selection than just surface expression of the receptor has to be employed. In fact, two "quality control" criteria apply: (1) the cells have to be able to induce a signal upon binding to "self" MHC/peptide ligand(s) in the thymus, and (2) the signal cannot be too strong. Two processes that assure compliance with the above criteria are positive and negative selection (Sebzda et al., 1999).

Signaling proteins in thymocyte development: positive vs negative contributions

The major requirement of thymic selection is the ability of a thymocyte to signal, initially through the pre-TCR, and then through the mature TCR. Both receptors use almost the same set of signaling proteins. Because efficient signal transduction is required for developmental progression, the role of a particular protein in signaling can be assessed by its effect on development. Indeed, functions of many signaling proteins have been identified by the developmental phenotype of their genetic deficiencies.

(i) Examples of positive contributions: Src and ZAP-70/Syk kinases

The importance of pre-TCR signaling in β-selection is clearly illustrated by the genetic studies. Targeted disruption of genes encoding the receptor components, including the *preTα*, *TCRβ*, *CD3γ*, *CD3ε*, and *TCRζ*, lead to a complete or partial arrest at the DN stage (Figure 2) (DeJarnette et al., 1998; Fehling et al., 1995; Haks et al., 1998; Love et al., 1993; Malissen et al., 1995; Mombaerts et al., 1992). Likewise, ablation of genes encoding known signaling proteins leads to similar, although of variable degree, blockade. Targeted inactivation of both members of Src kinases expressed in thymocytes, Lck and Fyn, leads to complete arrest at the DN3 stage (van Oers et al., 1996). Lck seems to be more important of the two, since the pre-TCR signaling is severely compromised in mice singly deficient for Lck, but not for Fyn (Molina et al., 1992; Stein et al., 1992). In the Lck-deficient mouse, however, Fyn activity is sufficient to rescue the development of some mature T cells, implying partial redundancy of the two kinases.

Functional redundancy is also observed in the ZAP-70/Syk family: (i) inactivation of *Syk* does not significantly perturb T cell development (Cheng et al., 1995; Turner et al., 1995); (ii) deletion of *ZAP-70* has no effect on β-selection, but thymocytes do arrest at the DP stage (Kadlecek et al., 1998; Negishi et al., 1995); (iii) doubly deficient mutant thymocytes are arrested at the DN3 point of development (Cheng et al., 1997). The observed phenotypes imply that ZAP-70 is sufficient for both, pre-TCR and mature TCR signaling, but Syk can contribute only to the pre-TCR signaling. This functional difference between ZAP-70 and Syk can be attributed to the differential expression of the kinases during development (Chu et al., 1999).

(ii) Examples of negative contributions: Csk and c-Cbl

Unlike that of Lck/Fyn and ZAP-70/Syk kinases, genetic deficiencies of the negative regulators of TCR signaling enhance selection processes and promote thymic development. Csk is a protein tyrosine kinase which negatively regulates TCR signaling by phosphorylating tyrosine 505 of Lck, thus inactivating this kinase (Figure 3C, and see below). Csk is a strong negative regulator of thymic selections since its genetic deficiency allows for T cell development even in the absence of both, pre-TCR- and TCR-dependent signaling (Schmedt et al., 1998; Schmedt and Tarakhovsky, 2001). c-Cbl is another negative regulator of TCR signaling which has an effect on thymocyte development. It has been shown to function as an E2-dependent ubiquitin-protein ligase in EGF and PDGF systems (Joazeiro et al., 1999; Levkowitz et al., 1999). In T cell lines, c-Cbl gets tyrosine phosphorylated upon antigen receptor triggering and interacts with a number of signaling molecules, including Src and ZAP-70/Syk families of kinases

(Howlett et al., 1999; Hunter et al., 1999; Lupher et al., 1997; Miyake et al., 1997). The interaction of c-Cbl with ZAP-70 is mediated by c-Cbl SH2 domain binding to the negative regulatory phosphorylation site (Y292) present in the linker region between the ZAP-70 C-terminal SH2 and kinase domains. The targeted deletion of c-Cbl confirms its negative role in TCR signaling. As expected, c-Cbl deficiency promotes positive selection of thymocytes and results in their hyper-responsiveness. Unexpectedly, however, DP thymocytes from the KO mice express significantly higher levels of the TCR, implying that c-Cbl might play a previously unappreciated role in regulation of the antigen receptor level (Murphy et al., 1998; Naramura et al., 1998).

Src-family kinases

Nine genes, *Blk, Fgr, Fyn, Hck, Lck, Lyn, Src, Yes, and Yrk,* have been classified into the family of Src kinases based on their similar size, ranging from 52 to 62 KDa, similar intron/exon structure, and the identical arrangement of the seven functional domains (Figure 3A, and see below). These non-receptor protein tyrosine kinases have been implicated in a variety of intracellular pathways regulating cell adhesion, migration, cell cycle, proliferation, apoptosis and differentiation (for review see Abram and Courtneidge, 2000; Tatosyan and Mizenina, 2000). Some Src-family members are expressed in many tissues, whilst others are restricted to particular cell types (Corey and Anderson, 1999). As described above, Lck and Fyn are the two members of Src-family most highly expressed in T cells.

Structural features of the Src family

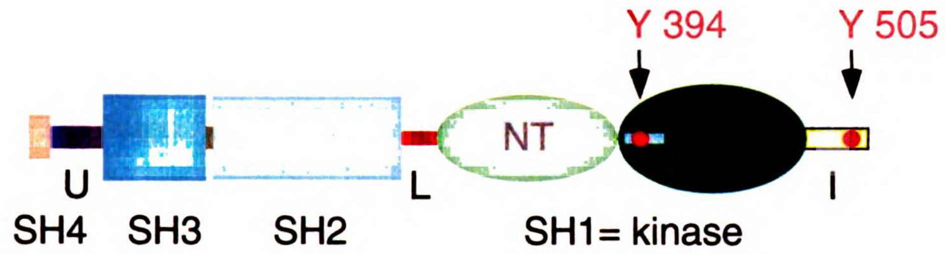
The initial comparison of Src family kinases revealed four functional domains arranged in the identical manner: an N-terminal unique (U) domain, followed by an SH3, an SH2, and an SH1 domains. A more refined structure has emerged with 3 additional common functional motifs: an N-terminal SH4 domain, a linker region (L) located between the SH2 and the SH1 domains, and a C-terminal inhibitory (I) domain. For the sake of clarity, I will discuss the structure of the Src-family kinases as having 7 distinct domains in the following order: SH4, U, SH3, SH2, L, SH1, and I (Figure 3A).

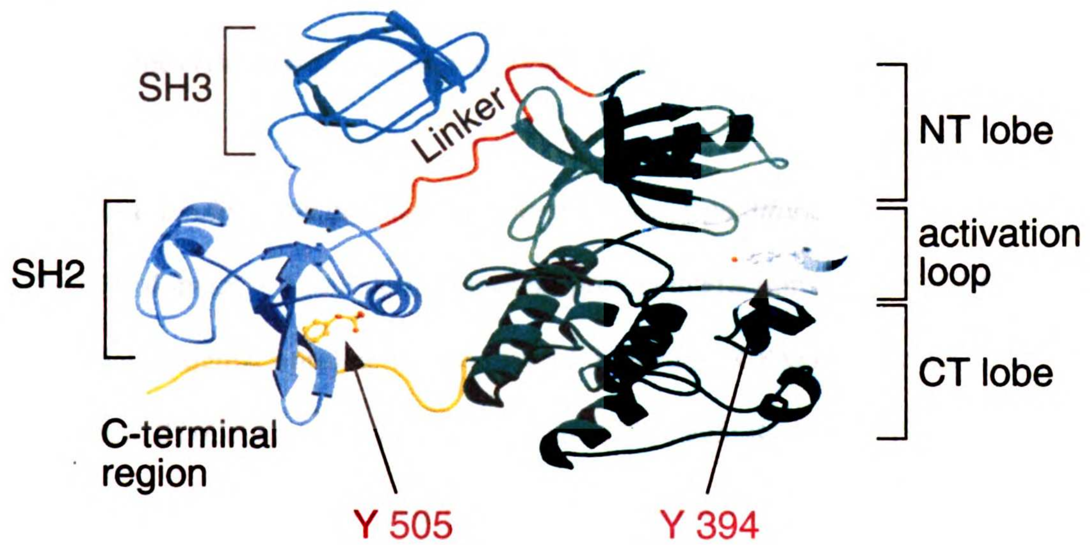
(i) SH4 domain binds to molecular membranes

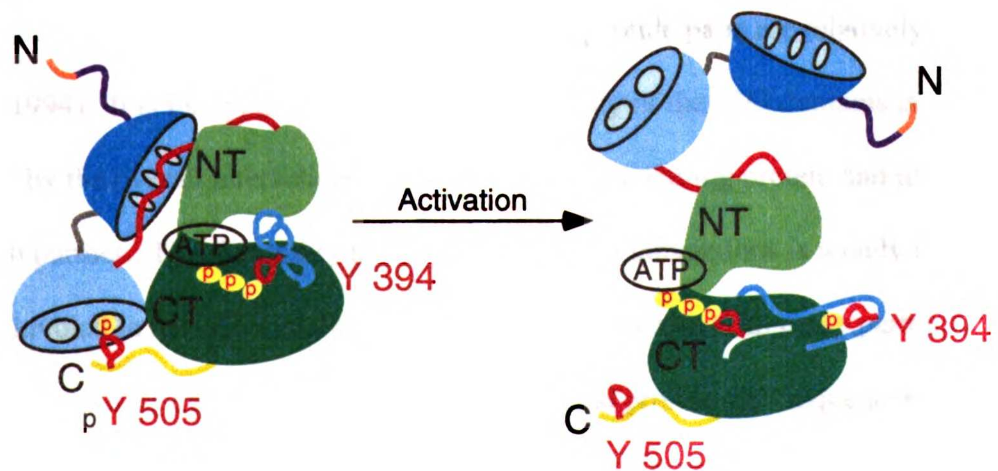
The most NH_2-terminal SH4 domain is comprised of 14-16 amino acids which contain signals for lipid modification(s) (Resh, 1993).. The consensus sequence for myristylation has been described as MGxxxS/T, but three kinases (Fyn, Yrk and Fgr) do not contain S/T within the SH4 domain (Resh, 1994). Despite that, all members of the family undergo co-translational attachment of a 14-carbon myristic acid to the NH_2-terminal glycine. This covalent attachment through the amide bond is considered permanent, and is necessary for localization of the kinases to cellular membranes (Resh, 1994). In addition to myristylation, one of two other motifs is necessary for the efficient membrane localization: the consensus sequence (myr-G-C-) for palmitylation (Fgr, Fyn, Hck, Lck, Lyn, Yes, and Yrk), or a cluster of positively charged amino-acid residues (Src and Blk).

(ii) U domain: potential contribution to target specificity

Figure 3. Functional domains, structural features, and the mechanism of activation of a Src-family kinase. **(A)** A cartoon representation of the 7 functional domains of Lck: N-terminal Src-homology 4 (SH4, orange), unique region (U, violet), SH3 (dark blue), SH2 (light blue), linker region (L, red), SH1=kinase domain (N-terminal lobe {NT}, light green; C-terminal lobe {CT}, dark green), and the C-terminal inhibitory region (I, yellow). Y^{394} is a part of the activation loop of the kinase domain, and Y^{505} resides at the C-terminus of the I region. **(B)** Ribbon diagram showing the structure and the organization of the "closed" conformation of c-Src (numbering of tyrosine residues corresponds to that of Lck). Adapted from Xu et al., "Three-dimentional structure of tyrosine kinase c-Src" Nature 385, 595-602 (1997). **(C)** The model for activation of the Src-family kinases based on the displacement of the SH2 and/or the SH3 domains. Interactions with the "activators" containing the high-affinity ligands for the SH2 (e.g. a tyrosine-phosphorylated YEEI) and/or the SH3 (e.g. a polyproline PxxP) domains relieves the intramolecular inhibitions. De-phosphorylation of the Y^{505} contributes to the stability of this "open" form. The conformational changes force the activation loop away from the active site, allowing the substrate to access the catalytic site. Adapted from Young et al., "Dynamic coupling between the SH2 and SH3 domains of c-Src and Hck underlies their inactivation by C-terminal tyrosine phosphorylation" Cell 105, 115-126 (2001).


A.
Y 394
Y 505
NT
U
L
I
SH4
SH3
SH2
SH1= kinase


B.
SH3
Linker
NT lobe
SH2
activation
loop
CT lobe
C-terminal
region
Y 505
Y 394


C.
N
NT
ATP
Y 394
CT
C
pY 505
Activation
N
NT
ATP
Y 394
CT
C
Y 505

The unique domain, varies in length from 50 to 70 amino acids, and is specific for each protein. This region was proposed to interact with particular receptors and target proteins (Thomas and Brugge, 1997). For example, Lck unique domain contains two cysteine residues (C 20 and 23) that serve as binding sites for the -CxCP- motif of cytoplasmic domains of CD4 and CD8 co-receptors (Shaw et al., 1990; Turner et al., 1990). The unique domain of Fyn does not contain such di-cysteine motif but instead has a stretch of five positively charged amino acid residues that might enhance its association with the membranes (Bijlmakers and Marsh, 1999).

(iii) SH3 domain: binding specificities and contribution to signaling

The SH3 domain is a well defined protein-protein interaction module of 40 to 70 amino acids that recognizes proline-rich sequences defined as R/KxxPxxP (class I), or PxxPxR/K (class II) (Cohen et al., 1995; Mayer and Eck, 1995; Ren et al., 1993). Even though individual SH3 domains display some preferences for unique proline-contaning peptides, for example Src SH3 domain prefers XXXRPLPPLPXP, whereas Fyn SH3 domain prefers XXXRPLPP(I/L)PXX (Rickles et al., 1994), the differences in the dissociation constant (Kd) of various SH3-proline-rich peptide pairs are relatively small (Yu et al., 1994). It is likely, therefore, that the specificity of the SH3 domains is determined by the overall interactions between the SH3-containing protein and its proline-rich partner. This flexibility in the protein-protein interactions is widely used by molecules involed in the cell signaling, enabling them to interact with many partners. For example, the Lck SH3 domain have been reported to bind proline-rich sequences within

c-Cbl, the p85 subunit of PI3K, Ras-GAP, HS1 and CD2 (Bell et al., 1996; Briggs et al., 1995; Prasad et al., 1993; Reedquist et al., 1994; Takemoto et al., 1995).

In addition to facilitating the interactions with multiple proteins, structural data on Src and Hck revealed that the SH3 domain contributes to the inhibition of the kinase domain by binding to the L region between the SH2 domain and the kinase domain (Sicheri et al., 1997; Xu et al., 1997). This finding was surprising since the L region of Src contains only a single proline in this region. The functional significance of the SH3 domain of Lck has been underscored by the data showing that its presence was necessary for the ability of an oncogenic form of Lck to enhance IL-2 production (Caron et al., 1992). Another group showed that the SH3 domain of Lck is obligatory to the activation of the MAPK pathway in TCR signaling (Denny et al., 1999).

(iv) SH2 domain: binding specificities and contribution to signaling

The SH2, like the SH3 domain, is an autonomous protein-protein interaction module widely distributed among signaling proteins. Crystal structures of several SH2 domains have been solved, including the one of Lck (Eck et al., 1993; Waksman et al., 1993). Approximately 100 amino acid-long, this globular domain consists of a central four-stranded β-sheet core and two flanking α-helices (Figure 3B). Crystal structures revealed that phosphopeptides bind in an extended conformation almost perpendicular to the edge of the central strand, making contacts with the fifth amino acid of the strand. On the basis of this β-D5 amino acid, Songyang et al. classified the SH2 domains into four groups (Songyang et al., 1993). Interestingly, all members of the Src family have a tyrosine residue at this position, and therefore were classified into group I. the

experimentally determined consensus sequence of the Src-family SH2 ligand, phospho-YEE(I/V), agrees with the theoretical considerations which predicted that P1 position would be occupied by a hydrophilic residue.

A large number of proteins interacting with the Lck SH2 domain has been reported: tyrosine phosphorylated ZAP-70 and Syk kinases (Straus et al., 1996; Thome et al., 1995), CD45, PLC-γ, Vav, Sam68, and Lad (Autero et al., 1994; Choi et al., 1999; Duplay et al., 1994; Fusaki et al., 1997; Gupta et al., 1994; Weber et al., 1992). In addition to these classical interactions between SH2 and phospho-tyrosine residues, a non-phosphorylated protein of 62 kDa was reported to interact with SH2 domain of Lck (Joung et al., 1996; Vadlamudi et al., 1996). A similar exception to the rule was reported for the SH2 domain of SAP which can bind to non-phosphorylated tyrosines (Kuriyan and Cowburn, 1997; Li et al., 1999; Poy et al., 1999). In addition to binding to various signaling proteins, the crystal structure showed that SH2 domain of Src kinases interacts with the negative regulatory site in an intramolecular interaction (Cooper and Howell, 1993).

Experimental evidence supports both, negative and positive roles of the SH2 domain in the function of Src kinases. An increased oncogenic potential of the mutant of Src lacking SH2 domain expressed in fibroblasts supports the negative role (Veillette et al., 1992). However, the same mutation in the activated form of Src inhibited tyrosine phosphorylation of proteins in T cells (Caron et al., 1992). In addition, a point mutation in the phospho-tyrosine binding pocket of the SH2 domain prevented Lck from reconstituting TCR signaling in an Lck-deficient Jurkat cell line (Straus et al., 1996).

These seemingly contradictory findings support the proposed mechanism for activation of Src-family kinases (see below).

(v) SH2-kinase domain linker: intracellular inhibition

Structural studies have described a functionally conserved linker (L) region of 15 amino acids located between the SH2 and the SH1 domains (Sicheri et al., 1997; Xu et al., 1997). This region forms a left handed polyproline (PP) II helix that binds the SH3 domain of the same molecule in a characteristic class II orientation (Figure 3B). The experimental data confirm that the intracellular interaction between the linker region and the SH3 domain negatively regulates the kinase activity (see Figure 3C for the model). For example, addition of the SH3 domain-binding peptides (a peptide containing the PXXP motif or a peptide derived from the HIV-1 Nef protein) to an *in vitro* kinase reaction resulted in substantial increase of Hck kinase activity (Moarefi et al., 1997). In addition, point mutations in the linker regions of c-Src and Lck that interfered with this intracellular interaction created the kinases which were unresponsive to the negative regulation by Csk (Gonfloni et al., 1997).

(vi) SH1 or the kinase domain: structure and the potential substrates

Following the L region is the catalytic domain of Lck. The crystal structure of Src and Hck revealed striking similarities between kinase domains of the Src- family and the corresponding domains of cyclic-AMP-dependent serine kinase (cAPK) or protein kinase A (PKA) (Sicheri et al., 1997; Xu et al., 1997). The two globular lobes of the kinase domain are connected by a flexible "hinge". The smaller N-terminal lobe contains

five anti-parallel β-strands and a single α-helix (helix C), whilst the C-terminal domain is mostly α-helical. The ATP-binding site is located between the lobes. The single helix C of the N-terminal lobe contains the conserved Glu residue, which in an active conformation projects into the catalytic cleft to form a salt bridge with the conserved Lys, an important coordinator of the α- and β-phosphates of ATP. It is the exclusion of this conserved glutamate from the active site, caused by a combination of the steric hindrance created by the activation loop and by the outward rotation of the helix C stabilized by the SH3-L interaction, that preserves the inactive conformation of the kinase. The identification of direct substrates of a particular kinase is a difficult task because the *in vitro* kinase assays eliminate strict substrate specificity, and the *in vivo* read-outs of the kinase activity might reflect the indirect involvement through some other kinase(s). Nevertheless, combination of the *in vitro* and the *in vivo* approaches identified c-Cbl, PI3K, Crk, Shc, Vav, Paxillin, Fak, Tec, SOS, and RasGap as the potential Src substrates (Corey and Anderson, 1999), and PLCγ1, Vav, PI3K, ZAP-70, and CD3ζ as the potential Lck substrates (Weil and Veillette, 1996).

(vii) C-terminal inhibitory (I) domain

Another common feature of the Src kinases is the presence of a tyrosine residue at the C-terminus which, when phosphorylated, can bind to the SH2 domain in an intramolecular interaction resulting in inhibition of the tyrosine kinase activity (MacAuley and Cooper, 1989). Structural analysis showed that this region of 12-15 amino acids forms an unstructured, flexible tether (Sicheri et al., 1997; Xu et al., 1997). Binding studies, combined with structural considerations, suggest that the sequence

Q(pY)QPQ of the Lck inhibitory region binds its SH2 domain with low affinity (Kuriyan and Cowburn, 1997; Payne et al., 1993).

Structural considerations for the regulation of the kinase activity

The crystal structures have provided a clear picture of how the Lck kinase can be regulated (Figure 3C). In the inactive conformation, cooperative binding of the SH3 domain to the linker region, the SH3 domain to the N-terminal lobe of the kinase domain, and the SH2 domain to the phosphorylated Y505, forces the N-terminal segment of the activation loop to block the active site of the kinase. Upon binding of the activators, the SH2 and SH3 domains are released from these intramolecular interactions, thus presenting Y394 in the activation loop for phosphorylation. In effect, this phosphorylation triggers an electrostatic switch that forces both the movement of the activation loop away from the active site and the realignment of the helix C into the active site. This is the active conformation of the kinase.

Subcellular localization of Lck

All members of the Src-family are targeted to biological membranes by a two-signal mechanism: a covalent attachment of myristic acid at the NH_2-terminal glycine (all members) and either the presence of a polybasic region (Src and Blk) or palmitylated cysteines (all but Src and Blk). Intact signals for both modifications seem to be essential for proper functioning of the kinases. For example, the lack of myristylation or palmitylation signals in the SH4 domain of Lck prevents this kinase from participation in

TCR signaling (Caron et al., 1992; Kabouridis et al., 1997; Yasuda et al., 2000). Despite having very similar membrane attachment mechanism, and the overall structure, various Src-family members are found in different regions of the cell.

The initial studies showed that v-Src was found predominantly in focal adhesion plaques (Rohrschneider, 1980). In contrast, the majority of c-Src was found to co-localize with the endosomal marker, cation-independent mannose 6-phosphate receptor (CI-M6PR), in the perinuclear regions of the fibroblasts (Kaplan et al., 1992). Further confirming this localization, c-Src was co-enriched with endosomes following biochemical fractionation of cellular membranes (Kaplan et al., 1992). The available data indicates that the distribution of a given protein depends also on cellular environment. For example, c-Src does not show endosomal localization in the neuroendocrine cell line PC12, but instead it is found associated with synaptic vesicles (Kaplan et al., 1992).

Lck and Fyn display differential localization despite using almost identical attachments to the membrane through the myristic (G2) and the palmitic (C3/6 in Lck and C3/5 in Fyn) acids (Alland et al., 1994; Ley et al., 1994; Yurchak and Sefton, 1995). In Jurkat T cells, Lck was detected at the plasma membrane in all of the cells. In addition, the majority of cells showed perinuclear, vesicular staining; this staining coincided with the CI-M6PR, a marker for endosomes and the *trans*-Golgi network. The relevance or significance of this perinuclear localization of Lck is unclear, especially in view of the finding that no such staining was seen in human T lymphoblasts (Ley et al., 1994).

In contrast to Lck, no Fyn could be detected at the plasma membrane, but instead this kinase was localized to the perinuclear region. The lack of plasma membrane staining was surprising, since Fyn-associated kinase activity was detected in association with cell-surface TCR (Samelson et al., 1990; Timson Gauen et al., 1992). One possible explanation for this discrepancy is the lower sensitivity of immunofluorescence microscopy as compared to kinase assays used in the coimmunoprecipitation studies. The perinuclear region that stained with anti-Fyn antibody was distinct from the endosomes, and corresponded to the centrosome. One explanation for the non-overlapping subcellular distribution of these Src kinases in T cells might be the ability of Lck, but not Fyn, to associate with cell surface molecules CD4 and CD8. This explanation is unlikely, however, because Lck localizes to the plasma membrane in nonlymphoid cells, which do not express CD4 or CD8 (Bijlmakers et al., 1997).

Src-like Adaptor Protein (SLAP)

Cloning of SLAP cDNA

In an effort to identify molecules interacting with the Eph family of receptor tyrosine kinases a novel molecule containing an SH3 and an SH2 domain was cloned (Tang et al., 1999). Sequence analysis revealed a high degree of homology to Src-family kinases, but lack of the kinase domain itself. Hence, the authors named the novel protein Src-like Adapter Protein or SLAP. This molecule interacted specifically with a cytoplasmic domain of Eph A2 (Eck) in a yeast two-hybrid screen (Tang et al., 1999). This interaction also occurred in vitro between GST-SLAP fusion protein and an

endogenous Eph A2 receptor present in the lysates from activated primary vascular smooth muscle cells. The authors did not, however, study the endogenous SLAP. Northern blot analysis indicated ubiquitous expression of *SLAP*. Interestingly, spleen, the only immune tissue used in the analysis, showed the highest level of *SLAP* mRNA.

Structural features of SLAP and Potential function of SLAP in TCR signaling

Like Src kinases, SLAP contains an NH_2-terminal SH4 domain, followed by a unique, an SH3, and an SH2 regions. Unlike Src family, SLAP lacks a kinase domain (Figure 4). Without knowing a crystal structure, it is difficult to evaluate whether SLAP has a region corresponding to the SH2-kinase linker region of Src proteins. It also difficult to predict whether SLAP contains a functional auto-inhibitory peptide at the C-terminus, although it does not contain a consensus sequence Q(pY)QPQ of Src family members.

The SH4 domain of SLAP has a required glycine at position 2, but does not have a perfect consensus sequence for myristylation: MGxxxS/T. As mentioned above, Fyn, Yrk and Fgr contain neither S nor T within the first 10 amino acids of its NH_2-terminus, yet they undergo efficient myristylation. In fact, SLAP has been shown recently to be myristylated (Manes et al., 2000). However, SLAP has no cysteine residues for the attachment of a second fatty acid, nor does it contain any clusters of polybasic amino acids in the entire SH4 and the unique domains. In summary, SLAP does not contain the required two signals for membrane attachment and therefore it either does not bind to the biological membranes or it may employ a novel mechanism to do so. SLAP SH3 and

Figure 4. SLAP shares high degree of sequence similarity with Lck and other members of the Src-family kinases.

The amino acid sequence of murine SLAP (mSLAP) and murine Lck (mLck) are aligned using "ClustalW Alignment" of the Mac Vector software (version 6.5.3). The identical residues are in grey boxes and the similar residues are in white boxes. The functional domains of Lck are color-coded as follows: SH4 is orange, unique region is violet, SH3 is dark blue, SH2 is light blue, SH2-kinase linker is red, N-terminal lobe of the kinase domain is light green, C-terminal lobe of the kinase domain is dark green, and the C-terminal inhibitory region is yellow. The Y^{394} is the activation loop, and the Y^{505} in the C-terminal inhibitory region are colored in red.

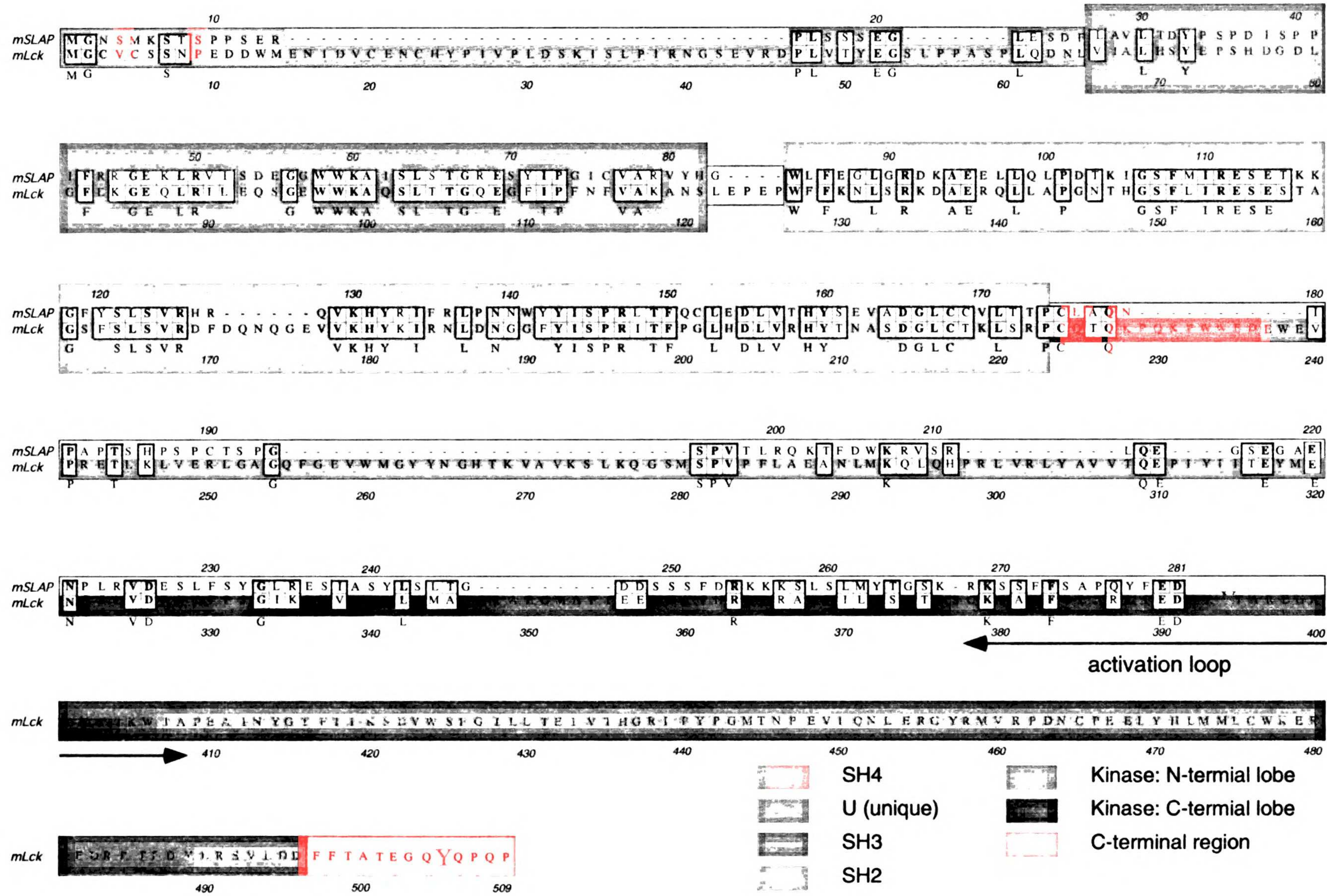


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Figure 5. SLAP sequence is highly conserved between mouse and human. The amino acid sequence of murine SLAP (mSLAP) and human SLAP (hSLAP) are aligned using "ClustalW Alignment" of the Mac Vector software (version 6.5.3). The identical residues are in grey boxes and the similar residues are in white boxes. The functional domains of SLAP are color-coded as follows: SH4 is orange, unique region is violet, SH3 is dark blue, SH2 is light blue, C-terminal region is light green.

```
                10          20                 30         40         50         60         70         80
mSLAP  MGNSMKSTSPPSERPLSSSEGLESDFLAVLTDYPSPDISPPIFRRGEKLRVISDEGGWWKAISLSTGRESYIPGICVARV
hSLAP  MGNSMKSTPAPAERPLPNPEGLDSDFLAVLSDYPSPDISPPIFRRGEKLRVISDEGGWWKAISLSTGRESYIPGICVARV
       MGNSMKST  P ERPL   EGL SDFLAVL DYPSPDISPPIFRRGEKLRVISDEGGWWKAISLSTGRESYIPGICVARV
                10          20

                90         100        110        120        130        140        150        160
mSLAP  YHGWLFEGLGRDKAEELLQLPDTKIGSFMIRESETKKGFYSLSVRHRQVKHYRIFRLPNNWYYISPRLTFQCLEDLVTHY
hSLAP  YHGWLFEGLGRDKAEELLQLPDTKVGSFMIRESETKKGFYSLSVRHRQVKHYRIFRLPNNWYYISPRLTFQCLEDLVNHY
       YHGWLFEGLGRDKAEELLQLPDTK GSFMIRESETKKGFYSLSVRHRQVKHYRIFRLPNNWYYISPRLTFQCLEDLV HY
                90         100        110        120        130        140        150        160

                170        180        190        200        210        220        230        240
mSLAP  SEVADGLCCVLTTPCLAQNIPAPTSHPSPCTSPGSPVTLRQKTFDWKRVSRLQEGSEGAENPLEVDESLFSYGLRESIAS
hSLAP  SEVADGLCCVLTTPCLTQSTAAPAMRAS----SSPVTLRQKTVDWRRVSRLQEDPEGTENPLGVDESLFSYGLRESIAS
       SEVADGLCCVLTTPCL Q   AP   S     SPVTLRQKT DW RVSRLQE  EG ENPL VDESLFSYGLRESIAS
                170        180            190        200        210        220        230

                250        260        270        281
mSLAP  YLSLTGDDSSSFDRKKKSLSLMYIGSKRKSSFFSAPQYFED
hSLAP  YLSLTSEDNTSFDRKKKSISLMYGGSKRKSSFFSSPPYFED
       YLSLT D   SFDRKKKS SLMY GSKRKSSFFS P YFED
           240        250        260        270    276
```

SH4
U (unique)
SH3
SH2
C-terminal region

SH2 domains are highly homologous to the respective domains of Lck. The amino acid compositions of SLAP and Lck SH3 domains are 55 percent identical. This degree of homology is higher than that for Src (42%), or other unrelated proteins (those of PLCγ1 and the p85α subunit of phosphatidylinositol 3-kinase share only 20 and 24% identity, respectively). The SH2 domain of SLAP is also highly homologous to the one present in Lck, sharing with it 50% identity. Src and other unrelated SH2 domains are less homologous, with Src being 43 percent identical, and Shc and Grb2 only 25 and 27%, respectively (Pandey et al., 1995). The crystal structural analysis identified eleven residues coordinating binding of the consensus phospho-peptide to Lck SH2 domain (Tong et al., 1996). All these residues are conserved in the sequence of SLAP SH2 domain (Figure 4).

Besides SH4, SH3 and SH2 domains the sequences of SLAP and Src family are divergent. SLAP has a very short unique region of 12 amino acids as compared to 48 amino acids present in Lck. The lack of a di-cysteine motif in SLAP U domain suggests that unlike Lck, SLAP does not bind to CD4 or CD8. It is difficult to evaluate if SLAP contains an SH2-kinase linker (L) since the sequence of this region is divergent even among the Src-family kinases, and not all the members contain the minimum consensus SH3 binding sequence PXXP. However, it is worth noting that SLAP has five prolines within the first 20 amino acids following the SH2 domain, which suggests that despite of the lack of the PxxP motif, SLAP L region might potentially interact with its own SH3 domain. It is intriguing to note that the C-terminal residues of SLAP have several stretches of homology to the N-terminal lobe and half of the activation loop of Lck. In addition, SLAP C-terminus contains a tyrosine residue that might become

phosphorylated, and thus might regulate the accessibility of its own SH2 domain. Although only the crystal structure can confirm the domain organization of the protein, it is interesting to speculate that the structure of SLAP may resemble that of the Src-family kinases.

General organization of the thesis

SLAP and Lck share the same arrangement and a high degree of amino acid identity between their respective SH2 and SH3 domains. Because of these structural similarities SLAP, which does not contain a kinase domain, might potentially interfere with Lck functions by binding to the same set of proteins. Therefore, I hypothesize that SLAP is a negative regulator of TCR signaling.

In the following three chapters I present the biochemical, functional, and genetic data supporting the negative regulatory role of SLAP in TCR signaling. In Chapter II, I show that SLAP is expressed in T cells, it interacts with similar subset of phosphoproteins that Lck does, and that it can inhibit TCR signaling. In Chapter III, I describe generation and the phenotype of SLAP deficient mice. In Chapter IV, I present genetic evidence supporting the notion that SLAP and c-Cbl participate in the same pathway leading to downregulation of TCR on DP thymocytes. Finally, I summarize my findings in the context of the current knowledge and propose future experiments.

CHAPTER II

SLAP IS A NEGATIVE REGULATOR OF T CELL RECEPTOR SIGNALING

Summary

Initiation of T cell antigen receptor (TCR) signaling is dependent on Lck, a Src-family kinase. The Src-like adaptor protein (SLAP) contains SH3 and SH2 domains, which are highly homologous to those of Lck and other Src-family members. Because of the structural similarity between Lck and SLAP we studied its potential role in TCR signaling. Here we show that SLAP is expressed in T cells and, when expressed in Jurkat T cells, can specifically inhibit TCR signaling leading to NFAT-, AP-1-, and IL-2-dependent transcription. The SH3 and SH2 domains of SLAP are required for maximal attenuation of TCR signaling. This inhibitory activity can be bypassed by the combination of PMA and ionomycin, suggesting that SLAP acts proximally in the TCR signaling pathway. SLAP colocalizes with endosomes in Jurkat and in HeLa cells, and is insoluble in mild detergents. In stimulated Jurkat cells, SLAP associates with a molecular signaling complex containing CD3ζ, ZAP-70, SLP-76, Vav, and possibly LAT. These results suggest that SLAP is a negative regulator of TCR signaling. Work presented in this chapter has been published in the Journal of Experimental Medicine: 191(3):463-473, February 2000.

Introduction

Activation of the T cell receptor (TCR) is a critical event for T cell development in the thymus as well as for initiating immune responses in mature T cells. The TCR is devoid of intrinsic catalytic activity, yet its engagement leads to significant induction of protein tyrosine kinase activity, resulting in phosphorylation of tyrosine residues on many proteins. Src-family protein tyrosine kinases, and especially in T cells one of its members, Lck, have been shown to be responsible for initiating this process by phosphorylating tyrosines within immunoreceptor tyrosine-based activation motifs (ITAMs) present within CD3 and ζ chains of the TCR (Weiss and Littman, 1994). Subsequently, tyrosine phosphorylated ITAMs serve as docking sites for SH2 domains present in the two members of another family of tyrosine kinases, ZAP-70 and Syk. Recruitment of ZAP-70/Syk to the TCR complex activates these kinases which, in concert with Lck, transduce the TCR signal downstream by phosphorylating a number of targets. This activation process ultimately leads to the transcription of genes such as interleukin-2 (IL-2), a hallmark of T cell activation.

The essential role of Lck in TCR signaling has been underscored by the demonstration that a Jurkat T cell line lacking expression of this kinase is unresponsive to TCR stimulation (Straus and Weiss, 1992). Moreover, mice deficient in or expressing a dominant-negative form of Lck, display a profound blockade in thymocyte development in the transition to the CD4/CD8 double positive (DP) stage (Levin et al., 1993; Molina et al., 1992). ITAM phosphorylation is deficient in both the Lck Jurkat line and in Lck deficient thymocytes (Iwashima et al., 1994; van Oers et al., 1996).

Lck, like other Src-family members, has a distinctive structure. It has a unique N-terminal region followed by Src homology 3 (SH3), SH2, catalytic (SH1), and C-terminal regulatory domains. Two functions have been attributed to the unique region of Lck: 1) a myristylation and palmitylation of this region targets Lck to the plasma membrane, and 2) a cysteine-containing motif enables it to associate with CD4 or CD8 coreceptors (Abraham and Veillette, 1990; Paige et al., 1993; Shenoy-Scaria et al., 1993; Turner et al., 1990). The SH3 and SH2 domains are well-defined protein-protein interaction modules (Pawson and Gish, 1992) and, as such, facilitate binding of Lck to many signaling molecules. The SH3 domain can recognize proline-rich sequences within c-Cbl, the p85 subunit of PI3K, Ras-GAP, HS1 and CD2 (Bell et al., 1996; Briggs et al., 1995; Prasad et al., 1993; Reedquist et al., 1994; Takemoto et al., 1995). The SH2 domain can interact with tyrosine phosphorylated ZAP-70 and Syk kinases (Straus et al., 1996; Thome et al., 1995) or with the negative regulatory site of Lck in an intramolecular interaction (Cooper and Howell, 1993). Following the SH2 domain is the catalytic domain of Lck. Activation of the kinase is dependent on autophosphorylation of a single tyrosine residue within the activation loop of the kinase domain. Dephosphorylation of a well-conserved tyrosine present in the C-terminal regulatory region is also required for kinase activation (reviewed in (Chow and Veillette, 1995)).

A molecule structurally resembling Src family protein tyrosine kinases was cloned using yeast two-hybrid screen (Pandey et al., 1995). Like Lck, this Src-like adaptor protein (SLAP) contains a short unique N-terminal domain, followed by SH3 and SH2 domains. However, it does not contain a tyrosine kinase domain. Both the SH3 and SH2 domains of SLAP are highly homologous to those of Lck and share with them 55

and 50 percent identity, as compared to 42 and 43 percent with c-Src, respectively (K best non-intersecting alignments, EXPASY). The SLAP SH3 and SH2 domains share less homology with other SH3 domains (those of PLCγ1 and the p85α subunit of phosphatidylinositol 3-kinase share only 20 and 24% identity, respectively), and SH2 domains (those of Shc and Grb2 share only 25 and 27% identity, respectively) (Pandey et al., 1995). Instead of a kinase domain SLAP contains a unique C terminus of 104 residues. The initial study where SLAP was identified suggested ubiquitous expression of SLAP mRNA among tissues tested. In a subsequent study, microinjected SLAP inhibited a platelet-derived growth factor receptor (PDGFR)-dependent mitogenic response in fibroblasts (Roche et al., 1998). Biochemical analysis revealed that SLAP associated with activated PDGFRs and that it could efficiently compete with Src for PDGFR binding.

Since the original report suggested the possibility of its expression in T cells, we studied the potential role of SLAP in regulating TCR signaling. Here we report that SLAP is expressed in T lineage cells, and, when expressed in the Jurkat T cell line, it can inhibit TCR signaling.

Results

Tissue- and cell-type specific expression of SLAP mRNA

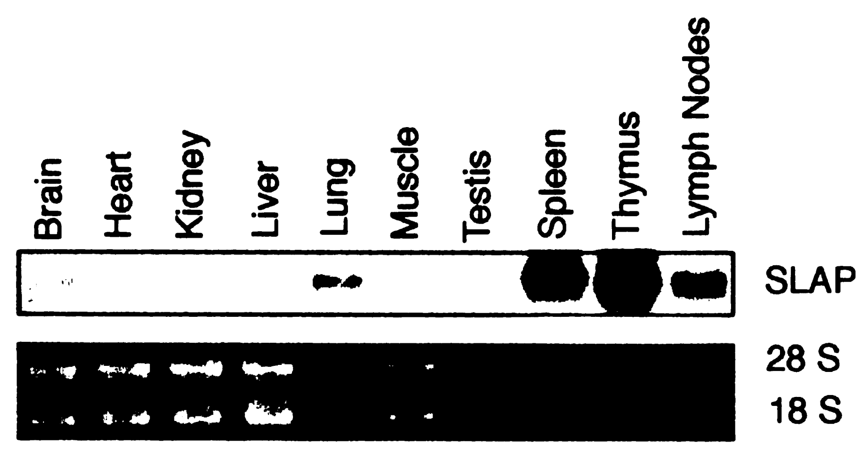
As the first step in our analysis of SLAP, we studied its tissue specific expression in mouse tissues and cell lines. SLAP mRNA is predominantly expressed in lymphoid tissues, though low levels can be detected in lung and brain (Figure 1A). To further elucidate the cell types that express SLAP mRNA, we compared message levels in purified splenic T cells, purified splenic B cells, and in various cell lines representing fibroblasts, macrophages, mast cells, B cells, and T cells (Figure 1B). Note that EL-4 thymoma was the only murine cell line we analyzed that expressed significant levels of the message. Upon longer exposure of the Northern blot, Raw264.7 and P815 had detectable levels, but NIH 3T3 cells did not. Strikingly, both primary T cells and primary B cells expressed significant levels of SLAP mRNA. Based on these data, we conclude that T lineage cells and B lineage cells are the predominant cell types expressing SLAP.

Effect of SLAP expression on TCR-induced transcriptional responses

Having demonstrated endogenous SLAP transcripts in thymocytes and primary T cells, we wanted to study the functional consequences of the presence of SLAP protein in T cells. We analyzed the effects of SLAP expression in Jurkat-TAg cells on one measure of TCR signaling - the transcriptional activity of the IL-2 promoter and its two elements, NFAT and AP-1. Although Jurkat and Jurkat-TAg cells expressed SLAP mRNA, we have not been able to detect SLAP protein in these cell lines (data not shown).

Figure 1. SLAP is predominantly expressed in the lymphoid tissues. Northern blot analysis was performed on multiple tissues (Figure 1A), various cell lines {fibroblast (NIH 3T3), macrophage (Raw264.7), mastocytoma (P815), B cell lines, and T cell lines}, and enriched splenic B and T cells (more than 92% and 98% pure, respectively; Figure 1B). A ^{32}P-labeled DNA fragment from the unique C terminus of SLAP cDNA was used to detect SLAP mRNA present in 10 μg of total RNA. Equal loading and complete transfer of RNA was confirmed by ethidium bromide staining and quantitation of ribosomal 28S and 18S RNA using BIORAD Gel Doc 1000 system (see the lower panel in A and B).

A



B

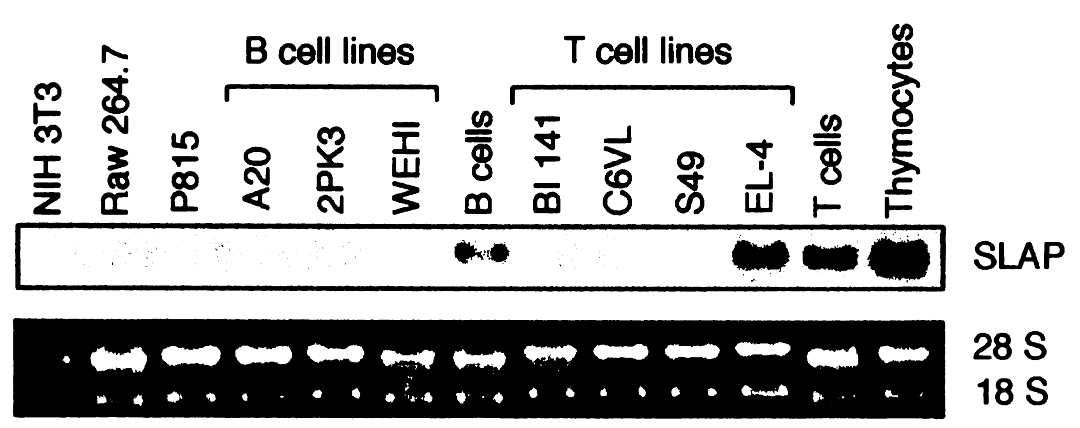
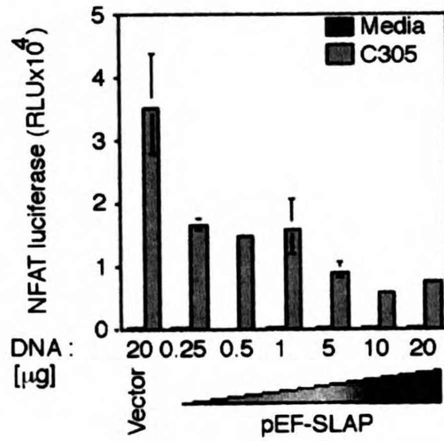
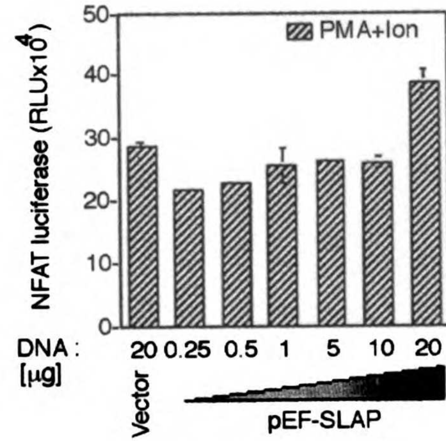
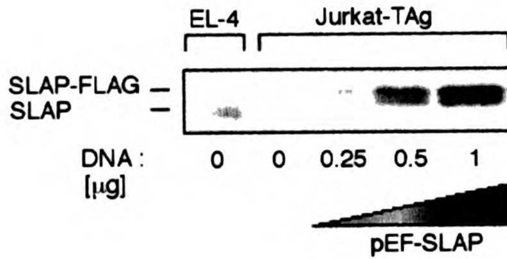


Figure 2. SLAP inhibits TCR induction of NFAT-driven transcription in a dose dependent manner. Jurkat T-antigen cells were cotransfected with four different plasmids: (1) a luciferase reporter under the transcriptional control of a multimerized NFAT element (NFAT luciferase), (2) a transfection efficiency indicator (pRc/βgal), (3) a transfection tag (pEF-TacT), and (4) an effector plasmid (either pEF-SLAP or an empty vector, pEF-Bos). The total amount of DNA was kept constant by supplementing the various amounts of pEF-SLAP plasmid with an empty vector. After 16-24 hours, cells from each transfection were tested for the transfection efficiency by β-galactosidase assay (data not shown), or incubated for additional 8 hours with medium alone (Media), soluble anti-TCR mAb (C305) or with PMA and ionomycin (PMA+ Ion), and assayed for luciferase activity. Luciferase activity was normalized to β-galactosidase and is presented in relative light units (RLU) (2A and 2B). Cells were also tested for the level of SLAP protein: transfections containing 0.25 μg, 0.5 μg, or 1.0 μg of pEF-SLAP were enriched for Tac^+ cells by magnetic beads (Figure 2 D), and used for immunoprecipitations of the ectopically expressed SLAP (Figure 2C, see materials and methods). As a positive and negative controls for the presence of murine SLAP, EL-4 and nontransfected Jurkat T-Ag cells were used, respectively.


A
Media
C305
NFAT luciferase (RLUx10^4)
5
4
3
2
1
0
DNA :
[μg]
20 0.25 0.5 1 5 10 20
Vector
pEF-SLAP


B
PMA+Ion
NFAT luciferase (RLUx10^4)
50
40
30
20
10
0
DNA :
[μg]
20 0.25 0.5 1 5 10 20
Vector
pEF-SLAP


C
EL-4
Jurkat-TAg
SLAP-FLAG
SLAP
DNA :
[μg]
0 0 0.25 0.5 1
pEF-SLAP

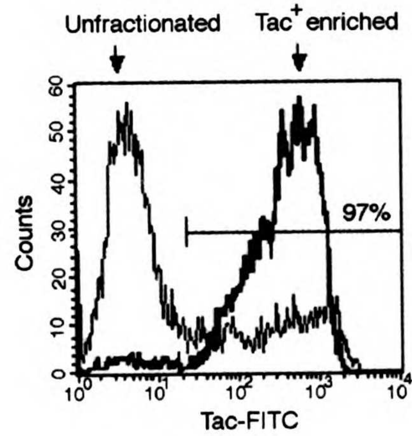
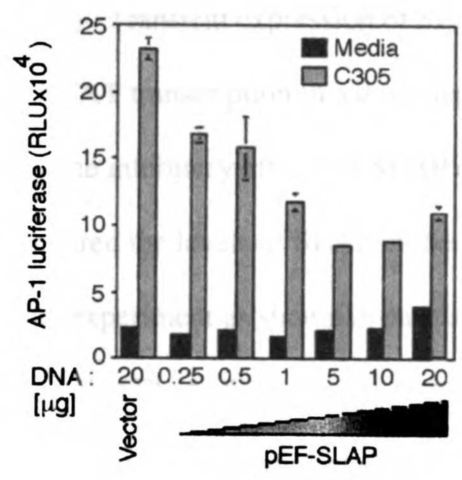
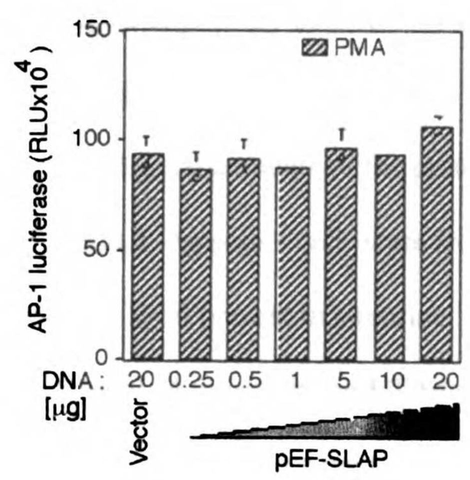
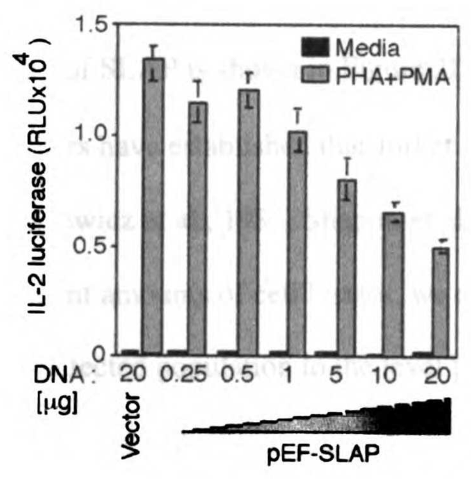
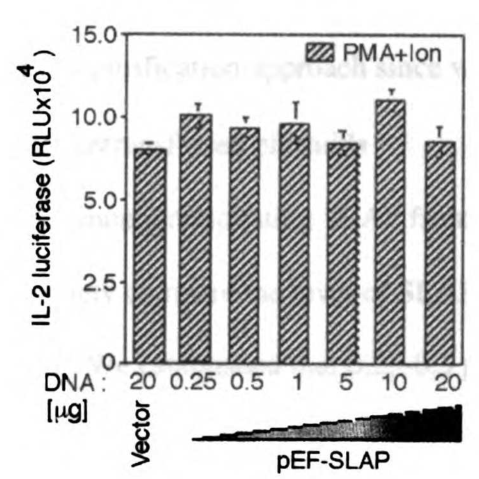

D
Unfractionated
Tac+ enriched
Counts
60
50
40
30
20
10
0
97%
10^0 10^1 10^2 10^3 10^4
Tac-FITC

Figure 3. SLAP inhibits TCR induction of both AP-1- and IL-2 promoter-driven transcription in a dose dependent manner. Cells were cotransfected with three different plasmids: (1) a luciferase reporter under the transcriptional control of either AP-1 element (3A and 3B) or IL-2 promoter (3C and 3D), (2) a transfection efficiency indicator (pRc/βgal), and (3) an effector plasmid (either pEF-SLAP or an empty vector, pEF-Bos). After 16-24 hours, transfected cells were assayed for β-galactosidase activity, and stimulated with either C305 (3A), PHA and PMA (3C), or combination of PMA and ionomycin as indicated (see materials and methods). The activity of each reporter is presented as described in Figure 2.


A
Media
C305
AP-1 luciferase (RLUx10^4)
25
20
15
10
5
0
DNA :
[μg]
20
0.25
0.5
1
5
10
20
Vector
pEF-SLAP


B
PMA
AP-1 luciferase (RLUx10^4)
150
100
50
0
DNA :
[μg]
20
0.25
0.5
1
5
10
20
Vector
pEF-SLAP


C
Media
PHA+PMA
IL-2 luciferase (RLUx10^4)
1.5
1.0
0.5
0
DNA :
[μg]
20
0.25
0.5
1
5
10
20
Vector
pEF-SLAP


D
PMA+Ion
IL-2 luciferase (RLUx10^4)
15.0
10.0
7.5
5.0
2.5
0
DNA :
[μg]
20
0.25
0.5
1
5
10
20
Vector
pEF-SLAP

We started our analysis by using a very sensitive reporter of TCR signaling, the luciferase gene under the control of an NFAT element arranged in triplicate (NFAT luciferase). Transient expression of SLAP in Jurkat T-Ag cells potently inhibited TCR-driven NFAT transcription in a dose-dependent manner (Figure 2A). To determine whether the inhibitory effects of SLAP can occur at physiological amounts of the protein we compared the levels of SLAP in these transfected Jurkat T-Ag cells (cells were from the same experiment as shown in panels A and B), with the level present in a murine thymoma line EL-4. To ensure appropriate comparison of EL-4 to only those J-TAg cells that were successfully transfected with SLAP, we also had cotransfected a tag to mark the transfected cells - a plasmid encoding the extracellular and transmembrane domains of Tac (IL-2 Receptor α-chain). By enriching for Tac-expressing cells using magnetic beads, we separated SLAP-expressing cells from the nontransfected cells (populations were more than 97% Tac-positive, e.g. the purification profile for cells transfected with 0.25 μg of SLAP is shown in Figure 2D). We used this purification approach since we and others have established that Jurkat cells coexpress cotransfected plasmids (Grynkiewicz et al., 1985; Shapiro et al., 1996). By immunoprecipitating SLAP from equivalent amounts of cell lysates, we could appropriately compare the level of SLAP in the transfected population to the level present in EL-4. We established that 0.25-0.5 μg of transfected pEF-SLAP plasmid resulted in comparable levels of SLAP protein to that present in EL-4 cells (2C). Significantly, even the transfection of 0.25 μg of pEF-SLAP plasmid significantly inhibited the NFAT reporter. These results indicate that SLAP can negatively regulate TCR signaling when expressed at approximately physiological (EL-4 cells) levels (Figure 2A and 2C).

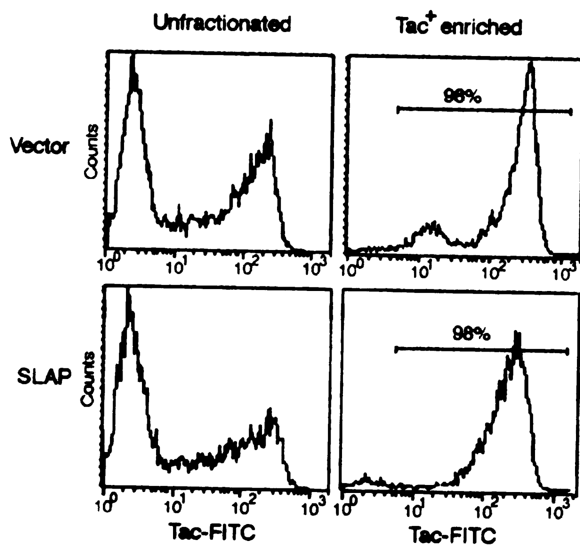
This suppressive effect was not limited to the NFAT element since SLAP could also inhibit TCR-dependent activation of AP-1 and IL-2 luciferase reporters (Figure 3A and 3C). To stimulate the IL-2 luciferase reporter we used a combination of PHA and PMA, an effective stimulus known to induce transcriptional activity of the IL-2 promoter in a TCR-dependent manner (Bram and Crabtree, 1994). SLAP did not interfere with the general transcriptional machinery in a nonspecific manner since it had no effect on NFAT, AP-1, or the IL-2 promoter in cells stimulated with PMA and the calcium ionophore (ionomycin) (Figure 2B, 3B, and 3D), nor did it suppress the luciferase activity under the control of the constitutive Rous sarcoma virus (RSV) promoter (data not shown). The ability of PMA and ionomycin, pharmacological agents that bypass proximal TCR signaling by activating Ras and increasing intracellular calcium, respectively, to activate transcription in the presence of SLAP places the SLAP-imposed blockade more proximal in the TCR signaling pathway than Ras activation and calcium increases. Moreover, TCR-dependent activation of both the Ras and Ca^{2+} pathways are inhibited by SLAP since stimulation of cells with anti-TCR antibody together with either PMA alone or ionomycin alone did not bypass the blockade (data not shown).

Functional effect of SLAP expression on TCR-induced Ca^{2+} flux and protein tyrosine phosphorylation

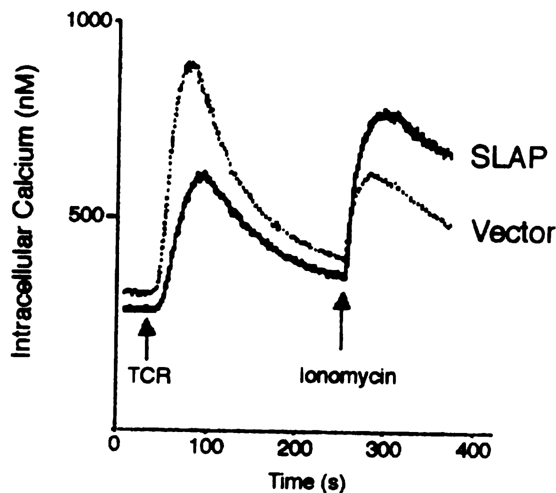
Since PMA and ionomycin treatment bypassed SLAP-dependent inhibition of NFAT induction, but anti-TCR plus PMA stimulation did not, we reasoned that SLAP might suppress the TCR-dependent Ca^{2+} flux. To examine this, we cotransfected SLAP or an empty vector, with a plasmid encoding Tac, and enriched for Tac-expressing cells

Figure 4. SLAP inhibits TCR-dependent Ca^{2+} mobilization in Jurkat cells. J-TAg cells were transiently cotransfected with three plasmids: (1) NFAT luciferase, (2) pEF-TacT, and (3) pEF-SLAP or an empty vector. 16 hours later, transfected cells were enriched based on expression of Tac on the cell surface (see materials and methods). Highly enriched populations (A) were loaded with the fluorescent dye indicator Indo-1, stimulated with soluble anti-TCR mAb (C305) followed by stimulation with ionomycin (arrows indicate the time of stimulation), and the change in intracellular Ca^{2+} concentration was measured as function of time (B). Cells from the same experiment were also tested for TCR-dependent NFAT induction (C).

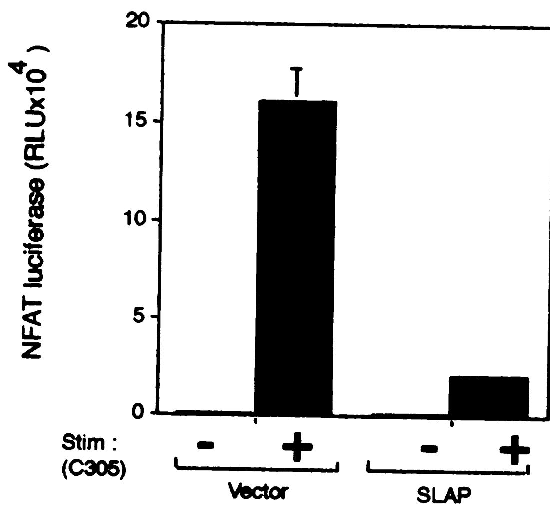
A



B



C

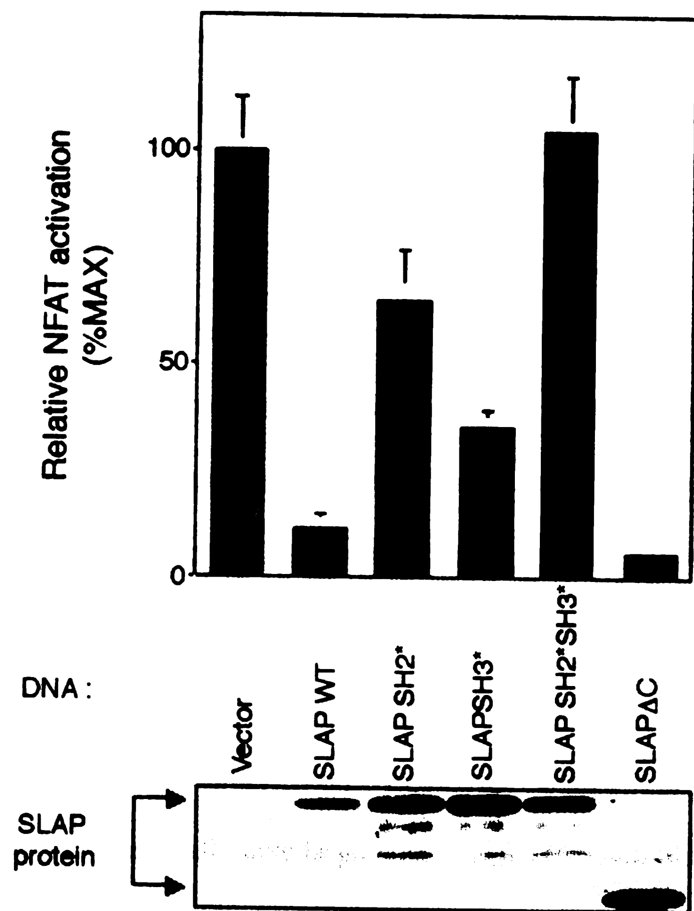


as described above (populations were more than 98% Tac-positive, Figure 4A). As shown in Figure 4B, cells transfected with SLAP, when compared to the vector transfected cells, showed a substantially reduced calcium flux response to anti-TCR stimulation. Importantly, this pattern of responsiveness was reversed upon subsequent stimulation of cells with ionomycin. Note that SLAP-transfected cells responded to the calcium ionophore by mobilizing more of intracellular Ca^{2+} than vector transfected cells. As a control in this same experiment, we also transfected the NFAT-luciferase reporter plasmid to show that SLAP was expressed at sufficient levels to inhibit the TCR signaling pathway leading to NFAT activation (Figure 4C). We employed the same approach of cotransfection and enrichment for Tac^+ cells to study the effect of SLAP on TCR-dependent increase in protein tyrosine phosphorylation. However, we failed to observe any differences in the pattern of tyrosine phosphorylation in total cell lysates from TCR-stimulated cells when SLAP was expressed (data not shown).

Contribution of the SH2, SH3, and the unique C-terminal domains to the inhibitory function of SLAP

To study the mechanism by which SLAP inhibits TCR signaling, we studied the role of the SH2, SH3, as well as the unique C-terminus of SLAP. As shown in Figure 5, inactivation of the SLAP SH2 domain, by replacing an arginine with a glutamic acid in the "FXXR" motif in the phosphotyrosine binding pocket, had a profound effect on the SLAP inhibitory activity. The SLAP SH2 mutant displayed only a weak inhibitory effect despite being expressed at higher levels than the wild type (WT) protein. Inactivation of

Figure 5. Both SH2 and SH3 domains of SLAP participate in inhibition of anti-TCR induced NFAT activity. Jurkat T-antigen cells were cotransfected with an NFAT luciferase reporter and various amounts of one of the following effector plasmids: a wild type SLAP (WT), an SH2 domain point mutant (SH2*), an SH3 domain point mutant (SH3*), an SH2 and SH3 domain double point mutant (SH2*SH3*), and a mutant with deleted C-terminus (ΔC). An empty vector was used as a control, and the total amount of DNA was kept constant by adding empty vector DNA. Individual transfections were matched based on the expression level of the various SLAP proteins (Figure 5, lower panel). TCR-dependent increase in NFAT activity was calculated as a fold induction, and represents a ratio of luciferase counts of anti-TCR- over media-treated cells. Fold induction obtained for vector transfected cells was defined as maximum (100% MAX).



Relative NFAT activation
(%MAX)
100
50
0
DNA :
Vector
SLAP WT
SLAP SH2*
SLAPSH3*
SLAP SH2*SH3*
SLAPΔC
SLAP
protein

the SH3 domain, by replacing a conserved proline with leucine at position 73 (Weiss et al., 1984), had a more modest effect on SLAP inhibitory function. However, when both the SH2 and the SH3 domains were mutated in the same construct, no inhibition of TCR signaling was observed, suggesting that both domains contribute to the SLAP inhibitory function in an additive manner. In contrast, the unique carboxyl terminus of SLAP did not play any role in the inhibition, since a mutant lacking this domain (SLAP ΔC) suppressed NFAT induction to the same extent as the WT protein.

Subcellular distribution of SLAP

We extended the structure-function analysis of SLAP to include subcellular localization of the WT protein and the various mutants. Indirect immunofluorescence staining showed perinuclear localization of SLAP in transiently transfected Jurkat T-Ag cells (Figure 6B). This staining was similar to that of an intracellular pool of Lck in Jurkat cells, which was reported to colocalize with the endosomal marker cation-independent mannose 6-phosphate receptor (CI-M6PR) (Eck et al., 1994). However, because Jurkat cells are small, have large nuclei, and a thin rim of cytoplasm, thereby preventing good resolution of cellular structures, we used HeLa cells to aid in a more precise SLAP cellular localization. Using indirect immunofluorescence to identify CI-M6PR (Figure 6D, green) and SLAP (Figure 6E, red), then overlaying the images, the appearance of yellow confirms that the two molecules colocalize (Figure 6F). Note, that of two cells present in the field (Figure 6D) only one was transfected and expressed SLAP (Figure 6E). The same pattern of staining was obtained with two different primary antibodies recognizing distinct epitopes on transfected SLAP (data not shown; see

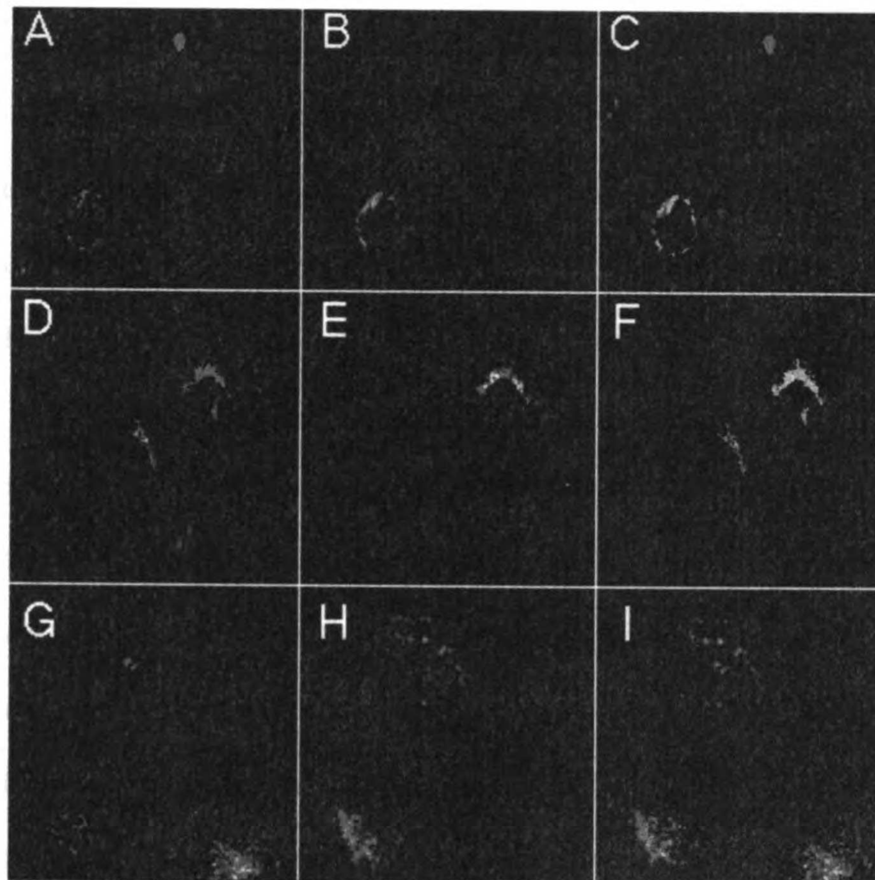
materials and methods). The observed SLAP staining was specific, since vector transfected cells did not stain with either antibody (data not shown).

Next, we asked which of the domains of SLAP played a role in its colocalization with endosomes by transfecting various mutants into Jurkat T-Ag or HeLa cells and staining for SLAP and CI-M6PR. Mutants with the inactivated SH2 domain (SH2* and SH2*SH3*) were the only ones that did not correctly colocalize with endosomes. Instead they displayed diffuse vesicular staining (Figure 6G, H, I, and data not shown). This difference in staining indicates that the endosomal localization of WT SLAP was not an artifact of overexpression since both WT and SH2 mutants were expressed at similar levels. The same pattern of staining was obtained for Jurkat cells (data not shown). Collectively, these data suggest that the SH2 domain of SLAP targets it, most likely by tyrosine phosphoprotein(s), to the cytoplasmic face of endosomes.

Insolubility of SLAP in mild detergents

During the course of our studies, we discovered that SLAP protein is insoluble in mild detergents (Figure 7). The insolubility of SLAP does not depend upon the actin cytoskeleton or on interactions with microtubules since its insolubility was not sensitive to cytochalasin D or nocodazole (data not shown). This property is dependent on the unique C-terminal domain, since the mutant lacking this domain partitioned to the 1 % NP-40 soluble fraction. The equal distribution of SLAP ΔC into soluble and pellet fractions in Figure 7 (experiment 1) was due to high levels of expression, since the same mutant was present predominantly in the soluble fraction when expressed at lower levels in experiment 2. SLAP insolubility does not correlate with its inhibitory function

Figure 6. The SLAP SH2 domain is required for its colocalization with endosomes. Jurkat (A, B, and C) and HeLa cells (D thru I) were transiently transfected with pEF-SLAP WT plasmid (A thru F) or pEF-SLAP SH2* mutant (G, H, and I), and the indirect immunofluorescence analysis was performed (see materials and methods). The endogenous endosomal marker, CI-M6P receptor, is present in all cells (green image), however, SLAP is present only in transiently transfected cells (red image). To depict the degree of colocalization of SLAP and CI-M6PR, the red and green channels were merged (C, F, and I). Each image represents a single section thru a field of cells.



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because, as shown in Figure 4, SLAP ΔC was as potent in inhibiting NFAT induction as the WT SLAP.

A specific subset of phosphoproteins interacts with SLAP

Given the critical importance of the SH2 domain for SLAP function, we wished to identify the tyrosine phosphorylated proteins that interact with SLAP. Since SLAP WT is insoluble in the mild detergents that are used for solubilization and are necessary to preserve protein-protein interactions, we were unable to perform direct immunoprecipitations of WT SLAP protein. Instead, we used two different approaches to identify the phosphoproteins that interact with SLAP. First, we used a GST-pulldown experiment to detect *in vitro* interactions. Among many inducibly tyrosine phosphorylated proteins present in Jurkat lysates, phosphoproteins of 24 KDa molecular weight (pp24), pp36/38, pp76, and pp95 were consistently bound by GST-SLAP (Figure 8, see arrowheads and a bracket). Other bands present in Figure 8 , notably the bands corresponding to putative pp34, pp46, and pp60, were not consistently seen. GST did not retain any substantial amounts of phosphoproteins by itself, indicating the specificity of the interactions. Notice that an almost identical set of proteins was retained by the GST-Lck SH2 domain, but not by the GST-Grb2 construct. These results suggest that a subset of TCR-induced tyrosine phosphoproteins interact specifically with SLAP *in vitro*.

Our second approach was aimed at identification of *in vivo* interactions and took advantage of the solubility of the SLAP ΔC mutant. We reasoned that this mutant should bind to the same target phosphoproteins as WT SLAP since both proteins similarly

Figure 7. The unique C-terminus of SLAP determines its insolubility in mild detergents. Jurkat cells were transiently transfected with an expression plasmid encoding either WT SLAP or the indicated mutants. After 24 hours cells were lysed in 1% NP-40 lysis buffer, and the detergent soluble (S) and the pellet (P) fractions were separated by standard centrifugation procedure (see materials and methods). Pellet fractions were solubilized in the SDS-PAGE loading buffer and cell-equivalent amounts were run on SDS-PAGE. SLAP protein was detected by Western blotting using rabbit anti-SLAP C1661 Ab. EXP, experiment; MWM, molecular weight marker.

inhibited TCR-induced NFAT activation, and they both localized to the same cellular structures. We compared anti-SLAP immunoprecipitations (IPs) from cells transfected with SLAP ΔC with anti-Lck IPs from cells transfected with an empty vector (Figure 9). Lack of phosphoproteins in anti-SLAP IPs from cells transfected with vector alone indicates the specificity of the interactions. In addition to the phosphoproteins already identified by GST-pulldown experiments (pp24, pp36/38, pp76, pp95; Figure 8), we consistently observed three new species in both SLAP and Lck IPs: pp50, pp56, and pp70 (Figure 9, see arrowheads and a bracket). To identify the proteins detected by anti-phosphotyrosine blotting, we reprobed the blot with antibodies specific for the proteins known to participate in TCR signaling and having similar mobility on SDS-PAGE: pp24 was confirmed to contain CD3-ζ chain; pp50 contained α-tubulin; pp56 contained Lck; pp70 contained ZAP-70; pp76 contained SLP-76; and, pp95 contained Vav. pp36/38 is likely to represent LAT, but we could not confirm this by Western blotting with anti-LAT heteroserum, perhaps due to the difficulty in detecting phospho-LAT with this reagent. Although pp36/38 and pp50 were predominantly present in SLAP ΔC IPs, we could also detect pp36/38 in Lck IPs on longer exposure of the blot. These results demonstrate that SLAP interacts, both *in vitro* and *in vivo*, with many proteins known to participate in TCR signaling.

Figure 8. SLAP interacts in vitro with a specific subset of phosphoproteins. Lysates from unstimulated (-) or stimulated (+) Jurkat T- Ag cells were used in the GST-pulldown experiment with the indicated fusion proteins (see materials and methods). Tyrosine phosphorylated proteins which interacted with the fusion proteins were resolved by SDS-PAGE and detected with the anti-PTyr mAb (4G10). Soluble whole cell lysates (WCL) were used as a control for stimulation. Molecular weight markers are indicated on the left and the phosphoproteins consistently present in GST-SLAP pulldowns are marked by arrowheads and a bracket to the right.

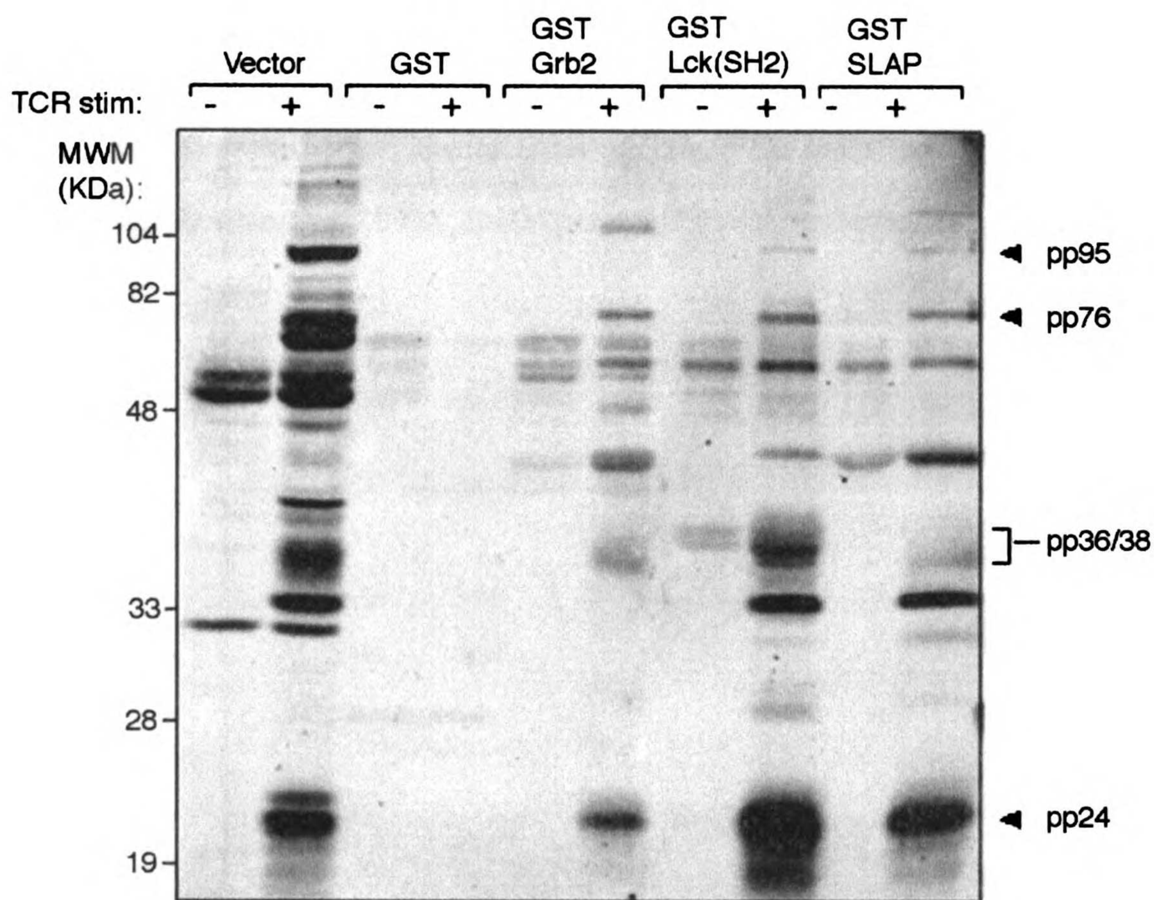

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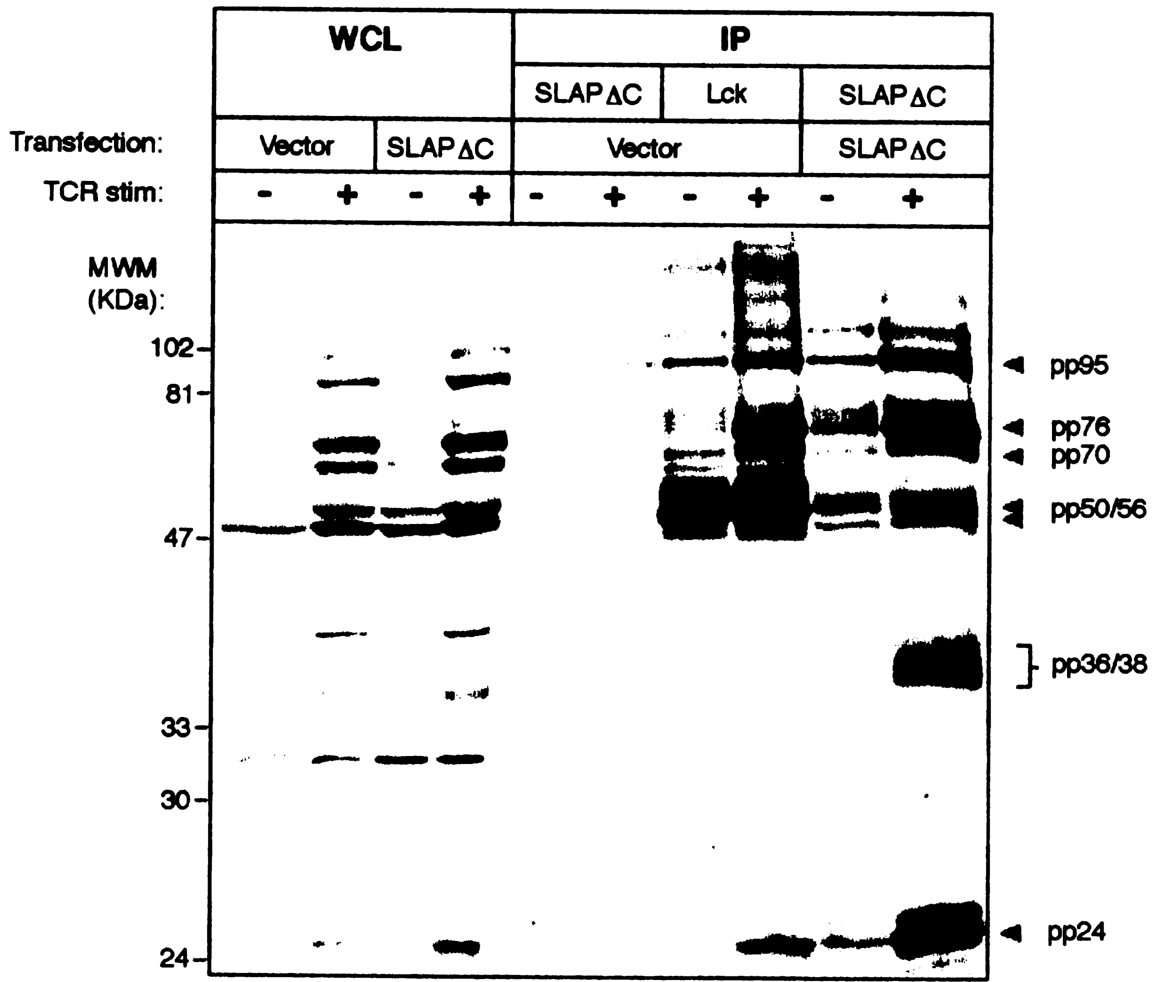
Figure 9. SLAP interacts in vivo with a specific subset of phosphoproteins. Jurkat T-Ag cells were transiently transfected with either an empty vector or pEF-SLAP ΔC. After 12-16 hours, cells were either left unstimulated (-) or stimulated with soluble anti-TCR Ab (C305) for 2 minutes (+), and lysed in 1% NP-40 lysis buffer. Equivalent amounts of lysates were used in anti-Lck and anti-SLAP IPs (see materials and methods). Subsequently, whole cell lysates or washed immunoprecipitates were separated by SDS-PAGE and probed with anti-PTyr mAb (4G10). MWM, molecular weight marker.


WCL
IP
SLAP ΔC
Lck
SLAP ΔC
Transfection:
Vector
SLAP ΔC
Vector
SLAP ΔC
TCR stim:
- + - + - + - + - +
MWM
(KDa):
102
81
47
33
30
24
pp95
pp76
pp70
pp50/56
pp36/38
pp24

Discussion

In this report, we found that SLAP can function as a negative regulator of TCR signaling. The T cell is a relevant cell type for studying SLAP function since SLAP is expressed in T and B lineage cells. Overexpression of SLAP in Jurkat T cells potently inhibited the TCR-dependent increase in NFAT-driven transcription. Inhibition of AP-1 and IL-2 transcription was also observed. A structure-function analysis revealed that both the SH3 and SH2 domains contribute to this inhibitory function. Wild type SLAP protein colocalized with the cation-independent mannose-6-phosphate receptor associated with endosomes, but SLAP mutants with inactive SH2 domain exhibited an altered diffuse, vesicular pattern. SLAP was insoluble in mild detergents and this property depended on the unique carboxyl terminus. In stimulated Jurkat cells SLAP associated with many proteins from the TCR signaling complex. Collectively, these results suggest that SLAP interacts with the stimulated TCR complex to down-regulate TCR-mediated signals.

It was originally thought that SLAP RNA is expressed ubiquitously among various tissues (Pandey et al., 1995) and endogenous protein was detected in NIH 3T3 fibroblasts (Roche et al., 1998). Here, we extended the study of SLAP expression to immune tissues and several cell lines representing different cell types. We confirm the finding of Pandey et al. that spleen expresses high level of SLAP mRNA, but we find that high level of expression is restricted to the immune tissues, with the highest level present in thymus. In addition, among various cell types tested only thymocytes, the purified primary T cells and B cells, and a T cell line (EL-4), expressed high levels of SLAP

message. Our studies used Northern blots containing 10 μg of total RNA (Figure 1) instead of 2 μg of enriched poly-A messenger RNA (21). Therefore, we could detect only relatively high level of expression in lymphoid tissues. Unlike the data of Roche et al., who used a heterologous rabbit serum and reported that NIH 3T3 fibroblast expressed endogenous SLAP protein, we were unable to detect SLAP RNA in NIH 3T3 cells. This difference might be due to clonal variability. However, we would suggest that high level of SLAP expression is specific for the immune tissues, and that T cells and B cells are predominant cell types expressing it.

Structural resemblance of SLAP to Src-family kinases and the recent finding that microinjected SLAP can negatively regulate Src-dependent receptor signaling pathway of the PDGFR in fibroblasts (Roche et al., 1998) suggested that SLAP could be a common negative regulator of signaling pathways dependent on activity of Src-family members. Since TCR signaling requires Src-family (Lck or Fyn) kinase activity (Straus and Weiss, 1992; van Oers et al., 1996), we tested the effects of SLAP overexpression in Jurkat cells on TCR signaling. Consistent with the role of SLAP in PDGFR signaling, we find that SLAP overexpression in Jurkat T cells significantly inhibited TCR-induced signal transduction as measured by transcriptional activity of IL-2 promoter and two of its integral elements, NFAT and AP-1. SLAP did not inhibit general transcription in a nonspecific manner since a constitutively active promoter of Rous sarcoma virus was not affected. These results suggest that the presence of adequate levels of SLAP protein in T cells could render them unresponsive to TCR signaling. Consistent with this hypothesis we found that Jurkat cells, which respond TCR stimulation very well, do not express detectable endogenous SLAP protein. However, EL-4 cells, which respond to anti-TCR

engagement very poorly, express SLAP at the level which, when ectopically expressed in Jurkat, is sufficient to significantly inhibit NFAT induction. Preliminary studies suggest that the level of SLAP protein in T cells may be post-transcriptionally regulated. We found that thymocytes, despite expressing high level of RNA, express very low level of SLAP protein (data not shown). Moreover, resting T cells, a population of T cells in which majority of cells can be activated by engagement of their TCRs, express SLAP RNA but no detectable SLAP protein (as determined by immunoprecipitations followed by Western blotting; data not shown). Stimulation of both populations of cells has little effect on RNA levels, but protein levels are induced. Further studies will be required to understand the exact mechanism responsible for the regulation of SLAP protein levels.

The inhibitory effects of SLAP appear to influence proximal events involved in TCR signaling since ionomycin- and PMA-mediated stimulation of IL-2, AP-1 and NFAT directed transcription was insensitive to SLAP inhibition. The ability of SLAP to uncouple TCR signaling from transcriptional activation of NFAT may, in part, be due to reduced levels of TCR-induced calcium fluxes. Though incomplete, an attenuated TCR-induced Ca^{2+} flux was observed in the presence of SLAP. Note, anti-TCR stimulation in conjunction with PMA, which should bypass the SLAP-imposed blockade of the PKC/Ras pathway, could not restore NFAT-driven transcription (data not shown). This result indicates that TCR signaling did not generate a sufficient calcium signal in the presence of SLAP to synergize with PMA. Interestingly, J14 cells, a Jurkat mutant deficient in SLP-76 expression, displayed a similar partial reduction in TCR stimulation-induced Ca^{2+} flux and were also unable to upregulate NFAT-driven transcription when stimulated with anti-TCR antibody and PMA (Yablonski et al., 1998). Similar epistasis

studies suggest that SLAP blocks the function of the PKC/Ras pathway (data not shown). However, we have failed to detect any influence of SLAP on the pattern of TCR induced protein tyrosine phosphorylation (data not shown). This is not a completely surprising finding since analysis of protein tyrosine phosphorylation in the whole cell lysates is a very crude way to assess the integrity of TCR signaling pathway. For example, the only difference observed in SLP-76 deficient J14 cells was the lack of tyrosine phosphorylation of the band corresponding to SLP-76 itself, despite the fact that PLC-γ1 phosphorylation was also impaired (Yablonski et al., 1998). Additional studies will be required to determine whether SLAP prevents tyrosine phosphorylation of specific substrates or serves to relocalize them, for example by targeting them to endosomes.

Our structure-function analysis showed additive contributions of both the SH3 and SH2 domains of SLAP to its inhibitory function, with the SH2 domain having a stronger effect. Even though no functional effect of the C-terminus of SLAP on TCR signaling was observed, we speculate that this unique 104 amino acid region might play a role in regulating the protein stability since it contains potential PEST sequences (Rechsteiner, 1990), thereby influencing the level of SLAP protein available to regulate the TCR response. Curiously, this unique C-terminal part renders SLAP insoluble in mild detergents.

SLAP protein accumulated in the perinuclear structures in Jurkat cells. Indirect immunofluorescence analysis of ectopically expressed SLAP in HeLa cells enabled a better resolution of cellular structures and confirmed colocalization of SLAP with an endosomal marker, the cation independent mannose-6-phosphate receptor (CI-M6PR). Interestingly, similar localization was reported for an intracellular pool of Lck (Ley et al.,

1994). Because our functional data place SLAP-dependent blockade upstream of effects caused by PMA and ionomycin, we were surprised that, unlike Lck, no plasma membrane staining was observed for SLAP. However, recruitment of SLAP to the plasma membrane might require TCR stimulation and it would be difficult to detect this in transfected Jurkat cells where only a thin rim of cytoplasm is seen in immunofluorescent microscopic studies. Moreover, the indirect immunofluorescence analysis might not be sensitive enough to detect low, yet functionally significant, amounts of SLAP in the plasma membrane. A similar observation was reported for CTLA 4 protein where only the intracellular, but not the plasma membrane, localization was detected by immunofluorescence staining (Thompson and Allison, 1997). Therefore, like CTLA 4, SLAP protein may rapidly shuttle from the plasma membrane to endosomes. Such shuttling to the plasma membrane may depend upon TCR signaling. Since the inhibitory effects of SLAP and its localization depended upon its SH2 domain, SLAP could play a role in downregulating the stimulated TCR complex.

Having established that SLAP interferes with the proximal events of TCR signaling and knowing that it structurally resembles Lck, we hypothesized that both proteins could interact with a similar set of targets. Indeed, the pattern of phosphoproteins interacting *in vitro* and *in vivo* with both proteins is very similar. Notably, tyrosine phosphorylated TCR ζ chain, ZAP-70, SLP-76, and Vav, as well as a 36/38 KDa protein which likely represents LAT, were all present in the SLAP-interacting complexes. We were unable to confirm these results with an endogenously expressed SLAP in EL-4 cells since SLAP protein is insoluble in mild detergents necessary to

preserve protein-protein interactions. These data showed that SLAP and Lck can interact with TCR-containing signaling complexes of similar composition.

Since the original submission of this manuscript, another study examining the role of SLAP in T cells was reported by Tang et al. (Tang et al., 1999). Although both studies used transient transfection system in Jurkat cells, Tang et al. found that expression of a GST-SLAP fusion protein potentiated TCR-induced NFAT responses, in marked contrast to the inhibitory effects we observed. The apparent difference could relate to the use of a GST-fusion of SLAP by Tang et al., rather than epitope-tagged proteins used by us. We confirmed our results by using a different construct encoding a myc-tagged human SLAP (data not shown). Moreover, Tang et al. used only a single dose of GST-SLAP plasmid, whereas we used a broad range of SLAP-encoded plasmid and found a dose-dependent inhibitory effect on IL-2, NFAT and AP-1 reporters. Nonetheless, despite the differences, our results are in agreement with findings of Tang et al. that SLAP interacts with a set of proteins relevant to TCR signal transduction.

In conclusion, the data presented here demonstrate that SLAP can function as a negative regulator of TCR function by interfering with the proximal signaling events. Therefore, the differential expression of SLAP protein in T cells could potentially regulate sensitivity to TCR stimulation thus influencing thymocyte development and mature T cell responses to antigenic stimulation. We are currently testing this hypothesis using SLAP deficient mice.

Experimental Procedures

Cells lines and reagents

Jurkat cells or Jurkat cells transfected with the simian virus 40 (SV40) large T-antigen (J-TAg cells; G. Crabtree, Stanford University) and murine cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin, streptomycin, and glutamine. HeLa cells were passaged in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% FCS. The antibody used for TCR stimulation of J-TAg cells was C305, an anti-Ti β chain mouse monoclonal antibody (mAb) (Weiss and Stobo, 1984). Blotting antibodies were as follow: an anti-FLAG M2 mAb (SIGMA); an anti-phosphotyrosine (PTyr) mAb, 4G10 (Upstate Biotechnology, INC); an anti-CD3ζ mAb, 6B10.2 (van Oers et al., 1995); rabbit anti-LAT serum (Zhang et al., 1998); sheep anti-SLP-76 serum (Motto et al., 1996); anti-ZAP-70 mAb, 2F3.2 (Iwashima et al., 1994); rabbit anti-Vav serum (Santa Cruz Biotech.); rabbit anti-α-Tubulin serum (SIGMA); and anti-Lck mAb, 1F6 (kindly provided by Dr. J. Bolen, Bristol-Meyers Squibb). Rabbit serum (C1661) directed against the N-terminal amino acids (6-25) of murine SLAP was generated in our laboratory. Two secondary reagents were used: a Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Southern Biotech. Associates Inc.), and HRP-conjugated protein-A (Amersham). For immunofluorescence studies, we used rabbit anti-murine SLAP C1661 polyclonal Ab or the M2 anti-FLAG mAb to detect FLAG-tagged murine SLAP, or rabbit anti-CI-M6PR polyclonal antiserum (kind gift of F.Brodsky) to detect CI-M6PR. FITC-conjugated goat anti-rabbit IgG (Gibco BRL) or

Texas Red-conjugated goat anti-mouse IgG (CALTAG) were used as secondary antibodies.

DNA constructs

All expression constructs used to express SLAP protein contained murine SLAP cDNA fused to the FLAG epitope tag at its C-terminus. SLAP cDNA-FLAG inserts were flanked by BamHI/XhoI sites, and were subcloned into BamHI/SalI sites of pEF-BOS X-C, a mammalian expression vector (Mizushima and Nagata, 1990). The pEF-SLAP SH3* had a CCA to CTC mutation changing a P to L at position 73; the pEF-SLAP SH2* had a CGA to GAA mutation changing an R to E at position 144; pEF-SLAPΔC expressed a truncated version of SLAP including the N-terminus, the SH3 and SH2 domains, and 10 amino acids of the unique C-terminus (i.e. aa 1-187). GST-SLAP, GST-Lck(SH2), and pEF-TacT were constructed as described (Pandey et al., 1995; Straus et al., 1996) (Lynch, K. and Weiss, A., in press, MCB). The Nuclear Factor of Activated T cells (NFAT) luciferase and IL-2 luciferase reporters were provided by G. Crabtree (Stanford University). AP-1 luciferase was previously described (Lynch and Weiss, 2000). pRc/βgal was a kind gift from M. Karin (University of California, San Diego).

Northern blot analysis

Tissues were derived from C57BL/6 mice. Total RNA was extracted using RNAzol (Tel-Test, INC.). 10 μg of total RNA per sample were stained with ethidium bromide, resolved on agarose gel, and transferred onto Magna Charge Nylon membrane (Micron Separations INC.). The blot was hybridized with a [^{32}P]dCTP-labeled

XcmI/ScaI fragment encompassing 330 nts encoding the unique C-terminus of murine SLAP.

Transfections and luciferase assays

$2x10^7$ Jurkat-TAg cells were resuspended in 400 μl of serum free RPMI media containing plasmid DNA, placed in a 0.4 cm cuvette, and electroporated at 250 V, 960 μF using the BioRad Gene Pulsar Electroporator. After 16-24 hours, cells were harvested, and stimulated with soluble anti-TCR antibody C305 (1:1000 dilution of ascites), PHA (1μg/ml), PMA (50 ng/ml), or ionomycin (1μM) or the indicated combination of reagents. After 8 hours, cells were lysed and assayed for luciferase activity as described (Shapiro et al., 1996). All transfections included the pRc/βgal plasmid (Wu et al., 1995) to normalize for differences in gene transfer by assay of β-galactosidase activity, which was performed using chemiluminescence assay system (TROPIX, Inc.).

Cell enrichment and immunoprecipitations

J-TAg cells were transiently cotransfected with pEF-TacT and the indicated effector plasmids. After 12 hours, cells were harvested, washed in PBS and resuspended in MACS purification buffer (PBS, 0.5% BSA, 2mM EDTA). Cells were then stained with FITC-conjugated anti-Tac mAb (anti-CD25-FITC, Becton Dickinson) followed by anti-FITC MultiSort MicroBeads (Miltenyi Biotec), and purified on a VS+ column according to manufacturer's protocol (Miltenyi Biotec). The purity of isolated cells was determined using flow cytometry (FACScan, Becton Dickinson). Tac^+ enriched populations were lysed in lysis buffer containing 1% NP-40, 10 mM Tris (pH 7.8), 150

mM NaCl, 2 mM EDTA, and protease and phosphatase inhibitors. Lysates were normalized for protein concentration using the Bradford assay (Bio-Rad). After addition of SDS to a final concentration of 1%, lysates were incubated on ice for 10 minutes, boiled for 5 minutes, diluted to final 0.5% SDS with 1% NP-40 lysis buffer, briefly sonicated to shear the genomic DNA, and centrifuged at 100,000g for 30 minutes. Soluble lysates were incubated with 1 μg of rabbit anti-murine SLAP C1661 polyclonal Ab crosslinked to Protein A-Sepharose beads (Pharmacia Biotech AB) for 2 hours. Beads were washed four times with 1% NP-40 buffer and boiled 5 minutes in SDS sample buffer. A standard protocol for Western blotting was followed: proteins were separated by SDS-PAGE, transferred to Immobilon-P (Millipore) membrane and immunoblotted with C1661 antibody followed by a protein A-conjugated to horseradish peroxidase (Amersham). Enhanced chemiluminescence was used to detect immunoreactive proteins (Amersham).

Measurement of intracellular calcium

Tac-positive cells were selected as described above and cultured for 2 hours to allow cell recovery from the purification procedure. To assay intracellular calcium concentration, cells were loaded with Indo-1 (Molecular Probes), washed, then stimulated with C305 mAb (1:1000) followed by stimulation with ionomycin (1μM). The fluorescence intensities at 400 and 500 nm wavelengths were measured using a Hitachi F-4500 fluorescence spectrofluorimeter and were used to calculate concentration of intracellular calcium (Helmberg et al., 1995).

Immunofluorescence microscopy.

J-TAg were transiently transfected as above, incubated for 12-16 hours, and seeded onto poly-L-lysine -coated slides. HeLa cells were transiently transfected using the calcium phosphate method, and incubated on a chambered coverglass (Lab-Tek) to allow for cell adhesion. Next, J-TAg or HeLa cells were fixed in 3.4% paraformaldehyde at room temperature for 20 minutes, followed by a 4-minute permeabilization in 0.1% Triton X-100 in PBS, and 10 minutes of blocking in 0.2% BSA in PBS. Cells were incubated with primary antibodies for 30 minutes, washed twice with 0.2% BSA in PBS, and stained with secondary antibodies. Mowiol (CALBIOCHEM) supplemented with 2.5% DABCO (SIGMA) was used to mount the coverslips and prevent photobleaching. Confocal fluorescence microscopy was performed using a Bio-Rad MRC1000 confocal imaging system interfaced to a Zeiss inverted microscope equipped with a 100X NA 1.3 objective.

GST-pulldowns, immunoprecipitations (IPs), and Western blotting.

Jurkat cells were left untreated or stimulated with C305 mAb (1:1000 dilution of ascites) for 2 minutes and lysed in 1% NP-40 lysis buffer. Procedures for GST-pulldowns, and IPs were previously described in detail (Straus et al., 1996). For GST-pulldown experiments, 2 μg of each GST fusion protein were incubated with lysates obtained from 5 x 10^7 cells for 2 hour. For IPs, 1 μg of rabbit anti-SLAP C1661 affinity purified Ab, or 1 μg of anti-Lck 1F6 mAb was incubated with lysates obtained from 3 x 10^7 transfected cells. Both antibodies were crosslinked to Protein A-Sepharose beads

Acknowledgments

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CHAPTER III

SLAP DOWNREGULATES THE T CELL RECEPTOR ON $CD4^{+}CD8^{+}$ THYMOCYTES AND REGULATES POSITIVE SELECTION

Summary

In this report we show that the Src-like adaptor protein (SLAP) plays an important role in thymocyte development. SLAP expression is developmentally regulated; it is low in $CD4^-CD8^-$ thymocytes, it peaks in the $CD4^+CD8^+$ subset, and it decreases to low levels in more mature cells. Disruption of the *SLAP* gene leads to a marked upregulation of TCR and CD5 expression at the $CD4^+CD8^+$ stage. The absence of SLAP was also developmentally significant as it enhanced positive selection in mice expressing the DO11.10 transgenic T cell receptor. Moreover, SLAP deletion at least partially rescued the development of *ZAP-70* deficient thymocytes. These results demonstrate that SLAP participates in a novel mechanism of TCR downregulation at the $CD4^+CD8^+$ stage, and regulates positive selection. Work presented in this chapter has been accepted for publication in Immunity: 15(3), September, 2001.

Introduction

Thymic development is responsible for generating mature T cells capable of recognizing a diverse set of pathogen-derived peptides. Three major populations of developing thymocytes can be defined on the basis of their ordered expression of CD4 and/or CD8 coreceptors (Sebzda et al., 1999; Wiest et al., 1999). These populations are: (1) an early double negative (DN) lacking both coreceptors; (2) an intermediate double positive (DP) expressing both; and, (3) the most mature single positive (SP) marked by either CD4 or CD8. *TCRβ* gene rearrangement occurs at the DN stage and leads to the expression of a pre-T cell antigen receptor, expression of which is required for progression to the DP stage. *TCRα* gene rearrangement takes place at the DP stage, and, when successful, leads to expression of T cell receptor (TCR) αβ heterodimers. It is the specificity of the individual clonally distributed TCRs that determines the fate of thymocytes during the subsequent process of selection.

TCR diversity is generated by random rearrangements at the *TCRα* and *β* gene loci. While this creates a large repertoire of thymocytes with unique antigen receptors, many thymocytes express either non-functional or self-reactive TCR. Such clones are subsequently eliminated by apoptosis; thymocytes with non-functional receptors fail to be positively selected, while cells expressing self-reactive TCR undergo negative selection. At the same time, potentially useful clones are positively selected. The quantitative selection model proposes that positive selection results from low avidity interactions between TCR and MHC-peptide complexes (Ashton-Rickardt et al., 1994; Ashton-Rickardt and Tonegawa, 1994; Williams et al., 1997). Avidity depends directly on both

the affinity of TCR for the MHC-self peptide complexes, and on the number of such interactions. Thus, according to this model, the level of TCR surface expression is of critical importance for the generation of thymocyte repertoire.

Positive selection occurs within the population of DP thymocytes that expresses a low level of TCR relative to more mature thymocytes and T cells (Shortman et al., 1991). This low level of TCR expression has been proposed to be regulated by post-transcriptional mechanisms (Bonifacino et al., 1990; Kearse et al., 1994; Maguire et al., 1990). Several reports have shown that certain experimental conditions, as well as some genetic deficiencies, can interfere with this regulation. For example, injection of anti-CD4 mAb (Bonifacino et al., 1990; McCarthy et al., 1988); prolonged incubation of thymocytes in single-cell suspension (Marrack et al., 1988); and, disruption of *MHC class II* (Gosgrove et al., 1991) or *Lck* genes (Molina et al., 1992), all resulted in upregulation of the TCR on DP thymocytes. Lck, a member of the Src-family of protein tyrosine kinases, has been reported to participate in induction of TCR downregulation (D'Oro et al., 1997). Based on these observations, it has been proposed that the low level of TCR on DP thymocytes is under the control of Lck, which is persistently activated in DP thymocytes due to MHC class II-CD4 interactions (Wiest et al., 1993).

Src-like adaptor protein (SLAP), a protein structurally resembling Lck, was identified in yeast 2-hybrid screens by using both the cytoplasmic domain of the receptor tyrosine kinase Eph A2 (Pandey et al., 1995), and a portion of the N-terminus of c-Cbl as bait (Tang et al., 1999). Like Lck, it has a short N-terminal region that can be myristylated (Manes et al., 2000), followed by an SH3 and an SH2 domain, each of which share 51 and 50 percent identity with the respective domains present in Lck.

Unlike Lck, SLAP has no kinase domain, containing instead a unique C-terminus of 104 amino acids.

SLAP mRNA is predominantly expressed in lymphoid tissues, with the thymus expressing the highest levels (Sosinowski et al., 2000). Confocal microscopy has shown that SLAP colocalizes with endosomes. Though not detected at the plasma membrane, SLAP has significant effects on cellular signaling. Ectopic expression of epitope-tagged SLAP inhibited TCR signaling in Jurkat T cells (Sosinowski et al., 2000), and PDGF signaling in NIH 3T3 fibroblast cells (Roche et al., 1998). Based on these observations, we proposed that SLAP is a negative regulator of TCR signaling (Sosinowski et al., 2000).

Here we show that SLAP is predominantly expressed at the DP stage of thymic development, where it appears to participate in the downregulation of cell surface TCR levels. Positive selection of SLAP-deficient thymocytes expressing a MHC class II-restricted transgenic (Tg) DO11.10 TCR was significantly improved over that of WT cells. Significantly, SLAP deficiency was able to partially rescue thymocyte development in ZAP-70 deficient mice. We propose a model in which SLAP plays an important role in thymocyte development by regulating TCR levels.

Results

Generation of SLAP-Deficient Mice

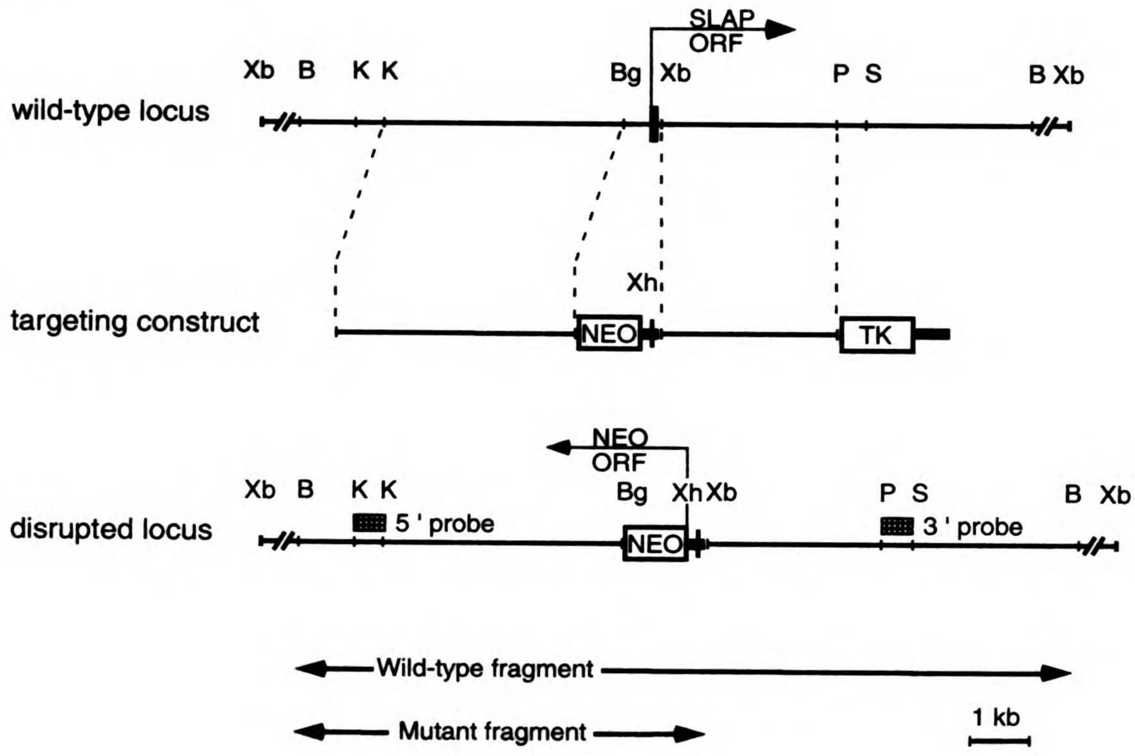
The *SLAP* gene was disrupted in embryonic stem (ES) cells by replacing part of the promoter region and the nucleotides encoding amino acids 1-20 with a neomycin resistance gene (Figure 1A). The expected mutation of the *SLAP* locus was confirmed by Southern blotting with the indicated 5' and 3' probes (Figure 1B and data not shown). *SLAP* $^{-/+}$ ES cells were used to generate chimeric mice, which subsequently transmitted the targeted allele to their progeny. Homozygous SLAP-deficient mice were born at Mendelian frequencies, indicating that the *SLAP* gene is not essential for embryonic development. SLAP-null mice were fertile, appeared healthy, and did not display any obvious physical abnormalities. Northern blot and RT-PCR analyses confirmed that *SLAP* gene had been functionally inactivated (Figure 1C, and data not shown).

SLAP Deficiency Results in Upregulation of TCR, CD4, CD5, CD8, and CD69 on DP thymocytes

We focused our analysis on thymocytes and mature T cells since these cell types express the highest level of SLAP mRNA (Sosinowski et al., 2000). The numbers and distribution of the major thymic and peripheral T cell subsets, as defined by the expression of CD4 and CD8 markers, were grossly normal (Figure 2 and data not shown). Levels of CD3 and TCR β, however, were markedly upregulated. In addition, SLAP deficient thymocytes displayed more moderate increases in CD4, CD5, CD8, and CD69 expression (Figure 3A). These differences were present only at the DP stage of thymic

Figure 1. Generation of SLAP deficient mice. (A) Partial restriction map of the *SLAP* locus. A 470 nucleotide long Bgl II – Xba I fragment containing a part of the promoter region and the first 21 amino acids of *SLAP* open reading frame (ORF), was replaced with the neomycin resistance (NEO) cassette. Boxes correspond to nucleotides defining ORFs of *SLAP*, *NEO*, and *thymidine kinase (TK)* genes; arrows indicate the direction of transcription; B, BamH I; K, Kpn I; Bg, Bgl II; Xb, Xba I; Xh, Xho I; P, Pst I; S, Sfi I. (B) Southern blot analysis of the genomic DNA from wild-type (WT), heterozygous (Het), and homozygous (KO) SLAP deficient mice. Genomic DNA was digested with BamH I/Xho I restriction enzymes, and probed using the 5' probe. (C) Northern blot analysis of the total RNA from WT, Het, and SLAP KO mice. SLAP mRNA was detected using a probe corresponding to the unique C-terminus of SLAP. The blot was reprobed with anti-actin probe to test for equal amount of total RNA. RT-PCR analysis, followed by sequencing of the product, revealed that the weak signal detected in the SLAP KO lane represents a splice variant of anti-sense NEO fused out-of-frame to a truncated SLAP message.

A



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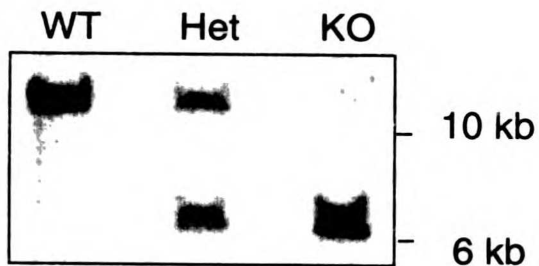

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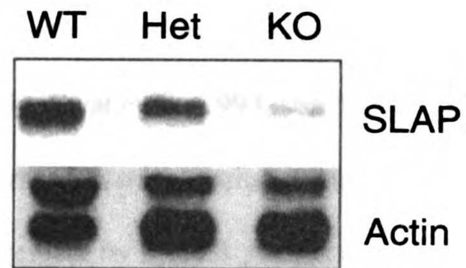

development and not evident in mature T cells in the periphery (Figure 3A and data not shown).

CD5 is a negative regulator whose expression level rises steadily during thymic maturation (Azzam et al., 1998; Pena-Rossi et al., 1999; Tarakhovsky et al., 1995). Genetic studies showed that the level of CD5 not only correlates with maturation, but also reflects the amount of signaling received by the cells (Azzam et al., 1998). In particular, both the level of TCR surface expression and the intensity of the TCR signal influence CD5 surface expression on DP thymocytes. The increase in CD5 expression on SLAP-null thymocytes, therefore, might be indicative of stronger TCR signaling. Although a direct role of SLAP in regulating CD5 levels is possible, we favor the indirect effect through the regulation of signaling intensity. In support of this hypothesis, SLAP deficiency had only minimal effect on upregulation of CD5 on DP cells in Lck-deficient mice, a genetic background where TCR signaling is severely compromised (Figure 3B). Indeed, the level of CD5 expression on DP thymocytes in the $Lck^{-/-}/SLAP^{-/-}$ doubly deficient mice is still considerably reduced when compared to the DP thymocytes of WT mice.

CD69 is another marker of developmental maturity in the thymus (Anderson et al., 1999; Bendelac et al., 1992; Bhandoola et al., 1999; Swat et al., 1993; van Meerwijk and Germain, 1993; Yamashita et al., 1993). This early activation marker is induced on DP thymocytes within 24 h of productive TCR engagement by ligands present on thymic stromal cells (Merkenschlager et al., 1997). Upregulation of CD69 occurs during both positive and negative selection (Bendelac et al., 1992; Kishimoto and Sprent, 1997).

Figure 2. Development of thymocytes in SLAP null mice. Thymocytes were stained for CD4 and CD8 and analyzed by FACS. (A) CD4 vs CD8 staining profiles of thymocytes from wild type (WT) and SLAP deficient ($SLAP^{-/-}$) littermates. (B) Frequency of DN, DP, and SP thymocytes. Results from individual WT (open circle) or $SLAP^{-/-}$ (filled circle) mice, and the average for each population (thick bar), are shown.

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staining profiles of ...
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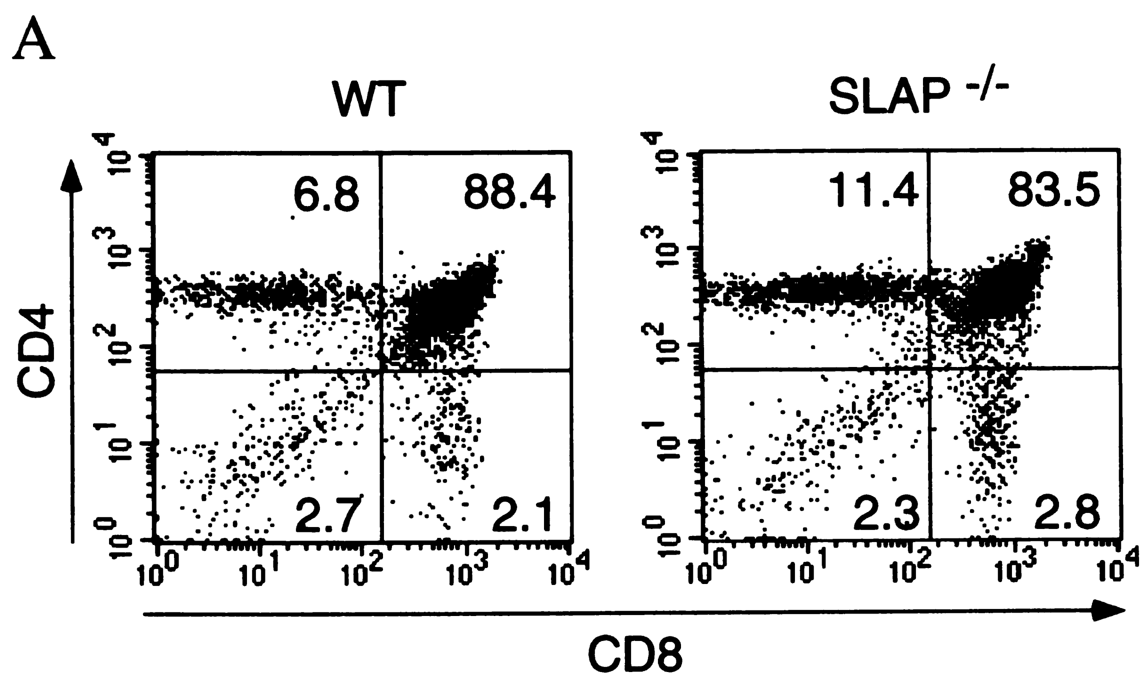
. are shown.

A



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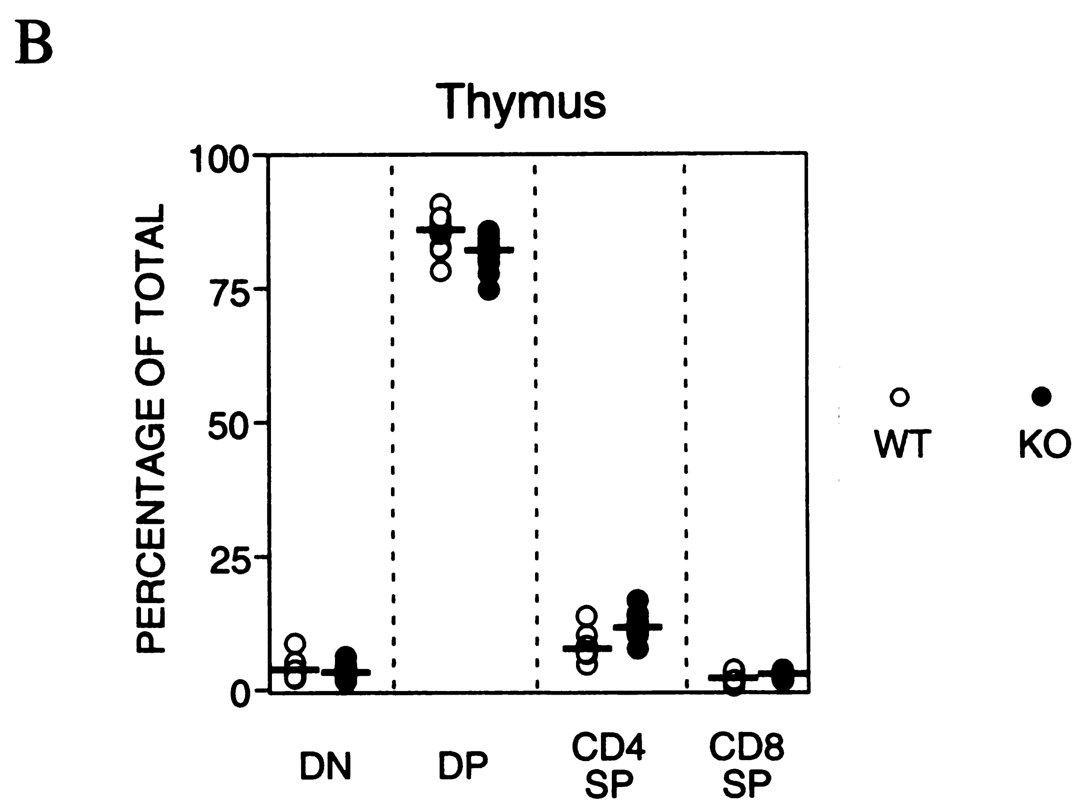


Figure 3. Upregulation of CD3, CD4, CD5, CD8, and CD69 on DP thymocytes in SLAP deficient mice. (A) Ungated (Total) or gated on DP, CD4 SP, and CD8 SP populations from a representative WT (solid grey) or SLAP KO (line) mice were assayed for surface expression of CD3, CD4, CD5, CD8 or CD69. (B) DP thymocytes from WT (dotted thin line), Lck deficient (solid grey), or Lck/SLAP doubly deficient mice (thick black) were stained for CD3 and CD5 expression. Data are represented as overlaid histograms.

A

CD3 CD4 CD5 CD8 CD69

Total

CD4/CD8 DP

M1

CD4 SP

CD8 SP

Fluorescence Intensity

SLAP WT SLAP -/-

B

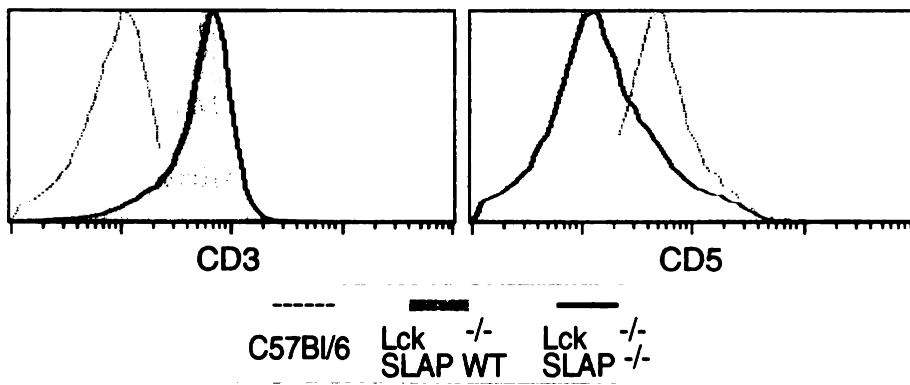


Figure 3 shows upregulation of CD69 expression on DP, SLAP-deficient thymocytes. In particular, the proportion of CD69^{high} cells is significantly higher among DP thymocytes in the SLAP deficient background (p-value <0.001; the mean ± standard deviation were: WT = 3.4 % ± 0.8, SLAP KO = 8.0 % ± 0.7). This might indicate that in the absence of SLAP more DP thymocytes receive strong TCR signals leading to positive selection or deletion.

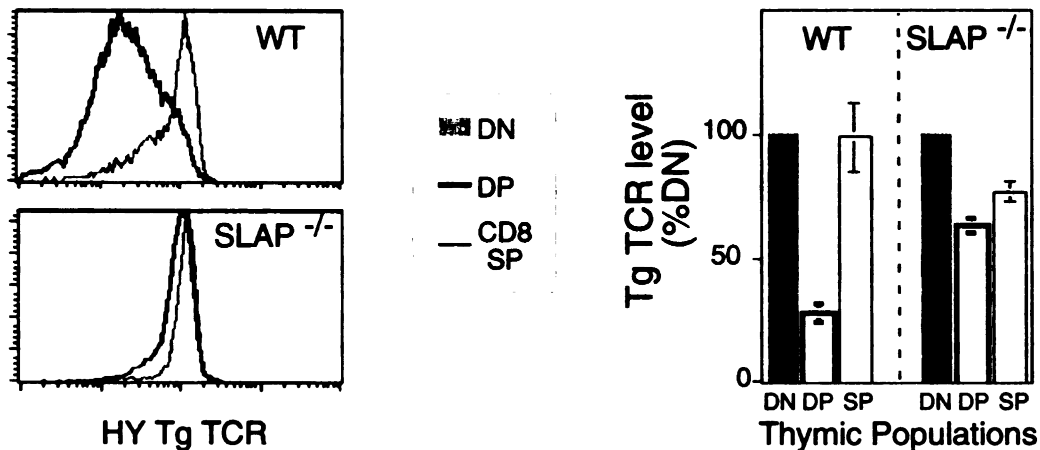
SLAP Downregulates TCR at the DP Stage Independently of the Positive Selecting Signal

Increased TCR density on SLAP-null DP thymocytes implicates SLAP in the regulation of TCR levels at this developmental stage. Post-transcriptional polyclonal downregulation of the TCR on DP thymocytes has previously been described (Bonifacino et al., 1990). Interestingly, this process is preserved during thymic development of cells expressing transgenic TCR α and β chains. For example, despite unusually high amounts of the TCR on DN thymocytes in HY TCR Tg mice, the level of the TCR decreases to non-transgenic levels upon transition to the DP stage (Borgulya et al., 1992). This active process of TCR downmodulation at the DN to DP transition has been observed in other TCR transgenic models (Crompton et al., 1993; Hogquist et al,, 1994; Wilson et al., 1992).

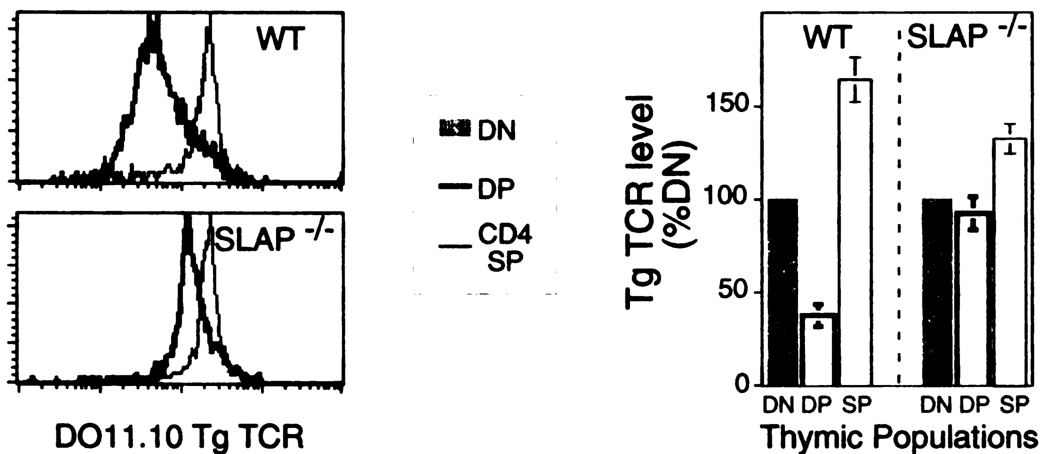
We exploited this observation to study the influence of SLAP on the downregulation of the TCR in MHC class I- and class II-restricted Tg TCR models of HY and DO11.10 mice, respectively. Our analysis confirmed that there was high surface expression of these transgenic TCRs on DN cells (Figure 4). In both cases we observed a

Figure 4. SLAP-dependent downregulation of the transgenic (Tg) TCR on DP thymocytes. Three types of Tg TCR models were analyzed: (A) class I restricted on a selecting MHC background (females expressing HY Tg on H-2^{b}); (B) class II restricted on a selecting MHC background (DO11.10 Tg on H-2^{d}); and (C) class I restricted on a nonselecting MHC background (HY Tg on H-2^{k}). Freshly isolated thymocytes were stained for CD4, CD8, and the Tg TCRs. DN (solid gray), DP (thick line), and SP (thin line) populations were gated based on CD4 and CD8 expression and analyzed for the Tg TCR density. Only relevant SP populations are shown: CD8 SP for class I restricted TCR (A and C) or CD4 SP for class II restricted TCR (B). Bar graphs depict the level of a transgenic TCR (mean fluorescence intensity), relative to the level expressed on DN, which is defined as 100%. Histograms represent individual mice, whereas bar graphs represent averages and standard errors for at least 3 animals.

A



B



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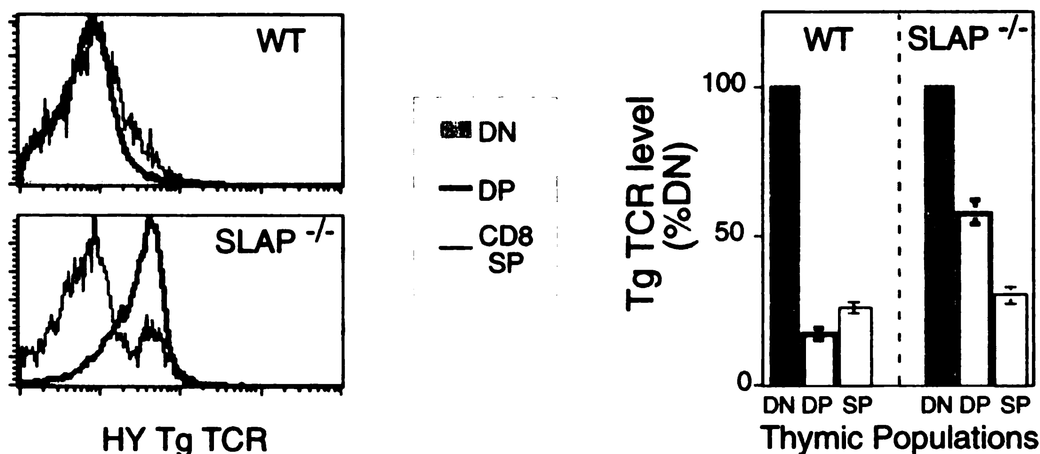


significant downregulation of the TCR at the DP stage, with subsequent upregulation of the receptors on the positively selected SP cells ("WT" histograms, Figure 4A and B). To compare the degree of TCR downmodulation at the DN to DP transition in various genetic backgrounds, we normalized the TCR levels to the DN population (Figure 4, bar graphs). This quantitative representation of the data shows clearly that in contrast to the WT cells, thymocytes from SLAP-null littermates displayed either no (Figure 4B), or only weak (Figure 4A) downregulation of the TCR on the DP population.

Because productive engagement of the TCR by MHC-peptide complexes leads to reduction in TCR surface density (Liu et al., 2000; Reinherz et al., 1982; Viola and Lanzavecchia, 1996), one could argue that the polyclonal TCR downregulation observed in the selecting WT backgrounds (Figure 4A, B) was an artifact of Tg expression. Specifically, in these genetic backgrounds all thymocytes express Tg receptors capable of interacting with the selecting MHC-peptide ligands expressed in the thymus. This might lead to positive selection and, consequently, to TCR depletion from cell surface. To address this concern, we repeated the experiment in mice expressing the HY-reactive TCR transgene in a non-selecting H-2^k MHC background, where no productive engagements leading to positive selection can occur (Kisielow et al., 1988). Once again, there was efficient TCR downregulation (80 %) upon transition from the DN to the DP stage in WT mice (Figure 4C). However, this downregulation of the HY TCR was much less efficient in the setting of SLAP deficiency. Taken together, the data in Figure 4 show that SLAP influences down-regulation of transgenic TCRs on DP thymocytes in a manner independent of positive selection.

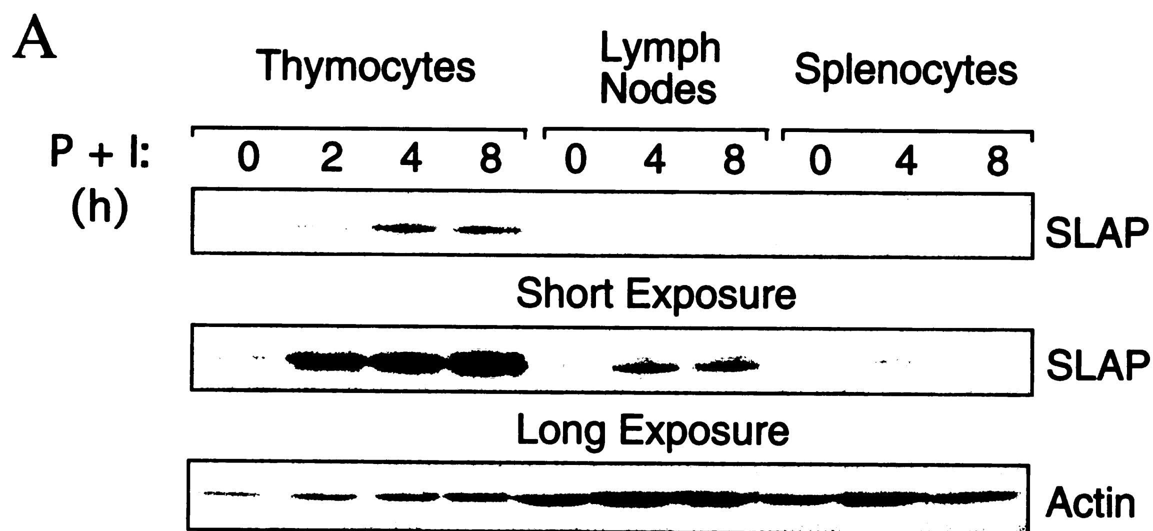
SLAP Protein is Expressed Predominantly in DP Thymocytes

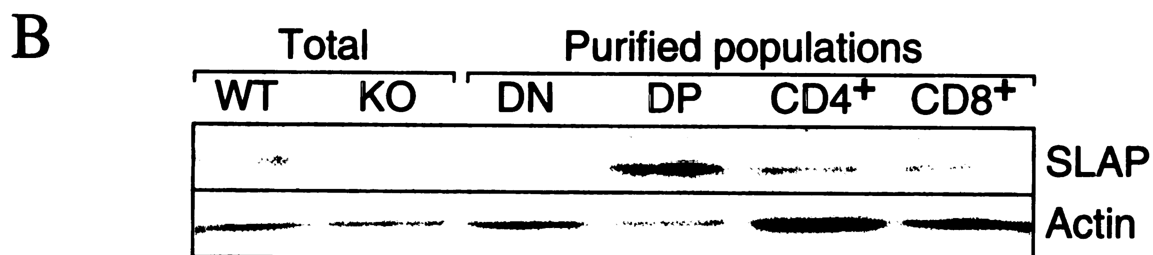
We previously characterized SLAP as being lymphoid-specific based on its mRNA expression pattern (Sosinowski et al., 2000). Here we demonstrate that thymus and lymph node cells, while at rest, express low levels of SLAP protein (Figure 5A, long exposure, P + I = 0 h). Notice that in order to emphasize the differences in SLAP expression in the thymus versus the periphery, we used twice as much lymph node cell and splenocyte lysate, as compared to thymocytes (Figure 5A; compare the actin signal). Short-term *in vitro* stimulation with phorbol ester (PMA) and/or the calcium ionophore, Ionomycin, modestly induced SLAP in the peripheral tissues represented by lymph nodes and spleen. In contrast, the same stimulation substantially induced SLAP protein in the thymus (Figure 5A). ConA-mediated stimulation of thymocytes also caused induction of SLAP (data not shown). This upregulation was of lesser magnitude, however, likely due to the functional dependency of ConA on TCR expression (Weiss et al., 1987), which may be limiting on the majority of thymocytes.

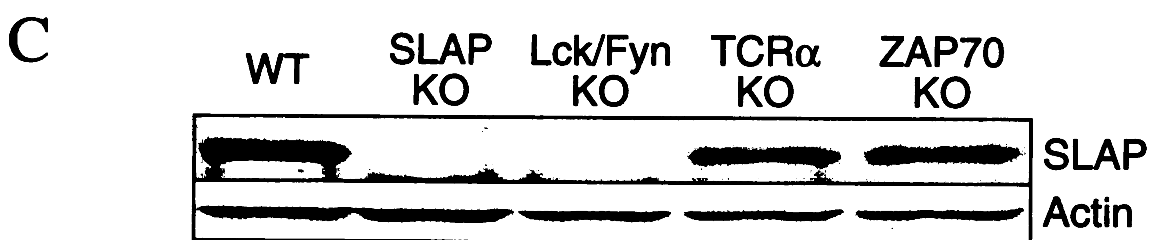
Because the presence of SLAP dramatically influenced TCR levels on DP cells, we characterized SLAP protein expression during thymic development. Highly enriched DN, DP, and SP thymic populations were analyzed for SLAP by immunoprecipitation. SLAP protein is present at higher levels in DP compared to DN or SP cells (Figure 5B, compare SLAP and actin expression). This finding is consistent with SLAP deficiency affecting the phenotype of DP thymocytes (Figures 3 and 4).

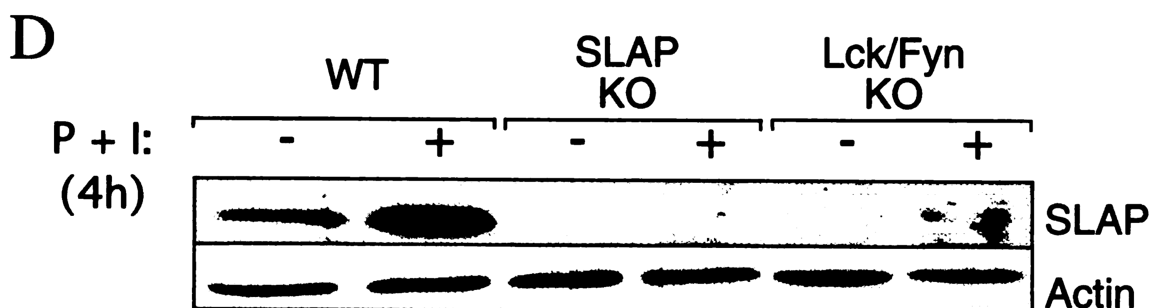
Since SLAP expression is inducible in thymocytes (Figure 5A), we postulated that the signal responsible for protein expression could be delivered either through the pre-TCR or the αβ TCR. To distinguish between these two possibilities, we analyzed the

Figure 5. Developmental regulation of SLAP expression. The indicated populations of cells were lysed, and SLAP was immunoprecipitated, Western blotted, and probed with anti-SLAP antibody (see Experimental Procedures). To reflect the relative amount of lysates used for immunoprecipitations, the same lysates were analyzed by Western blotting for actin. (A) Thymocytes, lymph node cells, and splenocytes were either unstimulated (0), or stimulated with PMA and Ionomycin (P + I) for the indicated period of time (h). Two exposures of the same blot are shown. (B) Freshly isolated thymocytes were enriched for the four major thymic populations: double negative (DN), double positive (DP), CD4 single positive ($CD4^+$), and CD8 single positive ($CD8^+$). Lysates were normalized for total protein expression, and the amount equivalent to 10^8 of unfractionated thymocytes was used for anti-SLAP immunoprecipitations. (C) Lysates from freshly isolated thymocytes of the indicated backgrounds were normalized for protein expression, and analyzed for the SLAP expression by immunoprecipitation. (D) Lysates from 10^8 of freshly isolated (-), or stimulated for 4h with PMA and Ionomycin (+), thymocytes from the indicated genetic backgrounds were normalized for protein expression, and analyzed for the SLAP expression by immunoprecipitation.


A
Thymocytes
Lymph Nodes
Splenocytes
P + I:
(h)
0 2 4 8 0 4 8 0 4 8
SLAP
Short Exposure
SLAP
Long Exposure
Actin


B
Total
Purified populations
WT KO DN DP CD4+ CD8+
SLAP
Actin


C
WT
SLAP KO
Lck/Fyn KO
TCRα KO
ZAP70 KO
SLAP
Actin


D
WT
SLAP KO
Lck/Fyn KO
P + I:
(4h)
- + - + - +
SLAP
Actin

level of SLAP protein in mutant mice that have impaired signaling through either receptor. Thymocytes lacking a pre-TCR signal, due to genetic deficiency of Src-family kinases Lck and Fyn (van Oers et al., 1996), do not express the SLAP (Figure 5C). Since Lck/Fyn deficient thymocytes are arrested at the DN3 stage of development ($CD25^{+}CD44^{-}$), the lack of SLAP expression might reflect either the direct dependency on pre-TCR signaling or the indirect requirement for transition to the DP stage. The ability of Lck/Fyn null thymocytes to upregulate SLAP upon a four-hour treatment with PMA and Ionomycin, a stimulation which is too short to induce the developmental progression of DN3 cells into DP compartment, is suggestive of the direct pre-TCR involvement (Figure 5D). However, since the magnitude of this response was at best modest, future studies are necessary to resolve this issue.

In contrast to Lck/Fyn null cells, thymocytes defective in αβ TCR signaling, due to lack of the receptor itself (TCRα KO), or lack of the tyrosine kinase ZAP-70 (ZAP-70 KO) which is required for propagation of the signal, express WT levels of SLAP (Figure 5C). This rules out the necessity of signaling through the mature αβ TCR for SLAP expression. Taken together, the data in Figure 5 show that SLAP is predominantly expressed in DP thymocytes (Figure 5B), its expression is inducible (Figure 5A and D) and depends on the activity of Lck and Fyn, but not on signaling through the αβ TCR or on pathways dependent on ZAP-70 (Figure 5C).

SLAP Deficiency Improves Positive Selection of DO11.10 TCR Transgenic Thymocytes

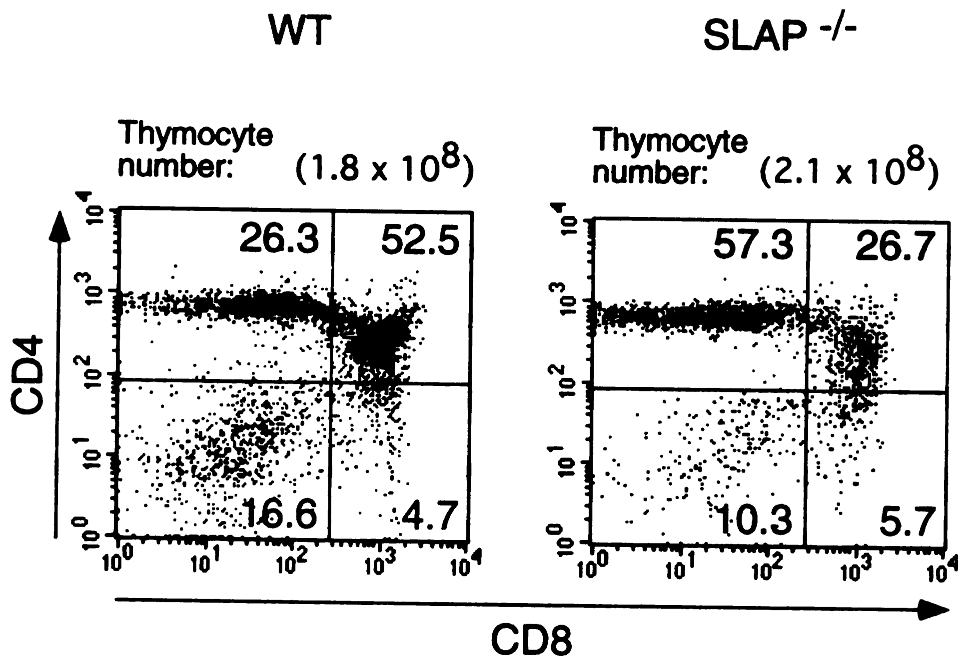
Thymic selection is critically dependent on the interaction between the TCR and a selecting peptide presented by a MHC molecule (Sebzda et al., 1999). Significant

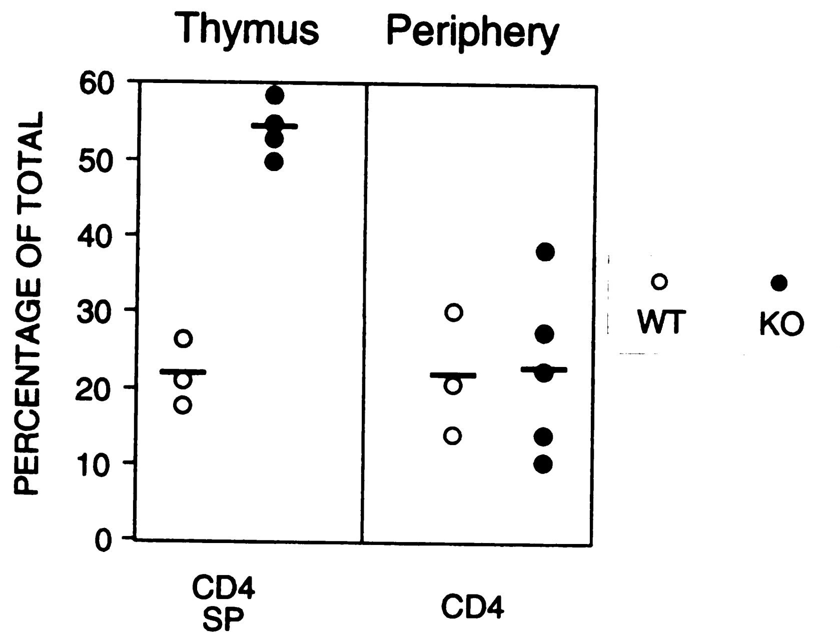
progress in understanding of this process has been made by analyzing TCR transgenic mice. These studies have demonstrated the influence of MHC-peptide density on both positive and negative selection. We reasoned that the SLAP-dependent regulation of TCR levels on DP might also influence the outcome of selection. Further analysis of DO11.10 mice on the H-2^d (selecting) background demonstrated that the lack of SLAP allowed twice as many cells to mature into the CD4 SP compartment, as compared to WT (Figure 6). Note that the total number of thymocytes was unchanged, while the number of DP cells decreased proportionally to the increase of SP cells. The increased number of positively selected CD4 SP thymocytes, however, did not translate into increased numbers of the corresponding mature T cells in the periphery (Figure 6B). Analysis of mice expressing the DO11.10 TCR on the H-2^b deleting background (Liu et al., 1996), showed that SLAP deficiency did not affect negative selection (data not shown). We conclude that positive selection of DO11.10 TCR Tg cells on the H-2^d background was potentiated by the absence of SLAP. In contrast to the studies with the class II MHC-restricted DO11.10 TCR transgenic system, similar studies employing class I MHC-restricted HY TCR Tg mice did not reveal alterations in either positive (females on H-2^b) or negative (H-2^d) selection (data not shown). It remains to be determined whether the absence of an effect in these mice reflects a unique role for SLAP in the selection of $CD4^+$ cells, or whether it relates to a specific TCR : ligand affinity.

SLAP Deficiency Rescues T Cell Development in the Absence of ZAP-70

Our initial report showed that SLAP functions as a negative regulator of TCR signaling when ectopically expressed in Jurkat T cells (Sosinowski et al., 2000). The

Figure 6. Increased positive selection of SLAP deficient thymocytes expressing a class II restricted DO11.10 Tg TCR. Cells were stained for CD4, CD8, and the Tg TCR, and analyzed by flow cytometry. (A) Total thymocyte numbers, and the percentages of the four thymic populations, are shown for individual representative littermates. (B) The average (bars) and individual (circles) frequencies of SP thymocytes, or splenic CD4 T cells, are shown.

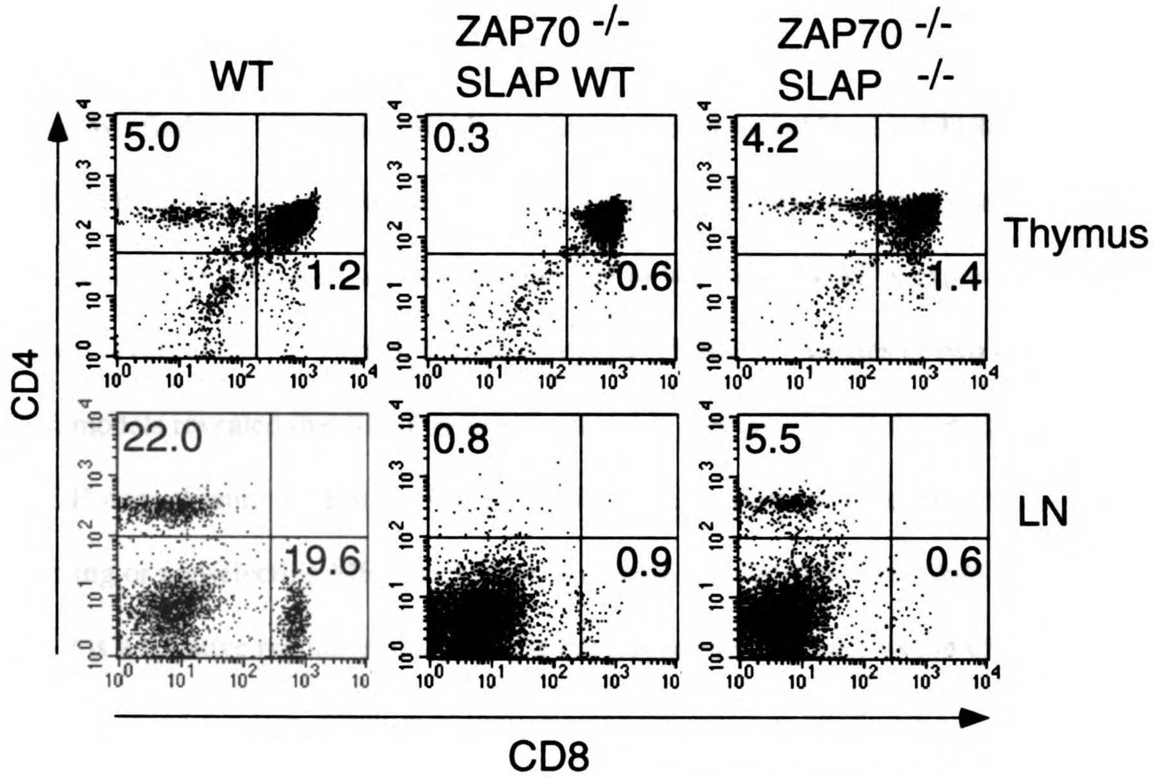

A
WT
SLAP -/-
Thymocyte number: (1.8 x 10^8)
Thymocyte number: (2.1 x 10^8)
26.3
52.5
16.6
4.7
57.3
26.7
10.3
5.7
CD4
CD8


B
Thymus
Periphery
PERCENTAGE OF TOTAL
CD4 SP
CD4
WT
KO

finding that SLAP deficiency can improve positive selection in DO11.10 mice (Figure 6) supports this notion. To assess the robustness of SLAP's influence on positive selection, we asked whether deletion of SLAP could overcome the complete lack of positive selection observed in ZAP-70-null mice (Kadlecek et al., 1998; Negishi et al., 1995). As previously reported, ZAP-70-deficient thymocytes are arrested at the DP stage, having neither SP cells in the thymus, nor $\alpha\beta$ T cells in the periphery (Figure 7). Ablation of SLAP expression enables ZAP-70 deficient thymocytes to progress not only to the SP stage of development, but also to become mature T cells in the periphery (Figure 7). The maturity status of SP thymocytes, and the peripheral T cells, was confirmed by anti-CD3, -CD5, -CD69 and -HSA staining (data not shown). The observed rescue, although impressive, was still incomplete, as only a low number of mature $CD4^+$ T cells was present in the periphery. This could reflect alterations in homeostatic mechanisms that control T cell survival or expansion in the periphery, perhaps due to impaired TCR signaling. The finding that SLAP elimination allows for positive selection of cells with a severe impairment of TCR signaling provides convincing evidence for SLAP functioning as a negative regulator of thymocyte development. The mechanism for the rescue of development in ZAP-70-deficient mice is novel since we failed to observe a similar effect in ZAP-70$^{-/-}$ mice lacking CD5, another negative regulator whose absence enhances signaling through the DO11.10 Tg TCR in response to H-2^d ligands (data not shown and Pena-Rossi et al., 1999).

Figure 7. SLAP deficiency rescues T cell development in ZAP-70 deficient mice. (A) Thymocytes (upper panel), or lymph node cells (lower panel), of the indicated genotypes were stained for CD4 and CD8 markers, and analyzed by flow cytometry. The percentages of SP thymocytes and mature T cells are indicated in the quadrants. (B) The average (bars) and individual (circles) frequencies of the indicated populations present in ZAP-70 KO/SLAP WT (WT) or ZAP-70 KO/SLAP KO (KO) mice are shown.

A



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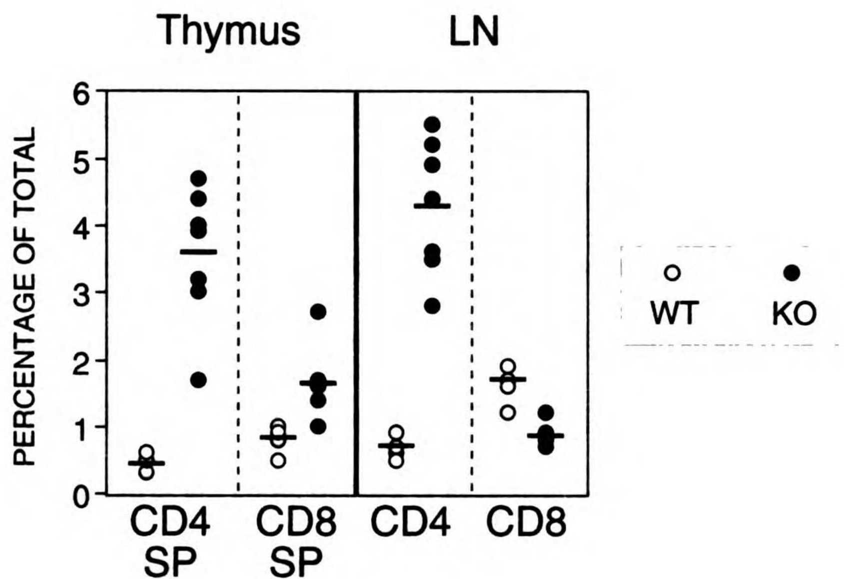

Discussion

In this report we describe the generation and the phenotype of SLAP-deficient mice. We find that DP cells in SLAP null mice have increased levels of TCR, CD4, CD5, CD8 and CD69 expression (Figure 3). That SLAP deficiency affects only DP thymocytes correlates well with our finding that in WT mice, SLAP is expressed at highest levels at the DP stage of thymic ontogeny (Figure 5). Studies of two transgenic TCR models revealed that SLAP regulates an active process of TCR downregulation at the DP stage (Figure 4). For a given TCR, this process occurs in either positively selecting or nonselecting MHC backgrounds (Figure 4). Our functional data show that SLAP is a negative regulator of thymocyte development since inactivation of the SLAP gene enhanced positive selection of thymocytes expressing an MHC class II-restricted Tg TCR (Figure 6), and it rescued the development of ZAP 70-deficient thymocytes (Figure 7).

Thymocyte development is a process of stepwise events that can be followed by monitoring changes in the surface expression of several molecules (Sebzda et al., 1999). Expression of the TCR on DP thymocytes is the key regulatory step in this process, for it is the interaction between the TCR and its ligands in the thymus that determines the fate of cells. Although the nature of this interaction remains controversial, several studies have shown that changes in MHC/peptide density can modify the outcome of selection (Ashton-Rickardt et al., 1994; Cook et al., 1997; Delaney et al., 1998; Fukui et al., 1997; Girao et al., 1997; Kersh et al., 2000; Sebzda et al., 1996; Sebzda et al., 1994). Moreover, the extent of thymocyte development in TCRζ-deficient mice reconstituted

with transgenic ζ chains directly paralleled the amount of TCR surface expression (Shores et al., 1994). Taken together, these findings indicate that thymic selection is controlled by the extent to which the TCR is engaged by MHC/peptide ligands. By downregulating the TCR, SLAP functions to decrease the number of such TCR-MHC/peptide interactions. Therefore, the upregulation of TCR levels on SLAP-deficient thymocytes may account for the observed changes in thymic selection.

Previous work has provided evidence for the existence of an active process of TCR downregulation that occurs normally at the DP stage. Disruption of this process was triggered in an *in vivo* system by the injection of anti-CD4 mAb, and in an *in vitro* system by the culture of thymocytes in single-cell suspension (Marrack et al., 1988). The abnormally elevated levels of TCR were thought to be a consequence of the disruption of the CD4-MHC class II interaction by antibody blockade or physical separation. Consistent with this interpretation, mice deficient in MHC class II, and CD4, also displayed upregulation of TCR on the DP thymocytes (Gosgrove et al., 1991). CD4, by localizing Lck to the engaged TCR, enables Lck to initiate a signal cascade that is required for T cell activation. Importantly, DP thymocytes deficient in Lck expression also upregulate TCR (Molina et al., 1992). It has been argued, therefore, that Lck activity is necessary for TCR downregulation on DP cells (Wiest et al., 1993). The same group proposed that the relevant interactions leading to TCR downregulation might occur between MHC/peptide and CD4, but not TCR itself (Wiest et al., 1996). This might explain the downregulation of both class I-, and class II-restricted Tg TCRs in nonselecting backgrounds (Figure 4C, and data not shown). Taken together, we postulate that the abnormal upregulation of TCR on DP thymocytes achieved either through

physical separation, with anti-CD4 mAb and single-cell suspension, or by genetic inactivation of *MHC class II*, *CD4*, *Lck*, and now *SLAP*, might reflect interference with the same developmental pathway.

The predominant expression of SLAP at the DP stage fits well with the observed phenotype of SLAP-null mice (Figure 5A and 5B). Lck/Fyn deficient thymocytes were previously reported to be arrested at the DN 3 stage ($CD25^{+}CD44^{-}$) due to a lack of pre-TCR signaling (van Oers et al., 1996). Figure 5C clearly shows the absence of SLAP at this stage, implying either a direct role of pre-TCR signaling in the induction of SLAP expression, or an indirect role through its influence on the developmental progression leading to the stage at which SLAP is expressed. The presence of the SLAP in TCRα- and in ZAP-70- deficient thymocytes argues that intact signaling through the mature αβ TCR is dispensable for upregulation of SLAP expression. In addition, this pattern of protein expression suggests that SLAP is in a position to influence αβ TCR, but not pre-TCR, signaling. Consistent with this hypothesis is the finding that SLAP deficiency did not alter thymocyte development in Lck or Lck/Fyn null mice (data not shown).

Our functional data support the proposed negative role of SLAP in thymocyte development. SLAP deficiency in an otherwise WT genetic background allows higher proportions of DP thymocytes to receive strong TCR signals leading to upregulation of CD69. Our data also show that loss of SLAP can more than double the efficiency of positive selection of cells bearing an MHC class II-restricted Tg DO11.10 TCR (Figure 6), and that it can rescue thymocyte development in ZAP-70 deficient mice (Figure 7). ZAP-$70^{-/-}$ thymocytes are arrested at the DP stage due to severe impairment in TCR signaling, and thus, lack of positive selection. Elimination of SLAP, which is indeed

expressed in ZAP-70-null thymocytes (Figure 5C), could allow a weaker signal, perhaps delivered by the closely related protein tyrosine kinase Syk, which is expressed at lower levels in DP cells, to partially restore positive selection. Two lines of evidence support this hypothesis: (1) overexpression of Syk can restore development in ZAP-70-deficient thymocytes (Gong et al., 1997); (2) there is an incomplete blockade in the development of ZAP-70$^{-/-}$ human thymocytes, which, unlike murine thymocytes, express substantial amounts of Syk at the DP stage (Arpaia et al., 1994; Chan et al., 1994; Chu et al., 1999; Elder et al., 1994). Remarkably, as observed in ZAP-70-null patients, only CD4 T cells are present in the periphery of ZAP-70/SLAP doubly deficient mice (Figure 7).

Finally, the role of SLAP as a negative regulator is supported by the fact that the phenotype of SLAP-null mice is strikingly similar to the phenotype caused by absence of a negative regulator c-Cbl. c-Cbl null DP thymocytes upregulate CD3, CD4, CD5, and CD69 to a similar extent as SLAP null cells (Murphy et al., 1998; Naramura et al., 1998, and data not shown). Likewise, in c-Cbl deficient mice, enhancement of positive selection was limited to an MHC class II-restricted Tg TCR model, with no alteration of either positive or negative selection in an MHC class I-restricted HY Tg TCR transgenic animals (Naramura et al., 1998). Despite these similarities, we did not observe significant hyperphosphorylation of multiple proteins examined in response to antigen receptor stimulation of SLAP-deficient thymocytes, a phenotype previously described for c-Cbl KO mice (Naramura, et al., 1998, and data not shown).

How might these molecules alter TCR levels? We have previously shown that SLAP can function as a negative regulator of TCR signaling, and that it can physically interact with the stimulated TCR complex (Sosinowski et al., 2000). SLAP was found to

bind the N-terminus of c-Cbl in a yeast 2-hybrid screen and in GST pull-down experiments (Tang et al., 1999). In addition, both c-Cbl and SLAP were shown to localize to endosomes, making endosomes a likely compartment where both proteins could interact (Levkowitz et al., 1998; Sosinowski et al., 2000). Endosomes have also been found to contain ubiquitinated protein conjugates, which suggests that this compartment might play a role in protein degradation (Doherty et al., 1989; Laszlo et al., 1990). There are data demonstrating ubiquitination of the TCR ζ-chain (Cenciarelli et al., 1992). Interestingly, c-Cbl has recently been identified as the ubiquitin-ligase for PDGF and EGF receptors (Joazeiro et al., 1999; Levkowitz et al., 1999).

Taken together, these results suggest the following model for SLAP function. Interactions between MHC class II and the αβ TCR, and/or between MHC class II and CD4-Lck, lead to TCR triggering. Engaged, phosphorylated receptors form signaling complexes that are subsequently downregulated. Once in the endocytic pathway, the interaction of these complexes with c-Cbl/SLAP results in their retention, ubiquitination, and possibly degradation. This model is especially exciting in the light of a recent report showing that stimulation-dependent TCR downmodulation results from intracellular retention and degradation of the receptor by lysosomes and proteasomes (Liu et al., 2000). This model also reconciles the contradiction that genetic disruption of positive regulators of signaling, such as MHC class II, CD4 and Lck, yields the same phenotype as inactivation of negative regulators, such as c-Cbl and SLAP. According to this model, positive regulators are necessary to "mark" the activated receptors by phosphorylation, while SLAP and c-Cbl could be required for their retention and degradation.

Although the proposed model concentrates on the SLAP-dependent regulation of TCR levels, it is possible that SLAP might also affect thymocyte development by directly inhibiting TCR signaling. In fact, overexpression of SLAP in Jurkat cells inhibited TCR signaling without influencing TCR expression (Sosinowski et al., 2000). Similar bimodal effects have been proposed for c-Cbl (Naramura et al., 1998). Further studies are in progress to differentiate between these two possibilities.

Experimental Procedures

Preparation of Targeting Construct

A bacterial artificial chromosome (BAC) containing a fragment of the murine *SLAP* gene (clone DMPC-HFF#1-255-A7) was obtained from Genome Systems Inc. (St. Louis, MO). Digestion of the BAC DNA with the XbaI restriction enzyme yielded an 11 kb fragment hybridizing with a 5'-probe, and a 10 kb fragment hybridizing with a 3'-probe (Figure 1). Both XbaI fragments were subcloned into pBluescript KS(+) vector (Stratagene), and used to generate the restriction enzyme map (Figure 1A). To enrich for clones with homologous recombination we used the positive/negative selection approach (Mansour et al., 1988). The targeting construct, containing a 5' homology region (KpnI-Bgl II), neomycin-resistance (NEO), a 3' homology region (XbaI-PstI), and an HSV-thymidine kinase cassette (TK), was prepared using standard methods.

Generation of SLAP-deficient Mice

The linearized targeting construct was transfected into 129-derived ES cells. Targeted ES cells were selected and processed by standard techniques (Ramirez-Solis et al., 1993). Homologous recombination was confirmed by Southern blotting (Figure 1B). Positive clones were injected into the blastocysts of C57BL/6 mice in order to generate chimeric male mice. Animals containing the disrupted *SLAP* allele in germ cells were obtained by breeding the chimeric males to C57BL/6 females. Further genotyping was accomplished by PCR using a mixture of three primers: 5' SLAP KO primer (5'-cactatgccctttaccttct-3'), 3' SLAP KO primer (5'-acagtgggctattacaggac-3'), and a 3' NEO

primer (5'-tggctacccgtgatattgctgaaga-3'). PCR amplified a fragment of 480 bp from the WT locus, while a larger fragment of 537 bp was amplified from the disrupted *SLAP* allele.

Southern and Northern Blot Analysis

Genomic DNA was digested with XbaI and XhoI restriction enzymes. Total RNA was extracted using RNAzol (Tel-Test, Inc.). 10 μg of either nucleic acid was resolved on a 0.7% agarose gel, and transferred onto a nylon membrane. The blots were hybridized with [^{32}P]dCTP-labeled (Rediprime II, Amersham) DNA probes: (i) a 5' (KpnI-KpnI), and a 3' (PstI-SfiI) genomic fragment (Southern blotting); (ii) a XcmI-ScaI fragment encoding the unique C-terminus of SLAP cDNA (Northern blotting).

Flow Cytometry

For each sample, 10^6 cells were stained with antibodies and analyzed on a FACS Calibur using CellQuest software (Becton Dickinson). The following antibodies were used: anti-CD3 (145-2C11)-FITC, anti-TCR β (H57-597)-FITC, anti-CD5-FITC, anti-CD69-FITC, anti-HSA-FITC, anti-B220-FITC, anti-IgD-FITC, anti-IgM-PE (Pharmingen); anti-DO11.10 TCR (KJ1-26)-FITC, anti-CD4-PE, and anti-CD8-TC (Caltag); anti-HY TCR (T3.70)-FITC (kind gift from J. Allison, UC Berkeley).

Animals

C57BL/6 and TCRα KO mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The Lck/Fyn (Kadlecek et al., 1998; van Oers et al., 1996), ZAP-70

(Kadlecek et al., 1998), and CD5 (Tarakhovsky et al., 1994) deficient mice, as well as DO11.10 (Murphy et al., 1990) and HY (Bluthmann et al., 1988) TCR transgenic mice, were previously described.

Purification of Thymic Populations

Cells were purified by the combination of a complement-dependent lysis and an antibody-directed purification using the MACS system (Miltenyi Biotec). For depletions, cell were labeled with either anti-CD4 Ab RL 172 (Ceredig et al., 1985), or anti-CD8 Ab 3.155 (Sarmiento et al., 1980) and then lysed using guinea pig (Gibco BRL; 1:6 dilution) or rabbit (Cedarlane Lab; 1:50 dilution) complement. Enriched populations were then stained with anti-CD4- or anti-CD8-FITC (Caltag), followed by anti-FITC beads, and purified on VS+ columns (Miltenyi Biotec). Cells purified from 16 experiments were pooled to obtain 10^8 cells of each type. The enriched populations were approximately 95% pure, as determined by flow cytometry.

Preparation of Cell Lysates, Immunoprecipitations, and Western Blotting

Preparation of cell lysates, immunoprecipitations, and Western Blotting were described previously (Sosinowski et al., 2000). Actin was detected in the whole cell lysates using a mouse anti-Actin C4 mAb (Chemicon International) followed by a goat anti-mouse IgG-HRP (Southern Biotech). Enhanced chemiluminescence was used to detect immunoreactive proteins (Amersham).

Acknowledgements

We thank Jeff Critchfield, and other members of the Weiss lab for critical review of the manuscript and for useful comments. This work was supported by the Howard Hughes Medical Institute, and the National Institute of Health grants GM39553 and PO1 A145865 to A.W.

CHAPTER IV

SLAP AND C-CBL PARTICIPATE IN THE SAME GENETIC PATHWAY LEADING TO DOWNREGULATION OF TCR ON DP THYMOCYTES

Summary

The data in Chapter 3 lead me to conclude that SLAP is a negative regulator of thymic selection. A similar function has been proposed for c-Cbl. In addition, SLAP has been reported to directly interact with c-Cbl. Here, I conduct a genetic analysis to test whether SLAP and c-Cbl participate in the same pathway. I found that deletion of either SLAP or c-Cbl leads to similar upregulation of CD3, CD4, CD5, and CD69, and rescues the development of thymocytes in the absence of ZAP-70. Analysis of SLAP/c-Cbl doubly deficient mice does not reveal any potentiation of the phenotype caused by either single mutation. Such additive effects would have been expected if both genes contributed equally to the same phenotype through different genetic pathways. Therefore, I conclude that SLAP and c-Cbl participate in the same pathway leading to downregulation of CD3, CD4, CD5, and CD69 on DP thymocytes, and preventing thymic maturation in the absence of ZAP-70.

Introduction

The *c-Cbl* (Casitas B-lineage Lymphoma) gene was originally identified as a cellular homologue of a viral oncogene *v-cbl*, which induces pre-B lymphomas and myeloid leukemias in mice (Langdon et al., 1989). To date, four genes homologous to c-Cbl have been identified: (1) *SLI-1* expressed in *Caenorhabditis elegans*, *D-Cbl* expressed in *Drosophila melanogaster* (occurring as two splice forms), and two mammalian members, *Cbl-b* and *Cbl-SL/Cbl-3* (Figure 1A). All five genes are classified now to the Cbl-family based on the high degree of similarity residing within the stretch of about 450 amino acids of their N-terminus. Evolutionary conservation of this region is impressive, since even such distant relatives as C. elegans and D. melanogaster share 55% and 68% identity with human c-Cbl, respectively. Recently identified Cbl-SL/Cbl-3 is 65% identical, and the most similar protein, Cbl-b, shares 98% of amino acids within the N-terminal region.

The common structural elements of the Cbl proteins are : a four helical bundle (4H), a Ca^{2+}-binding EF hand, an atypical SH2 domain, and a RING finger (RF) domain. The structurally simplest member of the family, D-Cbl (short), is composed entirely of these four elements. SLI-1 and Cbl-SL/Cbl-3 contain an additional proline-rich (PRO) domain at the C-terminus. The two most homologous members, c-Cbl and Cbl-b, as well as the D-Cbl (long), have more extensive PRO domains followed by a C-terminal region containing a leucine zipper (LZ) (Figure 1A). The recently solved crystal structure of the core showed that the 4H, the EF hand, and the atypical SH2 domain together form a

Figure 1. Cbl family: functional domains and the interacting proteins. **(A)** Functional domains of the c-Cbl protein include: four-helical bundle (4H, aa 50-147), two calcium-binding EF-hands (EF, aa 149-250), an atypical SH2 (SH2, aa 250-351), a zinc-binding RING finger (RF, aa 365-428), a proline-rich region (PR, aa 481-690), and a leucine zipper (LZ, aa 857-892). In addition to the centrally located proline-rich region, there are several PR stretches present in the C-terminal half of the protein. The Cbl family includes three mammalian proteins (c-Cbl, Cbl-b, and Cbl-3), two alternatively spliced *D. malanogaster* homologues (D-Cbl short and D-Cbl long), and a single C. elegans homologue (Sli-1). **(B)** Signal transducing proteins known to associate with c-Cbl. C-terminus of SLAP, and tyrosine-phosphorylated residues in ZAP-70 and Syk binds to the TKB domain. E2 protein ubiquitin ligases (e.g. UbcH7), and Sprouty (SPR2) bind to the RF. Two oncogenic forms of c-Cbl do not contain the RF domain: the 70Z mutant has a deletion of 17 amino acids (aa 366-382) and the v-Cbl contains only TKB domain (aa 1-357). The central PR region binds to SH3 domains of Src-kinases (Fyn and Src), Grb2, Nck, the Cbl-associated protein (CAP) (also known as Cbl-interacting protein {CIN 85} or SH3-encoding expressed in tumorigenic astrocytes {SETA}), whereas the short PR region in the C-terminus binds phosphatidylinositol-3-OH kinase (CMS). A phosphoserine located proximally to the PR region interacts with 14-3-3 protein. Several phosphotyrosines in the C-terminal part of c-Cbl bind to SH2 domains of Vav, p85 subunit of PI3K, and CrkL. The LZ is indicated in the homodimerization of c-Cbl. Modified from C. B. F. Thien and W. Y. Langdon, "Cbl: Many Adaptations to Regulate Protein Tyrosine Kinases", Nature Reviews Molecular Cell Biology 2; 294-307 (2001).

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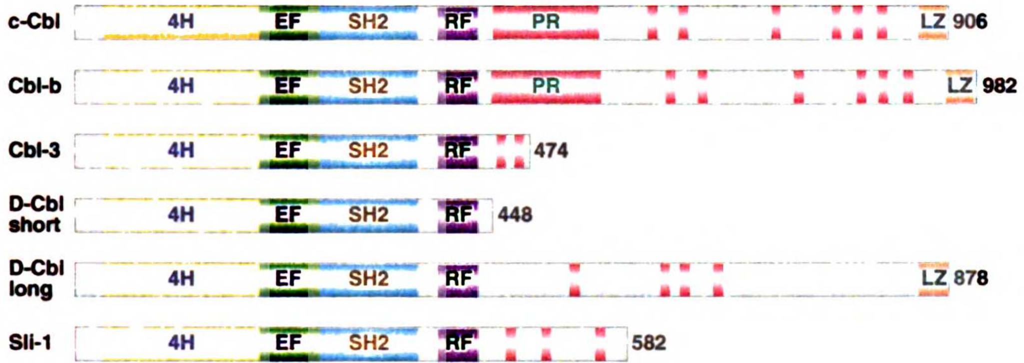

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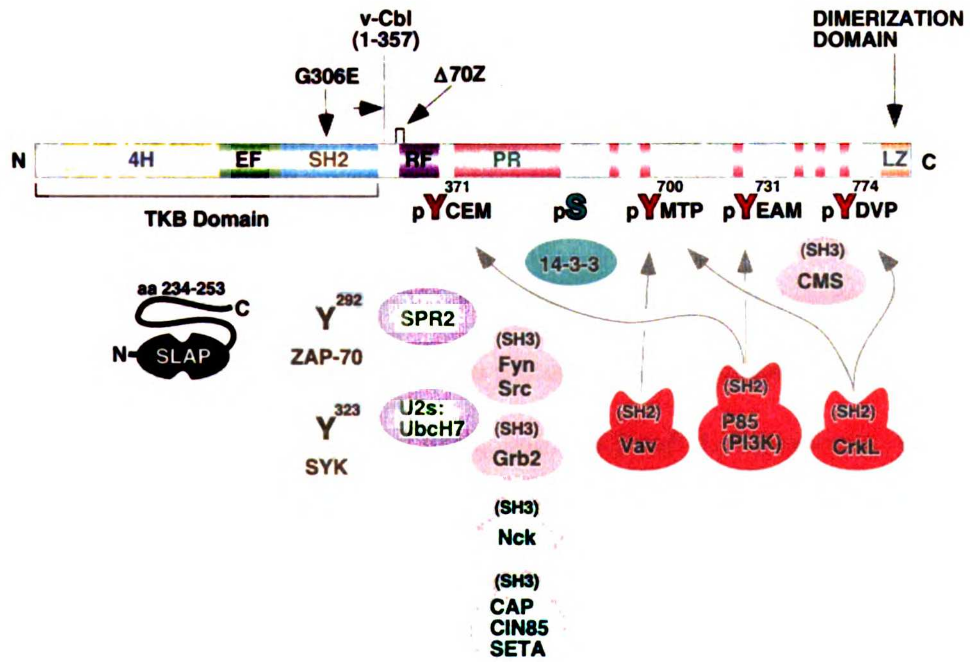

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Tyrosine Kinase Binding (TKB) domain which can recognize and bind to peptides containing phosphorylated tyrosine residues (Figure 2A, and Meng et al., 1999).

The most extensively studied member of the family, c-Cbl, has been implicated in signaling pathways initiated by growth factor receptors (epidermal growth factor {EGF}, platelet-derived growth factor {PDGF}, fibroblast growth factor {FGF}, colony-stimulating factor {CSF}, granulocyte-macrophage colony-stimulating factor {GM-CSF}, nerve growth factor {NGF}), cytokines (interleukin-2, -3, and -4, interferon-α, erythropoietin, thrombospondin), and immunoreceptors (T cell receptor {TCR}, B cell receptor {BCR}) (Liu and Altman, 1998; Lupher et al., 1998). Following engagement of each of these receptors this 120 kDa protein becomes rapidly phosphorylated on tyrosine residues, and binds to a large number of signaling proteins. c-Cbl has been reported to interact with a variety of proteins through its respective domains: the TKB domain binds to tyrosine phosphorylated EGFR, PDGFR, Syk and ZAP-70; the RF domain can interact with the components of the ubiquitination machinery (UbcH7, Ubc4, E2 ubiquitin), and with hSPRY2; the PRO region associates with SH3 domains of Grb2, Nck, CAP, the Src- and the Btk-families of protein tyrosine kinases; phosphorylated serine residues in PRO can retain 14-3-3 protein; the three major in vivo phosphorylated tyrosines, Y^{700}, Y^{731}, and Y^{774}, serve as docking sites for Vav, p85 of PI3K, and Crk I and II, respectively; finally, the LZ domain facilitates dimerization of c-Cbl (Figure 1B; for a comprehensive list of proteins interacting with Cbl see Lupher et al., 1998; Thien and Langdon, 2001). c-Cbl can form a trimolecular complex with ZAP-70 and the E2 ubiquitin ligases,UbcH7 (Figure 2B, and Zheng et al., 2000).

Figure 2. Crystal structure of the N-terminal regions of c-Cbl. **(A)** The tyrosine kinase binding (TKB) domain of c-Cbl is composed of three structural domains: 4H (colored in yellow), EF (colored in green), and an atypical SH2 (colored in blue). The Ca^{2+} ion coordinated by the EF domain, and the conserved arginine (R^{294}), are represented as a red star and a red fork, respectively. Secondary structural elements are labeled according to the established conventions ("α" represents α-helical regions, and "β" denotes β-sheets). The cartoon representation of the N-terminal part of c-Cbl present in this structure is depicted at the bottom of the Figure. Adapted from Meng et al., "Structure of the amino-terminal domain of c-Cbl complexed to its binding site on ZAP-70 kinase", Nature 398; 84-90 (1999). **(B)** c-Cbl forms a ternary complex with the protein ubiquitin-ligase UbcH7 and a phosphopeptide of ZAP-70. The three domains of TKB (4H, EF, and SH2) are colored green, the sequence of the SH2-RF linker is yellow, and the RF domain is red. The ZAP-70 phosphopeptide is colored pink and the UbcH7 is blue. The cartoon representation of the N-terminal part of c-Cbl characterized in this structure is depicted at the bottom of the Figure. Adapted from Zheng et al., "Structure of a c-Cbl-UbcH7 Complex: RING Domain Function in Ubiquitin-Protein Ligases", Cell 102; 533-539 (2000).

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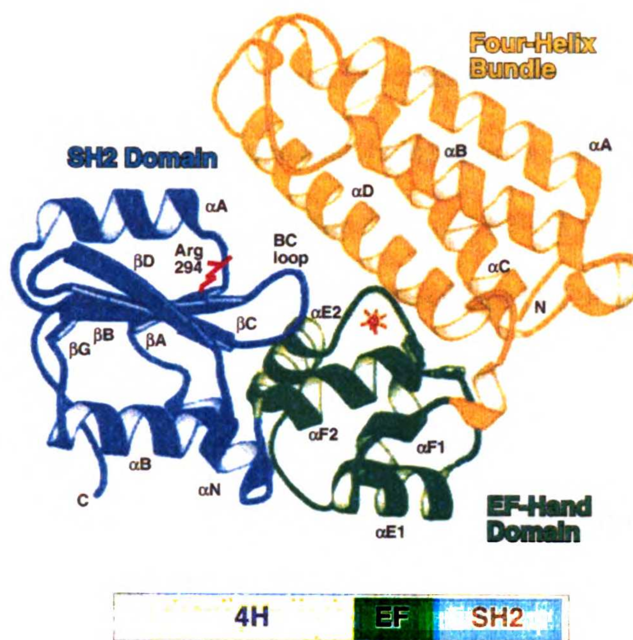

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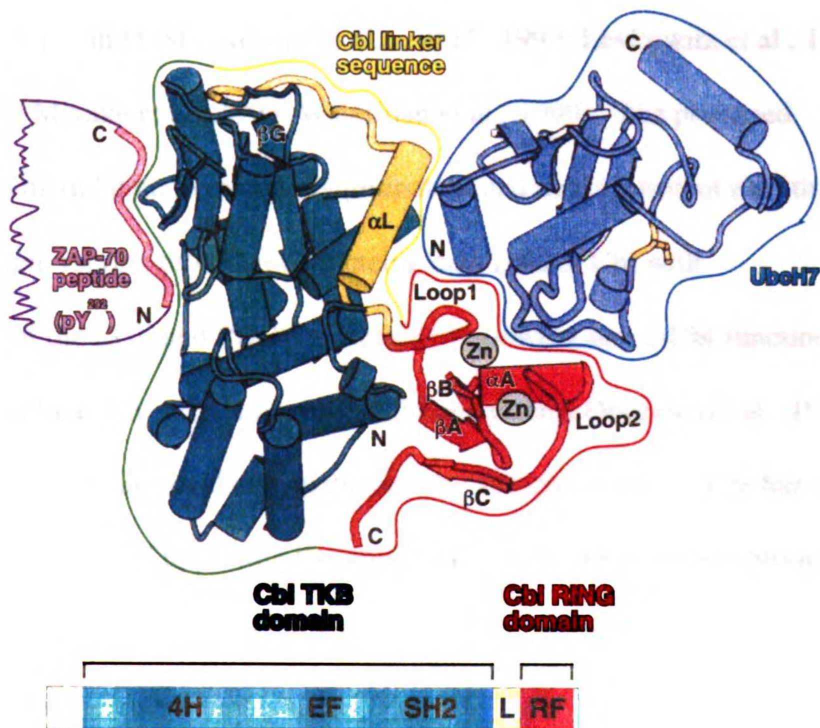


The initial clues to Cbl function came from the genetic studies in worms and in insects. SLI-1 was identified in a genetic screen as a negative regulator of EGF receptor signaling by virtue of its loss-of function mutation being able to rescue vulval development in worms expressing a hypomorphic form of LET-23 (EGFR homologue) (Jongeward et al., 1995). A similar function was proposed for D-Cbl, after its overexpression in the Drosophila eye resulted in a suppression of the Sevenless-dependent (EGFR homologue) development of the R7 photoreceptor cell. Experiments in NIH 3T3 fibroblasts confirmed the inhibitory role of Cbl in the EGFR signaling in mammals. Overexpression of the protein resulted in decreased phosphorylation of the EFGR and the JAK-STAT proteins, whereas antisense oligo treatment resulted in increased phosphorylation of these molecules (Ueno et al., 1997).

Recently, several groups have demonstrated Cbl-dependent downregulation of the stimulated EGF, PDGF, and CSF-1 receptors (Lee et al., 1999; Levkowitz et al., 1998; Miyake et al., 1998; Miyake et al., 1999; Waterman et al., 1999). The proposed mechanism for this modulation involved ubiquitination and degradation of the stimulated receptors. Two recent reports provided convincing data linking Cbl with polyubiquitination of the EGF and PDGF receptors by showing that c-Cbl functions as an E3-ubiquitin protein ligase in an *in vitro* reconstitution systems (Joazeiro et al., 1999; Levkowitz et al., 1999). This novel enzymatic activity associated with c-Cbl has been attributed to the RF domain. Supporting the importance of the RF is the evolutionary conservation of this domain among all the members of the family, and the oncogenic propensity of the RF-deficient mutant isolated from 70Z/3 pre-B lymphoma cell line (Andoniou et al., 1994). The data on the direct role of Cbl RF domain in an *in vivo*

ubiquitination has also been presented (Waterman et al., 1999; Yokouchi et al., 1999). These findings, together with the data showing the essential role of Cbl TKB domain, lead to a model in which TKB domain targets the activated receptors by binding to the phosphorylated tyrosine residues. Thereafter, the RF domain facilitates polyubiquitination and subsequent degradation of the receptors.

In addition to the regulation of the receptor tyrosine kinases, Cbl participates in the signaling pathways initiated by the immunoreceptors. In fact, the highest level of c-Cbl mRNA is expressed in the thymus, suggesting an important role for this protein in the pre-TCR and/or the TCR signaling (Langdon et al., 1989). Cbl becomes rapidly tyrosine phosphorylated upon engagement of the TCR and it becomes one of the major phosphoproteins in activated T cells. Overexpression of c-Cbl in Jurkat cells suppressed anti-CD3 induced AP1 and NFAT reporter activity; conversely, the presence of Cbl-N or 70Z/3, two forms of Cbl considered to act as dominant negative mutants, induced NFAT activity (Ota et al., 2000; Rellahan et al., 1997; Zhang et al., 1999). These experiments also showed that the inactivating point mutation in TKB domain (G306E) abrogated function of the WT and the mutant forms of Cbl.

In search for the target proteins of the Cbl TKB domain, Lupher et al., screened a phosphopeptide library and identified D(D/N)x(pY) as an optimal binding sequence (Lupher et al., 1997). Additional binding experiments demonstrated that a peptide encompassing Y^{292} of ZAP-70, conformed to this sequence and was responsible for the *in vivo* interactions between c-Cbl and ZAP-70. This finding was especially interesting in the light of previous reports identifying Y^{292} as one of the major *in vivo* phosphorylation sites and that a conservative mutation of this site rendered ZAP-70 hyperactive (Watts et

al., 1994; Zhao and Weiss, 1996). A corresponding residue in Syk (Y^{323}) has also been shown to play an analogous role in both, binding to c-Cbl and suppressing Syk function (Lupher et al., 1998; Ota et al., 2000). Reconstitution of early components of the TCR signaling pathway in COS 7 cells demonstrated that Syk and ZAP-70 undergo stimulation-dependent degradation that completely depends on the intact TKB domain and the presence of the phosphorylated Y^{323} and Y^{292}, respectively. In addition, the activity of ZAP-70 ^{Y292F}mutant was resistant to the negative regulation by Cbl, implying that ZAP-70 is the major target for suppression in the TCR signaling pathway (Ota et al., 2000).

Src-family kinases, including Fyn, Lyn, Lck, Hck and Yes, have also been demonstrated to interact with Cbl (Howlett et al., 1999; Hunter et al., 1999; Miyake et al., 1997). Unlike ZAP-70/Syk, Src kinases bind Cbl in the unstimulated state through the interaction of Src SH3 domain and Cbl polyproline region. Detailed analysis of Fyn interactions with c-Cbl in 293T cells showed that this interaction is more complex, involving at least two proline rich regions in Cbl and the SH2 domain of Fyn (Lill et al., 2000). Interestingly, the same study showed stimulation-dependent degradation of Fyn that required the interaction with c-Cbl.

Stimulation-dependent degradation of both Src and ZAP-70/Syk kinases suggested a mechanism for the negative regulation of TCR signaling by Cbl. This model was especially attractive considering that Cbl mediated degradation of EGF and PDGF receptors, the most proximal kinases in their respective signaling pathways. However, most of these studies were performed by overexpressing separate components in the fibroblast cell lines, and were not reproduced in T cells. In addition, c-Cbl deficient DT

40 chicken B cells had normal levels of Syk in the resting and stimulated states (Yasuda et al., 2000). Moreover, anti-BCR stimulation resulted in the identical pattern of tyrosine phosphorylated proteins in the WT and Cbl$^{-/-}$ cells, an outcome not expected if the Src and/or Syk family members were differentially affected by c-Cbl.

The phenotype of c-Cbl deficient mice was perhaps surprising since it suggested a previously unappreciated role for Cbl in regulation of TCR levels on thymocytes (Murphy et al., 1998; Naramura et al., 1998). Consistent with the highest expression level of c-Cbl mRNA in the thymus, the most pronounced phenotype of c-Cbl KO mice was the upregulation of CD3, CD4, CD5, and CD69 molecules on the surfaces of DP thymocytes. This upregulation was developmental stage-specific since the SP thymocytes, as well as the mature T cells in the periphery, did not show detectable differences in their expression of these molecules. Functionally, the absence of c-Cbl improved the efficiency of positive selection of thymocytes expressing MHC class II restricted transgenic TCR (5C.C7) (Naramura et al., 1998). This finding, together with the observation that c-Cbl$^{-/-}$ thymocytes displayed hyperphosphorylation of multiple proteins and enhanced thymocyte proliferation, confirmed the inhibitory role of Cbl in TCR signaling. These studies, however, did not support the model in which c-Cbl contributed to rapid degradation of the upstream kinases, since the level of both, ZAP-70 and Lck, were unchanged in the thymocytes from c-Cbl KO mice.

The results of my studies on SLAP (chapters II and III), as well as the published data describing c-Cbl, show that the products of these two genes have very similar functional characteristics: (i) they are both expressed predominantly in the thymus; (ii) they can both negatively regulate TCR signaling when overexpressed in T cell lines; (iii)

their deficiencies cause very similar phenotypes in mice. In addition, c-Cbl was shown to interact with SLAP in a yeast 2-hybrid screen and when transiently transfected into COS 7 cells (Tang et al., 1999). Taking into consideration the above facts, we decided to test the hypothesis that SLAP and c-Cbl participate in the same genetic pathway.

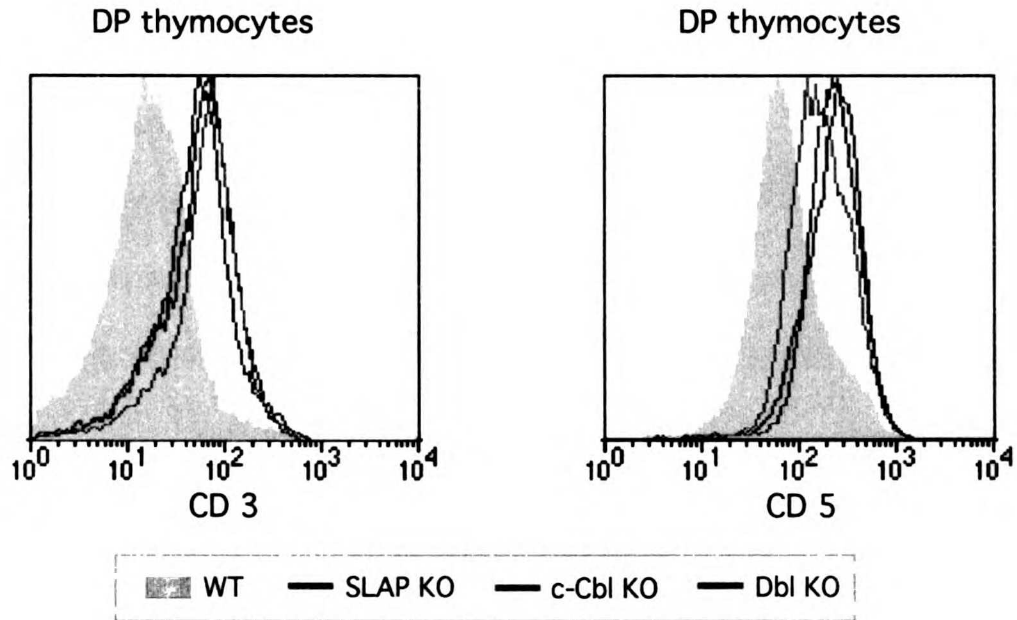
Results

I described the phenotype of SLAP deficient thymocytes in Chapter 3. A very similar phenotype was reported for c-Cbl null mice. Based on these observations I propose that SLAP and c-Cbl participate in the same pathway that influences the phenotype of DP thymocytes. In this chapter, I present genetic data supporting this hypothesis.

SLAP- and c-Cbl-deficient DP thymocytes upregulate CD3, CD4 and CD5

I began the genetic analysis by comparing cell surface phenotype of thymocytes developing in SLAP- or c-Cbl-deficient genetic backgrounds. Histogram overlays show that thymocytes from both mutant mice upregulate surface expression of CD3, CD4, and CD5 to similar extent, and that they express WT levels of CD8 (Figure 3, and data not shown). Importantly, deletion of both genes did not alter the phenotype already observed for either single mutation, suggesting these two genes share a common pathway. No differences were detected between the WT and the mutants at the SP stage (Figure 3B) as previously described (Chapter 3, and (Tang et al., 1999).

Figure 3. Comparison of cell-surface expression of CD3, CD4, CD5 and CD8 on WT, SLAP KO, c-Cbl KO, and SLAP x c-Cbl double KO thymocytes. Thymocytes were stained with anti-CD3, -CD4, -CD5, and -CD8 antibodies, and were analyzed by FACS. Each experiment was analyzed by overlaying histograms obtained for WT (grey), SLAP KO (red), c-Cbl KO (blue), and SLAP x c-Cbl double KO (green) thymocytes **(A)**. The resulting mean fluorescence intensity (MFI) values were plotted as the percentage of the MFI obtained for the WT cells **(B)**. For the sake of simplicity, only the expression of CD3 and CD5 is presented in the form of histograms. At least six animals of each type were analyzed in five independent experiments, and a single representative experiment is shown. .


A
DP thymocytes
DP thymocytes
CD 3
CD 5
WT
SLAP KO
c-Cbl KO
Dbl KO

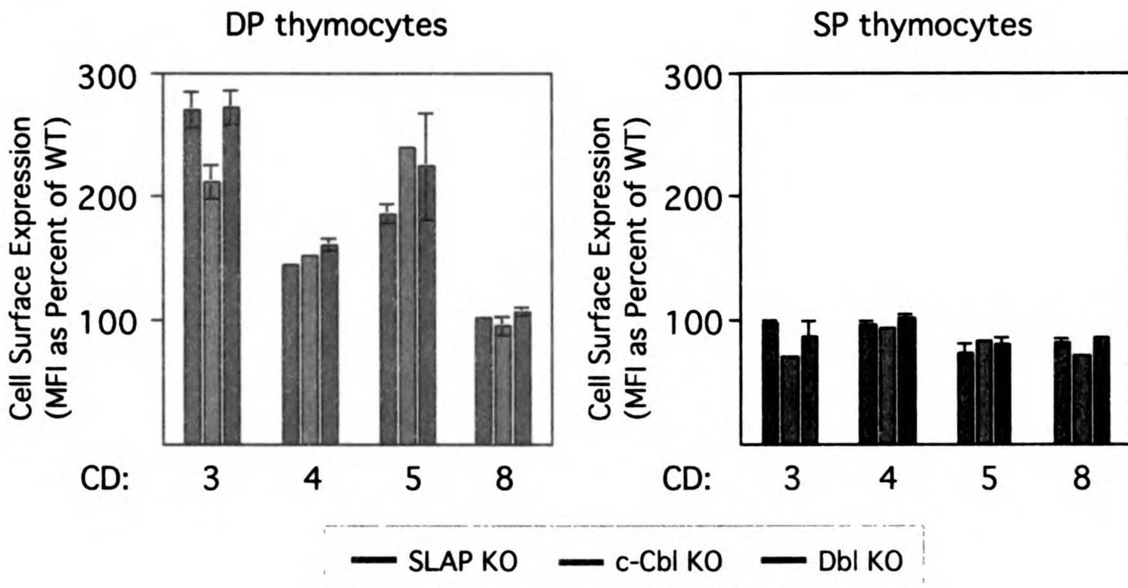

B
DP thymocytes
SP thymocytes
Cell Surface Expression (MFI as Percent of WT)
CD:
3
4
5
8
SLAP KO
c-Cbl KO
Dbl KO

Figure 4. Percentage of CD69^{high} DP thymocytes present in WT, SLAP KO, c-Cbl KO, and SLAP x c-Cbl double KO genetic backgrounds. Thymocytes from WT (grey), SLAP KO (red), c-Cbl KO (blue), and SLAP x c-Cbl double KO (green) mice were stained with anti-CD4, anti-CD8, and anti-CD69 antibodies, and analyzed by FACS. The M1 gate was defined arbitrarily based on the expression of CD69 on DP and SP thymocytes (**A**). The percentages of DP thymocytes falling within M1 gate are shown for each genetic background (**B**). At least six animals of each type were analyzed.

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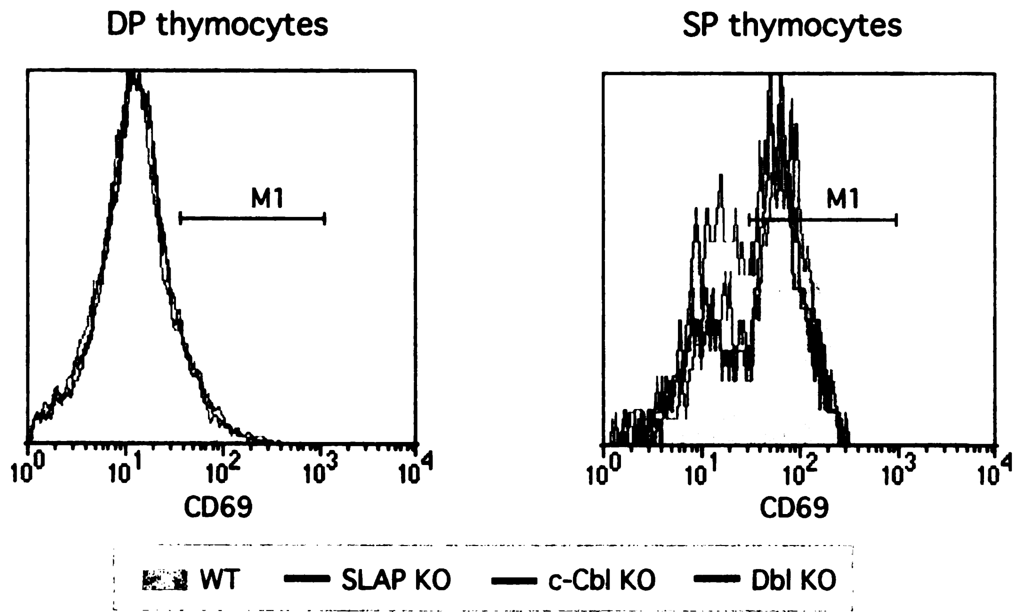

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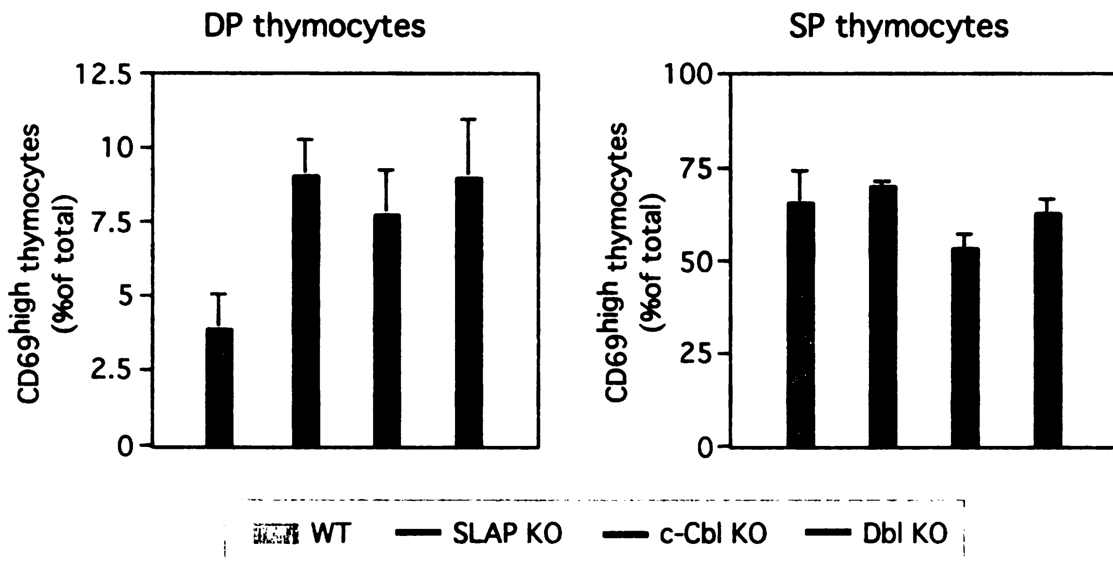


SLAP- and c-Cbl-deficiency result in increased proportion of CD69^{high} DP thymocytes

I reported in Chapter 3 that SLAP deletion resulted in a higher proportion of DP thymocytes expressing high level of CD69, presumably as a result of stronger signaling. Comparison of SLAP- and c-Cbl-deficiencies revealed the same percentage of CD69^{high} cells present in each genetic background (Figure 4). As in the case of CD3, CD4, and CD5 expression, no additive result was observed in the SLAP x c-Cbl double mutant mice.

SLAP- and c-Cbl-deficiencies rescue the development of ZAP-70-/- thymocytes

One of the most profound findings of Chapter 3 was that some SLAP null thymocytes could complete their thymic development without ZAP-70. The same phenotype is observed for Cbl deficiency, as illustrated by the appearance of the SP cells in the thymus (Figure 5A) and the mature T cells in the periphery (Figure 5B) of ZAP-70 x c-Cbl doubly null mice. Note that in both cases only the CD4 lineage was partially rescued.

The ability of SLAP and c-Cbl deficiencies to promote ZAP-70-independent thymic development was unique, since the deletion of CD5, another negative regulator of the development, did not result in appearance of the SP cells (data not shown). Quantitation of this phenomenon shows that, unlike upregulation of the surface markers (Figure 3 and 4), the efficiency of this developmental rescue was slightly higher when both mutations were combined (Figure 5C). This effect is less than additive, however, and more animals need to be analyzed to test its statistical significance. In addition, this

Figure 5. The effect of SLAP and c-Cbl deficiencies on thymic development in ZAP-70 deficient background. Thymocytes (**A**) or lymph node cells (**B**) with the indicated genetic backgrounds were stained with anti-CD4 and anti-CD8 antibodies, and analyzed by FACS. The percentage of CD4 and CD8 SP thymocytes (**C**), as well as CD4 and CD8 lymph node (LN) T cells (**D**) are present for the WT (grey), SLAP KO (red), c-Cbl KO (blue), and SLAP x c-Cbl double KO (green) genetic backgrounds. Circles represent individual animals, and bar graphs depict the averages. At least six animals of each type were analyzed.

difference was not seen in the periphery where the fractions of T cells were identical in all three genetic backgrounds (Figure 5D).

Discussion

My preliminary genetic analysis shows the same phenotypes of SLAP and c-Cbl deficient DP thymocytes: they upregulate CD3, CD4, and CD5, they display the same increase in the percentage of CD69^{high} cells, and they can progress to the SP stage and become mature T cells in the absence of ZAP-70. These striking similarities could be due to both genes participating in the same genetic pathway or due to the identical contributions of each gene to two separate pathways. A classical genetic approach to distinguish between these two possibilities is the analysis of the doubly deficient animals. This approach is commonly used in establishing genetic pathways leading to a given phenotype. For example, by comparing the phenotypes of single and double mutants, Ellis et al., classified six genes (ced-1, 2, 5, 6, 7, 10), which were responsible for engulfment of dead cells in Caenorhabditis elegans, into two distinct pathways. Combining two mutations within a group led to a phenotype no stronger than the stronger single mutant, suggesting the same genetic pathway. In contrast, double mutants carrying mutations in one gene in each class showed a vastly increased engulfment phenotype (i.e. vastly decreased ability to engulf dead cells), confirming additional contributions to the same phenotype from two separate pathways (Ellis et al., 1991). The analysis of the SLAP/c-Cbl double mutant thymocytes shows no such additive effects for surface expression of the CD 3, 4, 5, and 69 when both deficiencies were combined. Therefore, I

propose that SLAP and c-Cbl might participate in the same pathway leading to TCR downregulation on DP cells (see chapter 5 for the discussion of a model). The ultimate proof for this notion will require a complete understanding of the mechanism of action of these genes.

The ability of SLAP or c-Cbl deficient thymocytes to develop into mature single positive thymocytes and mature T cells in the absence of ZAP-70 is additional evidence supporting the notion that both genes share a common pathway. In this case, however, a small qualitative difference is observed in the phenotypes caused by each gene. In particular, the numbers of SLAP deficient SP thymocytes are lower than those observed for c-Cbl deficient cells (Figure 5C). In addition, the double mutants seem to have the highest number of SP thymocytes rescued. Yet, despite this higher number of SP thymocytes, the number of mature T cells in the periphery was the same (Figure 5D). This could reflect alterations in homeostatic mechanisms that control T cell survival or expansion in the periphery, perhaps due to impaired TCR signaling. The evaluation of the statistical significance of the above mentioned differences will require examination of more animals. Nonetheless, these data indicates that, unlike their effects on the expression of the cell surface markers on DP thymocytes, the contribution of SLAP and c-Cbl to the rescue of ZAP-70-deficient thymocytes might be through only partially overlaping pathways.

Experimental Procedures

Flow Cytometry

For each sample, 10^6 cells were stained with antibodies and analyzed on a FACS Calibur using CellQuest software (Becton Dickinson). The following antibodies were used: anti-CD3 (145-2C11)-FITC, anti-TCR β (H57-597)-FITC, anti-CD5-FITC, anti-CD69-FITC, (Pharmingen); anti-CD4-PE, and anti-CD8-TC (Caltag).

Animals

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The ZAP-70 (Kadlecek et al., 1998), were previously described. c-Cbl deficient mice were a kind gift from Dr. Hua Gu (National Institute of Allergy and Infectious Diseases, NIH, MD).

CHAPTER V

SUMMARY AND FINAL THOUGHTS

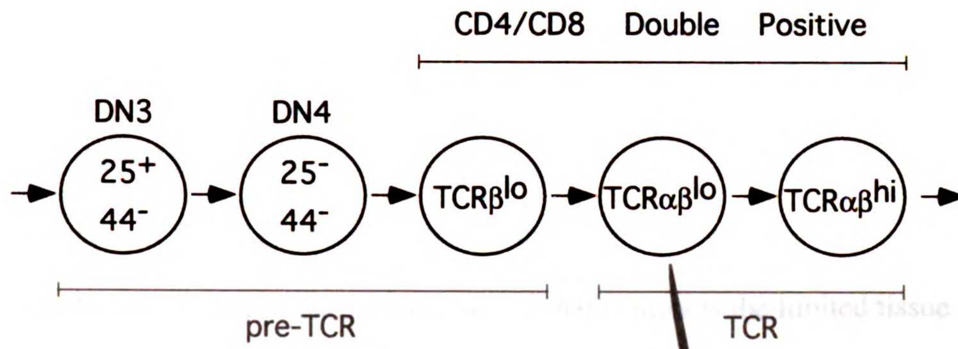
Summary

My analysis of the Src-like Adaptor Protein (SLAP) revealed that this adapter protein is a potent negative regulator of T cell receptor (TCR) signaling. I presented data showing that SLAP is expressed in T cells and can potently inhibit TCR derived signals leading to Il-2 promoter- and NFAT-driven transcription when overexpressed in Jurkat T cells (chapter II). I also generated SLAP deficient mice and conducted initial analyses of their phenotype. In particular, I have shown that SLAP plays a negative role in the surface expression of TCR on DP thymocytes. As a consequence, it negatively regulates positive selection of thymocytes expressing a MHC class II restricted transgenic TCR (chapter III). Finally, in chapter IV I presented genetic evidence that SLAP and c-Cbl are in the same pathway leading to downregulation of TCR on DP thymocytes. Here, I present a model for the regulation of SLAP expression and its potential functions in the thymus and in the periphery. I also speculate on the potential mechanism of SLAP action and propose future experiments and directions.

Preliminary Model for SLAP-Mediated Downregulation of TCR on DP Thymocytes

Based on the data presented in chapters II, III, and IV, I propose the following model for SLAP function in thymic development (see Figure 1). SLAP protein is induced by the pre-T cell receptor signaling during the transition from DN3 to DN4 stage and reaches the highest expression level in DP thymocytes. After a successful gene rearrangement at the *TCRα* locus, the mature αβTCR is gradually expressed on the cell surface of DP thymocytes. Despite synthesizing normal levels of the T cell receptor chains, immature DP thymocytes only express a few receptors on the cell surface. The signal(s) responsible for this downmodulation involves SLAP and is initiated by the interactions of the cell surface coreceptor CD4, and possibly TCR, expressed on DP thymocytes with the MHC class II molecules expressed on APCs. Ligation of CD4 facilitates trans-phosphorylation and activation of the CD4-associated Lck. In turn, activated Lck phosphorylates components of the TCR, which are thus "marked" by tyrosine phosphorylation for downregulation. The proposed downregulation requires both SLAP and c-Cbl, and involves intracellular retention and/or degradation of the TCR. In this process, SLAP and c-Cbl might bind as a complex to "marked" components of the internalized TCR. In addition, c-Cbl might catalyze ubiquitination of the TCR chains, while SLAP might shuttle the polyubiquitinated proteins to the endosomal/lysosomal compartment for degradation. Although this model is consistent with my data and the current literature, a more detailed biochemical analysis will be needed to confirm it.

Figure 1. Model for SLAP function during thymic development. The upper panel represents the pattern of SLAP expression during thymic development. The lower panel depicts the proposed mechanism of SLAP-dependent downregulation of TCR from the surface of DP thymocytes. According to this model, the interactions between the MHC class II molecules and the CD4 and/or TCR lead to activation of Lck, which in turn phosphorylates TCR. Tyrosine phosphorylation of the TCR chains marks them directly, or indirectly, for targeting by SLAP and c-Cbl. This results in the potential retention, ubiquitination, and the degradation of the TCR components.


CD4/CD8 Double Positive
DN3
DN4
25+
44-
25-
44-
TCRβlo
TCRαβlo
TCRαβhi
pre-TCR
TCR

SLAP
expression:
Antigen
Presenting
Cell
MHC with
antigen
CD4
T Cell
Receptor
Lck
Endosomal
recycling
compartment
ZAP-70
T Cell
Receptor
SLAP
c-Cbl
Ub
T Cell
Receptor
Early
endosome
Lysosome
Nucleus
AP-1
c-Jun
c-Fos
NF-AT
IL-2

Regulation of SLAP Expression

Expression of the mRNA

Expression of SLAP is controlled both transcriptionally and post-transcriptionally. The best evidence for transcriptional control is the limited tissue expression of SLAP mRNA (chapter II). In addition, even among the same cell types SLAP might be differentially expressed. For example, despite its presence in primary B and T cells, only one in four murine T cell lines, and none of the three tested B cell lines, expressed SLAP (chapter II). The differential expression of SLAP message might relate to the developmental stage at which these cells were transformed, as SLAP expression is developmentally regulated. Carrier et al., established a developmental pattern of gene expression during thymic development by screening 3000 mouse thymus cDNAs using complex-probe hybridization of DNA arrays (Carrier et al., 1999). In this study, SLAP mRNA was expressed in thymuses of WT, CD3ζ-, TCRδ-, and TCRα-deficient mice, but not detected in CD3ε-, RAG1-, and TCRβ-deficient animals. Since CD3ε-, RAG1-, and TCRβ-deficient thymocytes are arrested at the β-selection stage, these results indicated that SLAP mRNA is expressed in a post-DN3 population(s). The same study showed that SLAP mRNA appeared in the thymus at day 16 and afterwards during embryonic development. These results are in agreement with my conclusion about SLAP expression at the transition from DN3 to DN4 stage (chapter III).

Another aspect of SLAP expression is the nature of the signal leading to the transcriptional activity of the *SLAP* locus. In chapter III, I hypothesized that the pre-TCR signal, and in particular the activity of Lck and Fyn, is required either directly or

indirectly for SLAP expression. The lack of SLAP protein in Lck/Fyn double KO thymocytes, but its induction after 4-hour stimulation with PMA and ionomycin (chapter III) supports this hypothesis. However, since the observed upregulation of the protein expression was of a low magnitude, and the use of the pharmacological reagents may only approximate the pre-TCR signaling events, more direct evidence is needed. One line of experimentation might take advantage of the ability of RAG1 deficient thymocytes to progress from the DN3 to the DP stage upon in *in vivo* stimulation with anti-TCR antibody (Levelt et al., 1993). Monitoring the level of SLAP as a function of the developmental progression might provide important information not only on the initiation, but also on the persistence of SLAP expression at the DN4 and DP stages.

In addition to the pre-TCR, other receptors might induce SLAP expression. For example, Ohtsuki et al., reported induction of SLAP mRNA in U937 (histiocytic lymphoma), HL60 (acute myelocytic leukemia), and NB-4 (acute promyelocytic leukemia) upon treatment with all-trans retinoic acid (ATRA) (Ohtsuki et al., 1997). This induction was detectable after 3 hours and lasted for at least 2 days, during which time all three lines underwent ATRA-dependent differentiation. In contrast, stimulation of the same lines with TPA similarly led to differentiation, but did not result in SLAP expression. This is of interest since PMA stimulation of thymocytes induces some upregulation of SLAP message (data not shown). Expression of SLAP in tissues that do not express pre-TCR, for example lung, brain, muscle and kidney, also implicates other signaling pathways (Pandey et al., 1995). In the case of B cells and mature T cells one might speculate that the mature BCR and the TCR could deliver a signal leading to SLAP expression.

Expression of the Protein

There are several lines of evidence suggesting that, in addition to regulation of the mRNA, SLAP expression is controlled post-transcriptionally. First, the level of SLAP mRNA in the thymic populations enriched for DN, DP, and SP cells is approximately uniform (data not shown), yet the protein level is significantly different (Figure 5B, chapter III), with the DP cells expressing the highest level of the protein. Second, stimulation of thymocytes with PMA and/or ionomycin leads to a weak upregulation of SLAP mRNA (data not shown), but induces a several fold increase in the protein level (Figure 5A, chapter III). Third, a similar result was observed in mature T cells stimulated for 3 days with anti-TCR antibody. Finally, in Jurkat cells Northern blot analysis detected a strong SLAP-specific mRNA signal, but anti-SLAP immunoprecipitations, followed by anti-SLAP Western blotting, did not reveal any SLAP protein (data not shown). Taken together, the observed discordance between the mRNA and the protein levels implies a post-transcriptional mechanism controlling SLAP protein expression.

The nature of the signal leading to the expression of SLAP protein is still in question. Upregulation of the protein following treatment of thymocytes with PMA and/or ionomycin suggested that activation of Ca^{2+}, the PKC, or Ras pathway is sufficient to induce protein expression. This observation might explain expression of SLAP protein in EL-4 cells, since this line has been reported to have a persistently activated Ca^{2+}-dependent signaling pathway due to the gain-of-function mutation in the protein phosphatase calcineurin (Fruman et al., 1995). Mature T cells also upregulate SLAP protein upon prolonged (3 days) activation with anti-TCR antibody (data not shown).

Based on these observations, one might hypothesize that a persistent stimulation leads to protein expression. However, this might be a necessary but not a sufficient signal, since the same treatments of Jurkat cells did not result in the expression of detectable levels of SLAP protein (data not shown).

In contrast to my data, the presence of endogenously expressed SLAP protein has been reported in Jurkat and NIH3T3 cells (Roche et al., 1998; Tang et al., 1999). It is unclear whether the Jurkat clone used in that study expressed any endogenous protein since no pre-immune sera was used for control IP to confirm the specificity of the detected signal. In addition, the electrophoretic mobility of the band detected on a Western blot seemed to be too high to correspond to SLAP. The study using NIH3T3 used the appropriate pre-immune sera controls, and reported the presence of endogenous SLAP protein that could be easily detected in the whole cell lysates. Because SLAP IP from 5 x 10^7 thymocytes yields a barely detectable band on a Western blot (data not shown), this report implied that NIH3T3 cells express significantly higher level of the protein that thymocytes do. This is especially surprising in the light of my data presented in Figure 1 of Chapter II, showing a relatively high level of SLAP mRNA in the thymus and the lack of any detectable signal in NIH3T3 cells. At the moment the only explanation for the observed differences is a potential clonal variation or a reagent specificity problem.

Role of SLAP in Thymic Development

What is the function of SLAP during thymic development? As I have shown in chapter III, SLAP downregulates the TCR on DP cells and plays a negative regulatory role during selection. But, since overall development in SLAP deficient mice seems to be roughly normal, the reason why a low level of TCR expression on DP thymocytes is beneficial for the host is not apparent. One reason for limiting the expression of TCR might be to ensure that only thymocytes expressing TCR with relatively high affinity for self-MHC molecule-peptide complexes are selected. This thesis could be tested by limiting the specificity of the *in vivo* selecting ligands to a single MHC molecule-peptide complex (Ignatowicz et al., 1996), and by determining the affinities of the selected T cell repertoires for the selecting MHC molecule-peptide ligand. The affinity of any TCR for its selecting MHC molecule-peptide ligand is very low, however, and one would have to use a very sensitive surface plasmon resonance (SPR) technique which requires isolation and purification of many TCRα and β pairs (Davis et al., 1998).

Another reason for keeping TCR levels low at the DP stage might be related to the differential expression of Syk and ZAP-70 during thymic development. Chu et al., showed that Syk is expressed early during thymic development and becomes progressively downregulated during transition from DN3 to DN4, and onwards to DP stage (Chu et al., 1999). Moreover, Syk activation is less dependent on the Src-family kinases and CD45 than ZAP-70 (Chu et al., 1996; Zoller et al., 1997). Based on these findings, Chu hypothesized that Syk is important for pre-TCR signaling, since this process occurs in the absence of CD4 and CD8 co-receptors and the associated Lck. In contrast, ZAP-70 plays a role during positive selection because its activation critically depends on the presence of Lck brought to the TCR/MHC molecule-peptide complex by

CD4 or CD8 coreceptor. This dependence on the specific MHC molecule-coreceptor engagement enables CD4 and CD8 lineage commitment.

In the context of the differential expression of Syk and ZAP-70, the role of SLAP might be to downregulate the TCR on early DP thymocytes that still express significant amount of Syk, thus preventing a Syk-based and coreceptor-independent positive selection. In support of this hypothesis, such Syk-dependent positive selection is presumably responsible for the thymocyte maturation in the ZAP-70/SLAP double deficient mice (chapter III, Figure 7). It is difficult to test directly whether Syk participated in the development of the ZAP-70/SLAP double deficient T cells because ZAP-70/Syk double deficient thymocytes are arrested at the DN3 stage, before SLAP could be expressed (Cheng et al., 1997).

Function of SLAP in Mature T Cells

Expression of SLAP mRNA in mature T and B cells may imply a role for SLAP in the periphery (chapter II, Figure1). The level of SLAP protein in the resting lymph node (LN) and splenic cells is relatively low when compared to the amount of the protein expressed in thymocytes (chapter III, Figure 5A). However, upon *in vitro* stimulation of LN cells with anti-CD3 antibody SLAP expression was significantly increased at day 3, and then it returned to low levels on day 5 (data not shown). This transient upregulation of SLAP protein inversely correlated with the proliferative responses of the cells since they displayed the maximum rate of DNA synthesis between day 2 and day 3, and then became gradually less active. This suggested that SLAP might inhibit T cell proliferation

at the time when cells reach their maximum proliferative activities, and that SLAP deficient T cells might proliferate better at the later stages of stimulation. To normalize for the potential differences in the TCR affinities among cells selected with or without SLAP (see "The role of SLAP in Thymic Development" section), I compared proliferative responses of cells expressing the same DO11.10 transgenic TCR. WT and SLAP deficient T cells were stimulated with splenocytes loaded with the OVA 323-339 antigenic peptide and DNA synthesis was assayed on day 3, 5, and 7 (data not shown). In addition, I stimulated cells *in vitro* with a combination of anti-CD3, anti-CD28, and PMA and ionomycin (data not shown). Unfortunately, I did not observe any significant differences in proliferation of WT and SLAP KO T cells (data not shown). Therefore, I conclude that SLAP had no detectable influence on proliferative responses *in vitro*.

A formal possibility remains, however, that despite having the same transgenic TCR, the two populations of T cells differed in the signaling efficiency due to an adaptation that took place during selection. For example, SLAP KO thymocytes expressing the DO11.10 transgenic TCR had to express higher level of some inhibitory protein(s) (i.e. CD 5, chapter III) in order to survive the negative selection. Such higher level stimulation-dependent induction of this hypothetical protein(s) might be preserved in SLAP KO mature T cells, thus compensating for the lack of SLAP. To resolve this problem, one might generate an inducible SLAP KO mouse, where the deletion of *SLAP* gene occurs only after thymic selection.

A second potential role for SLAP in the periphery might be related to its effect on the TCR levels in the thymus. As proposed in my model, SLAP facilitates TCR downregulation in a process initiated by a chronic stimulation of Lck by a MHC and/or

TCR in DP thymocytes. A similar chronic stimulation of Lck may take place in T cells residing in the sites of chronic inflammation where they are exposed to high levels of MHC molecules due to secreted IFN-γ (Steeg et al., 1982; Sztein et al., 1984; Unanue, 1984). A similar chronic activation of Lck may take place in autoreactive T cells that escaped deletion in the thymus. Such potentially autoreactive cells were observed in the periphery of healthy mice expressing transgenic TCR specific for self-antigens. It was also observed that these potentially autoreactive cells downregulated expression of transgenic TCR and the coreceptors (Rocha and von Boehmer, 1991; Schonrich et al., 1991). The downregulation of these transgenic receptors correlated well with the decrease in antigen-specific proliferative responses. Therefore, the authors proposed that downregulation of TCR on autoreactive cells might be a novel mechanism for extrathymic tolerance induction. By the analogy to the SLAP function in the thymus, one might hypothesize that SLAP is responsible for this TCR downregulation on self-reactive T cells. This hypothesis could be tested by assessing the level of SLAP protein in the autoreactive T cells, and their ability to downregulate TCR.

Role of SLAP in Post-Translational Regulation of TCR on DP Thymocytes

The level of TCR on DP cells is Regulated Post-Transcriptionally

One of the major unanswered questions is the mechanism of TCR downregulation at the DP stage. Northern blot analysis showed clearly that DP thymocytes synthesized at least as much of the mRNAs encoding various TCR chains as did SP cells (Bonifacino et al., 1990). Examination of the translational rates for all the TCR components also failed

to detect any significant difference between DP and SP thymocytes (Bonifacino et al., 1990; Kosugi et al., 1992). However, pulse-chaise analysis revealed that some TCR components, and especially TCRα and ζ chains, were unstable in DP cells (Bonifacino et al., 1990; Kearse et al., 1994; Kosugi et al., 1992). Taken together, these results showed that TCR level on DP thymocytes is regulated post-translationally. Two models explaining this post-translational mechanism of regulation were put forward. The first proposed that DP thymocytes had a cryptic assembly defect, and that the "free" TCR components were degraded in the reticulum (ER). The second model stated that the mature TCR complexes were assembled properly, but that they were unstable and degraded in the post-ER compartment.

Model#1: Defective TCR Assembly in the Endoplasmic Reticulum

The first model was proposed by Kearse et al., who studied the efficiency of mature TCR assembly in the ER by examining the rate of metabolically labeled TCRζ incorporation into the CD3ε-bound complexes (Kearse et al., 1994). Because the addition of TCRζ chain is the last step of the mature TCR assembly (Alarcon et al., 1988; Bonifacino et al., 1988; Ohashi et al., 1985; Saito et al., 1987), comparing the level of CD3ε-bound ζ with the "free" ζ reflects the efficiency of the assembly. By showing that only about 24 % of the nascent TCRζ was incorporated into mature TCR in DP thymocytes, as compared to 67 % in splenic T cells, this study concluded that TCR assembly is impaired proximally to TCRζ addition. In fact, they proposed that this defect prevents efficient incorporation of the TCRα chain, resulting in a dramatic instability of the protein (the median survival time of nascent TCRα in DP thymocytes was 15 min

comparing to 75 min in mature T cells). Based on these and other findings, a model was proposed in which CD4 coreceptor expressed on the surface of DP thymocytes becomes engaged by MHC class II, and sends a signal interfering with the proper folding or processing of TCRα. Misfolded α chains prevent proper assembly of mature TCRs, and leads to subsequent degradation of free TCRα and ζ chains in the ER.

Model#2: Decreased Stability of Assembled TCR

The second model proposes that the low TCR level on DP thymocytes is due to the instability of the mature TCR. Data published by Kosugi et al. confirmed a short half-live of TCR α and ζ chain (Kosugi et al., 1992). However, by estimating the amount of CD3ε-associated ζ chain, they showed that the degree of mature TCR assembly was equivalent in DP and SP cells, thus directly contradicting the "defective assembly" model. It is unclear why the two studies arrived at different conclusions using similar approaches, but perhaps this relates to the use of different control populations: Kearse used splenic T cells, whereas Kosugi used SP thymocytes as standard for "normal" assembly of the TCR. In the same study, Kosagi showed that NH_4Cl treatment inhibited TCRζ degradation, thus implicating an endosomal/lysosomal pathway rather than the ER-based degradation. Consequently, the authors concluded that one possible interpretation of their results was the instability of the completely assembled TCR at the cell surface.

Persistent Activation of Lck Leads to Downregulation of the TCR

That TCRs are transported to the cell surface of DP thymocytes and are then rapidly internalized and degraded in lysosomes, was also proposed based on the studies of Lck effects on TCR downregulation (D'Oro et al., 1997). In this study, the presence of a constitutively active form of Lck in T cell lines created a phenotype very reminiscent of the DP thymocytes: (i) The cell surface TCR level was significantly downregulated due to a post-transcriptional mechanism; (ii) TCR α, β, and ζ chains were rapidly degraded, whereas the CD3 chains (ε, γ, and δ) were relatively stable; (iii) Inhibition of the endosomal/lysosomal pathway by NH_4Cl treatment prevented the degradation of TCR α, β, and ζ chains. Interestingly, inhibition of endogenous Lck in DP thymocytes, with herbimycin A or geldamycin, resulted in significant upregulation of the TCRα and ζ proteins (Wiest et al., 1993). A similar rescue of the TCR chains was observed when cells were treated with *o*-phenanthroline, a metal chelator that dissociated Lck from CD4. Based on these observations the authors concluded that chronic activation of Lck by CD4-MHC class II interactions was responsible for downregulating TCR level on DP thymocytes (Wiest et al., 1993).

Role of SLAP in TCR downregulation: Stability of the TCR Chains and the Rates of Receptor Endocytosis and Recycling

The phenotype of SLAP deficient mice led me to propose in Chapter III that SLAP participates in the same pathway of TCR downregulation in DP thymocytes as do MHC class II, CD4, Lck, and c-Cbl. In Chapter IV, I presented genetic evidence that SLAP and c-Cbl might indeed be in the same pathway. In addition to the genetic analysis, one might consider examining the biochemical effects of SLAP and c-Cbl

deficiencies. By using similar approaches to the ones used by Singer's group, one might not only strengthen the argument for the same pathway for SLAP and c-Cbl, but also for MHC class II molecule, CD4, and Lck (Kearse et al., 1994). For example, metabolic labeling and pulse-chase experiments, combined with 2D nonreducing-reducing SDS-PAGE, would test whether the upregulation of TCRα and ζ chains occurs in SLAP and c-Cbl deficient DP thymocytes. In fact, if my model is correct, all the deficiencies (i.e. MHCII molecule, CD4, Lck, c-Cbl, and SLAP) should display similar accumulation of these proteins (see below for the discussion of the model). Another important aspect of TCR downregulation is the rate of endocytosis and the extent of receptor recycling to cell surface. By using the reversible surface biotinylation approach of Liu et al., one might test if these processes are also similar in all of the genetic backgrounds tested (Liu et al., 2000). Here, one might expect to see some differences between SLAP and c-Cbl thymocytes, depending on the precise role of each protein. For example, if SLAP is responsible for the retention of the stimulated TCR in the endosomal/lysosomal compartment, but c-Cbl is only responsible for the ubiquitination of the complexes, then the degree of retention might be higher in c-Cbl KO cells since they will have SLAP to retain them.

The Role of Ubiquitin in the Degradation of TCR chains

It is well established that the degradation of TCRα and ζ, and to a lesser degree of the other TCR chains, is enhanced in DP thymocytes. However, the mechanism of this process, and its subcellular localization, is quite controversial. Experimental evidence implicated degradation in the endoplasmic reticulum (Bonifacino et al., 1990), lysosomes

(D'Oro et al., 1997; Kosugi et al., 1992; Liu et al., 2000; Valitutti et al., 1997), and proteosomes (Liu et al., 2000) One study even suggested that two distinct pathways degrade different TCR chains, with the initial step involving the proteosomal degradation of the ubiquitinated TCRζ followed by the lysosomal degradation of TCRαβ chains (Liu et al., 2000).

At present it is unclear what is the consequence of a covalent attachment of ubiquitin (Ub) molecule(s) to the TCR chains, a process known as ubiquitination. Cenciarelli et al., showed that TCRζ and CD3δ can become polyubiquitinated upon TCR stimulation in 2B4 hybridoma and splenic T cells (Cenciarelli et al., 1992). They estimated that approximately 10 % of TCR-associated ζ became polyubiquitinated within 5 minutes of TCR stimulation and, surprisingly, this persisted for at least 2 hours. This result suggested that 2B4 cells were unable to degrade the polyubiquitinated ζ protein, perhaps because they did not express SLAP. Recently, Wang et al. analyzed the effects of c-Cbl and ZAP-70 on the degree of polyubiquitination of TCRζ in Jurkat cells, and concluded that ZAP-70 plays an essential role in c-Cbl mediated ubiquitination by bridging c-Cbl and TCRζ (Wang et al., 2001). Although suggestive, the physiological relevance of this study is not obvious, since the results were obtained by overexpressing all four proteins (c-Cbl and ZAP-70 TCRζ, and Ub), and by using pervanadate to stimulate the cells. In addition, this study did not present any evidence of the Ub-dependent degradation of TCR chains. Taken together, there is limited data in the literature on the role of ubiquitination in TCR degradation, and in particular there is no direct evidence indicating that downregulation of TCR on DP thymocytes depends on ubiquitination.

Despite the lack of any direct evidence for Ub-dependent TCR degradation, the fact that c-Cbl, an E3 protein ubiquitin ligase, participates in SLAP-dependent TCR downregulation strongly suggests that at least some receptor chains might become ubiquitinated and possibly degraded by a Ub-dependent proteolysis. One potential problem in detecting ubiquitinated endogenous TCR chains might be a low level of such proteins at the steady state. To increase the amount of the substrates for ubiquitination, one might induce accumulation of the TCRs on DP cells by treating the cells with the Lck inhibitor PP1, as reported by D'Oro et al., (D'Oro et al., 1997). Upon washing away these inhibitors, the amount of triggered TCRs would potentially increase to a detectable level. In the same experiment one might follow the fate of surface TCR by immunoprecipitation of surface-biotinylated receptors, thus increasing the signal to noise ratio. Another potential problem might be the limiting amount of the endogenous Ub. That Ub is limiting for protein ubiquitination was deduced from experiments in which inhibition of proteosomes with MG-132 led to reduction in the level of free Ub, presumably due to inhibition of the Ub recycling pathway (Mimnaugh et al., 1997; Schubert et al., 2000). This exhaustion can be quite rapid, with more than 95 % of free Ub disappearing within 90 minutes of MG-132 treatment (Patnaik et al., 2000). Therefore, to enhance the amount of available Ub and its detection, one might generate mice overexpressing an epitope-tagged version of the protein. The two possible ways of accomplishing this are the generation of transgenic mice or the reconstitution of bone marrow with cells transduced with mouse stem cell virus expressing Ub. To avoid any potential nonspecific effects of Ub on development, the Ub ORF could be placed under control of an inducible promoter.

SLAP-Mediated Inhibition of TCR Signaling that is Independent of the Receptor Downregulation

The model for SLAP function in thymocyte development was based on the fact that SLAP deficient thymocytes were unable to downregulate TCR on DP cells. However, it is also possible that SLAP might be able to inhibit TCR signaling without affecting the cell surface level of the receptor. In fact, SLAP overexpression in Jurkat T cells significantly interfered with TCR signaling but had no effect on TCR level (chapter II and data not shown). Since SLAP has high sequence homology to Src-family kinases, it is possible that it could act as a competitive inhibitor of Lck. As described in Chapter I, SLAP N-terminus lacks the di-cysteine motif, making it an unlikely competitor for Lck binding to CD4. Therefore, SLAP might not be able to act upstream from Lck and suppress its initial activation. This prediction is supported by the apparently normal induction of protein tyrosine phosphorylation in cells overexpressing SLAP (chapter II, Figure 9, and data not shown). Instead, it is more likely that SLAP interferes with Lck-generated signals by binding to its substrate(s) or the interacting proteins. For example, SLAP might interfere with Lck binding to ZAP-70, an interaction proposed to be mediated by the Lck SH2 domain and the phosphorylated Y^{319} in the interdomain B of ZAP-70 (Pelosi et al., 1999). However, at present we do not have any data showing impairment of Lck or ZAP-70 activities, and the identity of any Lck substrate(s) affected by SLAP protein is unknown.

Finally, it is still possible that SLAP inhibits signaling in Jurkat T cells by facilitating TCR degradation. My preliminary analysis of the effects of SLAP

overexpression was limited to the examination of the TCR levels on unstimulated cells, and the rates of TCR downregulation during the first 2 hours of stimulation. If my model is correct, one might not expect to see any effect of SLAP on the level of unstimulated receptors because they would not be "marked" for the retention and/or degradation. Moreover, SLAP might not have any effect on the initial rate of TCR downregulation, and instead it might influence the level of TCR components several hours post stimulation. This delayed effect of SLAP on the level of TCR might still have functional consequences, as a continuous TCR signal is required for at least 2 hours for efficient IL-2 production (Weiss et al., 1987). In conclusion, more studies are needed to resolve the question of whether SLAP might inhibit TCR signaling without influencing TCR level.

Can SLAP inhibit TCR signaling independently of c-Cbl?

Although the data presented in chapter III show that SLAP and c-Cbl are in the same pathway leading to downregulation of TCR on DP thymocytes, each protein may exploit additional means to interfere with the TCR signaling. In fact, expression of either SLAP or c-Cbl in Jurkat cells inhibited TCR signaling without causing any apparent downregulation of the TCR (see above). In this system, one might ask if the inhibitory effects of SLAP and c-Cbl are inter-dependent. Since Jurkat does not express endogenous SLAP protein (data not shown) one might speculate that the inhibitory effects of c-Cbl observed in this cell line are independent of SLAP. Another possibility is that Jurkat expresses a protein that can functionally substitute for SLAP function. This

possibility is especially intriguing since a SLAP-like protein has been recently cloned (J. Wu, personal communications).

A direct testing of the complementary hypothesis on whether SLAP-induced inhibition of the TCR signaling depends on the function of c-Cbl is more difficult because Jurkat cells express endogenous c-Cbl protein. However, one may exploit the observation that c-Cbl-induced inhibition of TCR signaling critically depends on the interactions between c-Cbl and ZAP-70. Therefore, one might test indirectly if c-Cbl participates in the SLAP-dependent inhibition of TCR signaling by comparing the effects of SLAP in a ZAP-70 deficient Jurkat cell line (p116) stably reconstituted with either the WT ZAP-70 or ZAP-70 mutant, ZAP-70 (Y292F), which cannot bind to c-Cbl. The inability of SLAP to suppress TCR signaling in Jurkat cells expressing ZAP-70 (Y292F) would show that the interaction between c-Cbl and ZAP-70 is necessary for the SLAP function, thus implicating c-Cbl in SLAP inhibitory function.

Identification of a SLAP-Like Adapter Protein

A very exciting recent development is the identification of an adaptor protein structurally resembling SLAP (Jun Wu, personal communication). Because the report describing this protein has not been published yet, for the sake of simplicity I will call this protein SLAP-2. Like SLAP, this protein has a short N-terminus, followed by an SH3 and an SH2 domains, and a relatively short unique C-terminus. Preliminary data indicate that SLAP-2 can inhibit BCR-dependent induction of CD69 expression in B

cells, and TCR-dependent induction of NFAT expression in T cells. These findings suggest that SLAP and SLAP-2 might constitute a new family of adapter proteins. One might speculate that both family members are expressed in the same cell types, and therefore that the relatively mild phenotype of SLAP KO is due to a partial functional redundancy of the two proteins. This might also explain why SLAP deficiency did not result in any phenotype in mature T cells. The exciting future experiments might involve the analysis of the immune system in mice rendered deficient in expression of both genes.

Final thoughts

The data presented in this thesis indicates that SLAP plays an important role in the development and function of T cells. It is clear that SLAP can potently inhibit TCR signaling, and that it is necessary for downregulation of TCR on immature DP thymocytes. SLAP may also play an important role in the periphery, but this function of SLAP might be substituted for by another member of the SLAP family, a recently cloned adaptor protein SLAP-2. Base on my data showing functional interdependence of SLAP and c-Cbl, it is likely that there is an intimate functional relationship between the Cbl family of protein ubiquitin ligases and SLAP family of adaptor proteins. In fact, an exciting possibility exists that the Cbl-dependent negative regulation of signaling in other receptor systems (e.g. PDGF, EGF) depends on the interactions with the SLAP proteins. The research presented in this thesis provides further insight into the mechanism of T cell receptor signaling. In particular, my data shows the importance of negative regulation in the generation of mature T cells. More research is obviously needed to fully understand the regulation of SLAP expression and its function. One may hope that in the future we will be able to specifically suppress the function of the immune system by inducing SLAP expression in T and/or B cells.

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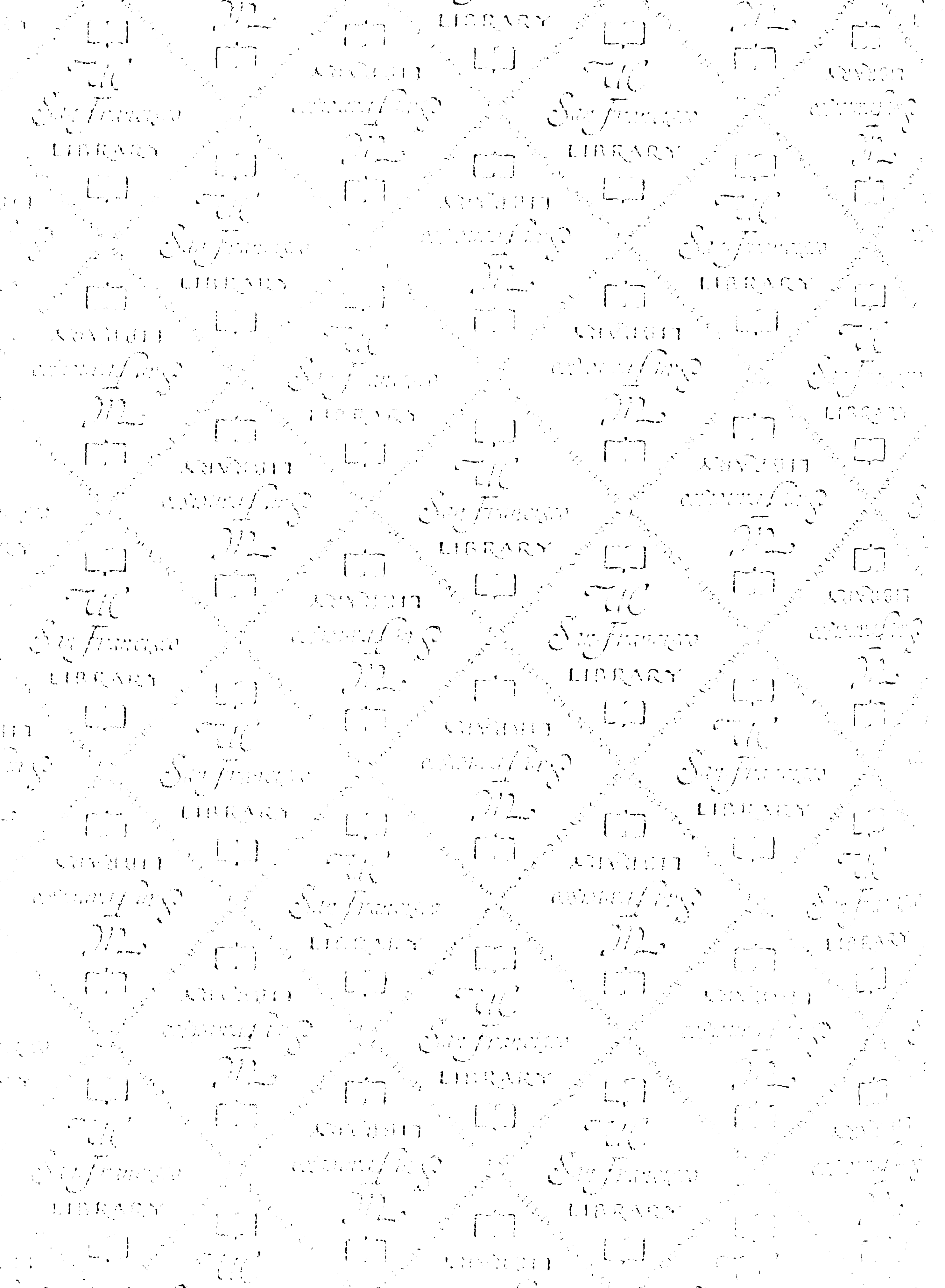
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