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Src-like adaptor protein: A molecule that regulates TCR/CD3 expression by adapting the E3 ubiquitin ligasec-Cbl to the TCR/CD3 complex

Author
Myers, Margaret D

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Src-Like Adaptor Protein: A Molecule that Regulates TCR/CD3 Expression by Adapting the E3 Ubiquitin Ligase c-Cbl to the TCR/CD3 Complex

by

Margaret D. Myers

DISSERTATION

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The studies presented in this thesis were performed predominantly by myself under the guidance of Dr. Arthur Weiss. Dr. Weiss was also the principal investigator supporting this research. A detailed description of author contributions is listed below.

Chapter 2:

M.D. Myers, L. L. Dragone and A. Weiss. Src-Like Adaptor Protein downregulates TCR/CD3 expression by targeting TCRζ for degradation (2005). *Accepted for publication in the Journal of Cell Biology.*

The studies presented in Chapter 2 were carried out predominantly by myself under the guidance of Dr. Arthur Weiss. L.L. Dragone aided in the propagation and analysis of C57BL/6 and SLAP deficient mice. L.L. Dragone and A. Weiss provided intellectual and editorial input.

Chapter 3:

M.D. Myers, T. Sosinowski, L.L. Dragone, C. White, H. Band, H. Gu and A. Weiss (2005). Src-like Adaptor Protein down regulates T cell receptor expression by adapting c-Cbl to the TCR/CD3 complex. *Manuscript in preparation.*

The studies presented in Chapter 3 are currently being written up to submit for publication. These studies were carried out predominantly by myself under the guidance of Dr. Arthur Weiss. T. Sosinowski generated the SLAP$^{-/-}$c-Cbl$^{-/-}$, SLAP$^{-/-}$ZAP-70$^{-/-}$, c-Cbl$^{-/-}$ZAP-70$^{-/-}$ and SLAP$^{-/-}$c-Cbl$^{-/-}$ZAP-70$^{-/-}$ mice. In addition, T. Sosinowski performed

the initial characterization of SLAP$^{-/-}$, c-Cbl$^{-/-}$ and SLAP$^{-/-}$c-Cbl$^{-/-}$ mice shown in Figure 1a-b and provided the thymus and lymph node analyses shown in Figure 2c-d. L.L. Dragone and C. White aided in the propagation and the subsequent analysis of all mouse lines with the exception of the experiments presented in Figure 1c-d. H. Band provided the c-Cbl constructs utilized in Figure 5. H. Gu donated the c-Cbl$^{-/-}$ mice. L.L. Dragone and A. Weiss provided intellectual and editorial input.

Chapter 4:

M.D. Myers, C. Geisler and A. Weiss. Identification of a dileucine-based motif in SLAP that is required to downregulate the T cell receptor. *Manuscript in preparation.*

The studies presented in Chapter 4 were carried out predominantly by myself under the guidance of Dr. Arthur Weiss. The CD3$\gamma^{-/-}$ Jurkat T cell line was provided by C. Geisler. Intellectual and editorial input was provided by A. Weiss.

Abstract

Src-like Adaptor Protein: A Molecule that Regulates TCR/CD3 Expression by Adapting the E3 Ubiquitin Ligase c-Cbl to the TCR/CD3 Complex

Margaret D. Myers

Src-Like Adaptor Protein (SLAP) downregulates expression of the T cell receptor (TCR)/CD3 complex during a specific stage of thymocyte development when the TCR repertoire is selected. Consequently, *SLAP*$^{-/-}$ thymocytes display alterations in thymocyte development. Interestingly, mice deficient in either SLAP or c-Cbl have very similar thymic phenotypes. We have studied the mechanism of TCR/CD3 downregulation by SLAP and c-Cbl. Using mice deficient for SLAP and/or c-Cbl, we demonstrate that thymocytes deficient in SLAP and/or c-Cbl have increased TCRζ chain expression due to a defect in TCRζ degradation. Failure to degrade TCRζ leads to an increased pool of fully assembled TCR/CD3 complexes that are capable of recycling back to the cell surface. SLAP functions in a pathway that requires the phosphorylated TCRζ chain and the Src-family kinase Lck, but not ZAP-70 or Slp-76. In addition, TCR/CD3 downregulation requires multiple domains in SLAP as well as the ring finger of c-Cbl. Moreover, both SLAP and c-Cbl are required to target the TCRζ chain for ubiquitination and degradation. Finally, we describe a previously uncharacterized dileucine-based motif present in the SH2 domain of SLAP that is required for TCR/CD3 downregulation and TCRζ degradation by SLAP and c-Cbl. These studies reveal a unique mechanism by which SLAP and c-Cbl contribute to the regulation of TCR expression during a distinct stage of thymocyte development.

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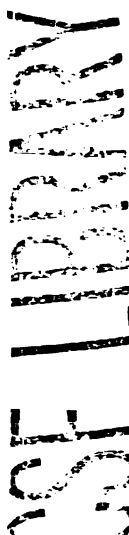
Chapter 1:

The TCR/CD3 Complex: Signaling, Trafficking and its Role in Thymocyte Development

Introduction:

In mammals, the immune system has evolved to include a variety of effector cell types, each of which plays a specific, non-redundant role in the generation of an immune response (Delves and Roitt, 2000). Cells of the immune system can be broadly distributed between two categories: cells of the innate immune system (macrophages and neutrophils, e.g.) and cells of the adaptive immune system (B and T lymphocytes). Innate immune cells recognize a limited number of conserved molecular patterns that are expressed only by pathogens, including components of bacterial cell walls and viral RNA. A family of non-variable receptors, many of which are expressed simultaneously on a single innate immune cell, mediates recognition of these conserved epitopes. The broad distribution of receptor expression present on innate immune cells in addition to the large number of cells present in the blood and peripheral tissues ensures that the innate immune system is capable of responding rapidly to infections.



Many pathogens can be cleared by the innate immune system alone. However, clearance of certain infections requires the additional contribution of the adaptive immune system. During such infections, the innate immune system continues to provide two essential functions. First, innate immune responses are required for the subsequent activation of the adaptive immune response. Second, innate immunity is required to control the early stages of infections. In contrast to innate immune cells, B and T lymphocytes do not express multiple non-variable receptors for pathogen recognition, but instead express a B cell or T cell antigen receptor (BCR or TCR) with a single antigenic specificity. The wide

range of receptor specificities present in the lymphoid population increases the possibility that at least one B and/or T cell can recognize a given antigenic peptide. However, the size of the lymphoid compartment is not limitless. Therefore, a wide range of receptor specificities leads to a decrease in the frequency of antigen-specific cells present in the lymphoid compartment. Consequently, activation of the adaptive immune system occurs much more slowly than activation of the innate immune system.

During development, each individual lymphocyte independently rearranges the gene segments encoding the antigen receptor, leading to the generation of an antigen receptor which is unique to that cell (Sebzda et al., 1999). The ability to generate such a diverse pool of receptors does not come without a price. Since gene segments are randomly rearranged, the receptors generated are not limited to binding only pathogenic epitopes, but may be capable of binding to self-peptides as well. Any lymphocyte expressing a self-reactive receptor is potentially dangerous, and is capable of causing autoimmune disease if not deleted from the lymphoid compartment. Therefore, mechanisms must exist to remove autoreactive B and T cells from the lymphocyte pool.

For T cells, one mechanism of removing autoreactive cells occurs in the thymus. T cells are unique in their recognition of foreign antigens in that they do not recognize a specific pathogenic epitope in isolation. Instead, T cells recognize short peptides derived from the pathogen which are displayed by major histocompatibility (MHC) molecules expressed on the surface of antigen presenting cells (Delves and Roitt, 2000). The T cell receptor must bind not only to the pathogenic peptide, but to self MHC molecules as well.

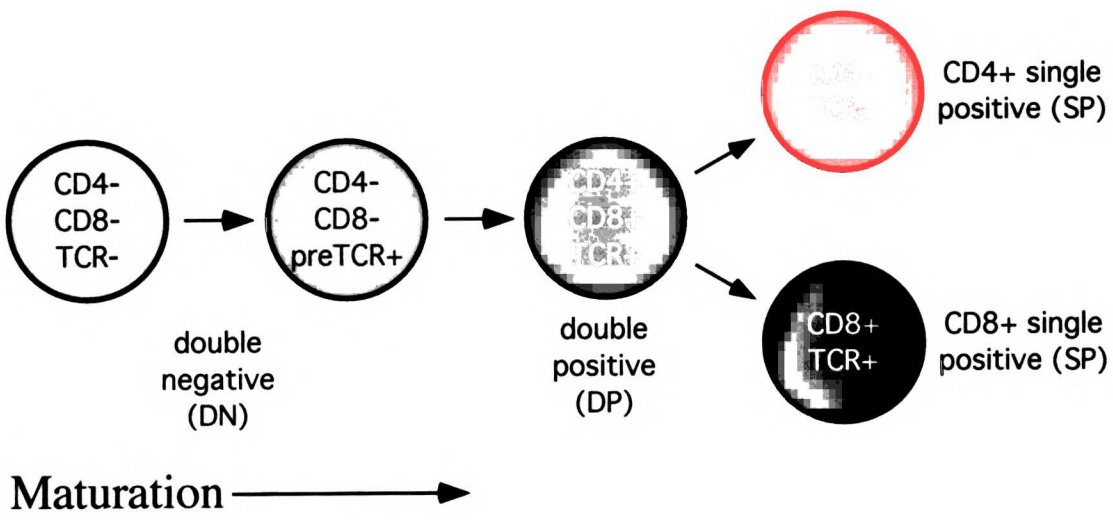
Consequently, T cells must be inherently autoreactive to some extent due to the requirement for binding to MHC molecules. However, strong binding of the TCR to MHC alone or to self-peptides may lead to an inappropriate autoimmune response. Therefore, during development, immature T cells (thymocytes) undergo selection processes that are designed to select for thymocytes expressing TCRs with an intermediate affinity for peptide/MHC molecules. This results in the generation of a pool of peripheral T cells that can interact only weakly with MHC molecules but can be induced to bind tightly to peptide/MHC complexes in the context of the correct antigenic peptide.

Thymocyte Development:

During thymocyte development, each individual thymocyte independently rearranges the gene segments encoding the TCRα and TCRβ genes to create a unique αβTCR. The αβTCR is part of a multi-chain complex which is composed of the peptide/MHC binding TCRα and β chains that are non-covalently associated with the CD3γεδε and TCRζζ chains, together referred to as the CD3 complex (Exley et al., 1991). While the αβTCR is responsible for antigen recognition, the remainder of the CD3 complex, the TCRζ chains in particular, are required for coupling the TCR to downstream signaling molecules.

Generation of an αβTCR initiates at the most immature stage of thymocyte development (Sebzda et al., 1999). At this early stage of development, thymocytes express neither CD4 nor CD8 coreceptor molecules (Figure 1). Thymocytes that are destined to become αβ T cells rearrange their TCRβ genes. If rearrangement of TCRβ is successful (in

Figure 1: Thymocyte development. Thymocyte development can be divided into three stages, which can be distinguished based on expression of coreceptor molecules (CD4 and CD8). The most immature thymocytes are negative for both CD4 and CD8. Signals through the preTCR induce the upregulation of CD4 and CD8 and progression to the double positive stage of thymocyte development. Double positive thymocytes expressing mature αβ T cell receptors that can bind to peptide/MHC molecules downregulate either CD4 or CD8 to become single positive thymocytes. After further maturation, thymocytes exit the thymus to become naïve peripheral T cells.



frame), the TCRβ chain is assembled together with a nonvariant preTCRα chain and the CD3 complex and the preTCR complex is transported to the cell surface. Surface expression induces ligand-independent signals through the preTCR complex (Irving et al., 1998), which allows for thymocytes to proliferate and upregulate CD4 and CD8 expression, thereby progressing to the CD4+CD8+ double positive (DP) stage of development.

At the DP stage of development, thymocytes rearrange their TCRα genes. If rearrangement of TCRα is successful, low levels of the mature TCR/CD3 complex are expressed on the surface of the developing thymocyte. Expression of the TCR/CD3 complex is crucial at this stage of development, as signals through the TCR are required for either the deletion or the survival of DP thymocytes (Love and Chan, 2003; Sebzda et al., 1999). If positively selecting TCR signals are received, DP thymocytes downregulate either CD4 or CD8 to become CD8+ or CD4+ single positive (SP) thymocytes, respectively. Finally, SP thymocytes undergo further selection and development processes before exiting the thymus to become mature peripheral T cells.

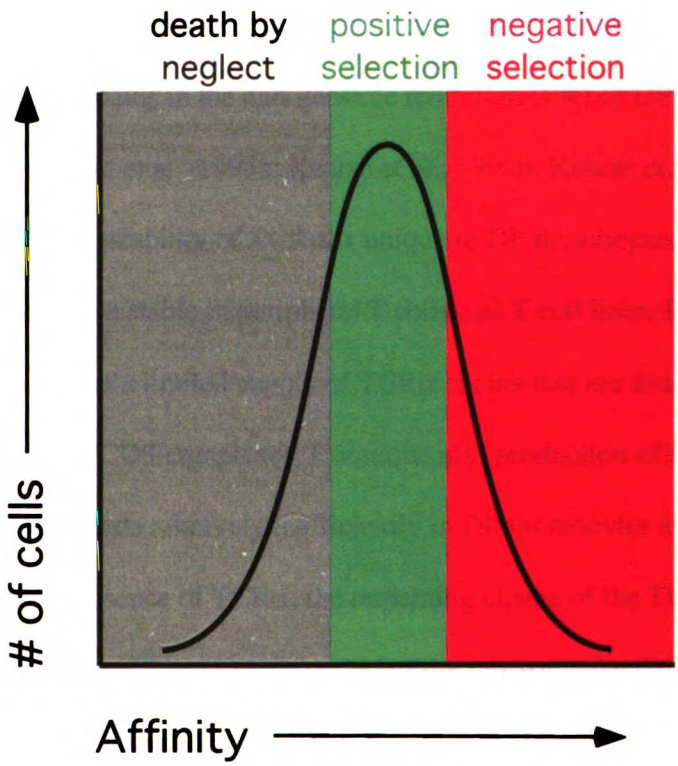
Unlike the preTCR, surface expression of the mature TCR/CD3 complex is not sufficient to initiate signaling. Instead, the TCR must interact with peptide/MHC molecules for signaling to occur. Therefore, the process of positive selection that occurs during the DP stage ensures that only thymocytes expressing TCRs that are capable of binding to peptide/MHC progress to the SP stage of development. In contrast, thymocytes that express TCRs with very low or no affinity for MHC molecules fail to receive positively

selecting signals and ultimately die. While this process selects for thymocytes expressing TCRs with some minimal reactivity with peptide/MHC, positive selection is unable to eliminate cells that interact too strongly to MHC and/or self peptides and could potentially become autoreactive. Therefore, thymocytes also undergo a negative selection process to eliminate thymocytes that express TCRs with too high affinity for either MHC or self-peptide molecules. During negative selection, strong signals through the TCR lead to the apoptosis of strongly autoreactive thymocytes. Consequently, only thymocytes with intermediate affinity for MHC molecules survive both negative and positive selection (Figure 2).

Regulation of TCR/CD3 Expression on DP Thymocytes

Signals through the TCR are required for positive selection. However, surface expression of the TCR/CD3 complex on DP is less than 10% of the level observed on SP thymocytes and peripheral T cells (Crispe et al., 1987; Finkel et al., 1987; Havran et al., 1987). The reason for low TCR/CD3 expression on DP thymocytes is unknown; however, we speculate that positive selection occurs more efficiently if TCR/CD3 levels are kept at a minimum. Low levels of TCR expression on DP thymocytes may function to prevent amplification of TCR signal transduction so that quantitative differences in signaling may be distinguished more accurately. Therefore, only thymocytes possessing TCRs with some minimum affinity for peptide/MHC molecules would receive positively selecting signals. After positive selection, TCR/CD3 expression is rapidly upregulated to levels similar to those observed on peripheral T cells. The increased TCR/CD3 expression on positively selected thymocytes thus reflects the level of TCR expression on mature T

Figure 2: Survival of thymocytes is dependent on T cell receptor affinity. Only thymocytes expressing T cell receptors with intermediate affinity for peptide/MHC molecules survive both positive and negative selection. Thymocytes expressing T cell receptors with low or no affinities for peptide/MHC fail to receive positively selecting signals and subsequently die via apoptosis (death by neglect). Thymocytes expressing T cell receptors with high affinity for peptide/MHC are potentially autoreactive and are deleted from the thymus by apoptosis (negative selection). Thymocytes expressing T cell receptors with intermediate affinity for peptide/MHC receive positive, but not negative selecting signals and develop into mature T cells (positive selection).



cells that encounter autoantigens outside of the thymus. The deletion of autoreactive T cells thus occurs at this higher physiologic TCR level. These observations suggest that strict regulation of TCR/CD3 expression during thymocyte development may be required to optimize receptor levels for both positive and negative selection. Therefore, alterations in TCR/CD3 expression during thymocyte development may dramatically alter the efficacy of both positive and negative selection.

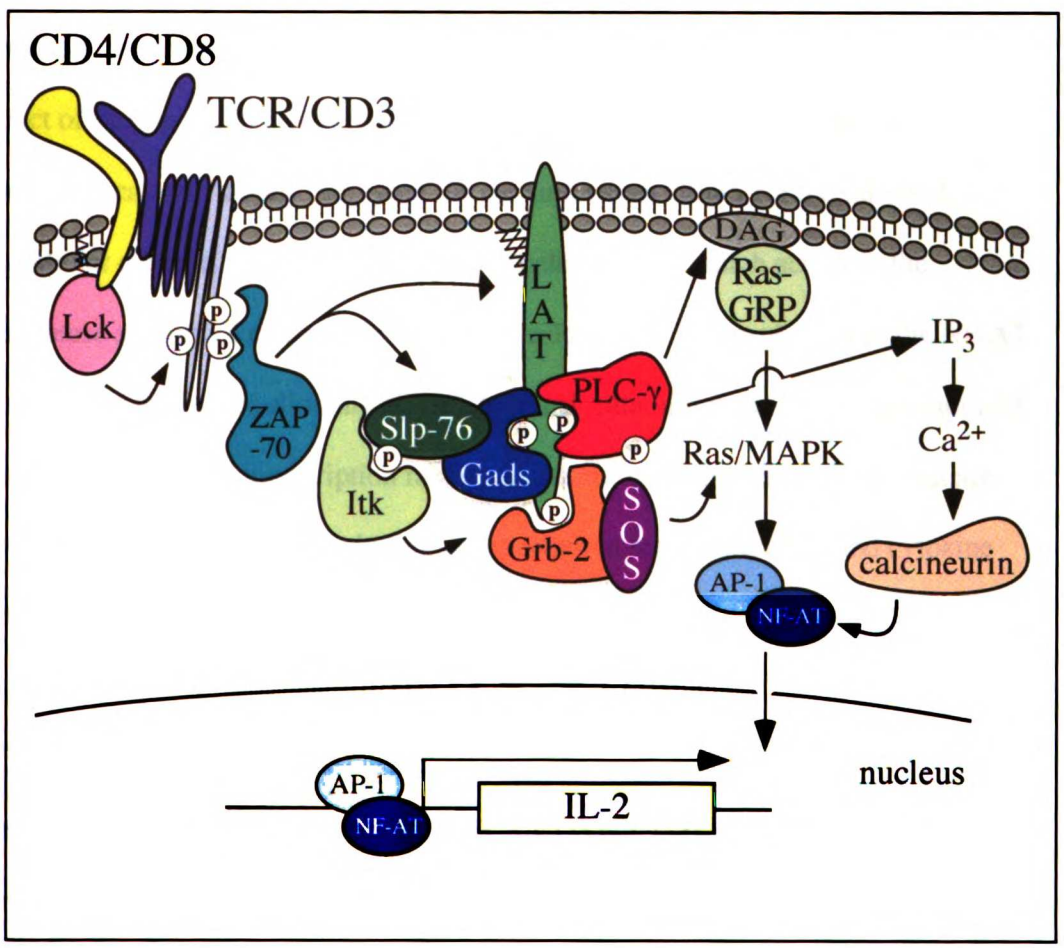
One mechanism regulating TCR/CD3 expression in the thymus involves the instability of newly synthesized TCRα chains in the endoplasmic reticulum (ER) of DP thymocytes (Kearse et al., 1994a; Kearse et al., 1995a; Kearse et al., 1995b; Kearse et al., 1995c; Kearse et al., 1994b). The instability of TCRα is unique to DP thymocytes, as TCRα chains are considerably more stable in peripheral T cells and T cell lines. Rapid degradation of TCRα causes a limited supply of TCRα chains that are available for assembly into mature TCR/CD3 complexes. Consequently, production of fully assembled TCR/CD3 complexes proceeds relatively inefficiently in DP thymocytes as compared to more mature cells. In the absence of TCRα, the remaining chains of the TCR/CD3 complex either remain in the ER or are transported to the lysosome for degradation (Bonifacino et al., 1990; Bonifacino et al., 1989; Klausner et al., 1990). Upon positive selection, however, newly synthesized TCRα chains are stabilized (Kearse et al., 1995c). Once TCRα chains are no longer limiting, assembly of the TCR/CD3 complex proceeds much more efficiently, resulting in increased TCR/CD3 expression at the cell surface.

TCR Signal Transduction:

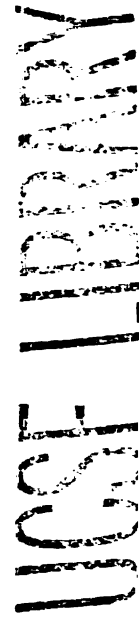
Development of T cells as well as peripheral T cell function is dependent on signaling through the TCR (Kane et al., 2000; Love and Chan, 2003; Weiss and Littman, 1994). The TCR/CD3 complex does not possess any intrinsic enzymatic activity. Therefore, engagement of the TCR alone is unable to optimally activate the TCR signaling pathway. Instead, co-ligation of the TCR together with CD4 or CD8 increases TCR sensitivity to antigen approximately 30-fold (Irvine et al., 2002). CD4 and CD8 bind to MHC molecules at non-polymorphic sites that are distinct from TCR binding. In addition, CD4 and CD8 are associated with the Src family tyrosine kinase, Lck, via their cytoplasmic domains. Simultaneous engagement of MHC by the TCR together with CD4 or CD8 brings Lck within close proximity of the cytoplasmic domains of the TCR/CD3 complex (Figure 3). Once recruited, Lck phosphorylates tyrosine residues present in the cytoplasmic domains of the CD3 chains, the TCRζ chain in particular. Phosphotyrosine residues in the CD3 and TCRζ chains function as docking sites for the SH2 domains of the ZAP-70 tyrosine kinase. Subsequently, ZAP-70 becomes activated and phosphorylates downstream substrates, including LAT and Slp-76.

LAT plays a central role in the signaling events that are induced by TCR engagement. Phosphorylated LAT binds to the SH2 domains of a number of downstream signaling molecules, including PLCγ, Gads and Grb-2, thus nucleating a TCR signaling complex. In addition to binding LAT, Gads also binds to Slp-76, which binds to and recruits the tyrosine kinase Itk into the signaling complex. Upon phosphorylation by Itk, PLCγ is activated and hydrolyzes PIP_2 present in the plasma membrane into diacylglycerol and IP_3, which are required for activation of the Ras/MAPK and calcium pathways,

Figure 3: Signal transduction through the TCR/CD3 complex. Co-ligation of CD4 or CD8 with the T cell receptor by MHC/peptide complexes brings Lck nearby to the TCR/CD3 cytoplasmic domains. Lck phosphorylates tyrosine residues in the TCRζ cytoplasmic domain, which allows for the recruitment of the Syk-family tyrosine kinase, ZAP-70 via the ZAP-70 SH2 domains. ZAP-70 phosphorylates downstream substrates, including the adaptor proteins LAT and Slp-76, which leads to the recruitment of additional adaptor (Grb-2, Gads) and effector (Sos, PLC-γ) molecules. Activation of Sos and PLC-γ leads to the activation of both the Ras/MAPK and calcium pathways. Together, the Ras/MAPK and calcium pathways activate critical transcription factors (AP-1 and NF-AT, respectively) that are required for induction of TCR-inducible genes, including IL-2.


CD4/CD8
TCR/CD3
Lck
ZAP
-70
LAT
DAG
Ras-
GRP
IP3
PLC-γ
Slp-76
Gads
Itk
Grb-2
SOS
Ras/MAPK
Ca2+
AP-1
NF-AT
calcineurin
nucleus
AP-1
NF-AT
IL-2

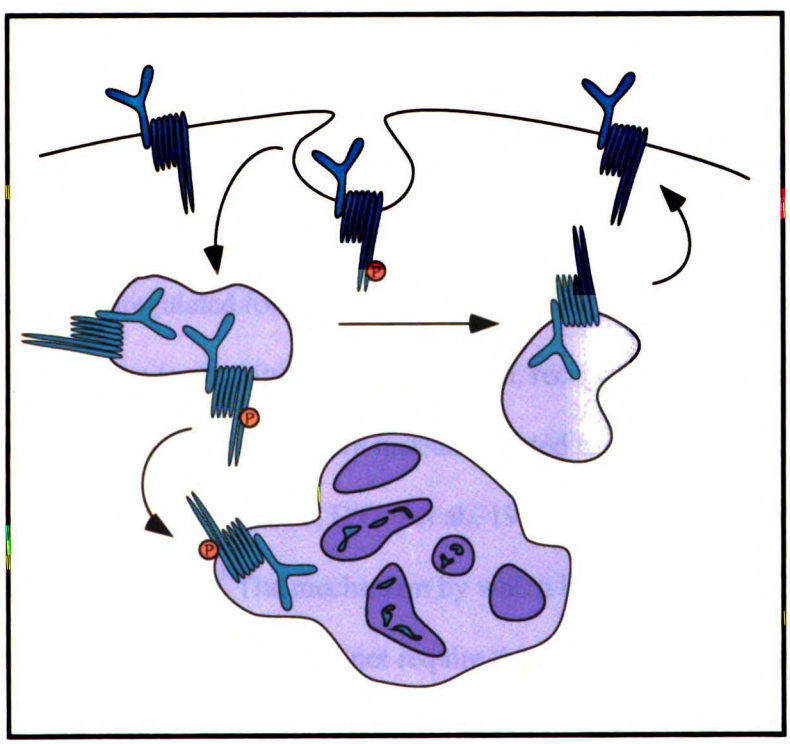
respectively. Diacylglycerol production activates PKC isozymes and the guanine nucleotide exchange factor, RasGRP, leading to the accumulation of Ras-GTP, and subsequently, activation of the Ras/MAPK pathway (Ebinu et al., 2000). In addition, LAT further functions to activate the MAPK pathway through binding to Grb-2, thereby recruiting Grb-2/SOS complexes to the membrane. SOS, like RasGRP is a guanine nucleotide exchange factor that also activates Ras. Activation of the Ras/MAPK pathway is required for the induction of the AP-1 transcription factors c-fos and c-jun. The other product of PLC-γ, IP_3, binds to receptors on the ER and induces the release of intracellular calcium stores. Emptying ER calcium stores allows for the influx of extracellular calcium, leading to a substantial calcium flux, which activates the phosphatase activity of calcineurin. Activated calcineurin dephosphorylates the NF-AT transcription factor, thus allowing NF-AT to translocate into the nucleus. Activation of both AP-1 and NF-AT transcription factors allows for transcription of genes that are involved in T cell activation, including genes required for proliferation and cytokine production.



Trafficking of the TCR/CD3 Complex:

Like many transmembrane receptors, the TCR/CD3 complex is not constitutively expressed at the plasma membrane of thymocytes and T cells (Figure 4). Instead, TCR/CD3 complexes are constitutively internalized and recycled back to the plasma membrane (Alcover and Alarcon, 2000; Krangel, 1987). Studies addressing the dynamics of TCR/CD3 recycling have demonstrated that the TCR/CD3 complex is rapidly internalized and recycled at a rate of 0.6-1.5% per minute (Liu et al., 2000; Menne et al.,

Figure 4: Trafficking of the TCR/CD3 complex. TCR/CD3 complexes are internalized via clathrin-coated pits. If TCRζ is not phosphorylated, the TCR/CD3 complex is sorted through recycling endosomes and is eventually recycled back to the plasma membrane. After TCR ligation, however, TCR/CD3 complexes containing phosphorylated TCRζ chain(s) not recycled and are instead targeted to the lysosome for degradation.

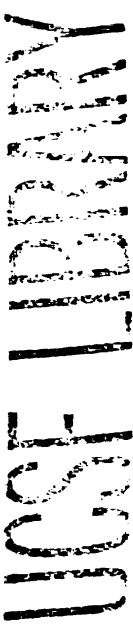


2002). Constitutive internalization of the TCR/CD3 complex requires a dileucine-based motif present in the cytoplasmic domain of CD3γ; mutation of either leucine within this motif prevents constitutive internalization of the TCR/CD3 complex (Dietrich et al., 2002). In addition, PKC-mediated phosphorylation of a serine residue immediately upstream of the dileucine motif increases the rate of TCR/CD3 internalization approximately ten-fold (Dietrich et al., 1994; Menne et al., 2002; Minami et al., 1987). However, this serine residue is not required for constitutive internalization mediated by the dileucine motif (Dietrich et al., 2002).

In addition to constitutive internalization, the TCR/CD3 complex has also been shown to undergo ligand-induced internalization, whereby receptors initially present on the cell surface are rapidly downregulated following stimulation through the TCR (Alcover and Alarcon, 2000). In contrast to constitutive internalization, receptors internalized upon receptor stimulation are not recycled, but are instead targeted to the lysosome for degradation (Alcover and Alarcon, 2000; D'Oro et al., 1997; Liu et al., 2000; Valitutti et al., 1997; von Essen et al., 2004). The mechanism by which ligand-induced TCR downregulation occurs is unclear, but does not require the dileucine motif present in CD3γ since mutations in this motif display normal rates of ligand-induced internalization (Dietrich et al., 2002). Previous studies have suggested that the rate of TCR/CD3 internalization is increased by activation of the TCR (Menne et al., 2002). In addition, subsequent studies have indicated that the rate of TCR/CD3 internalization does not change in response to TCR ligation, and that downregulation results from the failure of activated TCR/CD3 complexes to recycle back to the plasma membrane (Liu et al.,

2000). These models are not mutually exclusive; therefore, it is likely that both mechanisms contribute to ligand-induced TCR/CD3 downregulation.

Downregulation of activated TCRs plays an important regulatory role during signal transduction by physically separating the TCR from peptide/MHC molecules. Once internalized, the receptor can undergo one of two fates: the receptor can either be recycled back to the cell surface, or the TCR/CD3 complex may instead be targeted to the lysosome for degradation (Alcover and Alarcon, 2000). Transmembrane proteins can be targeted to the lysosome by a variety of sorting motifs (Bonifacino and Traub, 2003; Haglund et al., 2003; Heilker et al., 1999; Pelham, 2004; Umebayashi, 2003). Lysosomal targeting motifs function by binding to clathrin adaptor proteins, thereby facilitating entry of cargo molecules into clathrin coated vesicles that are eventually trafficked to the lysosome (Reviewed in 8, 27, 29, 63, 75). Several consensus lysosomal targeting motifs have been identified and include both tyrosine-based as well as dileucine-based motifs. In addition, ubiquitination of transmembrane proteins can also function as a lysosomal targeting motif. How the TCR/CD3 complex is targeted to the lysosome is unknown. However, it is possible that any one of the motifs described above may be required, as the TCR/CD3 complex not only contains consensus tyrosine and dileucine-based targeting motifs, but is also inducibly ubiquitinated following TCR stimulation (Cenciarelli et al., 1992; Cenciarelli et al., 1996; Hou et al., 1994; Wang et al., 2001).



Ubiquitination and the E3 Ubiquitin Ligase c-Cbl:

It is well known that ubiquitination targets soluble proteins for degradation via the 26S proteasome (Bonifacino and Weissman, 1998; Weissman, 1997). However, it is becoming increasingly clear that ubiquitination also plays an important role in receptor internalization, trafficking and degradation (Bonifacino and Traub, 2003; Haglund et al., 2003; Heilker et al., 1999; Pelham, 2004; Umebayashi, 2003). Ubiquitination of target proteins occurs through a series of steps that are catalyzed by three types of enzymes: E1 (ubiquitin activating) enzymes, E2 (ubiquitin conjugating) enzymes and E3 (ubiquitin ligase) enzymes. The molecular mechanism of ubiquitination is diagramed in Figure 5 (Bonifacino and Weissman, 1998; Weissman, 1997). In the first step, a single molecule of ubiquitin is covalently attached to an E1 in an ATP-dependent process. Next, the ubiquitin molecule is transferred from the E1 to an E2. Finally, the E3 binds to both the E2 and the target protein and catalyzes the transfer of ubiquitin from the E2 to a lysine residue present in the target protein, either directly or via a transient E3-ubiquitin intermediate. Therefore, by binding to both E2 enzymes and target substrates, E3 ligases play a crucial role in the ubiquitination process by providing substrate specificity for the ubiquitination machinery.

c-Cbl is a large, multi-domain protein that is inducibly tyrosine phosphorylated in response to stimulation of the TCR (Donovan et al., 1994). In addition, c-Cbl has been demonstrated to have E3 ubiquitin ligase activity (Duan et al., 2004; Rao et al., 2002a). The domain structure of the Cbl family of E3 ligases is depicted in Figure 6. The N-terminus of c-Cbl contains a tyrosine kinase binding (TKB) domain that binds to phosphotyrosine residues of both receptor tyrosine kinases and non-receptor tyrosine

Figure 5: The molecular mechanism of ubiquitination. Ubiquitination of target proteins occurs via an ATP-dependent process and involves the sequential action of an ubiquitin-activating (E1) enzyme, an ubiquitin conjugating (E2) enzyme and an ubiquitin ligase (E3). In the first step, a single molecule of ubiquitin is covalently linked to an E1. Next, the E1 transfers the ubiquitin molecule to the E2. Finally, the E3 ligase catalyzes the transfer of ubiquitin from the E2 onto the target protein.

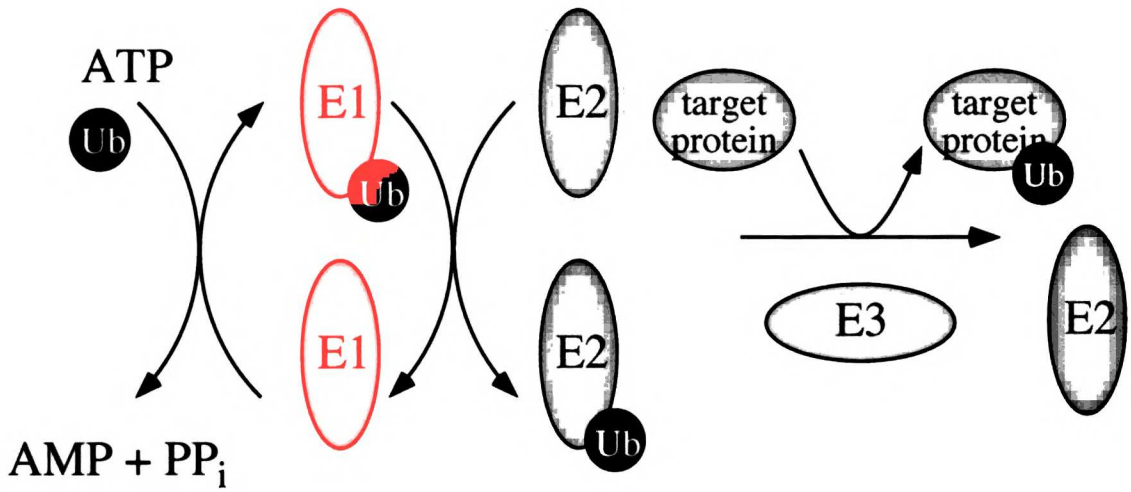
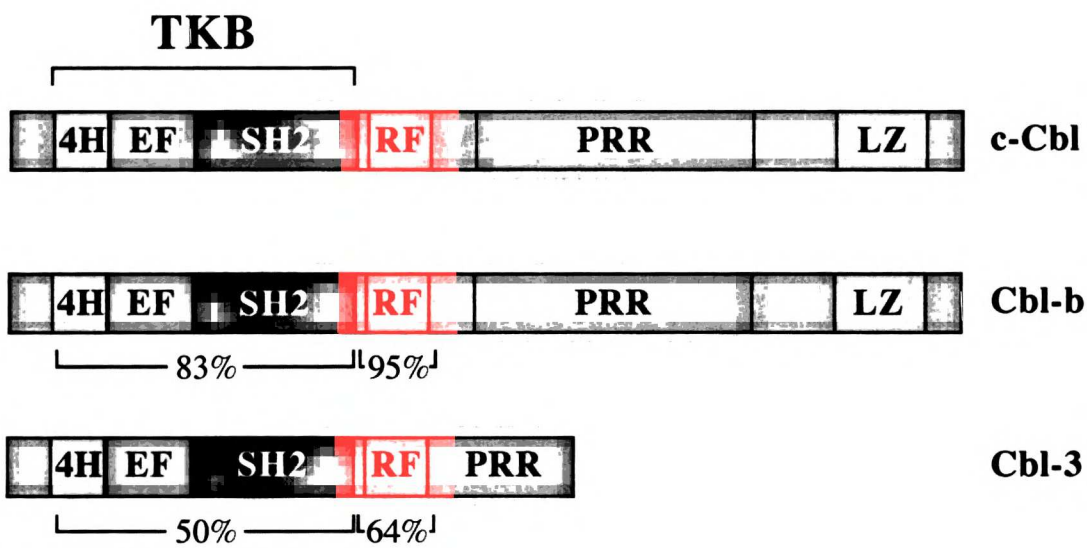

ATP
Ub
E1
Ub
E1
E2
E2
Ub
AMP + PPi
target protein
target protein
Ub
E3
E2

Figure 6: Domain structure of the murine Cbl family members. The Cbl family of E3 ubiquitin ligases is comprised of three members, each containing the highly conserved N-terminus consisting of a tyrosine kinase binding (TKB) domain and a ring finger (RF) domain, which is required for E3 ligase activity. The TKB is composed of a four-helix bundle (4H), a calcium-binding EF hand and a variant SH2 domain. In addition, c-Cbl and Cbl-b have a large C-terminal region, which contains an extensive proline-rich region (PRR) a leucine zipper (LZ) and multiple tyrosines can be inducibly phosphorylated. Numbers represent homology of murine Cbl-b and Cbl-3 as compared to c-Cbl.



kinases. The TKB domain of c-Cbl is composed of a four-helix (4H) bundle, a calcium binding EF hand and a partial SH2 domain. Together, these three domains structurally form an intact SH2 domain, with the 4H bundle completing the missing portion of the phosphotyrosine binding pocket and the EF hand positioning the 4H bundle and SH2 domain relative to one another (Meng et al., 1999). The C-terminus of c-Cbl contains a number of protein-protein interaction motifs, which includes multiple tyrosine residues that are inducibly phosphorylated in response to stimulation through the TCR, the Epidermal Growth Factor Receptor (EGF-R) and the Platelet-derived Growth Factor Receptor (PDGF-R). Phosphorylation allows c-Cbl to interact with the SH2 domains of numerous proteins, including Vav, members of the Crk family, Blk, Abl and several Src-family kinases, including Lck (Dikic et al., 2003). The C-terminus of c-Cbl also contains an extensive proline-rich region that is capable of binding to the SH3 domains of Grb-2, Nck, PLCγ, CIN85, CD2AP, Btk, Itk, the p85 subunit of PI3 kinase, CrkL, Tyk2, Abl as well as several Src-family kinases. The extreme C-terminus of c-Cbl contains a leucine zipper (LZ) that not only mediates dimerization of the molecule, but also contains homology to the ubiquitin-associated (UBA) domain, which could potentially mediate the binding of c-Cbl to ubiquitinated proteins (Duan et al., 2004). However, the ability of the LZ/UBA domain to bind ubiquitin has not been demonstrated.

Between the TKB and C-terminal domains of c-Cbl lies the ring finger (RF) domain. RF domains are zinc-binding domains that are composed of a linear series of conserved cysteine (C) and histidine (H) residues with the consensus motif: C-X_2-C-X_{9-39}-C-X_{1-3}-H-X_{2-3}-C/H-X_2-C-X_{4-48}-C-X_2-C, where X represents any amino acid (Freemont, 2000). Other

than the cysteine and histidine residues, there is little sequence conservation between RF domains. In addition to binding zinc, RF domains also bind to E2 enzymes, and are therefore present in a subset of E3 ligases. The RF of c-Cbl has been co-crystalized with the E2 UbcH7, and is required for the ubiquitin ligase activity of c-Cbl (Joazeiro et al., 1999; Zheng et al., 2000). Within the Cbl family, the RF domain has been highly conserved, especially between c-Cbl and Cbl-b (Figure 6). Furthermore, the Cbl family has been highly conserved throughout evolution, especially in the N-terminus, with expression of Cbl homologues dating as far back as *C. elegans*. In contrast, the C-termini of the Cbl family members are significantly less conserved, despite their similar domain organization. Therefore, the C-termini could potentially mediate a substantial degree of substrate specificity between Cbl family members.

Despite the high degree of homology between c-Cbl and Cbl-b, the two molecules are not interchangeable as evidenced by the differential phenotypes observed in c-Cbl as compared to Cbl-b deficient mice. Mice deficient in c-Cbl display alterations in thymocyte development and mammary gland formation (Naramura et al., 1998; Thien et al., 1999). In contrast, Cbl-b deficient mice display abnormalities in peripheral lymphocyte function (Bachmaier et al., 2000; Chiang et al., 2000). Furthermore, both B and T cells from Cbl-b deficient mice are hyperproliferative, and T cells lacking Cbl-b do not require costimulation for optimal T cell activation. Notably, the phenotypes of c-Cbl and Cbl-b deficient mice are consistent with their respective patterns of expression. Both c-Cbl and Cbl-b are ubiquitously expressed, with high levels of expression in hematopoietic cell lineages (Dikic et al., 2003). However, the highest levels of c-Cbl

expression are restricted to the thymus and testis, whereas Cbl-b expression appears to be highest in peripheral T cells. Despite low levels of expression, c-Cbl and/or Cbl-b are still required in non-hematopoietic tissues, as evidenced by the early embryonic lethality observed in mice lacking both c-Cbl and Cbl-b (Naramura et al., 2002). In contrast to c-Cbl and Cbl-b, Cbl-3 is not significantly expressed in hematopoietic cells and is instead primarily restricted to epithelial tissues (Dikic et al., 2003). Therefore, in addition to their unique C-termini, the differential tissue distribution of Cbl family members could contribute to a further degree of substrate specificity.

Early studies in mammalian and non-mammalian systems demonstrated that c-Cbl is an inhibitor of tyrosine kinases. Studies in *C. elegans* indicated that the worm homologue of c-Cbl functions as a negative regulator of the receptor protein tyrosine kinase, Let-23. Subsequently, many receptor protein tyrosine kinases, including the EGF-R (the mammalian homologue of Let-23) and the PDGF-R have been identified as substrates for negative regulation mediated by c-Cbl (Dikic et al., 2003; Duan et al., 2004; Rao et al., 2002a). Previous data haves demonstrated that c-Cbl binds to and downregulates both the activated EGF-R and PDGF-R. Downregulation of the EGF and PDGF receptors requires the RF domain of c-Cbl. In addition to receptor protein tyrosine kinases, several cytoplasmic tyrosine kinases, including Syk, ZAP-70 and Lck are also negatively regulated by c-Cbl (Rao et al., 2001; Rao et al., 2000; Rao et al., 2002b). Similar to the EGF-R and PDGF-R, downregulation of these cytoplasmic tyrosine kinases requires the RF domain of c-Cbl. Furthermore, overexpression of c-Cbl leads to increased

ubiquitination of the EGF-R, the PDGF-R, Syk, ZAP-70 and Lck, indicating that the negative regulation of these kinases by c-Cbl is due to ubiquitination and degradation.

Despite the evidence that c-Cbl regulates expression of ZAP-70 in cell lines, both *c-Cbl*$^{-/-}$ thymocytes and peripheral T cells express normal levels of ZAP-70 (Naramura et al., 1998; Thien et al., 1999). However, ZAP-70 is hyperactivated in c-Cbl deficient thymocytes, suggesting that c-Cbl may regulate ZAP-70 activity independently of ubiquitination and/or degradation (Thien et al., 1999). Previous studies have demonstrated that c-Cbl can also regulate Lck expression by ubiquitination and degradation (Rao et al., 2002b). Therefore, it is possible that c-Cbl may indirectly regulate the activity of ZAP-70 via its effects on Lck. Notably, expression of Lck is increased in *c-Cbl*$^{-/-}$ thymocytes (Thien et al., 1999), indicating that in thymocytes, c-Cbl may selectively regulate the expression of Lck, but not ZAP-70. Regardless, the enhanced signaling observed in *c-Cbl*$^{-/-}$ thymocytes significantly alters thymocyte development, since *c-Cbl*$^{-/-}$ mice expressing the DO11.10 transgenic TCR display increases in positive selection (Naramura et al., 1998).

In addition to increased Lck expression and perturbed ZAP-70 activity, c-Cbl deficient thymocytes also display alterations in the expression of a number of cell surface molecules. Specifically, *c-Cbl*$^{-/-}$ thymocytes display a selective upregulation of TCR/CD3, CD4, CD5 and CD69 expression on DP, but not SP thymocytes (Naramura et al., 1998; Thien et al., 1999). Notably, the upregulation of the TCR could contribute to the increased TCR signaling phenotype in c-Cbl deficient thymocytes. The mechanism by

which CD4, CD5 and CD69 are upregulated in the absence of c-Cbl has not yet been described. Previous studies have demonstrated that expression of CD5 and CD69 are upregulated on DP thymocytes in response to TCR signaling (Azzam et al., 1998; Merkenschlager et al., 1997). Therefore, the increased expression of CD5 and CD69 on *c-Cbl*$^{-/-}$ thymocytes is likely to be a secondary effect due to enhanced signaling in the absence of c-Cbl. In contrast, the mechanism by which CD4 is upregulated on *c-Cbl*$^{-/-}$ thymocytes is less clear. However, previous studies have demonstrated that surface expression of CD4 is stabilized by its association with Lck (Pelchen-Matthews et al., 1992). Therefore, upregulation of CD4 may be an indirect effect due to increased Lck expression in *c-Cbl*$^{-/-}$ thymocytes.

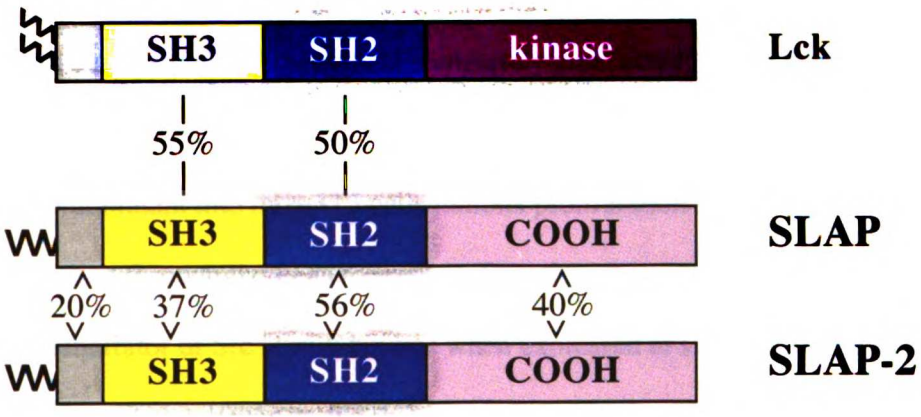
The observed increase in TCR/CD3 expression at first appeared counterintuitive, since hyperactivation of T cells typically induces a compensatory downregulation of TCR expression (D'Oro et al., 1997). Therefore, increased TCR levels on *c-Cbl*$^{-/-}$ thymocytes gave the first indication that c-Cbl may directly regulate TCR/CD3 expression on DP thymocytes by targeting component(s) of the activated TCR/CD3 complex for ubiquitination and degradation. Like many cell surface receptors, components of the TCR/CD3 complex are ubiquitinated upon activation (Cenciarelli et al., 1992; Cenciarelli et al., 1996; Hou et al., 1994; Wang et al., 2001). The mechanism by which the TCR becomes ubiquitinated in response to stimulation has remained elusive. Despite the numerous tyrosine residues present in the TCR/CD3 complex that become phosphorylated in response to TCR stimulation, the TCR/CD3 complex does not bind to c-Cbl directly. Overexpression studies have indicated that ZAP-70, through its

interactions with both TCRζ and c-Cbl could function as an adaptor, thus allowing for TCRζ ubiquitination (Wang et al., 2001). Alternatively, additional adaptor molecule(s) may be required to link c-Cbl to the TCR/CD3 complex. One such candidate adaptor molecule is the Src-Like Adaptor Protein (SLAP).

Src-Like Adaptor Protein:

SLAP was initially identified during a yeast-two-hybrid screen for proteins that interact with the cytoplasmic domain of the receptor protein tyrosine kinase EphA2 (Pandey et al., 1995). As its name implies, SLAP shares significant homology with the Src family of tyrosine kinases, the lymphocyte specific member, Lck in particular (Figure 7). Like Src kinases, SLAP has a unique N-terminal region which is lipid modified by myristic acid, thereby targeting SLAP to the cytoplasmic face of cellular membranes. The N-terminus of SLAP is followed by an SH3 domain, which, unlike Src kinases, is separated only briefly, from the downstream SH2 domain instead of being separated by a short linker region. This suggests that the positioning of the SH3 and SH2 domains of SLAP may be more rigid relative to one another and may account, at least in part, for the differential binding specificities of SLAP as compared to Src-family kinases (Manes et al., 2000; Sosinowski et al., 2000). Another significant difference between SLAP and Src kinases lies in the C-termini of the molecules. In contrast to Src kinases, SLAP does not contain a C-terminal kinase domain. Instead, the C-terminus of SLAP is a unique stretch of about 100 amino acids, and does not share significant homology with any other known protein, even the related family member, SLAP-2. The function of the SLAP C-terminus has

Figure 7: Domain structure of the SLAP family members as compared to the highly homologous tyrosine kinase, Lck. Lck, SLAP and SLAP-2 all possess unique N-termini containing lipid modifications, which targets the proteins to cellular membranes. Following the N-terminus are an SH3 and an SH2 domain, respectively. Finally, while the C-terminus of Lck contains a kinase domain, the C-termini of SLAP and SLAP-2 do not possess any known enzymatic activity. Numbers represent identity between the indicated domains.


SH3
SH2
kinase
Lck
55%
50%
WW
SH3
SH2
COOH
SLAP
20%
37%
56%
40%
WW
SH3
SH2
COOH
SLAP-2

remained enigmatic. However, previous studies have suggested that the SLAP C-terminus may be required for its interaction with c-Cbl (Tang et al., 1999).

Early studies in Jurkat T cells and the NIH/3T3 fibroblast cell line indicated that SLAP may function as an inhibitor of Src kinases, possibly by functioning in a dominant negative manner (Roche et al., 1998; Sosinowski et al., 2000). Overexpression of SLAP in Jurkat T cells inhibits the transcriptional activation via NF-AT and AP-1 sites as well as activation of the IL-2 promoter in response to TCR stimulation. Maximal inhibition of NF-AT activity requires both the SH3 and SH2 domains of SLAP; mutation of either domain alone leads to a partial restoration of NF-AT activity in response to TCR stimulation. In contrast, NF-AT activity is completely unaffected if both the SH3 and SH2 domains are mutated in combination. Furthermore, microinjection of WT SLAP, but not an SH2 point mutant into NIH/3T3 cells inhibits proliferation in response to Src-mediated signaling through the PDGF receptor. Therefore it is possible that SLAP can function as an inhibitor of Src family kinases when expressed at super-physiologic levels. However, it has yet to be demonstrated that SLAP functions in this capacity at physiological levels of expression *in vivo*.

Northern Blot analysis of SLAP mRNA expression in cell lines and primary tissues indicated that SLAP mRNA is expressed predominantly in hematopoietic cells, with highest levels of expression detected in thymus, spleen and lymph node (Sosinowski et al., 2000). Within the thymus, SLAP protein appears to be developmentally regulated, with SLAP protein expression confined almost entirely to DP thymocytes (Sosinowski et

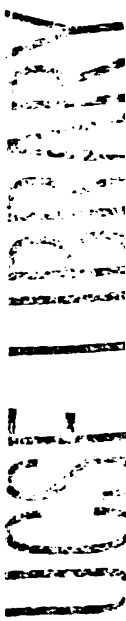
al., 2001). Furthermore, Lck-/-Fyn-/- doubly deficient thymocytes (which fail to progress to the DP stage of development due to the lack of preTCR signaling) do not express SLAP protein, suggesting that SLAP is upregulated in response to signals through the preTCR. Finally, studies in peripheral lymphocytes indicate that SLAP protein is also expressed in B and T cells, as well as during B cell development (L. Dragone et al., manuscript in preparation).

To gain a better understanding of SLAP function, thymic development was analyzed in mice deficient for the SLAP gene. Strikingly, *SLAP*$^{-/-}$ thymocytes display a very similar phenotype as thymocytes deficient in c-Cbl (Naramura et al., 1998); specifically, TCR/CD3, CD4, CD5 and CD69 expression were all upregulated on *SLAP*$^{-/-}$ DP, but not SP thymocytes (Sosinowski et al., 2001). Furthermore, *SLAP*$^{-/-}$ thymocytes expressing a transgenic TCR displayed increases in positive selection. As SLAP has no known enzymatic activity, the mechanism by which *SLAP*$^{-/-}$ downregulates TCR/CD3 expression was unclear. However, the similarities between *SLAP*$^{-/-}$ and *c-Cbl*$^{-/-}$ thymocytes led us to postulate that SLAP and c-Cbl may function in the same pathway to regulate TCR/CD3 expression on DP thymocytes. Therefore, we set out to elucidate the mechanism of SLAP-mediated TCR/CD3 downregulation and the potential role of c-Cbl in this pathway.

Topics to be Discussed:

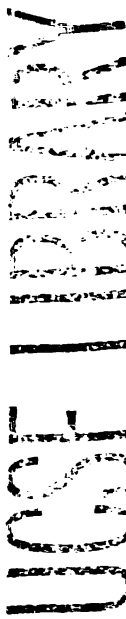
The following chapters will describe studies demonstrating how SLAP, together with c-Cbl regulates TCR/CD3 expression on DP thymocytes. Chapter 2 uses mice deficient in

SLAP to demonstrate that SLAP regulates the expression of the TCR/CD3 complex by targeting TCRζ chains present in fully assembled TCR/CD3 complexes for degradation. Chapter 3 uses a genetic approach to demonstrate that SLAP and c-Cbl function in the same pathway to regulate TCR/CD3 expression. Furthermore, in Chapter 3 we describe a cotransfection system that has been used to determine the cellular and molecular requirements for SLAP/c-Cbl mediated TCR/CD3 downregulation. In Chapter 4 we provide evidence for a dileucine-based motif within the SH2 domain of SLAP that may be required for targeting SLAP and TCR/CD3 complexes bound to SLAP to late endosomes and/or lysosomes. Finally, Chapter 5 will discuss the implications of these findings and propose some future directions for study.



Chapter 2:

SLAP Downregulates TCR/CD3 Expression by Targeting the TCRζ Chain for Degradation



Summary:

Src-Like Adaptor Protein (SLAP) downregulates expression of the T cell receptor (TCR)/CD3 complex during a specific stage of thymocyte development when the TCR repertoire is selected. Consequently, *SLAP*$^{-/-}$ thymocytes display alterations in thymocyte development. Here we have studied the mechanism of SLAP function. We demonstrate that thymocytes deficient in SLAP have increased TCRζ chain expression due to a defect in TCRζ degradation. Failure to degrade TCRζ leads to an increased pool of fully assembled TCR/CD3 complexes that are capable of recycling back to the cell surface. We also provide evidence that SLAP functions in a pathway that requires the phosphorylated TCRζ chain and the Src-family kinase Lck, but not ZAP-70. These studies reveal a unique mechanism by which SLAP contributes to the regulation of TCR expression during a distinct stage of thymocyte development.

Introduction:

T cells develop in the thymus, where immature thymocytes undergo a developmental program that ensures generation of T cells with a repertoire of TCRs that are capable of recognizing foreign antigens presented by major histocompatibility complex (MHC) molecules without being autoreactive (Love and Chan, 2003). The αβTCR is part of a multi-chain complex which is composed of the peptide/MHC binding TCRα and β chains that are non-covalently associated with the CD3γεδε and TCRζζ chains, together referred to as the CD3 complex (Exley et al., 1991). While the αβTCR is responsible for antigen recognition, the remainder of the CD3 complex, the TCRζ chains in particular, are required for coupling the TCR to downstream signaling molecules.

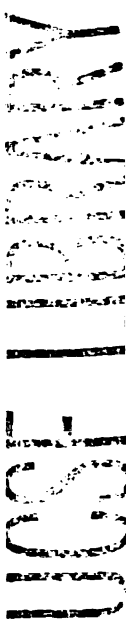
Generation of the αβTCR repertoire is initiated at the most immature stage of thymocyte development (Sebzda et al., 1999). At this early stage of development, thymocytes express neither CD4 nor CD8 coreceptors. Thymocytes that are destined to become αβ T cells stochastically rearrange their TCRβ genes. If rearrangement of TCRβ is successful (in frame), the TCRβ chain is transported together with a non-variant preTCRα chain and the CD3 complex to the cell surface as the pre-TCR complex. Surface expression of the preTCR complex induces ligand-independent signals (Irving et al., 1998) which allows for thymocytes to proliferate and upregulate CD4 and CD8 expression, thereby progressing to the CD4+CD8+ double positive (DP) stage of development. At the DP stage of development, thymocytes rearrange their TCRα genes. If rearrangement of TCRα is successful, low levels of the mature TCR/CD3 complex are expressed on the surface of the developing thymocyte. Expression of the TCR/CD3 complex is crucial at

this stage of development, as signals through the TCR are required for the survival (positive selection) or deletion (negative selection) of DP thymocytes (Love and Chan, 2003; Sebzda et al., 1999). If the TCR expressed by a DP thymocyte cannot bind to self-peptide/MHC molecules, the thymocyte fails to receive positively selecting signals and subsequently dies. Conversely, if the TCR interacts too strongly with self-peptide/MHC molecules, the cell is potentially autoreactive and is deleted via apoptosis . Thymocytes that express TCRs with an intermediate affinity for peptide-MHC downregulate either CD4 or CD8, thereby progressing to the single positive (SP) stage of development. Progression to this more phenotypically and functionally mature SP stage is also associated with a ten-fold increase in the level of the TCR/CD3 complex to that of mature peripheral T cells. At the SP stage, thymocytes are subjected to further selection and maturation processes before exiting the thymus as mature T cells.

Both positive and negative selection processes are dependent on the strength of signals received through the TCR/CD3 complex. Signal strength is dependent not only on the intrinsic affinity of the TCR for peptide/MHC molecules, but also the number of receptors that interact with peptide/MHC. TCR/CD3 expression on DP thymocytes is only approximately 10% of the level observed on SP thymocytes and mature T cells (Finkel et al., 1987; Havran et al., 1987). Previous studies have demonstrated that modulating levels of TCR/CD3 expression in developing thymocytes can lead to alterations in positive selection (Ericsson and Teh, 1995; Naramura et al., 1998; Sosinowski et al., 2001), suggesting that tight regulation of surface TCR/CD3 levels is required for normal TCR repertoire selection. Therefore, proteins that regulate surface

TCR/CD3 levels in the thymus are likely to be important determinants of thymocyte development.

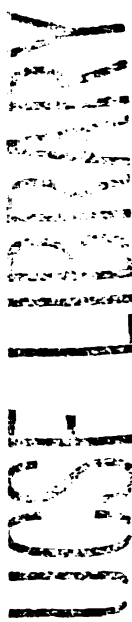
Recently, we have shown that Src-like adaptor protein (SLAP) regulates the level of TCR/CD3 expression on DP thymocytes (Sosinowski et al., 2001). SLAP was identified in a yeast-two-hybrid screen for proteins that interact with the cytoplasmic domain of the Eck receptor protein tyrosine kinase (Pandey et al., 1995). SLAP is highly homologous with Src family kinases, the T lymphocyte specific family member Lck, in particular. Like Src family kinases, SLAP has a unique N-terminus which is myristolated, thereby targeting SLAP to cellular membranes (Manes et al., 2000). The N-terminus of SLAP is followed by an SH3 and an SH2 domain, which share 55% and 50% amino acid sequence identity with these domains in Lck, respectively. Unlike Src-family kinases, however, SLAP lacks a kinase domain and, instead, contains a unique C-terminus whose function remains unclear.



Since SLAP is highly homologous to Lck but lacks a kinase domain, it was postulated that SLAP could negatively regulate Src-family kinases by functioning in a dominant negative manner. Overexpression and microinjection studies in non-lymphoid cells have shown that SLAP can inhibit Src-mediated signaling through the platelet-derived growth factor receptor (Manes et al., 2000; Roche et al., 1998). In Jurkat T cells, transient overexpression of SLAP can inhibit signaling downstream of the TCR at the level of NF-AT, AP-1 or IL-2 transcriptional reporter activity (Sosinowski et al., 2000). Maximal inhibition of NF-AT activity in Jurkat T cells requires both the SH2 and SH3 domains of

SLAP. Together, these data suggest that SLAP is an inhibitor of Src-family kinases. However, the mechanism by which SLAP inhibits signaling remains unclear, as no differences in overall tyrosine phosphorylation were observed in Jurkat T cells that overexpress SLAP or in *SLAP*$^{-/-}$ thymocytes (T. Sosinowski and A. Weiss, unpublished, and data not shown).

Studies in mice demonstrated that SLAP protein expression is developmentally restricted, and is most highly expressed in DP thymocytes (Sosinowski et al., 2001). Consistent with this restricted pattern of expression, targeted inactivation of the SLAP gene demonstrated that SLAP downregulates TCR/CD3 expression at the DP stage of thymocyte development. In addition to increased TCR/CD3 expression, *SLAP*$^{-/-}$ DP thymocytes also display increased levels of CD4, CD5 and CD69. Furthermore, *SLAP*$^{-/-}$ thymocytes display increases in positive selection in the presence of a transgenic TCR. Finally, SLAP deficiency can partially overcome a developmental block at the DP stage and rescue the development of CD4+ SP thymocytes and peripheral T cells in mice that lack the ZAP-70 tyrosine kinase. These alterations in thymocyte development in the absence of SLAP argue that control of surface TCR/CD3 levels on DP thymocytes is an important regulatory step in the generation of peripheral T cells. Therefore, we set out to elucidate the mechanism of SLAP-mediated TCR/CD3 downregulation on DP thymocytes. Here we show that SLAP deficient thymocytes have a defect in TCRζ chain degradation, which leads to an increased pool of fully assembled TCR/CD3 complexes that are capable of recycling back to the cell surface.



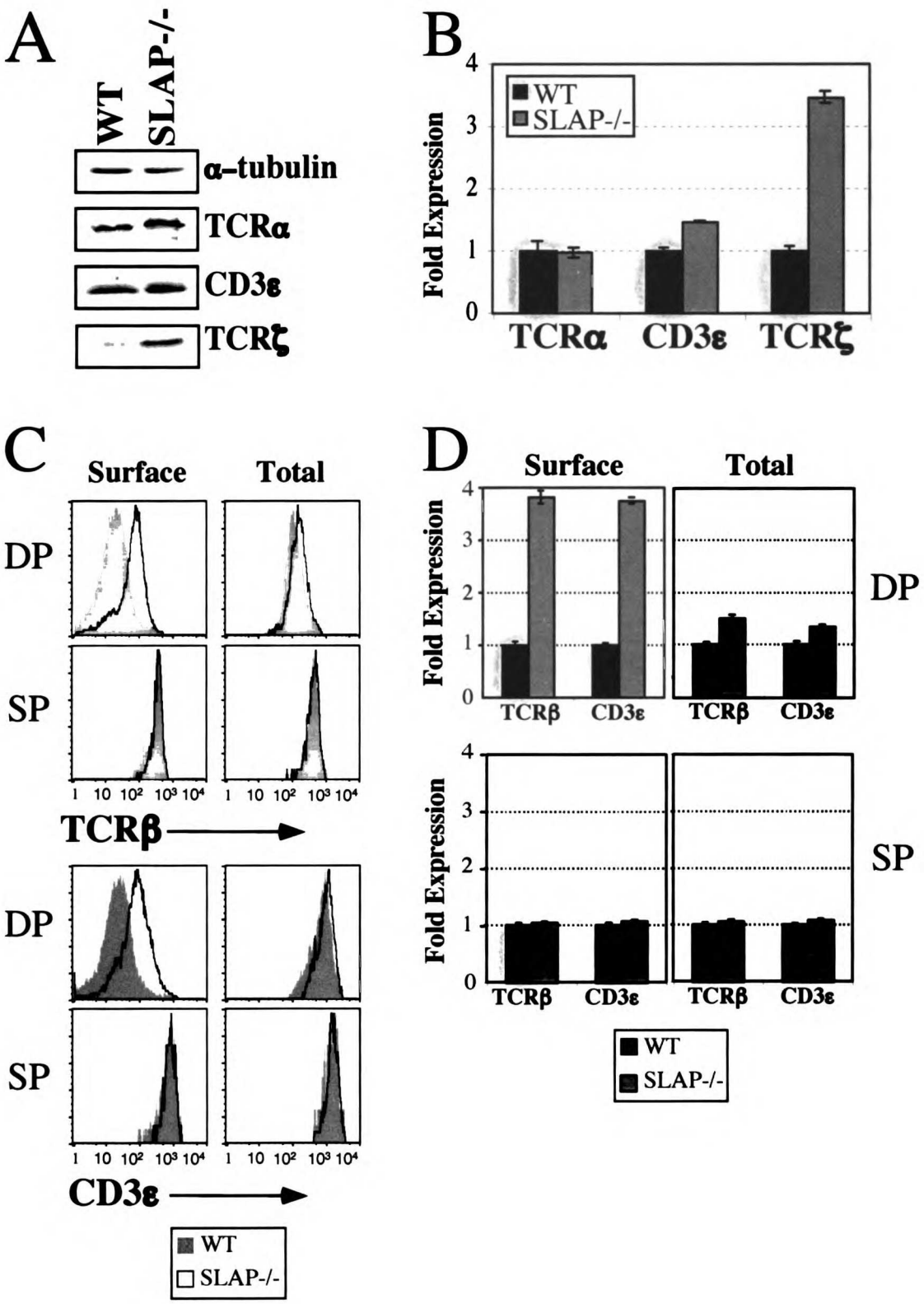
Results:

Total levels of TCRζ are increased in *SLAP*$^{-/-}$ DP thymocytes.

SLAP$^{-/-}$ mice have increased levels of surface TCR/CD3 on DP thymocytes (Fig. 1C, and (Sosinowski et al., 2001). We reasoned that if either synthesis or degradation is altered by the absence of SLAP, total levels of TCR/CD3 should also be upregulated. Total cell lysates were prepared from CD8+ thymocytes (which contained mostly DP thymocytes, but also contained small numbers of CD8+ SP thymocytes). Purified cells were > 95% DP as determined by FACS (data not shown). By western blot analysis, total levels of TCRζ chain were consistently increased 3-4 fold in *SLAP*$^{-/-}$ CD8+ thymocytes as compared to wild-type (WT) controls (Fig. 1A-B). In contrast, total levels of TCRα and CD3ε were not substantially increased. Although lysates contained mostly DP thymocytes, there was a small (< 5%) contaminating pool of CD8+ SP thymocytes in lysate preparations. Therefore, we used intracellular FACS staining to analyze total TCR/CD3 levels specifically in DP thymocytes (Fig. 1C-D). We were unable to stain for the TCRζ chain by FACS, but intracellular FACS analysis confirmed the results obtained by western blot analysis for CD3ε. While surface levels of CD3ε (as well as TCRβ) were consistently 3-5 fold higher on *SLAP*$^{-/-}$ DP thymocytes, total levels of TCR/CD3 were not significantly increased.

Increased TCRζ chain expression in the absence of SLAP suggests that SLAP modifies the rate of TCRζ synthesis and/or degradation. Metabolic labeling of CD8+ thymocytes demonstrated that synthesis of TCRζ as well as TCRα and CD3ε during a 30 minute pulse label was similar in WT and *SLAP*$^{-/-}$ thymocytes (Fig. 2A). To test whether the rate

Figure 1: TCRζ chain expression is increased in SLAP$^{-/-}$ mice. A) Western blot analysis of TCRα, CD3ε and TCRζ expression in RIPA lysates of purified CD8+ thymocytes from WT and SLAP$^{-/-}$ mice. Data are representative of 6 independent experiments. B) Quantitation of western blots represented in (A) using quantitative luminescence. Data represent mean expression of TCRα, CD3ε or TCRζ by SLAP$^{-/-}$ CD8+ thymocytes as compared to WT controls ± SEM. TCR/CD3 levels have been normalized to α-tubulin. C) Surface or total TCRβ and CD3ε expression by either surface or intracellular FACS staining. Data are representative of six mice per genotype. D) Quantitation of FACS staining shown in C. Data represent the average mean fluorescence intensity (MFI) ± SEM as compared to WT controls for six mice of each genotype.


A
WT
SLAP-/-
α-tubulin
TCRα
CD3ε
TCRζ
B
WT
SLAP-/-
Fold Expression
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TCRα
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TCRζ
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TCRβ
DP
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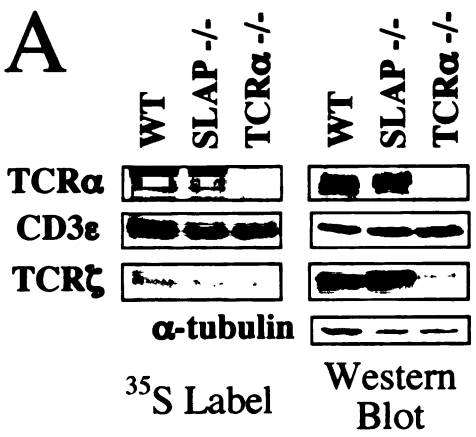
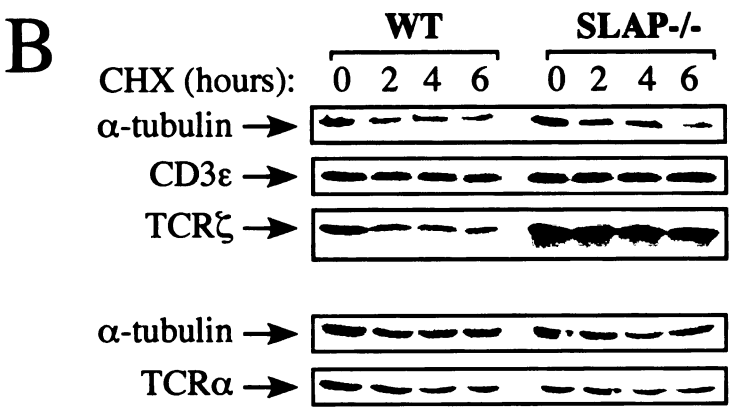
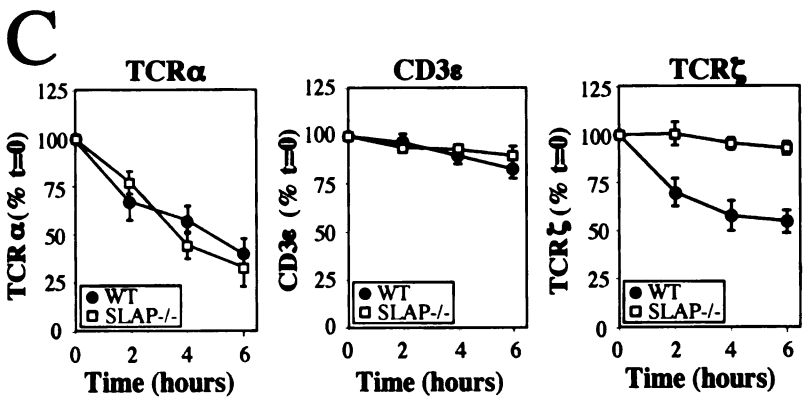
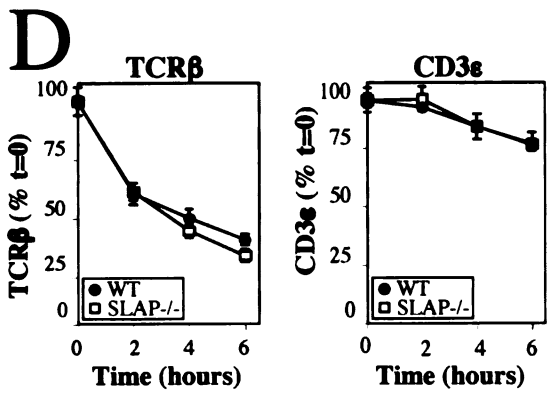
1 10 10² 10³ 10⁴
CD3ε
WT
SLAP-/-
D
Surface
Total
Fold Expression
TCRβ
CD3ε
DP
SP
WT
SLAP-/-

Figure 2: TCRζ degradation is impaired in SLAP$^{-/-}$ thymocytes. A) TCRα, CD3ε and TCRζ immunoprecipitates from WT and SLAP$^{-/-}$ thymocytes after 30 minutes of metabolic labeling. TCRα-/- thymocytes were used as a control for TCRα expression. Data are representative examples of three independent experiments. B) Degradation of TCRα, CD3ε and TCRζ by WT and SLAP$^{-/-}$ CD8+ thymocytes treated with cycloheximide (CHX) as assessed by western blot analysis of RIPA lysates. Data are representative of > 4 independent experiments. C) Quantitation of western blots shown in (C). Data are plotted as the mean percent expression relative to t = 0 and are the average of three mice per genotype ± SEM. D) Degradation of TCRβ or CD3ε in the presence of cycloheximide by DP thymocytes as assessed by intracellular FACS staining.


A
WT
SLAP -/-
TCRα -/-
WT
SLAP -/-
TCRα -/-
TCRα
CD3ε
TCRζ
α-tubulin
35S Label
Western Blot


B
WT
SLAP-/-
CHX (hours): 0 2 4 6 0 2 4 6
α-tubulin
CD3ε
TCRζ
α-tubulin
TCRα


C
TCRα
CD3ε
TCRζ
TCR α (% t=0)
CD3ε (% t=0)
TCRζ (% t=0)
125
100
75
50
25
0
WT
SLAP-/-
0 2 4 6
Time (hours)


D
TCRβ
CD3ε
TCRβ (% t=0)
CD3ε (% t=0)
100
75
50
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WT
SLAP-/-
0 2 4 6
Time (hours)

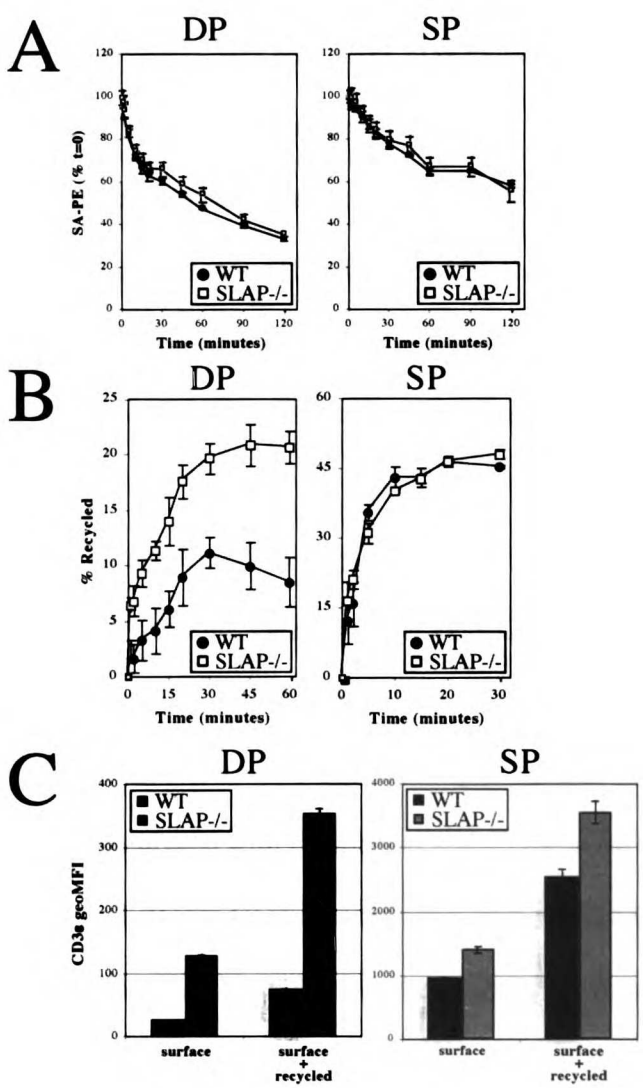
of TCRζ degradation was altered in the absence of SLAP, thymocyte single cell suspensions enriched for DP thymocytes (CD8+ purified) were treated with cycloheximide to inhibit synthesis of the TCR chains and the stability of CD3ε, TCRζ and TCRα over time was assessed by either western blot analysis or intracellular FACS staining. Degradation of TCRζ, but not CD3ε, TCRα or TCRβ was markedly impaired in *SLAP*$^{-/-}$ thymocytes as compared to WT controls (Fig. 2B-D). We have been unable to study TCRζ and TCRα degradation using a FACS-based assay. Therefore, we simultaneously investigated CD3ε degradation by intracellular FACS staining to determine whether the results obtained by western blot analysis of CD8+ purified thymocytes were similar to the results obtained by intracellular FACS staining of DP thymocytes. CD3ε degradation by western blot analysis was comparable to the FACS-based assay (Fig. 2B-D), demonstrating that contamination from SP thymocytes does not significantly contribute to the impaired degradation of TCRζ observed in *SLAP*$^{-/-}$ thymocytes.

Recycling TCR/CD3 complexes are increased in the absence of SLAP.

The failure of *SLAP*$^{-/-}$ thymocytes to degrade TCRζ may be due to alterations in the dynamics of TCR/CD3 internalization and/or recycling. However, *SLAP*$^{-/-}$ DP thymocytes pre-coated with anti-CD3ε monoclonal antibodies (mAb) internalized surface bound mAb with similar kinetics as WT thymocytes (Fig. 3A). We next tested whether SLAP regulates recycling of the TCR/CD3 complex. Thymocytes were incubated in culture with PE-labeled anti-CD3ε mAb for 30 minutes at 37°C to label an internalized pool of CD3ε. Cells were then transferred to ice, washed, and surface-bound antibody was

Figure 3: Increased TCR/CD3 recycling in SLAP$^{-/-}$ thymocytes.

A) Ligand-induced internalization of CD3ε from WT or SLAP$^{-/-}$ thymocytes pre-coated with biotinylated anti-CD3ε mAb. CD3ε remaining on the cell surface was detected by staining with PE-streptavidin. B) Recycling of previously internalized anti-CD3ε antibody by WT and SLAP$^{-/-}$ thymocytes. Data are presented as a percentage of total CD3ε internalized at t=0. C) Comparison of surface CD3ε with the total recycling pool of CD3ε. The surface and recycling pools were labeled by incubating thymocytes with a PE anti-CD3ε antibody for 60 minutes on ice or at 37°C, respectively. All data were determined by FACS and represent three mice per genotype ± SEM.



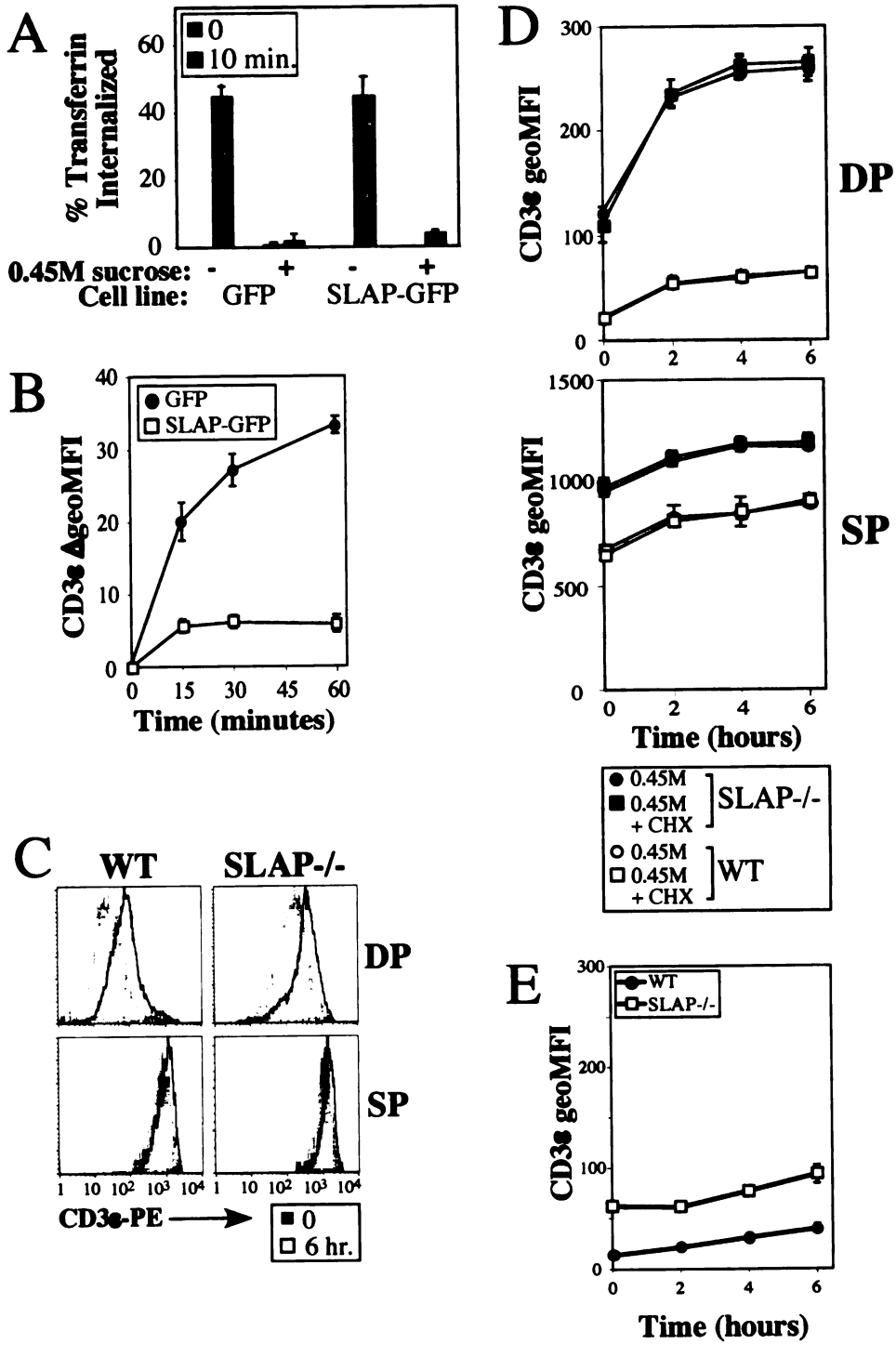
removed using two sequential low pH washes. Thymocytes were then resuspended in cell culture medium and incubated at 37°C for various timepoints. Anti-CD3ε mAb recycled back to the cell surface during the course of the assay were removed by a second series of low pH washes. The amount of CD3ε-PE fluorescence lost as compared to staining of the intracellular pool at t=0 was used to calculated the percentage of CD3ε recycled at each timepoint. SLAP deficient DP thymocytes recycled CD3ε more rapidly than DP thymocytes from WT mice (Fig. 3B). Furthermore, SLAP deficient DP thymocytes recycled more CD3ε over the course of the assay, suggesting that the size of the CD3ε recycling pool is increased in the absence of SLAP. Notably, the increase in CD3ε recycling was seen only in DP thymocytes, where SLAP is most highly expressed, since CD3ε recycling by SP thymocytes was similar regardless of genotype.

We next tested whether the size of the CD3ε recycling pool is altered in the absence of SLAP. We have been unable to measure the size of the TCR/CD3 recycling pool using Fab fragments, presumably due to the low level of CD3ε expression in DP thymocytes as compared to background staining and lower biotin labeling of the Fab fragments. Therefore, thymocytes were incubated with PE-labeled anti-CD3ε mAb either on ice to label only surface CD3ε or at 37°C for 60 minutes to label both surface CD3ε as well as CD3ε recycled to the cell surface during the 60 minute incubation. More recycling CD3ε was labeled in *SLAP*$^{-/-}$ DP thymocytes as compared to WT (Fig. 3C), indicating that the number of mature TCR/CD3 complexes present in the recycling pool is increased in the absence of SLAP. In contrast, the size of the CD3ε recycling pool was more similar in SP thymocytes, suggesting that consistent with expression in DP thymocytes, SLAP

regulates the size of the recycling pool specifically at the DP stage of thymocyte development.

Our lab has previously shown that SLAP-mediated TCR/CD3 downregulation on DP thymocytes does not depend on a positively selecting MHC allele (Sosinowski et al., 2001). Therefore, we were interested in studying CD3ε recycling in the absence of any TCR ligation. We reasoned that if we could block internalization of the TCR/CD3 complex, any new TCR/CD3 appearing on the cell surface would be due to newly synthesized or recycled TCR/CD3. To block TCR/CD3 internalization, we exploited the observation that the TCR is internalized via clathrin-coated pits (Telerman et al., 1987). Hypertonic medium (0.45M sucrose, e.g.) blocks clathrin-mediated endocytosis by inducing spontaneous clathrin lattice formation in the absence of cell membranes, thereby depleting the cell of clathrin monomers that would be used in vesicle formation (Daukas and Zigmond, 1985; Heuser and Anderson, 1989). Furthermore, hypertonic medium has been shown to inhibit the internalization of the TCR/CD3 complex (Dallanegra et al., 1988). To validate the use of hypertonic medium to inhibit TCR/CD3 internalization, we first analyzed the uptake of fluorescently labeled transferrin by the transferrin receptor, a process that requires clathrin-mediated endocytosis (Mellman, 1996; Schmid, 1997). Since DP thymocytes do not express the transferrin receptor, we first tested the effect of hypertonic medium on Jurkat T cell lines stably expressing either a SLAP-GFP fusion or GFP alone as a control. Transferrin uptake via the transferrin receptor was completely inhibited by hypertonic medium in both stable cell lines (Fig. 4A), demonstrating that hypertonic medium blocks clathrin-mediated endocytosis regardless of whether SLAP

Figure 4: Increased TCR/CD3 recycling is revealed in hypertonic medium. A) Uptake of Alexa647-labeled transferrin by Jurkat T cell lines expressing SLAP-GFP or control (GFP) in the presence or absence of hypertonic medium as assessed by FACS. B) Expression of CD3ε on Jurkat T cell lines expressing SLAP-GFP or control (GFP) incubated in hypertonic medium as assessed by FACS. Data are presented as the absolute increase in CD3ε MFI expression relative to t = 0. Data in A and B are the average of three experiments ± SEM. C) CD3ε expression on WT or $SLAP^{-/-}$ thymocytes incubated in hypertonic medium for 0 or 6 hours as assessed by FACS. Data are representative of three mice per genotype. D) MFI of CD3ε expression on WT or $SLAP^{-/-}$ thymocytes incubated in hypertonic medium (0.45M) for the indicated time in the presence or absence of cycloheximide (CHX). E) MFI of CD3ε expression on WT or $SLAP^{-/-}$ DP thymocytes incubated for the indicated time in cell culture medium only. Data in (D) and (E) are the average of three mice per genotype ± SEM.


A
% Transferrin Internalized
0
10 min.
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Cell line: GFP SLAP-GFP
B
GFP
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CD3ε ΔgeoMFI
Time (minutes)
C
WT
SLAP-/-
DP
SP
CD3ε-PE
0
6 hr.
D
CD3ε geoMFI
DP
SP
Time (hours)
0.45M
0.45M + CHX
SLAP-/-
WT
E
WT
SLAP-/-
CD3ε geoMFI
Time (hours)

is present or not. We next analyzed the effect of SLAP on CD3ε expression while clathrin-mediated endocytosis was blocked in Jurkat T cells. Strikingly, we were able to detect upregulation of CD3ε surface expression over the timecourse of the experiment in the control cell line; however, upregulation of CD3ε by Jurkat T cells was markedly inhibited in the presence of SLAP (Fig. 4B).

To investigate whether SLAP expression affects upregulation of CD3ε on DP thymocytes as it did in our model cell line, we incubated thymocyte single cell suspensions in hypertonic medium and followed CD3ε expression over time. Both WT and *SLAP*$^{-/-}$ DP thymocytes upregulate CD3ε on the cell surface over the course of the assay (Fig. 4C). However, quantitation demonstrated that CD3ε upregulation was substantially increased in *SLAP*$^{-/-}$ DP thymocytes as compared to WT controls (Fig. 4D). Interestingly, the observed upregulation of CD3ε in hypertonic medium is comparable to the estimated amount of CD3ε present in the recycling pool (Fig. 3C), indicating that increases in the size of the TCR/CD3 recycling pool can be revealed upon incubation of thymocytes in hypertonic medium.

Previous studies have reported that prolonged culture of DP thymocytes in the absence of TCR/MHC interactions results in upregulation of the TCR/CD3 complex by stabilization of newly synthesized TCRα chains (Bonifacino et al., 1990; Kearse et al., 1995c). Indeed, both WT and *SLAP*$^{-/-}$ DP thymocytes upregulate CD3ε when cultured in the absence of 0.45M sucrose (Fig. 4E). However, over this time course, the increase in CD3ε expression was markedly lower than the increase observed on thymocytes

incubated in hypertonic medium. In addition, most of the CD3ε upregulation observed in hypertonic medium was detected after only 2 hours in culture, a time at which little or no CD3ε upregulation has yet occurred in the absence of 0.45M sucrose. Furthermore, incubation of thymocytes in cycloheximide to inhibit protein synthesis had no effect on the upregulation of CD3ε in hypertonic medium (Fig. 4D). In contrast, in parallel experiments using thymocytes cultured in media without 0.45M sucrose, upregulation of CD3ε was sensitive to cycloheximide treatment (data not shown), demonstrating that cycloheximide effectively inhibited protein synthesis in our cultures.

It is possible that incubation of thymocytes in hypertonic medium has an effect on cell viability. However, Annexin V staining failed to demonstrate a decrease in thymocyte viability for thymocytes incubated in hypertonic medium (data not shown). In addition, since SLAP protein expression is predominantly restricted to DP thymocytes (Sosinowski et al., 2001), upregulation of CD3ε in hypertonic medium should not be altered in SP thymocytes. Notably, CD3ε upregulation by CD4+ SP thymocytes cultured in hypertonic medium was similar regardless of genotype (Fig. 4D). These data demonstrate that upregulation of TCR/CD3 expression in hypertonic medium can be used to analyze the pool of recycling TCR/CD3 complexes in the absence of receptor crosslinking.

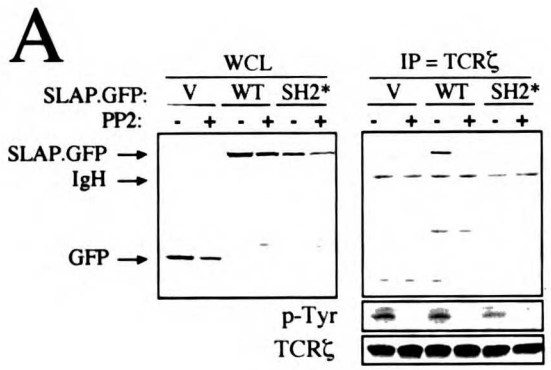
The TCRζ chain is the target of SLAP.

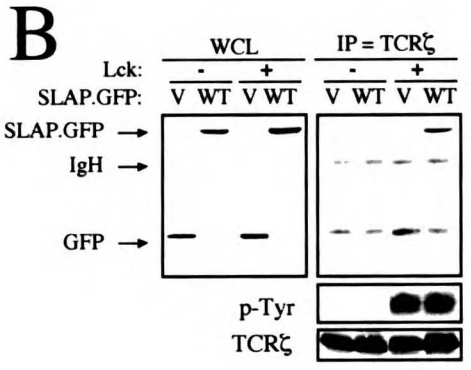
We have demonstrated that expression of TCRζ is increased in *SLAP*$^{-/-}$ thymocytes due to impaired degradation of TCRζ. A SLAP-GST fusion has previously been shown to interact with several phosphoproteins, including the TCRζ chain (Sosinowski et al., 2000;

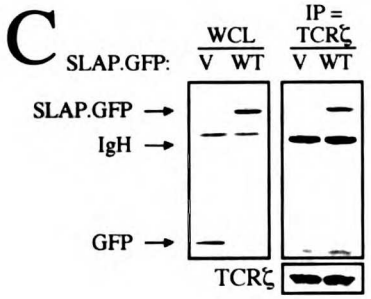
Tang et al., 1999). Therefore, we postulated that SLAP could bind to TCRζ leading to its subsequent degradation. To study the interaction between SLAP and TCRζ, we first examined Jurkat T cells that had been transiently transfected with SLAP-GFP to determine whether SLAP can interact with the endogenous TCRζ chain. SLAP-GFP but not the GFP control coimmunoprecipitated with TCRζ (Fig. 5A). Furthermore, SLAP-GFP containing a point mutation in the SH2 domain coimmunoprecipitated only weakly with TCRζ. The cytoplasmic domain of TCRζ is phosphorylated by Lck (Iwashima et al., 1994; van Oers et al., 1996). Therefore, inhibition of src-family kinase catalytic activity with PP2 caused a loss of basal TCRζ phosphorylation in Jurkat T cells (Fig. 5A). Interestingly, PP2 treatment also caused a corresponding loss in SLAP-GFP coimmunoprecipitating with TCRζ. The dependence on Src-family kinase activity prompted us to study whether SLAP can interact with TCRζ in the Lck deficient Jurkat T cell line J.CaM1. Transiently transfected SLAP-GFP failed to coimmunoprecipitate with TCRζ in J.CaM1; however stable reconstitution of Lck back into the J.CaM1 cell line resulted in restoration of basal phospho-TCRζ as well as the recovery of SLAP-GFP coimmunoprecipitating with TCRζ (Fig. 5B).

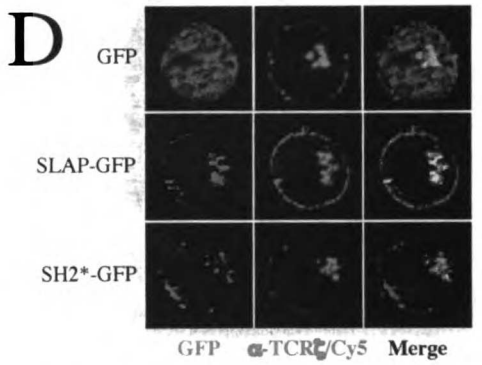
In addition to TCRζ, SLAP-GST has also been shown to interact with phosphorylated ZAP-70 (Sosinowski et al., 2000; Tang et al., 1999). ZAP-70 is also phosphorylated by Lck and can bind to the phosphorylated TCRζ cytoplasmic domain (Iwashima et al., 1994; van Oers et al., 1996). Therefore, SLAP-GFP may indirectly interact with TCRζ via ZAP-70. However, transiently transfected SLAP-GFP also coimmunoprecipitated with TCRζ in the ZAP-70 deficient Jurkat T cell line, P116 (Fig. 5C), indicating that the

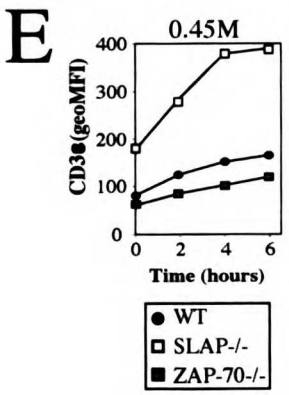
Figure 5: SLAP interacts with phospho-TCRζ via the SLAP SH2 domain. A) Immunoprecipitates of endogenous TCRζ from Jurkat T cells transiently transfected with GFP (V), SLAP-GFP (WT) or SH2*-GFP (SH2*) as assessed by western blot analysis. Half of each transfection was pretreated with the Src-family kinase inhibitor PP2 prior to immunoprecipitation. B) Immunoprecipitates of TCRζ from J.CaM1 (Lck-/-) or J.CaM1 stably reconstituted with Lck as assessed by western blot analysis. Cell lines were transiently transfected with either GFP or WT-GFP. Phospho-TCRζ observed in A and B is basal TCRζ phophorylation present in unstimulated cells. C) Immunoprecipitates of TCRζ from the ZAP-70-/- Jurkat T cell line P116 transfected with GFP or SLAP-GFP as assessed by western blot analysis. D) Localization of GFP fluorescence (green) and endogenous TCRζ (red) in Jurkat T cells transiently transfected with GFP, SLAP-GFP or SH2*-GFP as analyzed by deconvolution microscopy. Data are representative of > 10 cells analyzed per transfection for three independent experiments. E) MFI of CD3ε expression on ZAP-70-/- DP thymocytes incubated in hypertonic medium. Data are the average of three mice of each genotype ± SEM. Thymocytes from one WT and one $SLAP^{-/-}$ mouse were used for comparison. F) FACS analysis of CD3ε expression on DP and SP thymocytes in the presence (TCRζTg) or absence (TCRζD67-150) of most of the TCRζ cytoplasmic domain. Histograms are representative of three mice per genotype. G) MFI of CD3ε expression on DP and SP thymocytes expressing full-length (TCRζTg) or a cytoplasmic truncation (TCRζD67-150) of the TCRζ cytoplasmic domain incubated in hypertonic medium as assessed by FACS. Data are the average of three mice per genotype ± SEM.

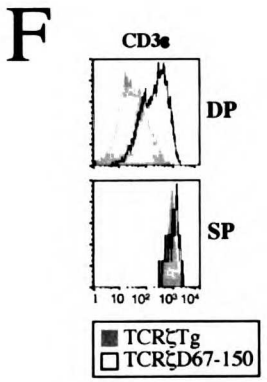


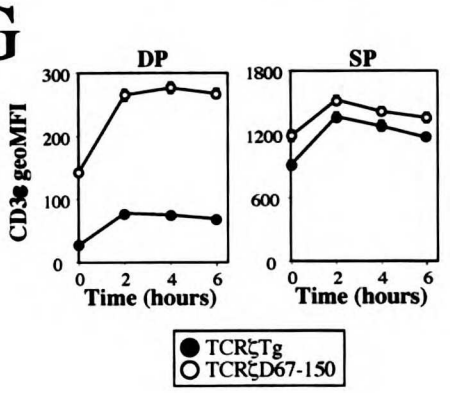












interaction between phosphorylated TCRζ and SLAP-GFP does not require ZAP-70 and may instead be a direct interaction. Furthermore, neither surface TCR/CD3 expression (Kadlecek et al., 1998; Negishi et al., 1995) nor upregulation of CD3ε by DP thymocytes incubated in hypertonic medium (Fig. 5E) were increased in the absence of ZAP-70, suggesting that ZAP-70 is not required for SLAP-mediated downregulation of the TCR/CD3 complex *in vivo*.

It has previously been shown that upon transient transfection, SLAP localizes to an intracellular compartment and displays partial colocalization with late endosomes (Sosinowski et al., 2000). Therefore, we predicted that SLAP would colocalize with TCRζ in an intracellular compartment. Extensive colocalization of SLAP-GFP with endogenous TCRζ was observed following transient transfection into Jurkat T cells (Fig. 5D). Colocalization occurred primarily in an intracellular compartment, as very little SLAP-GFP was detected at the plasma membrane. Interestingly, colocalization requires the SH2 domain of SLAP, as an SH2 point mutation of SLAP-GFP displayed very little colocalization with the TCRζ chain. These data suggest that the SH2 domain of SLAP is required for SLAP to interact with phosphorylated TCRζ, thus targeting TCRζ for degradation.

To investigate the requirement for the TCRζ cytoplasmic domains for the effects of SLAP on TCR expression *in vivo*, we obtained TCRζ-deficient mice that were reconstituted with transgenes encoding either full length TCRζ (TCRζ.Tg) or a cytoplasmic truncation of TCRζ (TCRζ.D67-150) (Shores et al., 1994). TCRζ.D67-150

is a deletion of amino acid residues 67 – 150 of TCRζ resulting in the loss of five of six tyrosines normally present in the TCRζ cytoplasmic domain. Interestingly, relative to mice expressing TCRζ.Tg, mice expressing TCRζ.D67-150 displayed increased surface levels of TCRβ and CD3ε on DP, but not on SP thymocytes (Fig. 5F and data not shown). Furthermore, mice expressing the truncated form of TCRζ displayed increased CD3ε upregulation in hypertonic medium on DP, but not SP thymocytes (Fig. 5G) suggesting that the TCR/CD3 recycling pool is increased in the absence of the TCRζ cytoplasmic domain. The observed increase was not due to new synthesis of the complex as cycloheximide had no effect on TCR/CD3 recycling in hypertonic medium (data not shown). Additionally, no significant increase in CD3ε expression was observed on thymocytes incubated in the absence of 0.45M sucrose (data not shown). These data indicate that the cytoplasmic domain of TCRζ is required to prevent the accumulation of fully assembled TCR/CD3 complexes in the recycling pool of DP thymocytes.

Discussion:

We have studied the mechanism by which TCR/CD3 expression is increased in the absence of SLAP. Our data demonstrate that *SLAP*$^{-/-}$ thymocytes have increased TCRζ expression due to a defect in TCRζ degradation. Failure to degrade TCRζ leads to an accumulation of fully assembled TCR/CD3 complexes that continue to recycle back to the plasma membrane instead of being retained and/or degraded. In addition, we have shown that Lck, but not ZAP-70 activity is required for SLAP to interact with the phosphorylated form of TCRζ. It has previously been shown that TCRζ is basally phosphorylated in DP thymocytes (Nakayama et al., 1989; van Oers et al., 1994) and that TCRζ phosphorylation is almost undetectable in Lck-/- thymocytes (van Oers et al., 1996). Interestingly, surface TCR/CD3 expression is also upregulated in Lck-/- DP thymocytes (Molina et al., 1992), suggesting that the inability of SLAP to bind TCRζ may contribute to the increase in TCR/CD3 expression observed on Lck-/- DP thymocytes. Thus, SLAP regulates the expression of the TCR in DP thymocytes by targeting tyrosine phosphorylated TCRζ for degradation in an Lck-dependent manner.

Previous data indicates that unassembled TCRζ chains are rapidly degraded in the ER of DP thymocytes (Bonifacino et al., 1990) and that newly synthesized TCRζ chains are stabilized by their incorporation into fully assembled TCR/CD3 complexes (Kearse et al., 1995c). This raised the possibility that SLAP could function to prevent assembly of the TCR/CD3 complex. Currently, it is believed that the rate of TCR/CD3 assembly is low in DP thymocytes due to the instability of the TCRα chain. However, since we failed to detect a difference in expression, synthesis or degradation of the TCRα chain in the

absence of SLAP, we must conclude that SLAP does not regulate assembly of the TCR/CD3 complex by the mechanism previously described. In addition, our data clearly demonstrate that SLAP interacts with the phosphorylated TCRζ chain, which has been shown to be present only in fully assembled, mature TCR/CD3 complexes (Kearse et al., 1993). Therefore, it is unlikely that SLAP plays a direct role in the assembly of TCR/CD3. We cannot exclude the possibility that SLAP somehow regulates TCR/CD3 assembly through an indirect mechanism that has yet to be described. However, our data suggests that a substantial proportion of TCRζ is present mainly in fully assembled TCR/CD3 complexes as evidenced by the large increase in TCRζ chain protein expression in WT thymocytes as compared to TCRα-/- thymocytes (Fig. 1A). Together, these data strongly indicate that the degradation of TCRζ observed in WT DP thymocytes is predominantly due to degradation of TCRζ derived from fully assembled TCR/CD3 complexes.

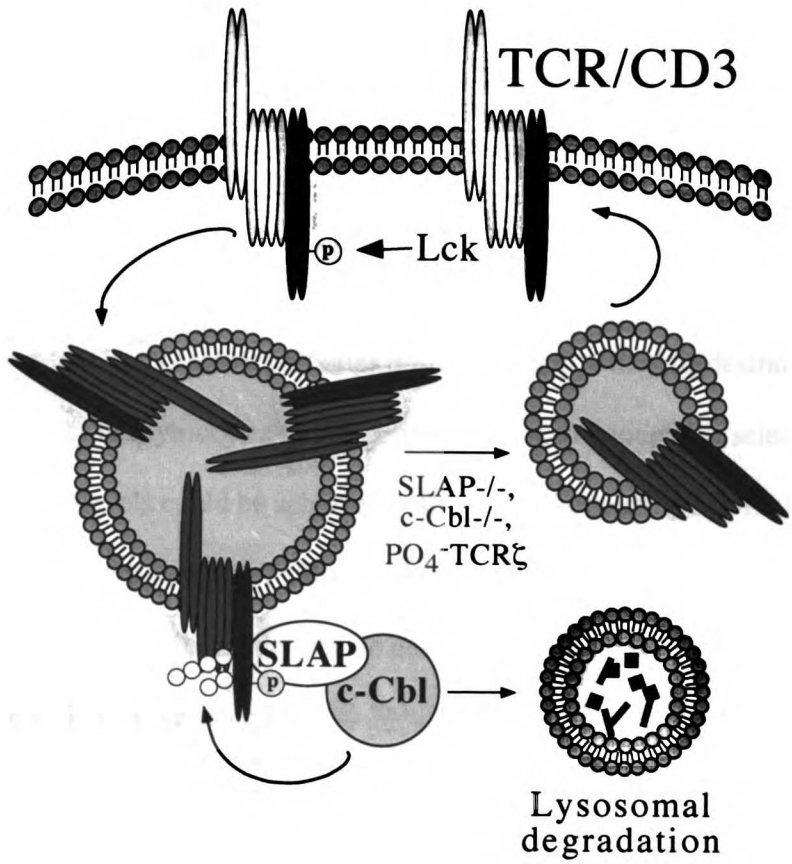
The TCR has been shown to undergo constitutive internalization in cell lines and T cells, with an estimate of 0.6 - 1.2% of TCRs internalized per minute (Liu et al., 2000; Menne et al., 2002). Therefore, subtle modifications to the rate of recycling or internalization could have large effects on the steady-state level of TCR expression on the cell surface. We consistently observed an increase in both the rate as well as the absolute amount of CD3ε recycled by *SLAP*$^{-/-}$ DP thymocytes. Notably, the increased amount of CD3ε recycled (5-9% as calculated from Fig. 3C) is consistent with the 5-7.5% of TCRζ chains that are estimated to be phosphorylated in the thymus (N. van Oers, personal communication). Furthermore, the small difference in CD3ε recycling can account for the

loss of TCRζ that is observed over time in WT but not *SLAP*$^{-/-}$ thymocytes when incubated in cycloheximide. Therefore, SLAP appears to target only a small proportion of the constitutively recycling TCR/CD3 complexes that contain phospho-TCRζ. However, this nonetheless has a substantial effect on the steady state level of TCR/CD3 expression in SLAP deficient thymocytes.

In contrast to TCRζ, we were unable to detect a substantial increase in the level of TCRα, TCRβ or CD3ε in the absence of SLAP. Likewise, degradation of TCRα, TCRβ and CD3ε were not noticeably altered in the absence of SLAP. One possible explanation for these observations is that DP thymocytes typically express a relatively large intracellular pool of unassembled and/or partially assembled TCR/CD3 chains, some of which are rapidly degraded (Bonifacino et al., 1990; Bonifacino et al., 1989; Chen et al., 1988; Lippincott-Schwartz et al., 1988). Therefore, the large pools of unassembled TCR/CD3 chains could mask any differences in TCR/CD3 expression or degradation. Alternatively, our results could also be interpreted to indicate that TCRζ separates from the rest of the mature TCR/CD3 complex and is independently degraded via a SLAP-dependent mechanism. Separation of the TCR/CD3 complex has previously been described (Kishimoto et al., 1995; La Gruta et al., 2004; Ono et al., 1995; Thien et al., 2003). Additional studies will be required to conclusively determine whether the remainder of the recycling TCR/CD3 complex is also degraded via a SLAP-dependent mechanism or is retained in an intracellular compartment.

The mechanism by which SLAP targets TCRζ for degradation has yet to be elucidated. Degradation of TCRζ is likely to involve the E3 ubiquitin ligase c-Cbl (Figure 6). SLAP has previously been reported to interact with the N-terminus of c-Cbl (Tang et al., 1999). In addition, c-Cbl deficient mice have a very similar phenotype as SLAP deficient mice with regard to TCR/CD3, CD4 and CD5 upregulation on DP thymocytes and increases in positive selection (Naramura et al., 1998). Finally, TCRζ has previously been shown to be ubiquitinated (Cenciarelli et al., 1992; Cenciarelli et al., 1996; Hou et al., 1994), perhaps via c-Cbl (Wang et al., 2001). Therefore, we suspect that the ubiquitin ligase activity of c-Cbl is required to target TCRζ for degradation and subsequently prevent the accumulation of recycling TCR/CD3 complexes. Indeed, our unpublished results indicate that c-Cbl and SLAP function in the same pathway to regulate TCR/CD3 expression on DP thymocytes by targeting the TCRζ chain for degradation (M. Myers and A. Weiss, manuscript in preparation). Therefore, we suggest the model shown in Figure 6. Surface TCR/CD3 complexes in DP thymocytes are constitutively phosphorylated on TCRζ chains by Lck. The TCR/CD3 complex is internalized and transported to an intracellular compartment where SLAP binds to phosphorylated TCRζ, thus targeting TCRζ for ubiquitination and degradation via a c-Cbl dependent mechanism. In the absence of TCRζ, the remainder of the TCR/CD3 complex is either degraded or retained in an intracellular compartment. Conversely, in the absence of SLAP, the TCRζ is neither ubiquitinated nor degraded, the TCR/CD3 complex remains intact and continues to recycle back to the plasma membrane.

Figure 6: Model. TCR/CD3 complexes are endocytosed via clathrin-coated pits and interact with SLAP in an intracellular compartment. TCRζ is ubiquitinated by c-Cbl and degraded. In the absence of TCRζ chains, the remainder of the TCR/CD3 complex is degraded. However, if SLAP fails to interact with TCRζ, the TCR/CD3 complex remains intact and continues to recycle back to the cell surface.



We have identified a novel mechanism by which TCR/CD3 expression is regulated in a developmentally restricted manner. Added to previous studies that have implicated regulation at the level of ER assembly of the TCR/CD3 complex is the current mechanism of SLAP-mediated degradation of phospho-TCRζ containing complexes. It is interesting to speculate why DP thymocytes possess multiple mechanisms to regulate surface TCR/CD3 expression. We suspect that receptor levels are kept low on DP thymocytes to ensure selection of developing thymocytes expressing TCRs with the appropriate avidity for self peptide/MHC complexes. Due to the massive amplification of the TCR signaling cascade, it may be difficult for a thymocyte to differentiate between strong and weak TCR signals if TCR levels are high. Therefore, quantitative differences in signaling may be more easily distinguished if levels of surface TCR/CD3 are kept low. Likewise, the increase in receptor levels after positive selection may be desirable for a heightened sensitivity of thymocytes to self peptides that lead to negative selection. Such increases in receptor levels could be achieved both by downregulating of SLAP expression as well as by promoting TCR assembly. Thus, SLAP may play a key role in regulating TCR/CD3 levels during thymocyte development to optimize receptor levels for both positive and negative selection.

Materials and Methods:

Cell lines and plasmids: Jurkat T cells, JCaM1, JCaM1+Lck and P116 cell lines were maintained in RPMI containing 5% FBS, 2 mM glutamine, penicillin and streptomycin. JCaM1 is a Lck-/- Jurkat T cell line (Goldsmith and Weiss, 1987); JCaM1+Lck has been stably reconstituted with Lck (Straus and Weiss, 1992). P116 is a ZAP-70-/- Jurkat T cell line (Williams et al., 1998). Plasmids encoding GFP, SLAP-GFP and SH2*-GFP were initially constructed by PCR amplification of WT or SH2 mutated murine SLAP from pEF-BOS (Sosinowski et al., 2000) and ligation into pN1-GFP (Clontech; Palo Alto, CA) via EcoRI and BamHI sites. GFP constructs were subcloned into pCDEF3 using EcoRI and NotI sites.

Mice: *SLAP*$^{-/-}$ mice have previously been described (Sosinowski et al., 2001) and have been backcrossed to a total of eight generations onto the C57BL/6 background. C57BL/6 mice (Taconic; Germantown, NY) were used as WT controls. ZAP-70-/- mice have previously been described (Kadlecek et al., 1998). TCRα-/- mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TCRζ.Tg and TCRζ.D67-250 were generously provided by Dr. Elizabeth Shores and have previously been described (Shores et al., 1994).

FACS staining: After washing in PBS, thymocytes were stained with CD4 (RM4-5, eBioscience; San Diego, CA), CD8α (53-6.7, Pharmingen; San Diego, CA), CD3ε (145-2C11, eBioscience; San Diego, CA) and TCRα (H57-197, eBioscience; San Diego, CA) in FACS buffer (PBS with 1%BSA and 0.01% NaN_3) for 30 minutes at 4°C. For

intracellular FACS staining, thymocytes were washed in PBS before fixation in 4% paraformaldehyde for 20 minutes at room temperature. After washing, thymocytes were permeabilized in 0.5% saponin in FACS buffer for 20 minutes at room temperature. Thymocytes were stained with the antibodies listed above in 0.5% saponin containing 1% goat serum for 30 minutes at 4°C. Jurkat T cells were stained with α-CD3ε (UCHT1, BD Biosciences; San Diego, CA) in FACS buffer for 30 minutes at 4°C. All FACS staining was performed in duplicate.

Internalization and Degradation Assays: For ligand-induced CD3ε internalization, surface CD3ε was labeled with biotinylated anti-CD3ε antibodies (145-2C11) for 30 minutes on ice. Thymocytes were washed, resuspended at 5 x 10^6/mL in primary cell culture medium (RPMI containing 10% FBS, 2 mM glutamine, 50 uM β-mercaptoethanol, penicillin and streptomycin) and incubated at 37°C for the indicated timepoints. Anti-CD3ε remaining on the cell surface was detected by staining with PE-conjugated streptavidin (Caltag Laboratories; Burlingame, CA). For degradation assays, thymocytes were cultured at 5 x 10^6/mL in primary cell culture medium containing 100 ug/mL cycloheximide. At each timepoint, cells were mixed with 100uL ice-cold PBS containing 1% BSA and 0.1% NaN_3. Cells were maintained on ice for the remainder of the assay until FACS staining.

CD3ε Recycling: Freshly isolated thymocyte single cell suspensions (20 x 10^6/mL) were cultured at 37°C in primary cell culture media containing PE-labeled anti-CD3ε (145-2C11; 5 ug/mL). After 30 minutes in culture, thymocytes were washed twice with PBS. Surface bound antibody was removed by washing thymocytes twice in ice-cold PBS

containing 1% BSA (pH 1.5) followed by immediate neutralization in FACS buffer. These conditions removed > 97% of cell surface staining. Thymocytes (5 x 10^6/mL) were incubated at 37°C in primary cell culture for the indicated timepoints. At each timepoint, cells were mixed with 100uL ice-cold PBS containing 1% BSA and 0.1% NaN_3 and maintained on ice for the remainder of the assay. After all timepoints had been collected, thymocytes were again washed twice in ice-cold PBS containing 1% BSA (pH 1.5) followed by immediate neutralization in FACS buffer to remove anti-CD3ε antibody bound to recycled CD3ε. Thymocytes were stained for CD4 and CD8 and analyzed by FACS. Recycled CD3ε was calculated using the formula:

$$\% \text{ Recycled} = 100 * \frac{(\text{MFI at } t = 0) - (\text{MFI at timepoint})}{\text{MFI at } t = 0}$$

Labeling of Recycling Pool: Freshly isolated thymocyte single cell suspensions were cultured at 20 x 10^6/mL in primary cell culture media containing 100ug/mL cycloheximide and PE labeled anti-CD3ε (145-2C11 at 5 ug/mL) for 30 minutes on ice to fully label surface CD3ε followed by a subsequent 60 minute incubation either on ice or at 37°C to achieve steady state labeling of the recycling pool. Cells were washed, stained for CD4 and CD8 expression and analyzed by FACS.

Hypertonic Recycling Assay: Jurkat T cells (10^6/mL in RPMI) or freshly isolated thymocyte single cell suspensions (5 x 10^6/mL in primary cell culture media) were incubated with 0.45M sucrose. At each timepoint, cells were mixed with 100uL ice-cold

PBS containing 1% BSA and 0.1% NaN_3. Cells were maintained on ice for the remainder of the assay until FACS staining.

Inhibition of clathrin-mediated endocytosis was measured by inhibition of transferrin uptake. Jurkat T cells were incubated on ice with Alexa647-labeled transferrin (Molecular Probes; Eugene, OR) for 20 minutes to allow for transferrin binding. Excess transferrin was washed off with three washes in ice-cold PBS. Cells were resuspended in RPMI ± 0.45M sucrose and were incubated either on ice or at 37°C for 10 minutes. After incubation, cells were transferred into ice-cold PBS containing 1% BSA and 0.1% NaN_3 and rested for 1 hour on ice. Surface bound transferrin was competed off using a 50 fold excess of unlabelled transferrin (Sigma; St. Louis, MO) in FACS buffer for 30 minutes at room temperature. Internalized transferrin was calculated using the formula:

$$\% \text{ Internalized} = \frac{\text{MFI of competed sample} - \text{background MFI}}{\text{MFI of uncompeted sample} - \text{background MFI}}$$

where background represents the remaining fluorescence present on competed samples incubated on ice.

Western blotting and immunoprecipitations: For total levels of TCRα, CD3ε and TCRζ, CD8+ thymocytes were purified using magnetic cell sorting (Miltinyi Biotec; Auburn, CA) according to the manufacturer's protocol. Recovered cells were ≥ 95% DP as assessed by FACS. CD8+ thymocytes were lysed at 200 x 10^6/mL in RIPA lysis buffer supplemented with protease inhibitors (leupeptin, aprotinin, PMSF, pepstatin A) for 30 minutes on ice. Postnuclear supernatants were prepared by centrifuging samples at

16,000 x g (4°C) for 30 minutes. For TCRα, postnuclear supernatants from 50 x 10^6 cell equivalents were immunoprecipitated with α-TCRα (H-142; Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) and protein G (Amersham Biosciences; Uppsla, Sweden) for 1 hour at 4°C. Samples were washed four times with lysis buffer, resuspended in SDS loading buffer and boiled for 5 minutes. For CD3ε and TCRζ, postnuclear supernatants from 5 x 10^6 cell equivalents were mixed with SDS loading buffer and boiled. Samples were electrophoresed in a 12.5% SDS-PAGE gel, transferred to Immobilon and blotted for α-tubulin (B-5-1-2, Sigma; St. Louis, MO), TCRα (H-142), CD3ε (M20, Santa Cruz Biotechnology Inc.; Santa Cruz, CA) or TCRζ (8D3, Pharmingen; San Diego, CA). Subsequently, membranes were incubated in secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences; Uppsla, Sweden) and were detected using enhanced chemiluminescence (Amersham Biosciences; Uppsla, Sweden). For TCR/CD3 degradation experiments, CD8+ thymocytes were cultured in primary cell culture media containing 100 ug/mL cycloheximide. At each timepoint, thymocytes were lysed in RIPA lysis buffer and maintained on ice for the remainder of the experiment. Lysates were blotted for α-tubulin, TCRα, CD3ε or TCRζ as described above. Western blots were quantitated on a Kodak Imaging Station using Kodak 1D image analysis software version 3.5 (Eastman Kodak Co.; Rochester, NY).

For SLAP/TCRζ coimmunoprecipitations, cells were transiently transfected with GFP, SLAP-GFP or SH2*-GFP overnight. Cells were washed twice in PBS and lysed at 50 x 10^6/mL in RIPA lysis buffer as described above supplemented with $NaVO_4$. Postnuclear supernatants from 60 x 10^6 cells were immunoprecipitated with TCRζ (6B10.2, Santa

Cruz Biotechnology, Inc.; Santa Cruz, CA) and protein G as described above. Samples were electrophoresed in a 12.5% SDS-PAGE gel, transferred to Immobilon and blotted with for GFP (JL8, Clontech) or phosphotyrosine (4G10, Upstate Biotechnology; Charlottesville, VA) as described above. Subsequently, membranes were stripped (30 minutes at 55°C in 5% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris, pH 6.8) and blotted for TCRζ (8D3, Pharmingen). In some experiments, transfected cells were incubated overnight with 20 uM PP2 (Calbiochem; San Diego, CA) to inhibit src-family kinase activity.

Metabolic Labeling: 80 x 10^6 freshly isolated thymocytes were cultured for 30 minutes in 4 mL cysteine and methionine free media (Biofluids; Rockville, MD) at 37°C. Thymocytes were labeled with 2 mCi ofTran^{35}SLabel (ICN; Irvine, CA) for 30 minutes at 37°C. Cells were washed, lysed in RIPA lysis buffer (as described above) and precleared for 30 minutes at 4°C with protein G. Lysates were immunoprecipitated for TCRα (H-142), CD3ε (M-20) or TCRζ (6B10.2), run on an SDS-PAGE gel and transferred to Immobilon as described above. Half of each immunoprecipitate was used to detect the ^{35}S label. The other half of the immunoprecipitate was western blotted (as described above) as loading controls.

Immunofluorescence:

Jurkat T cells were transfected with GFP, SLAP-GFP or SH2*-GFP overnight. Transfected cells were washed and allowed to settle onto poly-L-lysine coated plates.

Samples were fixed in 4% paraformaldehyde (20 minutes, room temperature) then permeabilized with 0.1% Triton-X-100 (10 minutes, room temperature). Samples were incubated in blocking buffer (PBS with 0.5% BSA, 0.5% milk, 1% goat serum) for 10 minutes at room temperature, then incubated in primary antibody (6B10.2, 1:50) in blocking buffer for 2 hours at 37°C. After washing, samples were incubated in secondary antibody (Cy5-goat-anti-mouse IgG, Jackson ImmunoResearch Laboratories; West Grove, PA) in blocking buffer for 20 minutes at room temperature. Samples were washed, coverslipped and visualized on a Marianas Turn-Key system from Intelligent Imaging (Denver, CO). Images were deconvolved using SlideBook software and exported as TIFF files.

Chapter 3:

SLAP Regulates TCR Expression on Developing Thymocytes by Adapting c-Cbl to the TCR Complex

Summary:

Src-Like Adaptor Protein (SLAP) regulates surface expression of the TCR/CD3 complex by targeting the TCRζ chain for degradation. Surprisingly, mice deficient in either SLAP or c-Cbl have very similar thymic phenotypes. We provide both genetic and functional evidence that SLAP and c-Cbl function in the same pathway to downregulate expression of the TCR/CD3 complex on DP thymocytes. TCR/CD3 downregulation requires Lck, multiple domains in SLAP and the ring finger of c-Cbl. Finally, SLAP and c-Cbl interact and are required to target the TCRζ chain for ubiquitination and degradation, thus preventing the accumulation of fully assembled TCR/CD3 complexes present in the recycling pool. These studies indicate that SLAP links the ubiquitin ligase activity of c-Cbl to the TCR/CD3 complex, thus allowing for regulation of TCR/CD3 expression on developing thymocytes in a stage specific manner.

Introduction:

Thymocytes progress through multiple developmental stages that ensures the generation of T cells expressing T cell receptors (TCRs) with an appropriate range of avidities for MHC (Love and Chan, 2003). At the CD4+CD8+ double positive (DP) stage of development, thymocytes that have productively rearranged both TCRα and TCRβ genes begin to express low levels of the mature TCRαβ on the surface together with the CD3 chains. Expression of the TCR/CD3 complex at this stage of development is crucial, since signals through the TCR/CD3 complex are required for either survival (positive selection) or deletion (negative selection) of developing thymocytes. Positively selected thymocytes downregulate either CD8 or CD4 expression as well as upregulate TCR/CD3 expression to become CD4+ or CD8+ single positive (SP) thymocytes. Finally, SP thymocytes undergo further maturation and selection processes before exiting the thymus as naïve T cells.

Whereas DP cells have a strict requirement for signals through the TCR for both positive and negative selection, TCR/CD3 expression on DP thymocytes is less than 10% the level expressed on single positive (SP) thymocytes and peripheral T cells (Finkel et al., 1987; Havran et al., 1987). This suggests that developing thymocytes actively maintain low levels of surface TCR/CD3 expression, potentially to decrease the sensitivity of thymocytes to interactions with self-peptide/MHC molecules so that quantitative differences in signaling can be distinguished more accurately. Previous studies have demonstrated that thymocytes employ multiple mechanisms to regulate TCR/CD3 expression during thymocyte development. First, the rate of TCR/CD3 assembly is kept

low due to rapid degradation of newly synthesized TCRα chains (Kearse et al., 1995b). In addition, our lab has previously shown that SLAP targets TCRζ chains present in mature TCR/CD3 complexes for degradation, thus preventing the accumulation of fully assembled complexes capable of recycling back to the plasma membrane (Myers, 2005).

SLAP is an adaptor which shares a significant degree of similarity to Src family kinases (Pandey et al., 1995). Like Src kinases, SLAP has a unique, myristolated N-terminus, followed by an SH3 and an SH2 domain, which are 55% and 50% homologous to the SH3 and SH2 domains of Lck, respectively. However, unlike Src kinases, the C-terminus of SLAP does not contain a kinase domain. Overexpression studies in cell lines indicated that SLAP inhibits signaling downstream of Src-family kinases following stimulation through either the TCR or the PDGF-R, perhaps by functioning in a dominant negative manner. It is unclear whether SLAP can inhibit Src kinase function when expressed at physiological levels. However, the C-terminus of SLAP is not required to inhibit Src kinase signaling, and the function of this domain is unclear.

Previous studies have suggested that the C-terminus of SLAP can bind to c-Cbl (Tang et al., 1999), indicating that SLAP and c-Cbl may function in the same pathway. A further link between SLAP and c-Cbl was suggested from the characterization of mice deficient in either molecule. Mice deficient in either SLAP or c-Cbl express increased levels of surface TCR/CD3, CD4, CD5 and CD69 expression on DP thymocytes (Naramura et al., 1998; Sosinowski et al., 2001). Moreover, thymocytes deficient in either SLAP or c-Cbl displayed an increase in positive selection in the presence of the DO11.10 transgenic

TCR. Notably, the stage-specific thymic phenotypes observed in *SLAP*$^{-/-}$ and *c-Cbl*$^{-/-}$ mice are consistent with the expression pattern of these molecules. c-Cbl is expressed primarily in hematopoietic cells and testis; however, the highest levels of expression are found in the thymus. Similarly, whereas SLAP mRNA is expressed in a variety of hematopoietic tissues, SLAP protein appears to be even more developmentally restricted, with expression highest in DP thymocytes.

We have previously shown that SLAP modulates TCR/CD3 expression on DP thymocytes by targeting TCRζ chains present in fully assembled TCR/CD3 complexes for degradation. In the absence of TCRζ, the remainder of the complex is either degraded or retained in an intracellular compartment. Although it is clear that SLAP is required to target the TCRζ chain for degradation, the mechanism by which TCRζ chains are targeted for degradation has not been tested. Similarly, it is unclear how c-Cbl modulates TCR/CD3 expression on DP thymocytes. c-Cbl is a large, multi-domain protein containing multiple tyrosine residues that become phosphorylated upon TCR stimulation (Donovan et al., 1994). c-Cbl is composed of an N-terminal tyrosine kinase binding (TKB) domain, a ring finger domain, several proline rich regions and a C-terminal leucine zipper (Rao et al., 2002a). The ring finger of c-Cbl has been demonstrated to have E3 ubiquitin ligase activity (Joazeiro et al., 1999). E3 ligases are a component of the ubiquitination machinery that catalyze the transfer of ubiquitin molecules to protein substrates, thus targeting the protein for proteasomal and/or lysosomal degradation (Weissman, 1997). Therefore, E3 ligases play a crucial role in the ubiquitination process by providing target specificity.

Multiple proteins expressed by T cells, including the TCRζ chain have been identified as targets for ubiquitination (Cenciarelli et al., 1992; Cenciarelli et al., 1996; Hou et al., 1994; Rao et al., 2002a; Wang et al., 2001), suggesting that c-Cbl may be required to ubiquitinate TCRζ in DP thymocytes. In an overexpression system, it has previously been shown that ZAP-70 can function to adapt c-Cbl to the TCRζ chain, thus targeting TCRζ for ubiquitination (Wang et al., 2001). However, TCR/CD3 expression is not upregulated on ZAP-70-/- DP thymocytes, suggesting that additional molecule(s) may be required to regulate TCR/CD3 expression. We hypothesized that SLAP functions as an adaptor for c-Cbl, and is required to recruit c-Cbl to the TCR/CD3 complex. Here we demonstrate that thymocytes deficient in both SLAP and c-Cbl have a similar phenotype as thymocytes deficient in either SLAP or c-Cbl alone. In addition, we provide evidence that SLAP and c-Cbl functionally cooperate in the same pathway to regulate TCR/CD3 expression by targeting the TCRζ chain for ubiquitination and degradation.

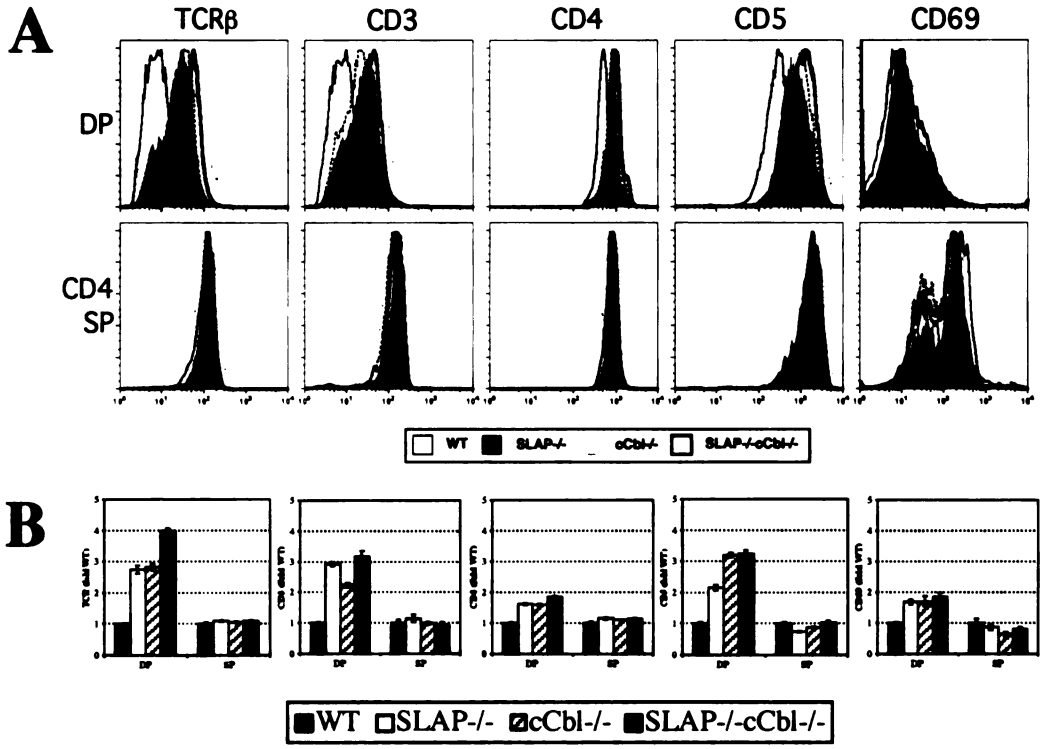
Results:

SLAP and c-Cbl function in the same pathway

To determine whether SLAP and c-Cbl function in the same genetic pathway, mice deficient in either SLAP or c-Cbl were crossed to generate *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ doubly deficient mice and surface receptor expression was determined by flow cytometry. As shown previously (Naramura et al., 1998; Sosinowski et al., 2001), TCRβ and CD3ε were approximately 3-4 fold higher on *SLAP*$^{-/-}$ or *c-Cbl*$^{-/-}$ DP, as compared to WT controls (**Fig. 1a,b**). In contrast, TCR/CD3 expression was not altered on SP thymocytes regardless of genotype. *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ DP thymocytes also expressed higher TCR/CD3 levels on DP thymocytes; however, the increase in TCR/CD3 expression was neither additive nor synergistic. In addition, *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ mice expressed a modest 1.5-fold increase in CD4 expression on DP, but not SP thymocytes that was similar to the levels observed on DP thymocytes deficient in either molecule alone (**Fig. 1a,b**).

CD5 and CD69 are cell surface molecules that are upregulated on thymocytes in response to TCR signals. CD5 is a negative regulator of signal transduction whose expression level reflects the amount of TCR signaling received by developing thymocytes (Azzam et al., 1998). CD69 is an early activation marker that is induced on thymocytes in response to

Figure 1 *SLAP*$^{-/-}$, *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$c-Cbl DP thymocytes have a similar phenotype. (**a**) Expression of the indicated cell surface molecules on DP and CD4+ SP thymocytes as assessed by flow cytometry. (**b**) Quantitation of FACS staining of multiple mice as represented in (**a**). Data indicate the fold mean fluorescent intensity (MFI) ± SEM compared to DP or SP thymocytes isolated from WT thymi. (**c**) Loss of SLAP and/or c-Cbl rescues the development of ZAP-70-/- SP thymocytes and peripheral T cells. Thymocytes (top) or lymph node (LN) T cells (bottom) of the indicated genotypes were stained for CD4 and CD8 and analyzed by flow cytometry. The frequency of SP thymocytes and LN T cells are indicated. (**d**) The average frequency ± SEM of CD4+ and CD8+ SP thymocytes or LN T cells for the indicated genotypes.


A
TCRβ
CD3
CD4
CD5
CD69
DP
CD4 SP
WT
SLAP-/-
cCbl-/-
SLAP-/-cCbl-/-
B
WT
SLAP-/-
cCbl-/-
SLAP-/-cCbl-/-
C
WT
ZAP-70-/-
ZAP-70-/- SLAP-/-
ZAP-70-/- c-Cbl-/-
ZAP-70-/- SLAP-/- c-Cbl-/-
7.8
1.2
0.8
0.2
2.5
0.7
4.7
0.6
6.5
0.6
Thymus
32.4
18.4
1.1
0.4
2.7
0.1
2.8
0.1
5.6
0.2
LN
CD4
CD8
D
Thymus
LN
Percent of total
CD4 SP
CD8 SP
CD4
CD8
WT
ZAP-70-/-
ZAP-70 -/- x SLAP-/-
ZAP-70 -/- x c-Cbl -/-
ZAP-70 -/- x SLAP-/- x c-Cbl -/-

TCR signaling and is associated with thymocytes that have been positively selected (Merkenschlager et al., 1997). Both *SLAP*$^{-/-}$ and *c-Cbl*$^{-/-}$ DP thymocytes have previously been shown to express higher levels of both CD5 and CD69 on DP, but not SP thymocytes (Naramura et al., 1998; Sosinowski et al., 2001). *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ doubly deficient mice expressed increased levels of CD5 and CD69 on DP, but not SP thymocytes that were similar to the levels observed on thymocytes deficient in either molecule alone (**Fig. 1a,b**).

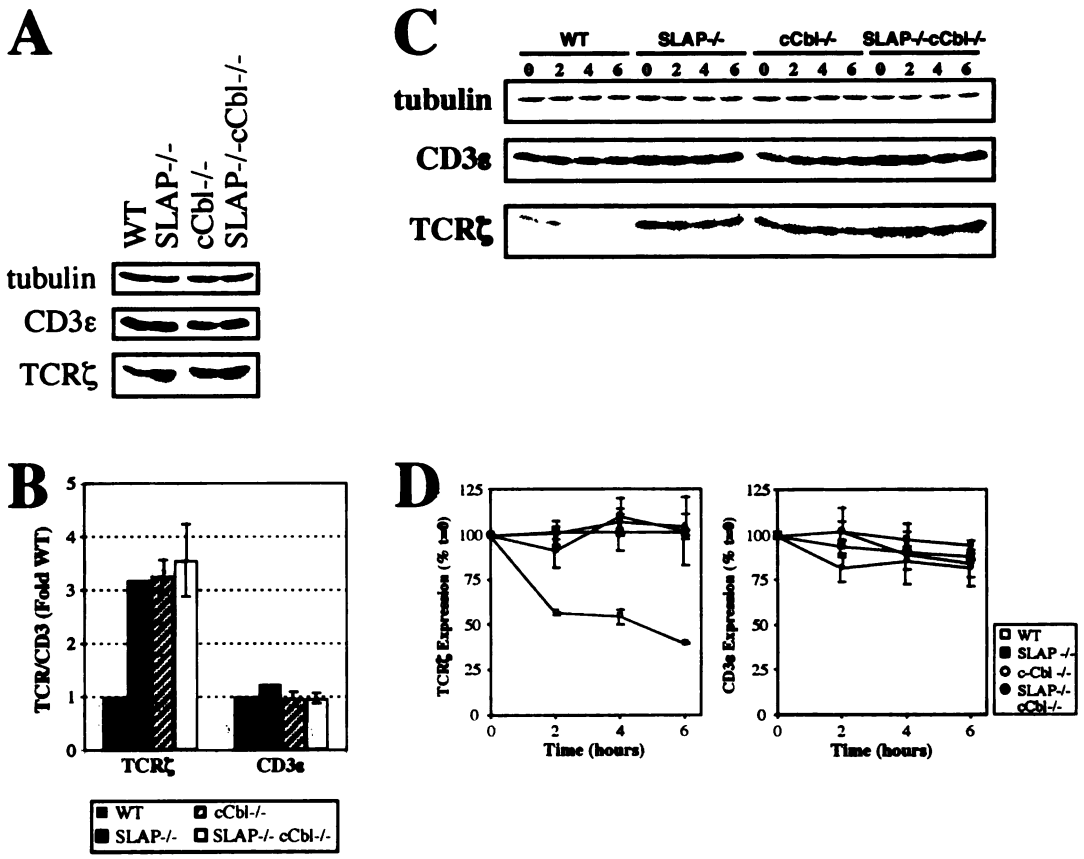
The ZAP-70 tyrosine kinase is essential for propagation of TCR signal transduction in both thymocytes and peripheral T cells (Kane et al., 2000). Since signals through the TCR are required for T cell development, ZAP-70-/- thymocytes are blocked at the DP stage of development (Kadlecek et al., 1998; Negishi et al., 1995). Consequently, ZAP-70-/- mice express few SP thymocytes and peripheral T cells. Previous studies have demonstrated that the loss of SLAP expression could partially rescue the developmental defect observed in the absence of ZAP-70 (Sosinowski et al., 2001). Development of ZAP-70-/- CD4+ SP thymocytes and peripheral T cells was also partially rescued in the absence of either c-Cbl alone or in the absence of both SLAP and c-Cbl (**Fig. 1c,d**). Rescue of CD4+ SP cells was somewhat additive in the thymus; however, loss of both molecules could not completely compensate for loss of ZAP-70, especially in the peripheral T cell compartment.

Both *SLAP*$^{-/-}$ and *c-Cbl*$^{-/-}$ thymocytes express increased levels of the TCRζ chain (Myers, 2005; Thien et al., 1999). Previous studies have demonstrated that the increase in TCRζ

expression by *SLAP*$^{-/-}$ thymocytes is due to a defect in TCRζ degradation. Western blot analysis of TCRζ expression by CD8+ *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ CD8+ thymocytes (cells were purified to enrich for DP thymocytes and were > 95% DP) demonstrated that total levels of TCRζ expression were also increased 3-4 fold as compared to WT controls (**Fig. 2a,b**). In contrast, total levels of CD3ε remained similar in all of the genotypes tested. To analyze degradation of the TCRζ chain in *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ mice, CD8+ thymocytes were incubated in the presence of the protein synthesis inhibitor cycloheximide and TCRζ expression was followed over time by western blot analysis. Similar to *SLAP*$^{-/-}$ thymocytes, *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ thymocytes failed to substantially degrade TCRζ over the 6 hour course of the assay (**Fig. 2c,d**). In contrast, the low level of CD3ε degradation observed in WT thymocytes was not substantially affected regardless of genotype.

We previously demonstrated that ligand-induced internalization of the TCR/CD3 complex is not altered in *SLAP*$^{-/-}$ thymocytes. Similarly, thymocytes deficient in c-Cbl or both SLAP and c-Cbl displayed normal rates of anti-CD3ε induced TCR/CD3 internalization (data not shown). Therefore, we next tested whether the size of the CD3e recycling pool was increased in the absence of c-Cbl or both SLAP and c-Cbl. Notably, failure to degrade TCRζ leads to increased number of TCR/CD3 complexes present in the recycling pool of *SLAP*$^{-/-}$ thymocytes. We have previously shown that the size of the recycling pool can be revealed by incubation of thymocytes in hypertonic medium, 0.45M sucrose, e.g. (Myers, 2005). Hypertonic medium inhibits clathrin-mediated endocytosis by depleting the cell of free clathrin monomers (Daukas and Zigmond, 1985;

Figure 2 Increased TCRζ expression and TCR/CD3 recycling pool due to a defect in TCRζ degradation. **(a)** Western blot analysis of CD3ε and TCRζ in purified CD8+ thymocytes from WT, *SLAP*$^{-/-}$, *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ mice. **(b)** Quantitation of western blots represented in **(a)** using quantitative luminescence. Data represent the mean of CD3ε or TCRζ expression by *SLAP*$^{-/-}$, *c-Cbl*$^{-/-}$, or *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ CD8+ thymocytes as compared to the WT control ± SEM. TCR/CD3 levels have been normalized to α-tubulin. For each experiment, one WT and one *SLAP*$^{-/-}$ mouse were used for comparison. **(c)** Western blot analysis of CD3ε and TCRζ expression in WT, *SLAP*$^{-/-}$, *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ CD8+ thymocytes following treatment with cycloheximide for the indicated time. **(d)** Quantitation of CD3ε and TCRζ expression represented in **(c)**. Data are expressed as the mean percent expression relative to t=0 ± SEM. One WT and one *SLAP*$^{-/-}$ mouse were included as controls. **(e)** MFI ± SEM of CD3ε expression on DP or SP thymocytes incubated in cell culture medium with or without 0.45M sucrose for the indicated genotypes.


A
WT
SLAP-/-
cCbl-/-
SLAP-/-cCbl-/-
tubulin
CD3ε
TCRζ
C
WT
SLAP-/-
cCbl-/-
SLAP-/-cCbl-/-
0 2 4 6 0 2 4 6 0 2 4 6 0 2 4 6
tubulin
CD3ε
TCRζ
B
TCR/CD3 (Fold WT)
TCRζ
CD3ε
WT
SLAP-/-
cCbl-/-
SLAP-/- cCbl-/-
D
TCRζ Expression (% t=0)
CD3ε Expression (% t=0)
Time (hours)
Time (hours)
WT
SLAP -/-
c-Cbl -/-
SLAP-/- cCbl-/-
E
RPMI
0.45M sucrose
CD3ε (geoMFI)
DP
SP
Time (hours)
Time (hours)
WT
SLAP -/-
c-Cbl -/-
SLAP-/- cCbl-/-

Heuser and Anderson, 1989). Since the TCR/CD3 complex is internalized via clathrin-coated pits (Telerman et al., 1987), incubation of cells in hypertonic medium blocks internalization of the TCR (Dallanegra et al., 1988). Over time, TCR/CD3 accumulation on the cell surface (in the absence of new receptor synthesis) is indicative of the number TCR/CD3 complexes present in the recycling pool. This methodology provides results that are consistent with an assay using intact antibodies to measure the size of the CD3ε recycling pool (Myers, 2005). However, the use of hypertonic medium to assess TCR/CD3 recycling is advantageous because it does not require ligation of the TCR and therefore does not induce signaling.

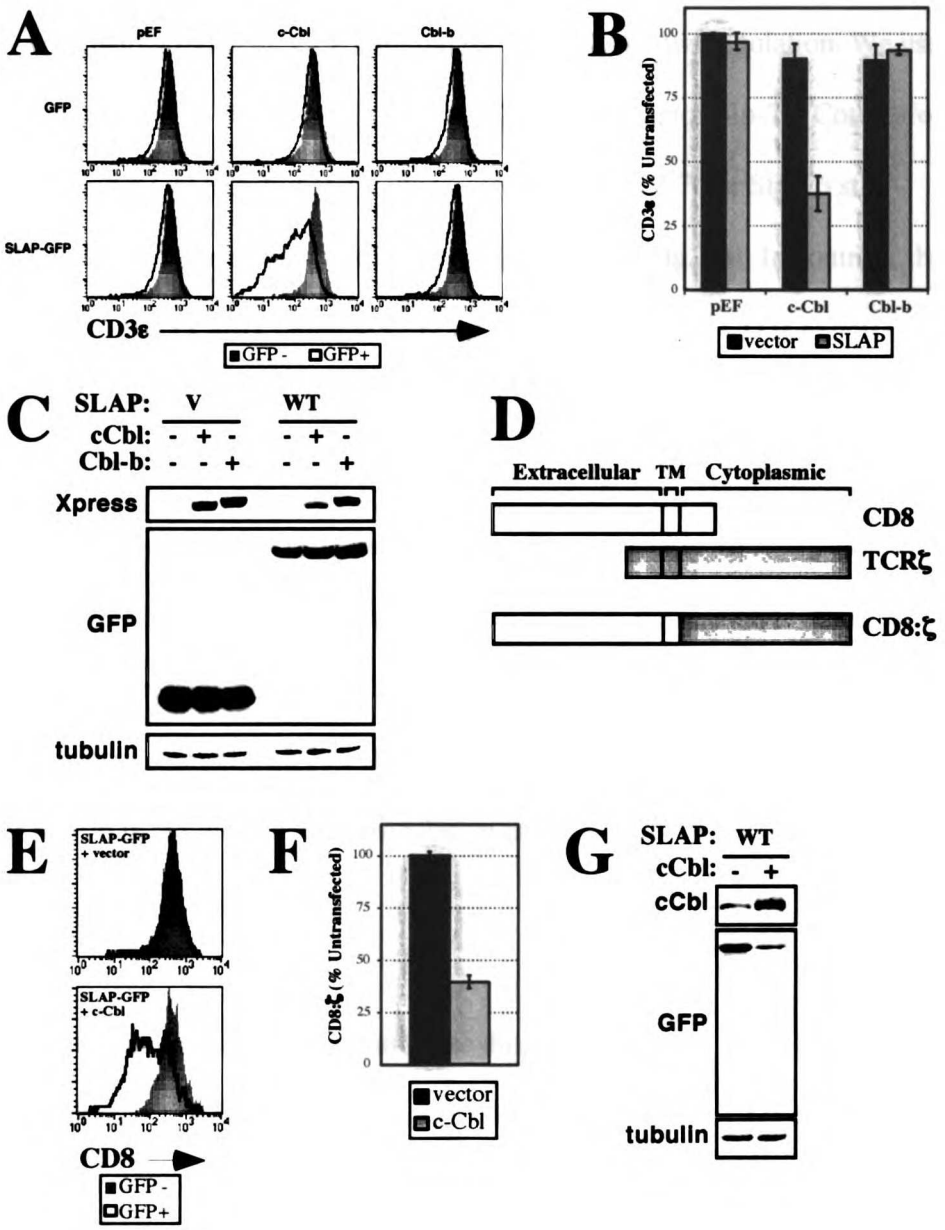
As previously observed (Myers, 2005), CD3ε upregulation by thymocytes incubated in hypertonic medium was increased on *SLAP*$^{-/-}$ DP, but not SP thymocytes as compared to WT controls (**Fig. 2e**). CD3ε upregulation in hypertonic medium was also increased in both *c-Cbl*$^{-/-}$ as well as *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ DP thymocytes, demonstrating that the number of fully assembled TCR/CD3 complexes present in the recycling pool of *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ DP thymocytes is increased to a similar extent as *SLAP*$^{-/-}$ DP thymocytes. The increase in CD3ε expression over the six hour assay was dependent on hypertonicity, as little TCR/CD3 upregulation was observed on DP thymocytes incubated in the absence of 0.45M sucrose (**Fig. 2e**). Furthermore, the upregulation of CD3ε on DP thymocytes was due to TCR/CD3 recycling as opposed to new synthesis, since the protein synthesis inhibitor cycloheximide had no effect on CD3ε expression (data not shown).

SLAP and c-Cbl downregulate TCR/CD3 expression on Jurkat T cells

To elucidate the mechanism of SLAP/c-Cbl mediated TCR/CD3 downregulation, we developed a model system to induce TCR/CD3 downregulation by SLAP and c-Cbl. Jurkat T cells were transfected by electroporation with SLAP which was fused to GFP at the C-terminus or with GFP alone as a control, and TCR/CD3 expression was assessed on transfected (GFP+) versus untransfected (GFP) cells. Transfection of either SLAP-GFP or c-Cbl alone into Jurkat T cells had no effect on CD3ε expression. In contrast, cotransfection of both SLAP-GFP and c-Cbl into Jurkat T cells substantially downregulated surface CD3ε expression on GFP+ cells in the absence of TCR ligation (**Fig. 3a,b**). CD3ε downregulation was specific for c-Cbl, as cotransfection of Cbl-b with SLAP-GFP had no effect on CD3ε downregulation, despite similar levels of Cbl-b expression (**Fig. 3c**).

We have previously shown that the TCRζ cytoplasmic domain is required to prevent the accumulation of TCR/CD3 complexes present in the recycling pool (Myers, 2005). Therefore, we tested whether the TCRζ cytoplasmic domain in isolation can be downregulated by SLAP-GFP and c-Cbl. JβCD8.ζ14 is a TCRβ deficient Jurkat T cell line that has been stably transfected with a chimeric CD8:ζ molecule (**Fig. 3d**). JβCD8:ζ14 T cells do not express the TCR/CD3 complex at the cell surface. However, surface expression of the CD8:ζ chimera occurs independently of the remaining TCR/CD3 chains and can recapitulate TCR signaling upon crosslinking of CD8 (Irving and Weiss, 1991). Cotransfection of SLAP-GFP and c-Cbl into JβCD8.ζ14 T cells downregulated surface expression of CD8:ζ (**Fig. 3e,f**), demonstrating that the TCRζ cytoplasmic domain is a sufficient target for downregulation by SLAP and c-Cbl.

Figure 3 Cotransfection of SLAP and c-Cbl downregulates TCR/CD3 expression. **(a)** FACS analysis of CD3ε expression on GFP+ versus GFP- Jurkat T cells transiently transfected with the indicated Xpress-Cbl or SLAP-GFP expression constructs. **(b)** Quantitation of data represented in **(a)**. Data are presented as percent CD3ε expression on GFP+ cells versus GFP- cells ± SEM and have been normalized to the vector control. **(c)** Western blot analysis of Xpress-tagged Cbl and SLAP-GFP expression in Jurkat T cells transfected with the indicated constructs. α-tubulin was included as a loading control. **(d)** Schematic representation of the CD8:ζ chimera, which includes the extracellular and transmembrane (TM) regions of CD8 fused to the cytoplasmic domain of TCRζ. **(e)** FACS analysis of CD8 expression on GFP+ versus GFP- JβCD8.ζ14 T cells that had been transiently transfected with either SLAP-GFP alone or SLAP-GFP and c-Cbl. **(f)** Quantitation of data represented in **(e)**. Data are presented as percent CD8 expression on GFP+ cells as compared to GFP- cells ± SEM and have been normalized to the vector control. **(g)** Western blot analysis of c-Cbl and SLAP-GFP expression in JβCD8.ζ14 T cells transfected with the indicated constructs. α-tubulin was included as a loading control.



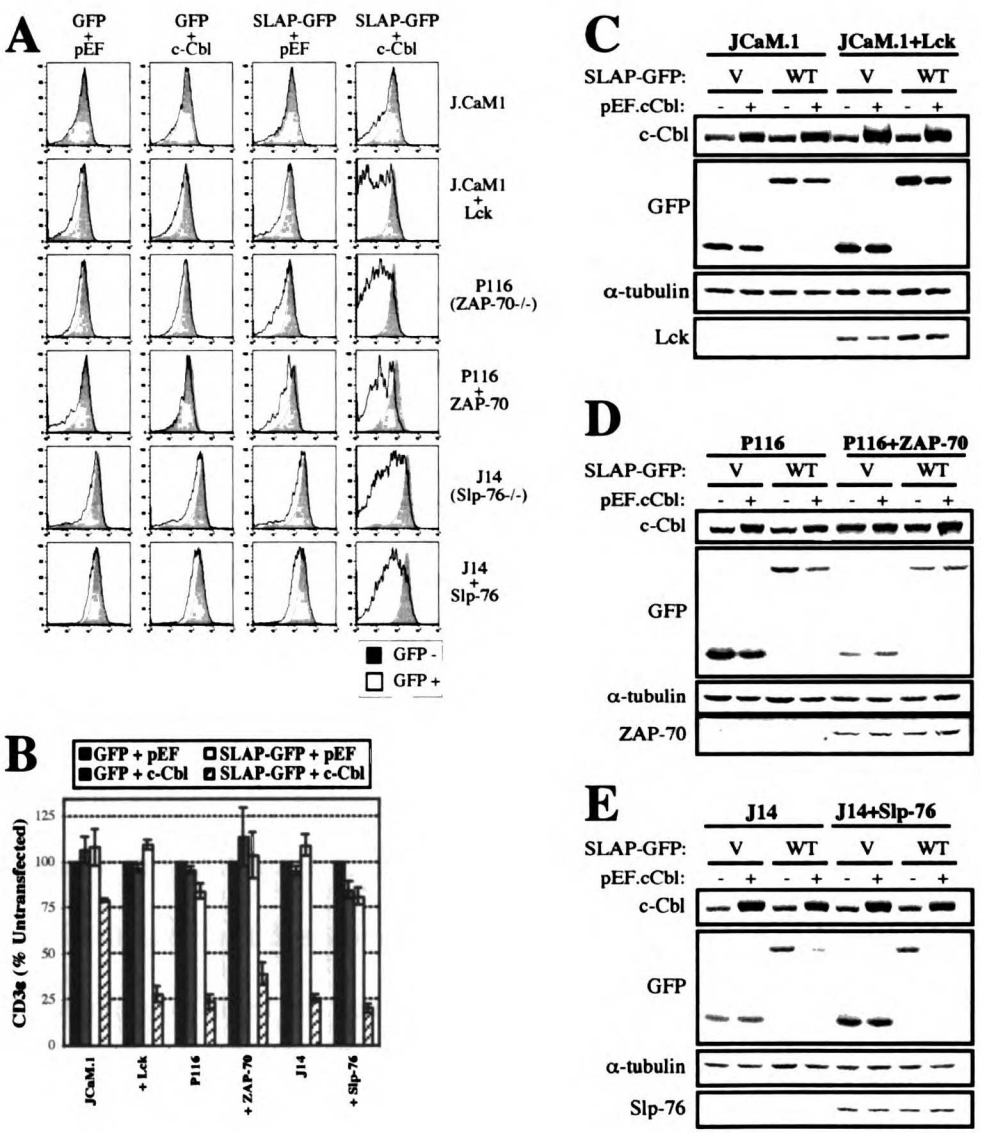
Lck is required to downregulate TCR/CD3 expression

The development of a Jurkat T cell system in which to study TCR/CD3 downregulation by SLAP and c-Cbl provided us with a powerful tool to study both the cellular as well as the molecular requirements for TCR/CD3 downregulation by SLAP and c-Cbl. We first tested which signaling molecules are required for TCR/CD3 downregulation. We used Jurkat T cell lines that are deficient in Lck, ZAP-70 or the adaptor Slp-76. Cotransfection of SLAP-GFP and c-Cbl into Jurkat T cells lacking either ZAP-70 or Slp-76 still downregulated CD3ε expression specifically on GFP+ cells (**Fig. 4a**). In contrast, the Lck deficient Jurkat T cell J.CaM1 failed to substantially downregulate CD3ε expression following cotransfection of SLAP-GFP and c-Cbl (**Fig. 4a**). However, stable reintroduction of Lck back into JCaM.1 restores the ability of SLAP and c-Cbl to downregulate CD3ε expression. The failure of SLAP and c-Cbl to downregulate expression of CD3ε in J.CaM1 T cells was not due to lower expression levels of SLAP and/or c-Cbl, as expression of both molecules was comparable to the levels expressed in J.CaM1 stably reconstituted with Lck (**Fig. 4b**). These data indicate that Lck, but not ZAP-70 or Slp-76 is required for downregulation of the TCR/CD3 complex by SLAP and c-Cbl.

TCR/CD3 downregulation requires multiple domains of SLAP

To test which domains of SLAP are required to downregulate TCR/CD3 expression in Jurkat T cells, a panel of GFP-tagged SLAP constructs (**Fig. 5a**) were cotransfected with WT c-Cbl, and CD3ε expression on GFP+ versus GFP- cells was determined by FACS. Mutation of the myristolation site (G2A) or the SH2 domain of SLAP (SH2*) eliminated

Figure 4 TCR/CD3 downregulation requires Lck. **(a)** FACS analysis of CD3ε expression on the indicated Jurkat T cell lines transiently transfected with GFP or SLAP-GFP ± c-Cbl expression constructs. **(b)** Quantitation of CD3ε expression on the indicated Jurkat T cell lines following transient transfection with GFP or SLAP-GFP ± c-Cbl. Data are presented as percent CD3ε expression on GFP+ versus GFP- cells ± SEM and have been normalized to the GFP control (V) for each cell line. **c-e)** Western blot analysis of c-Cbl and SLAP-GFP expression as well as expression of Lck **(c)**, ZAP-70 **(d)**, and Slp-76 **(e)** in the indicated cell lines. α-tubulin was included as a loading control.

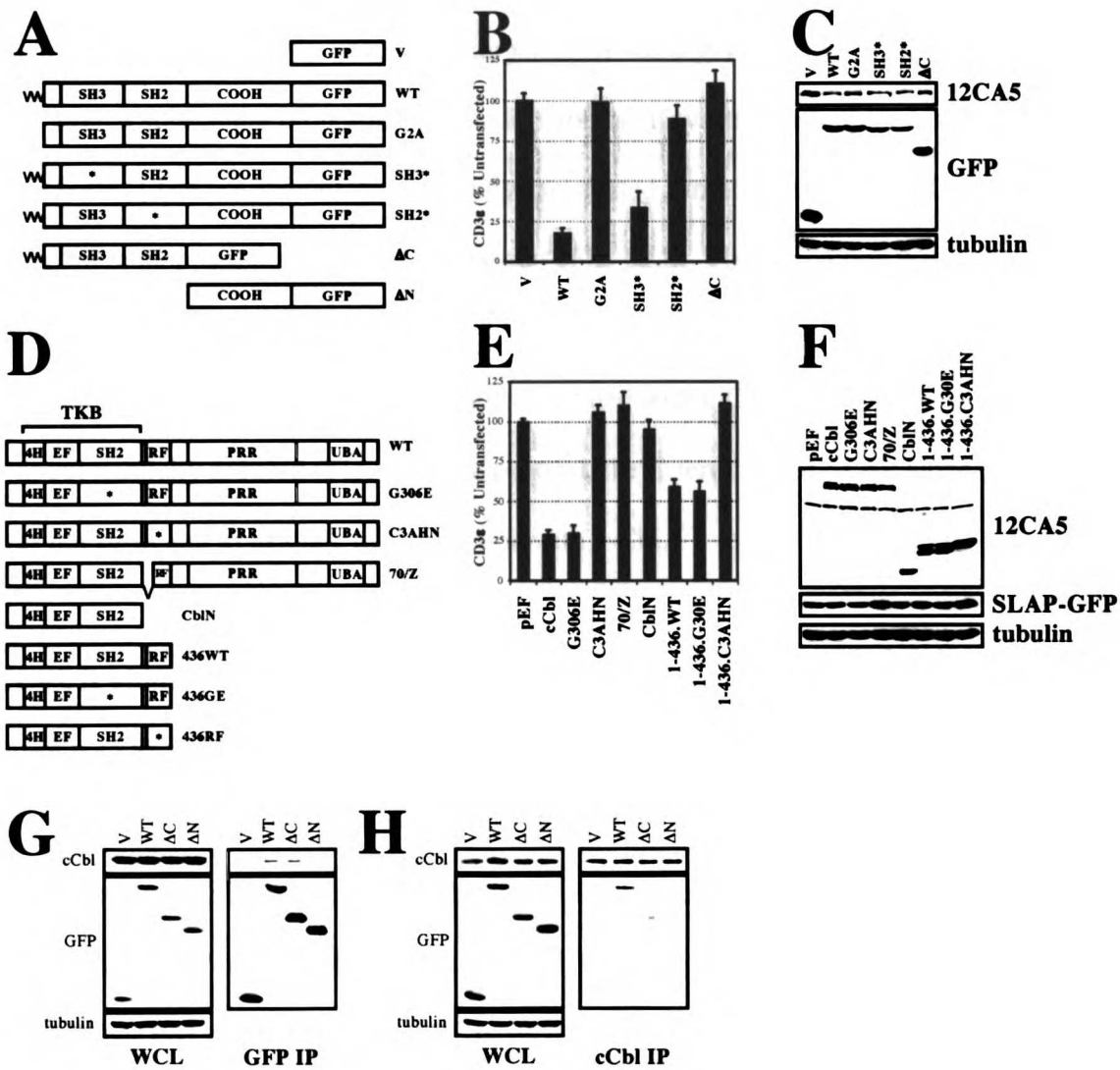


the downregulation of CD3ε following cotransfection with SLAP and c-Cbl (**Fig. 5b**), despite similar levels of expression of SLAP and c-Cbl (**Fig. 5c**). In addition, a C-terminal truncation of SLAP (ΔC) failed to downregulated CD3ε expression when cotransfected with c-Cbl despite markedly higher levels of expression (**Fig. 5b,c**). In contrast, mutation of the SH3 domain of SLAP (SH3*) had little, if any effect on CD3ε downregulation by SLAP and c-Cbl.

TCR/CD3 downregulation requires the ring finger domain of c-Cbl

A similar mutational approach was taken to determine which domains of c-Cbl are required to downmodulate TCR/CD3 expression in Jurkat T cells. A panel of HA-tagged c-Cbl constructs (**Fig. 5d**) were cotransfected with SLAP-GFP and CD3ε expression on GFP+ versus GFP- cells was determined by FACS. Mutation of the ring finger of c-Cbl, either by a partial deletion (70/Z) or by multiple point mutations in the ring finger domain (C3AHN), eliminated the ability of c-Cbl to downregulate CD3ε expression following cotransfection with SLAP-GFP (**Fig. 5e**). In contrast, mutation of the c-Cbl TKB domain (G306E) had no effect on the ability of c-Cbl to downregulate CD3ε expression when cotransfected with SLAP. Interestingly, the conserved N-terminus of c-Cbl encompassing the TKB domain and ring finger domains (1-436.WT) was sufficient to downregulate TCR/CD3 expression, albeit less completely, even if the TKB domain of c-Cbl was mutated (1-436.G306E). These data suggest that the ring finger domain of c-Cbl is absolutely required to downregulate TCR/CD3 expression in the presence of SLAP. The observed downregulation was not a consequence of higher expression levels, since a similar truncation mutant of c-Cbl containing multiple point mutations in the ring finger

Figure 5 TCR/CD3 downregulation requires multiple domains in SLAP and the ring finger of c-Cbl. **(a,d)** Schematic representation of the SLAP-GFP **(a)** or c-Cbl **(d)** constructs used in this study. Myristolation of SLAP is indicated by the N-terminal extension. Point mutation(s) in the SH3 or SH2 domains of SLAP and the TKB or ring finger domains of c-Cbl are indicated with an asterisk. **(b,e)** Quantitation of CD3ε expression on Jurkat T cells transiently transfected with WT c-Cbl and the indicated SLAP-GFP constructs **(b)** or WT SLAP-GFP and the indicated c-Cbl constructs **(e)**. Data are presented as percent CD3ε expression on GFP+ versus GFP- cells ± SEM and have been normalized to the vector control. **(c,f)** Western blot analysis of HA-c-Cbl and SLAP-GFP expression by Jurkat T cells transfected with the indicated constructs. α-tubulin was included as a loading control. **(g)** Coimmunoprecipitation of WT c-Cbl with GFP (V) or the indicated SLAP-GFP constructions. **(h)** Coimmunoprecipitation of GFP (V) or the indicated SLAP-GFP constructs with WT c-Cbl. Protein expression in whole cell lysates (WCL) is shown on the left side of each panel **(g,h)**.



domain (1-436.C3AHN) or a mutant of c-Cbl truncated N-terminal to the ring finger (CblN) expressed at similar levels had no effect on CD3ε expression (**Fig. 5e,f**).

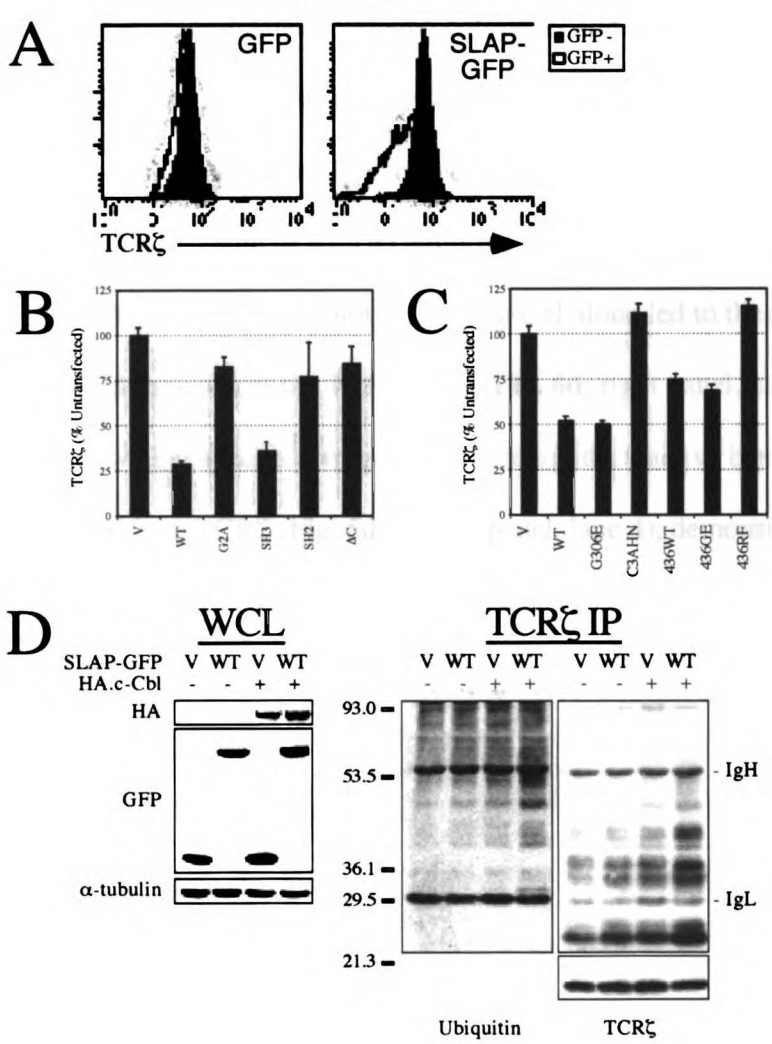
SLAP and c-Cbl interact in Jurkat T cells

We had previously been unable to detect an interaction between SLAP and c-Cbl due to the insolubility of SLAP in nonionic detergents, NP-40, e.g. (Sosinowski et al., 2000). However, a substantial proportion of SLAP is soluble if lysates are prepared in RIPA lysis buffer (data not shown). In transiently transfected Jurkat T cells, SLAP-GFP and c-Cbl coimmunoprecipitated from RIPA lysates (**Fig. 5g,h**). The interaction was specific, as no interaction was observed between c-Cbl and the GFP control. The C-terminal truncation of SLAP (ΔC) also coimmunoprecipitated with c-Cbl, although the interaction was substantially reduced (**Fig. 5g,h**). In contrast, the C-terminus of SLAP alone (ΔN) was not sufficient to mediate the interaction with c-Cbl.

TCRζ is ubiquitinated and degraded following SLAP/c-Cbl cotransfection

Since both SLAP (Myers, 2005) and c-Cbl (**Fig. 2c,d**) are required for efficient TCRζ degradation by DP thymocytes, we next tested whether TCRζ is degraded following cotransfection of SLAP-GFP and c-Cbl. Jurkat T cells were transfected with c-Cbl and either SLAP-GFP or with GFP as a control and TCRζ expression was assessed by intracellular FACS staining using a PE-labeled anti-TCRζ antibody. Expression of either SLAP or c-Cbl alone failed to downregulate expression of TCRζ. In contrast, TCRζ expression was substantially downregulated in cells expressing both SLAP-GFP and c-Cbl (**Fig. 6a**). Similar to TCR/CD3 downregulation, degradation of TCRζ required the

Figure 6 SLAP and c-Cbl target TCRζ for ubiquitination and degradation. (a) Intracellular FACS staining for TCRζ expression in GFP+ versus GFP- Jurkat T cells transiently transfected with SLAP-GFP alone or with c-Cbl. **(b,c)** Quantitation of TCRζ expression in Jurkat T cells transiently transfected with WT c-Cbl and the indicated SLAP-GFP constructs **(b)** or with SLAP-GFP and the indicated c-Cbl constructs **(c)**. Data are presented as percent CD3ζ expression on GFP+ versus GFP- cells ± SEM and have been normalized to the vector control. **(d)** Detection of TCRζ ubiquitination by western blot analysis. Left: Western blot analysis of HA-c-Cbl and GFP expression by Jurkat T cells transfected with the indicated constructs. α-tubulin was included as a loading control. Middle and right: TCRζ immunoprecipitates from Jurkat T cells transfected with the indicated constructs. The middle panel shows ubiquitin-reactive species that comigrate with higher molecular weight TCRζ species observed in the right panel. Molecular weight markers are indicated.


A
GFP
SLAP-GFP
GFP -
GFP+
TCRζ
B
TCRζ (% Untransfected)
V
WT
G2A
SH3
SH2
ΔC
C
TCRζ (% Untransfected)
V
WT
G306E
C3AHN
436WT
436GE
436RF
D
WCL
TCRζ IP
SLAP-GFP V WT V WT
HA.c-Cbl - - + +
HA
GFP
α-tubulin
V WT V WT V WT V WT
- - + + - - + +
93.0
53.5
36.1
29.5
21.3
IgH
IgL
Ubiquitin
TCRζ

myristolation site, the SH2 domain and the C-terminus of SLAP as well as the ring finger of c-Cbl (**Figure b,c**), indicating that downregulation of surface TCR/CD3 expression may be coupled to the degradation of TCRζ.

The ring finger of c-Cbl has E3 ubiquitin ligase activity (Rao et al., 2002a). Therefore, we tested whether TCRζ is ubiquitinated following cotransfection of SLAP and c-Cbl. TCRζ was immunoprecipitated from lysates of Jurkat T cells transfected with SLAP, c-Cbl or both SLAP and c-Cbl. Immunoprecipitates were immunoblotted with either an antibody specific for the TCRζ chain or with an antibody specific for ubiquitin. Transfection of both SLAP and c-Cbl, but not SLAP or c-Cbl alone led to the appearance of higher molecular weight species of the TCRζ chain (**Fig. 6d**; right panel, lane 4). These higher molecular weight species comigrated with ubiquitin reactive bands that were immunoprecipitated with TCRζ (**Fig. 6d**; middle panel, lane 4), demonstrating that the laddering of TCRζ that is observed upon SLAP/c-Cbl cotransfection is due to ubiquitin modification of the TCRζ chain.

Discussion:

We have provided both genetic and functional evidence that SLAP and c-Cbl cooperatively regulate TCR expression on DP thymocytes. Thymocytes deficient in SLAP and/or c-Cbl express increased expression of TCRβ, CD3ε, CD4, CD5 and CD69 at the DP stage of thymocyte development. In addition, the failure to express either SLAP and/or c-Cbl partially rescues the development of ZAP-70-/- SP thymocytes and peripheral T cells, presumably by increasing TCR avidity in a compromised signaling system. Finally, both SLAP and c-Cbl are required to target the TCRζ chain for degradation. Loss of either molecule leads to an increase in the number of fully assembled TCR/CD3 complexes present in the recycling pool (Myers, 2005). Importantly, the phenotype of doubly deficient mice is neither additive nor synergistic, indicating that SLAP and c-Cbl function in the same pathway to regulate surface expression of the TCR/CD3 complex.

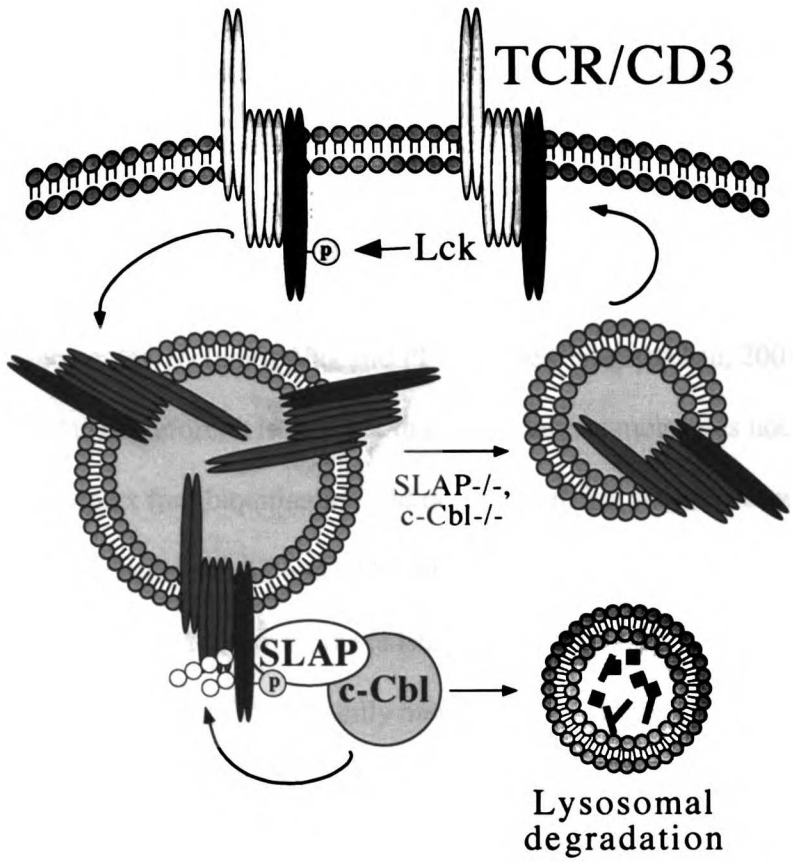
In addition to our studies in DP thymocytes, transfection of both SLAP-GFP and c-Cbl, but not SLAP-GFP or c-Cbl alone into Jurkat T cells downregulates surface CD3ε as well as total TCRζ expression, thus mimicking the phenotype of DP thymocytes. Notably, it is the DP stage of thymocyte development to which expression SLAP is primarily restricted. Moreover, the ring finger activity of c-Cbl is required not only for TCRζ ubiquitination and degradation, but also the downregulation of surface TCR/CD3 expression. Therefore, similar to what is observed in DP thymocytes, TCR/CD3 downregulation in Jurkat T cells following cotransfection of SLAP and c-Cbl appears to result from accelerated degradation of the TCRζ chain.

The SH2 domain of SLAP is also required for surface CD3ε downregulation and TCRζ degradation. Previous studies have shown that the SH2 domain of SLAP is required for the interaction of SLAP with phosphorylated TCRζ (Sosinowski et al., 2000; Tang et al., 1999). Furthermore, we have also shown that Lck is required for SLAP to interact with TCRζ. Phosphorylation of TCRz ITAMs is presumably mediated by tonic signaling via Lck (Qian et al., 1996; van Oers et al., 1994). Therefore, we hypothesize that the defect in CD3ε downregulation and TCRζ degradation in Lck deficient Jurkat T cells results from the inability of SLAP to interact with unphosphorylated TCRζ chains. However, previous studies have demonstrated that Jurkat T cells deficient in Lck have a defect in both constitutive and ligand-induced TCR/CD3 internalization due to a defect in clathrin heavy chain phosphorylation (Crotzer et al., 2004). Therefore, we cannot exclude the possibility that TCR/CD3 downregulation following cotransfection of SLAP and c-Cbl into Jurkat T cells may be due to a defect in TCR/CD3 internalization.

Based on our studies, we propose the model in **Figure 7**. TCRζ present in fully assembled TCR/CD3 complexes is phosphorylated by Lck and is internalized via clathrin-coated pits. TCR/CD3 complexes are transported to an endosomal compartment where phospho-TCRζ binds to the SH2 domain of SLAP. SLAP binds and recruits c-Cbl to the TCR/CD3 complex, whereupon c-Cbl ubiquitinates the TCRζ chain, thereby targeting TCRζ for degradation. In the absence of TCRζ, the remainder of the TCR/CD3 complex is either degraded or retained in an intracellular compartment. Notably, failure to ubiquitinate and/or degrade TCRζ in the presence of SLAP is sufficient for the

TCR/CD3 complex to recycle back to the cell surface, suggesting that the complex is not actively bound and retained by SLAP in the absence of c-Cbl. In contrast, SLAP appears to only be required to recruit c-Cbl to the TCR/CD3 complex. Thus, in DP thymocytes, SLAP functions as an adaptor to target the E3 ubiquitin ligase activity of c-Cbl to phosphorylated TCRζ chains.

Figure 7 Model. TCR/CD3 complexes are internalized via clathrin-coated pits. SLAP interacts with phosphorylated TCRζ chain and recruits c-Cbl. TCRζ is ubiquitinated by c-Cbl, and is subsequently degraded. In the absence of TCRζ, the remainder of the TCR/CD3 complex is either degraded or retained in an intracellular compartment. In the absence of either SLAP or c-Cbl, TCRζ is neither ubiquitinated nor degraded, the TCR/CD3 complex remains intact and continues to recycle back to the cell surface.


TCR/CD3
P
Lck
SLAP-/-,
c-Cbl-/-
SLAP
P
c-Cbl
Lysosomal
degradation

The ability of SLAP to regulate TCR/CD3 expression on Jurkat T cells is dependent on c-Cbl. In contrast, cotransfection of SLAP with Cbl-b fails to downregulate TCR/CD3 expression. This is somewhat surprising, since c-Cbl and Cbl-b are highly homologous (84% identity), especially in the N-terminal region that is sufficient to downregulate TCR/CD3 expression (Rao et al., 2002a). However, unlike c-Cbl, Cbl-b does not appear to regulate basal TCR/CD3 expression since neither thymocytes nor peripheral T cells have increased receptor expression in the absence of Cbl-b(Bachmaier et al., 2000; Chiang et al., 2000). Instead, Cbl-b may play a role in ligand-induced TCR downregulation; T cells deficient in both c-Cbl and Cbl-b have a reduced capacity to downregulate TCR/CD3 expression in response to TCRβ crosslinking (Naramura et al., 2002). In addition, Cbl-b has been shown to have E3 ubiquitin ligase astivity for a number of other molecules, including Vav and PI-3 kinase (Fang and Liu, 2001; Miura-Shimura et al., 2003). Therefore, it is possible that Cbl-b targets molecules not included in the TCR/CD3 complex for ubiquitination and degradation. Further studies will be required to determine whether SLAP and Cbl-b function together to regulate expression of other signaling molecules. Moreover, the existence of multiple SLAP (SLAP and SLAP-2) and Cbl (c-Cbl, Cbl-b, Cbl-3) family members raises the question of whether various permutations of SLAP and Cbl family members independently function to regulate the expression of unique protein targets.

The C-terminus of SLAP has previously been shown to interact with c-Cbl (Tang et al., 1999). We observed that the interaction between SLAP and c-Cbl is reduced, but not absent if the SLAP C-terminus is truncated. Our preliminary results suggest that the

interaction between SLAP and c-Cbl is complex, and may involve multiple domains in SLAP and/or c-Cbl. An N-terminal deletion of SLAP completely fails to interact with c-Cbl. Both the SH2 and the SH3 domains of Src family kinases have been shown to interact with c-Cbl (Andoniou et al., 2000; Donovan et al., 1994; Sanjay et al., 2001; Tsygankov et al., 1996). Therefore, it is possible that the SH2 and/or SH3 domain(s) of SLAP contribute to the interaction with c-Cbl. Regardless, it is clear that the residual interaction between SLAP.ΔC and c-Cbl is not sufficient for TCR/CD3 downregulation and TCRζ degradation. It is also possible that the C-terminus of SLAP may perform additional function(s) that are unique from its interaction with c-Cbl.

We have shown that SLAP and c-Cbl function in the same pathway to regulate TCR/CD3 expression on DP thymocytes. Loss of SLAP and/or c-Cbl leads to similar alterations in thymocyte development (Naramura et al., 1998; Sosinowski et al., 2001), emphasizing that strict regulation of TCR/CD3 expression levels on developing thymocytes is essential during thymic education. The function of SLAP and c-Cbl to maintain low levels of TCR/CD3 expression may be critical for the positive selection of an appropriate TCR repertoire. Consequently, the TCR repertoire in *SLAP*$^{-/-}$ and/or *c-Cbl*$^{-/-}$ T cells may be inappropriately biased for less efficient self –recognition. The outcome of SLAP and/or c-Cbl deficiency on the peripheral TCR repertoire in non-transgenic mice has not yet been tested. Therefore, future studies in this area may eventually lead to a greater understanding of T cell development and T cell repertoire selection.

METHODS

Cell lines and cell culture. Cell lines were maintained in RPMI supplemented with 10% FBS, 2 mM glutamine, penicillin and streptomycin. Cell lines are either wild type Jurkat T cells or have been derived from a parental Jurkat T cell line: JCaM1 (Lck-/-) (Goldsmith and Weiss, 1987), JCaM1+Lck (J.CaM1 stably reconstituted with Lck) (Straus and Weiss, 1992), P116 (ZAP-70-/-), P116.C39 (P116 stably reconstituted with ZAP-70) (Williams et al., 1998), J14 (Slp-76-/-), J14-76-11 (J14 stably reconstituted with Slp-76) (Yablonski et al., 1998) and JβCD8.ζ14 (TCRβ-/- stably expressing a chimeric CD8:ζ molecule) (Irving and Weiss, 1991).

Expression Constructs. Plasmids encoding GFP-tagged SLAP constructs were constructed by PCR amplification of murine SLAP from pEF-BOS (Sosinowski et al., 2000) and ligation into pN1-GFP (Clontech; Palo Alto, CA) via EcoRI and BamHI sites. GFP constructs were subcloned into pCDEF3 using EcoRI and NotI sites. Xpress-tagged c-Cbl and Cbl-b were a gift from Y.-C. Liu (La Jolla Institute for Allergy and Immunology, San Diego, CA). HA-tagged c-Cbl constructs (excluding 436WT, 436GE and 436RF) have previously been described (Ota et al., 2000; Rao et al., 2001; Rao et al., 2000). 436WT, 436GE and 436RF were constructed by PCR amplification from full length c-Cbl, G306E or C3AHN and ligation into pAlter-MAX. HA-tagged c-Cbl constructs were subcloned into pCDEF3 via EcoRI and NotI sites.

Mice. *SLAP*$^{-/-}$ mice have previously been described and have been backcrossed onto C57BL/6 at least 5 generations (Myers, 2005; Sosinowski et al., 2001). C57BL/6 mice

(Taconic; Germantown, NY) were used as WT controls. *c-Cbl*$^{-/-}$ mice have previously been described (Naramura et al., 1998) and were crossed with *SLAP*$^{-/-}$ mice to generate *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ mice. ZAP-70-/- mice have been described (Kadlecek et al., 1998) and have been crossed with *SLAP*$^{-/-}$, *c-Cbl*$^{-/-}$ and *SLAP*$^{-/-}$ *c-Cbl*$^{-/-}$ mice.

FACS staining. After washing in PBS, thymocytes were stained with antibodies against CD4 (RM4-5, eBioscience; San Diego, CA), CD8α (53-6.7, Pharmingen; San Diego, CA), TCRβ (H57-197, eBioscience), CD3ε (145-2C11, eBioscience), CD5 (53-7.3, Pharmingen) or CD69 (H1.2F3, Pharmingen) in FACS buffer (PBS with 1%BSA and 0.01% azide) for 30 minutes. Thymocytes were washed in FACS buffer and analyzed by flow cytometry. All data presented are representative of at least six mice per genotype.

Jurkat T cells transfected overnight with the indicated constructs were washed in PBS and stained with antibodies against human CD3ε (UCHT1, BD Biosciences; San Diego, CA) or CD8 (3B5, Caltag Laboratories; Burlingame, CA) as described above. For intracellular FACS staining, transiently transfected Jurkat T cells previously stained with α-CD3ε were washed in PBS and fixed in 4% paraformaldehyde for 20 minutes. After washing, Jurkat T cells were permeabilized in FACS buffer containing 0.5% saponin for 20 minutes at room temperature. Jurkat T cells were stained with a PE-conjugated antibody against TCRζ (6B10.2, Santa Cruz Biotechnology Inc.; Santa Cruz, CA) in 0.5% saponin containing 1% mouse serum for 1 hour at 4°C. Cells were washed in FACS buffer and analyzed by flow cytometry. All data presented are representative of at least six independent experiments.

Hypertonic Recycling Assay. Freshly isolated thymocyte single cell suspensions were cultured at 5 x 10^6/mL in primary cell culture media (RPMI containing 10% FBS, 2 mM glutamine, 50 μM β-mercaptoethanol, penicillin and streptomycin) in the presence or absence of 0.45M sucrose. At each timepoint, cells were mixed with 100μL ice-cold PBS containing 1% BSA and 0.1% NaN_3. Cells were maintained on ice for the remainder of the assay, stained and analyzed by flow cytometry.

Western blotting. For total levels of CD3ε and TCRζ, CD8+ thymocytes were purified using magnetic cell sorting (Miltinyi Biotec; Auburn, CA) according to the manufacturer's protocol. Recovered cells were ≥ 94% DP as assessed by FACS. CD8+ thymocytes were lysed at 200 x 10^6/mL in RIPA lysis buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM MOPS (pH 7.0), 150 mM NaCl, 2 mM EDTA) supplemented with protease inhibitors (leupeptin, aprotinin, PMSF, pepstatin A) for 30 minutes on ice. Postnuclear supernatants were prepared by centrifuging samples at 16,000 x g (4°C) for 30 minutes. Samples were electrophoresed in a 12.5% SDS-PAGE gel, transferred to Immobilon (Millipore Corporation; Billerica, MA) and blotted for α-tubulin (B-5-1-2, Sigma; St. Louis, MO), CD3ε (M20, Santa Cruz Biotechnology Inc.; Santa Cruz, CA) or TCRζ (8D3, Pharmingen). Membranes were incubated in secondary antibodies coupled to horseradish peroxidase (Amersham Biosciences; Uppsla, Sweden) and detected using enhanced chemiluminescence (Amersham Biosciences). Western blots were quantitated on a Kodak Imaging Station using Kodak 1D image analysis software version 3.5 (Eastman Kodak Co.; Rochester, NY).

For TCRζ degradation experiments, CD8+ thymocytes were cultured at 20 x 10^6/mL in primary cell culture media containing 100 ug/mL cycloheximide (Sigma). At each timepoint, thymocytes were lysed in RIPA lysis buffer and maintained on ice for the remainder of the experiment. Lysates were immunoblotted for α-tubulin (B-5-1-2), CD3ε (M20) or TCRζ (8D3). Lysis, western blotting and quantitation were performed as described above.

For cell line experiments, cells were transfected overnight with the indicated constructs, washed twice in PBS and lysed at 50 x 10^6/mL in RIPA lysis buffer as described above. Postnuclear supernatants were electrophoresed in a 10% SDS-PAGE gel, transferred to Immobilon and blotted for α-tubulin (B-5-1-2), GFP (JL8, Clonetech; Palo Alto, CA) or c-Cbl (12CA5, Boehringer Mannheim; Indianapolis, IN; Xpress, Invitrogen; Carlsbad, CA; C15, Santa Cruz Biotechnology Inc.) as described above.

SLAP/c-Cbl coimmunoprecipitations. 20 x 10^6 Jurkat T cells were transiently transfected with HA-tagged c-Cbl and the indicated SLAP-GFP constructs. 4.5 hours after transfection, cells were washed and lysed as described above. Lysates were immunoprecipitated with α-HA (12CA5) or α-GFP (JL8, Clontech) crosslinked to Protein G (Amersham Biosciences) for 30 minutes at 4°C. Immunoprecipitates were washed four times with ice-cold lysis buffer, resuspended in SDS-PAGE loading buffer and boiled. Samples were electrophoresed, transferred to Immobilon and immunoblotted as described above. For each transfection, postnuclear supernatants from 5 x 10^5 cell

equivalents were immunoblotted as described above to control for expression. Data presented are representative of six independent experiments.

TCRζ Ubiquitination. 80 x 10^6 Jurkat T cells were transiently transfected with the indicated SLAP-GFP and HA-tagged c-Cbl constructs. 4.5 hours after transfection, cells were washed and lysed in RIPA lysis buffer supplemented with 1.25 mg/mL N-ethylmalemide (Sigma). Lysates were immunoprecipitated for TCRζ (6B10.2) with protein G for 1 hour at 4°C. Samples were washed four times with ice-cold lysis buffer, resuspended in SDS loading buffer and boiled for 5 minutes. Samples were electrophoresed in a 10% SDS-PAGE gel, transferred to Immobilon and immunoblotted for HA (12CA5), GFP (JL8), α-tubulin (B-5-1-2), ubiquitin (P4D1, Santa Cruz Biotechnology, Inc.) or TCRζ (8D3) as described above. Data are representative of at least ten independent experiments.

Chapter 4:

Identification of a Novel Dileucine-based Motif in SLAP that is Required for Downregulating TCR/CD3 Expression

Summary:

Src-Like Adaptor Protein (SLAP), together with the ubiquitin ligase, c-Cbl, targets the zeta chain of the TCR/CD3 complex for degradation. Subsequently, the accumulation of fully assembled TCR/CD3 complexes is prevented, leading to decreased expression of the TCR/CD3 complex. Failure to express either SLAP or c-Cbl generates a population of double positive thymocytes expressing increased levels of the TCR/CD3 complex, which causes alterations in thymic development. We have investigated the mechanism by which TCRζ is targeted for degradation. Here we describe a previously uncharacterized dileucine-based motif present in the SH2 domain of SLAP that is required for TCR/CD3 downregulation by SLAP and c-Cbl. These studies suggest a novel mechanism by which SLAP targets substrates for degradation.

Introduction:

A striking feature of cellular membranes is that transmembrane proteins are not randomly distributed, but are instead maintained within restricted membrane domains. Maintenance of distinct domains requires the active sorting of transmembrane proteins to distinct regions within the cell and is known to occur in regions of cellular membranes that are associated with clathrin coats. Clathrin, when assembled into polymers provides the structure required for the formation of coated vesicles (Anderson et al., 1977; Roth et al., 1976). In addition to clathrin, protein sorting involves a number of accessory molecules that allow clathrin coated vesicles to be targeted to a variety of subcellular locations depending on the accessory molecule(s) bound to the protein of interest (Bonifacino and Traub, 2003; Heilker et al., 1999). Such accessory molecules include both adaptor protein (AP) complexes and Golgi-localized, gamma-ear-containing, Arf-binding (GGA) proteins. In addition to binding clathrin, AP complexes and the GGA proteins bind to the cytoplasmic tails of transmembrane proteins, thereby facilitating the entry of cargo proteins into clathrin coated regions of the membrane.

Clathrin adaptor proteins do not randomly bind to transmembrane proteins; instead, AP complexes and GGA proteins bind to protein targeting motifs within the cytoplasmic domains of cargo proteins (Bonifacino and Traub, 2003; Heilker et al., 1999). A number of protein targeting motifs have been identified and have been categorized on the basis of the direction of protein sorting mediated by the particular motif: golgi to endosome sorting, endosome to golgi sorting, internalization from the plasma membrane and lysosomal targeting. Three lysosomal targeting motifs have been identified, each of

which can also mediate internalization from the plasma membrane. Tyrosine-based motifs generally conform to the consensus sequence Yxxϕ (where x represents any amino acid and ϕ represents a bulky hydrophobic group); dileucine-based motifs have a consensus sequence of [D/E]xxxL[I/L]. Both Yxxϕ and [D/E]xxxL[I/L] motifs have been demonstrated to interact with the μ subunit of AP complexes but not with GGA proteins. Finally, in addition to tyrosine and dileucine motifs, ubiquitin can also function as a lysosomal targeting motif. However, the mechanism by which ubiquitinated proteins are targeted to the lysosome is less clear and is a topic of intense investigation.

The process of ubiquitination is most well known for its role in targeting cytoplasmic proteins for degradation by the 26S proteasome (Bonifacino and Weissman, 1998; Weissman, 1997). However, it is becoming increasingly clear that ubiquitination can also target transmembrane proteins for lysosomal degradation (Haglund et al., 2003; Pelham, 2004; Strous and Govers, 1999; Umebayashi, 2003). The type of ubiquitination required for lysosomal targeting differs from the type of ubiquitination that is required for proteasomal degradation. Specifically, proteasomal degradation requires that the target protein be modified with polyubiquitin chains formed by the sequential addition of ubiquitin molecules to Lys48 of the previous ubiquitin molecule (Bonifacino and Weissman, 1998; Weissman, 1997). In contrast, proteins targeted for lysosomal degradation can be modified by either monoubiquitination on one or more lysine residues or modified by ubiquitin chains formed by the sequential addition of ubiquitin molecules to Lys63 of the previous ubiquitin molecule (Umebayashi, 2003).

It is unclear how ubiquitination targets transmembrane proteins to the lysosome. However, the mechanism may require proteins that contain ubiquitin-binding motifs. The ubiquitin binding protein, Hrs, is believed to bind and sort ubiquitinated proteins from early endosomes into late endosomes/multivesicular bodies (Raiborg and Stenmark, 2002). Subsequently, the Endosomal Sorting Complex Required for Transport-I (ESCRT-I) complex can recognize ubiquitinated proteins. ESCRT-I sorts ubiquitinated proteins into the inner vesicles of multivesicular bodies (MVBs) where they are exposed to proteolytic enzymes for degradation (Babst, 2005). Thus, by sequential interactions with ubiquitin-interacting sorting proteins within the endocytic pathway, transmembrane proteins could eventually be delivered to MVB/lysosomes for degradation. However, the GGA proteins also contain ubiquitin-binding motifs (Pelham, 2004). Therefore, GGA proteins may also mediate sorting of ubiquitinated proteins to the lysosome.

The αβ T cell receptor (TCR) is part of a multi-chain complex composed of the TCRα and β chains which are non-covalently associated with the CD3γεδε and TCRζζ chains, together referred to as the CD3 complex (Exley et al., 1991; Schrum et al., 2003). While the α and β TCR chains are responsible for binding to ligand, the CD3 chains are required for coupling the αβTCR to intracellular signaling molecules. Like many other transmembrane receptors, the TCR/CD3 complex is dynamically regulated on the surface of thymocytes and T cells (Alcover and Alarcon, 2000). Previous studies have shown that a dileucine-based motif in the cytoplasmic domain of CD3γ (Figure 1A) mediates the constitutive internalization and recycling of the TCR/CD3 complex. Mutations in CD3γ that disrupt the dileucine motif do not undergo constitutive internalization (Dietrich et al.,

2002; Letourneur and Klausner, 1992). In addition to the dileucine-based motif, phosphorylation of a serine residue directly upstream of the dileucine by Protein Kinase C (PKC) induces the internalization of the TCR/CD3 complex by increasing the rate of receptor internalization via the dileucine motif approximately ten-fold (Dietrich et al., 1994; Menne et al., 2002; Minami et al., 1987). Once internalized, the receptor must be dephosphorylated before the TCR/CD3 complex is recycled back to the plasma membrane (Dietrich et al., 1998). While the serine residue is essential for PKC mediated internalization of the TCR/CD3 complex, this residue does not appear to be required for constitutive internalization of the complex (Dietrich et al., 2002).

In addition to constitutive internalization and recycling, the TCR/CD3 complex also undergoes ligand-induced internalization (Alcover and Alarcon, 2000; Schrum et al., 2003). TCR/CD3 complexes that are internalized in response to ligand are not recycled, but are instead predominantly targeted to the lysosome for degradation (D'Oro et al., 1997; Valitutti et al., 1997; von Essen et al., 2004). Interestingly, the dileucine-based motif is not required for ligand-induced internalization, suggesting that multiple sorting mechanisms may regulate the TCR/CD3 complex (Dietrich et al., 2002). In particular, the abundant tyrosine-based signaling motifs ($Yxx\phi x_{6-8}Yxx\phi$) present in the CD3γδε and TCRζ chains each contain two potential tyrosine-based sorting motifs ($Yxx\phi$) and may play a role in either constitutive or ligand-induced internalization of the TCR/CD3 complex. Finally, the TCRζ chain has been shown to be inducibly ubiquitinated following stimulation through the TCR. Therefore, it is likely that multiple motifs present

within the TCR/CD3 complex contribute to both the constitutive as well as ligand-induced internalization of the TCR/CD3 complex.

The dynamic internalization and recycling of the TCR/CD3 complex suggests that expression of the complex is highly regulated. TCR/CD3 expression is even more tightly regulated at the CD4+CD8+ double positive (DP) stage of thymocyte development. While signals through the TCR/CD3 complex are required for the development of DP thymocytes into mature T cells, TCR/CD3 expression is only about 10% of the level on more mature thymocytes and peripheral T cells (Finkel et al., 1987; Havran et al., 1987). The reason for low TCR/CD3 expression on DP thymocytes is unknown; however, we speculate that low levels of expression are required for optimal thymocyte development, as alterations in development are observed if TCR/CD3 expression is modified at the DP stage of development (Ericsson and Teh, 1995; Naramura et al., 1998; Sosinowski et al., 2001). Reduced TCR/CD3 expression is due in part to rapid degradation of nascent TCRα chains in the endoplasmic reticulum of DP thymocytes which impairs the assembly and, subsequently, the cell surface expression of the TCR/CD3 complex (Kearse et al., 1994a; Kearse et al., 1995a; Kearse et al., 1995b; Kearse et al., 1995c). In addition, we have previously shown that Src-like Adaptor Protein (SLAP) downregulates expression of fully assembled TCR/CD3 complexes by targeting the TCRζ chain present in fully assembled TCR/CD3 complexes for degradation.

SLAP is an adaptor molecule which shares both structural and sequence similarity with Src family kinases. Like Src-family kinases, SLAP contains a unique, lipid modified N-

terminal domain, an SH3 domain and an SH2 domain. However, instead of a C-terminal kinase domain, the C-terminus of SLAP is unique and may be required for SLAP to bind the E3 ubiquitin ligase, c-Cbl (Tang et al., 1999). In thymocytes, SLAP functions together with c-Cbl to regulate TCR/CD3 expression at the DP stage of thymocyte development. By interacting with both TCRζ and c-Cbl, SLAP functions as an adaptor for c-Cbl, thus targeting TCRζ for degradation. Degradation of TCRζ prevents the accumulation of fully assembled TCR/CD3 complexes, thereby preventing the complex from recycling back to the cell surface.

Cotransfection of SLAP and c-Cbl into Jurkat T cells downregulates TCR/CD3 expression and induces TCRζ degradation, mimicking the phenotype of DP thymocytes. The SH2 domain of SLAP is required for this function, most likely due to the requirement of the SH2 domain for binding phosphorylated TCRζ. Interestingly, immunofluorescence studies of transiently transfected SH2*SLAP has demonstrated that the SH2 mutant of SLAP is mislocalized (Sosinowski et al., 2000). WT SLAP has been shown to localize to a perinuclear vesicular compartment, which predominantly colocalizes with a late endosomal resident protein, the Mannose-6-Phosphate Receptor (M6P-R). In contrast, the SH2 mutant of SLAP localizes to punctate cytoplasmic vesicles that no longer colocalize with the M6P-R. These studies suggest that interactions via the SH2 domain of SLAP are required to target SLAP to late endosomes. Since previous studies have suggested that components of the TCR/CD3 complex, including TCRζ are degraded in lysosomes (D'Oro et al., 1997; Valitutti et al., 1997; von Essen et al., 2004),

we speculated that SLAP is targeted to late endosomes via its interactions with phosphorylated target proteins (TCRζ, e.g.) to the lysosome.

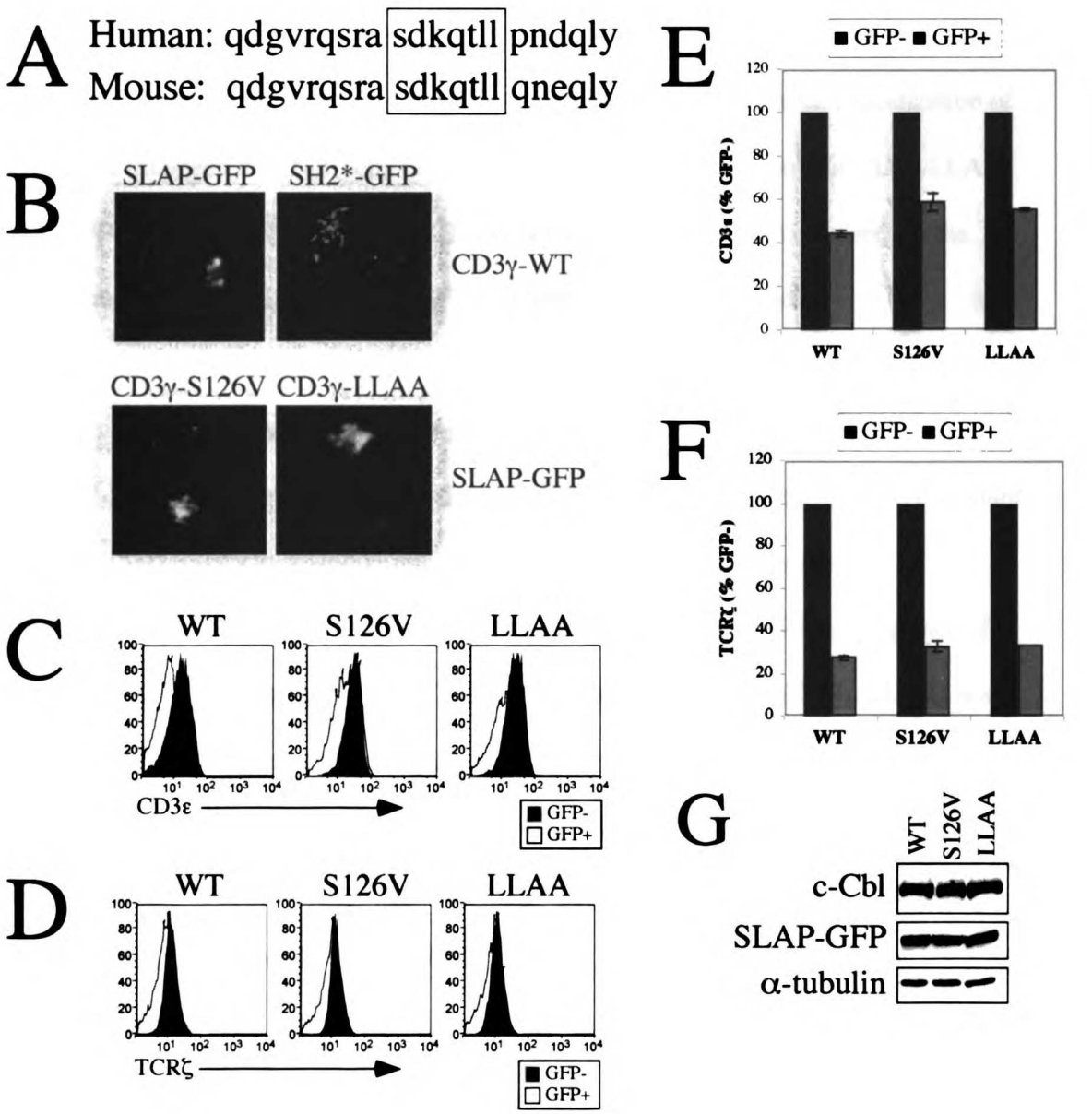
The CD3γ chain contains a dileucine-based motif that could mediate endosomal and/or lysosomal sorting of SLAP if bound to the TCR/CD3 complex. Therefore, we hypothesized that the dileucine-based sorting motif present in CD3γ may be required to target SLAP/TCR complexes to late endosomes and/or lysosomes. However, we show here that localization of SLAP is not affected by mutations in either the dileucine motif or the PKC phosphorylation site of CD3γ. Furthermore, TCR/CD3 downregulation following cotransfection of SLAP and c-Cbl occurs independently of the CD3γ dileucine motif, suggesting that additional motif(s) for TCR/CD3 downregulation must exist. Upon closer analysis of the primary amino acid structure of SLAP, we identified of a motif reminiscent of dileucine-based lysosomal targeting motifs. Here, we demonstrate that the dileucine motif of SLAP is required for TCR/CD3 downregulation following cotransfection of SLAP and c-Cbl. In addition, mutation of either leucine within this motif causes a mislocalization of SLAP similar to that observed upon mutation of the SLAP SH2 domain without impairing the ability of SLAP to bind phosphorylated TCRζ. These studies suggest a novel mechanism by which SLAP targets substrates for lysosomal degradation.

Results:

TCR/CD3 downregulation in CD3γ mutants:

Previous studies have shown that mutation of the SLAP SH2 domain prevented SLAP from binding to phosphorylated TCRζ (Sosinowski et al., 2000; Tang et al., 1999). In addition, the SH2 mutant of SLAP was mislocalized and no longer colocalized with the M6P-R. Therefore, we hypothesized that SLAP is targeted to late endosomes via its interaction with phosphoprotein targets. The CD3γ chain contains a dileucine-based motif that could target SLAP/TCR complexes to late endosomes upon the interaction of SLAP with TCRζ (Figure 1A). JGN is a Jurkat T cell derivative that does not express endogenous CD3γ and therefore does not express the TCR/CD3 complex on the cell surface (Geisler, 1992). Stable reconstitution of the JGN T cell line with WT CD3γ or CD3γ containing mutations in the dileucine-based motif (S126V or L131A,L132A, henceforth referred to as LLAA) restores TCR/CD3 expression at the cell surface (Dietrich et al., 1994). However, as constitutive endocytosis of the TCR/CD3 complex requires the dileucine in CD3γ, TCR/CD3 complexes expressing CD3γ-LLAA are not

Figure 1: The CD3γ dileucine motif is not required for SLAP/c-Cbl mediated TCR/CD3 downregulation. A) Partial sequence alignment of human and mouse CD3γ. The dileucine motif is indicated. B) Colocalization of SLAP-GFP or SH2*GFP (upper right) in JGN T cells reconstituted with CD3γ-WT (top), CD3γ-S126V (lower left) or CD3γ-LLAA (lower right) with the M6P-R (red). C) Surface CD3ε staining on JGN T cells reconstituted with CD3γ-WT, CD3γ-S126V or CD3γ-LLAA following cotransfection of SLAP-GFP and c-Cbl. Data are representative of 6 independent transfections. D) Intracellular FACS staining of TCRζ in JGN T cells reconstituted with CD3γ-WT, CD3γ-S126V or CD3γ-LLAA upon cotransfection of SLAP-GFP and c-Cbl. Data are representative of 6 independent transfections. E,F) Quantitation of the experiments represented in C and D. Data indicate the average percent CD3ε or TCRζ expression on transfected versus untransfected cells ± SEM. G) Representative western blot demonstrating equivalent expression of c-Cbl and SLAP-GFP in the indicated cell lines. α-tubulin was included as a loading control.


A
Human: qdgvrqsra sdkqtll pndqly
Mouse: qdgvrqsra sdkqtll qneqly
B
SLAP-GFP
SH2*-GFP
CD3γ-WT
CD3γ-S126V
CD3γ-LLAA
SLAP-GFP
C
WT
S126V
LLAA
CD3ε
GFP-
GFP+
D
WT
S126V
LLAA
TCRζ
GFP-
GFP+
E
GFP- GFP+
CD3ε (% GFP-)
WT
S126V
LLAA
F
GFP- GFP+
TCRζ (% GFP-)
WT
S126V
LLAA
G
WT
S126V
LLAA
c-Cbl
SLAP-GFP
α-tubulin

constitutively internalized, and instead remain stably expressed at the cell surface (Dietrich et al., 2002). In contrast, constitutive internalization of the TCR/CD3 complex proceeds normally in JGN T cells that have been reconstituted with either CD3γ-WT or CD3γ-S126V.

To test whether the dileucine motif in CD3γ is required for the subcellular localization of SLAP, we transfected JGN T cells expressing CD3γ-WT, CD3γ-S126V or CD3γ-LLAA with GFP-tagged SLAP (SLAP-GFP). Transfected cells were fixed, costained for the M6P-R and colocalization of SLAP-GFP and M6P-R was determined by immunofluorescence microscopy. As previously described, localization of WT SLAP-GFP was predominantly restricted to a perinuclear vesicular region that colocalized with the M6P-R in JGN T cells expressing CD3γ-WT (Figure 1B). In contrast, the SH2 mutant of SLAP (SH2*GFP) localized to punctate cytoplasmic vesicles that no longer colocalized with the M6P-R. Surprisingly, localization of SLAP-GFP in the absence of either the PKC phosphorylation site (CD3γ-S126V) or the dileucine (CD3γ-LLAA) was similar to CD3γ-WT, being primarily localized to a perinuclear vesicular region that partially colocalized with the M6P-R (Figure 1B). These data suggest that the subcellular localization of SLAP is not dependent on the CD3γ dileucine-based motif and that other targeting motif(s) present in the TCR/CD3 complex, SLAP or other unidentified protein(s) may be required.

While the dileucine-based motif of CD3γ is not required for SLAP localization, the motif may still be required for TCRζ degradation and/or downregulation of surface TCR/CD3

complexes following cotransfection of SLAP and c-Cbl. We have previously demonstrated that cotransfection of SLAP and c-Cbl together into Jurkat T cells downregulates surface CD3ε expression by targeting TCRζ for degradation by a mechanism similar to what is observed in DP thymocytes. Therefore, we tested whether these events still occur in JGN T cells expressing mutant CD3γ molecules. JGN T cells expressing CD3γ-WT, CD3γ-S126V or CD3γ-LLAA were cotransfected with SLAP-GFP and c-Cbl, and CD3ε expression on GFP positive versus GFP negative cells was determined by FACS. As previously observed, transfection of SLAP or c-Cbl alone had no effect on CD3ε expression in any of the cell lines tested (data not shown). However, cotransfection of SLAP-GFP and c-Cbl into JGN expressing WT CD3γ downregulated CD3ε expression specifically on GFP+ cells (Figure 1C-D). Notably, CD3ε expression was also downregulated on GFP+ JGN T cells that express either CD3γ-S126V or CD3γ-LLAA. CD3ε downregulation was slightly less robust for JGN T cells expressing CD3γ-S126V or CD3γ-LLAA as compared to CD3γ-WT, despite similar expression levels of SLAP-GFP and c-Cbl (Figure 1G). These data indicate that the lysosomal targeting motif in CD3γ may contribute to, but is not required for TCR/CD3 downregulation by SLAP and c-Cbl.

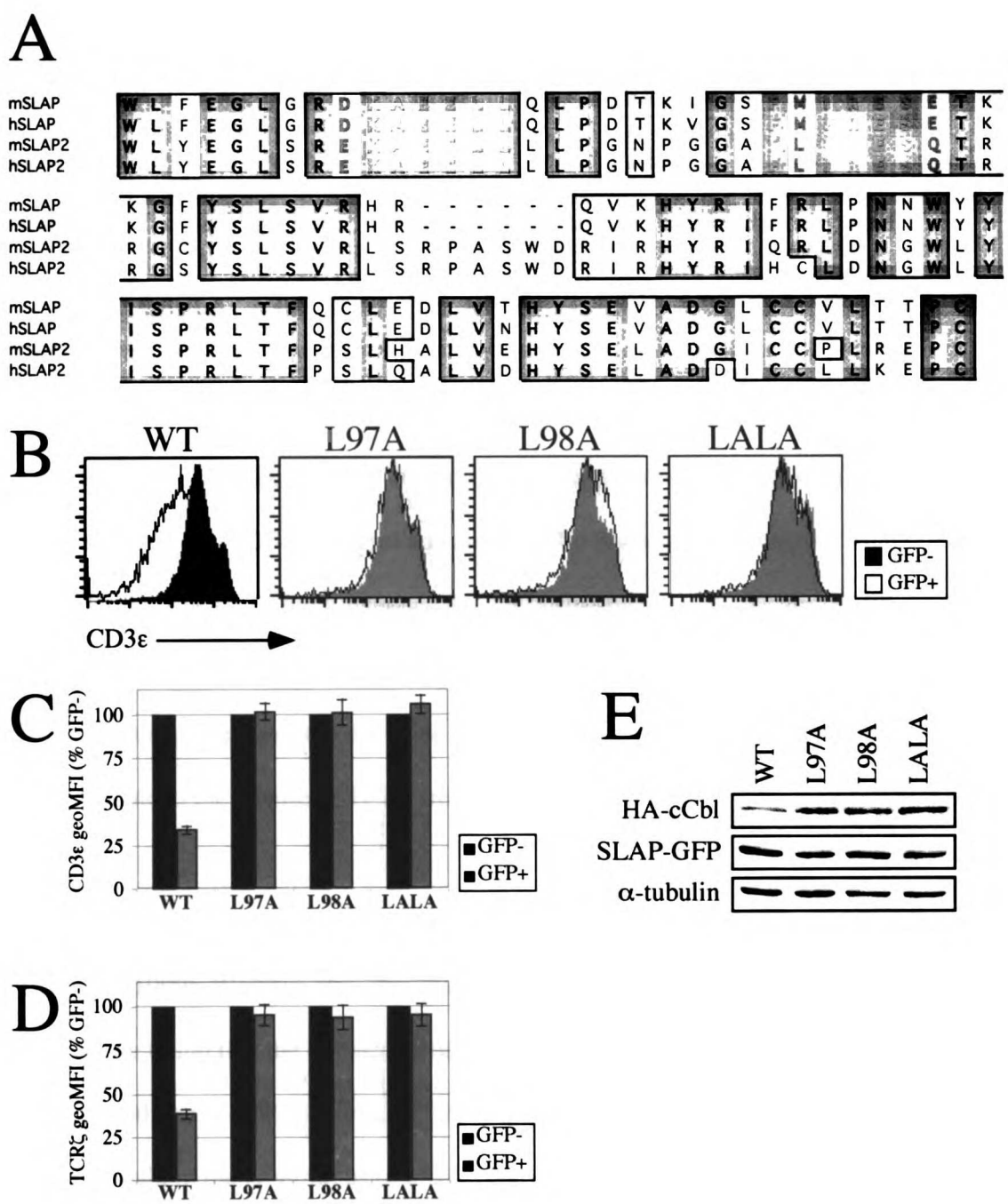
It is possible that in the absence of CD3γ-WT, SLAP and c-Cbl may prevent recycling of internalized TCR/CD3 complexes back to the cell surface independently of targeting TCRζ for degradation. Therefore, we tested whether degradation of TCRζ by SLAP and c-Cbl is dependent on the either the PKC phosphorylation site or the dileucine motif of CD3γ. JGN T cells expressing CD3γ-WT. CD3γ-S126V or CD3γ-LLAA were

cotransfected with SLAP-GFP and c-Cbl, and TCRζ expression by GFP positive versus GFP negative cells was determined by intracellular FACS staining. Neither the PKC phosphorylation site nor the dileucine motif of CD3γ were required for TCRζ degradation following cotransfection of SLAP and c-Cbl (Figure 1E-F). Unlike expression of CD3ε, degradation of TCRζ was not at all impaired in CD3γ-S126V and CD3γ-LLAA expressing T cell lines, indicating that additional motif(s) present in the TCRζ chain may contribute to the sorting of TCRζ to the lysosome.

A dileucine motif in SLAP is required for TCR/CD3 downregulation:

Upon closer examination of the primary amino acid structure of SLAP, a motif that resembles a dileucine-based sorting motif was identified within the SLAP SH2 domain (Figure 2A). This motif is 100% conserved between human and mouse SLAP and SLAP-2, suggesting that the dileucine-based motif of SLAP may be functionally important within the SLAP family. To determine whether the SLAP dileucine-based motif is required for TCR/CD3 downregulation, we cotransfected WT SLAP-GFP or GFP-tagged dileucine mutants of SLAP (L97A, L98A or L97,98A, henceforth referred to as LALA) together with c-Cbl and CD3ε expression on GFP+ versus GFP- cells was determined by FACS. Interestingly, mutation of either leucine alone or mutation of both leucines in combination completely prevented downregulation of CD3ε by SLAP and c-Cbl (Figure 2B-C). Furthermore, intracellular FACS staining of TCRζ demonstrated that the dileucine-based motif of SLAP was also required for degradation of TCRζ (Figure 2D). Failure to downregulate CD3ε and TCRζ expression was not due to reduced expression

Figure 2: A dileucine motif in SLAP is required for SLAP/c-Cbl mediated TCR/CD3 downregulation. A) Sequence alignment of both human and mouse SLAP and SLAP-2 SH2 domains. The putative dileucine (red) and the phosphotyrosine binding (green) motifs are indicated. B) Surface CD3ε staining on Jurkat T cells cotransfected with c-Cbl and the indicated SLAP-GFP constructs. Data are representative of 6 independent transfections. C,D) Quantitation of CD3ε or TCRζ expression following cotransfection of SLAP and c-Cbl. Data indicate the average percent CD3ε or TCRζ expression on transfected versus untransfected cells ± SEM. E) Representative western blot demonstrating equivalent expression of c-Cbl and SLAP-GFP in the indicated cell lines. α-tubulin was included as a loading control.

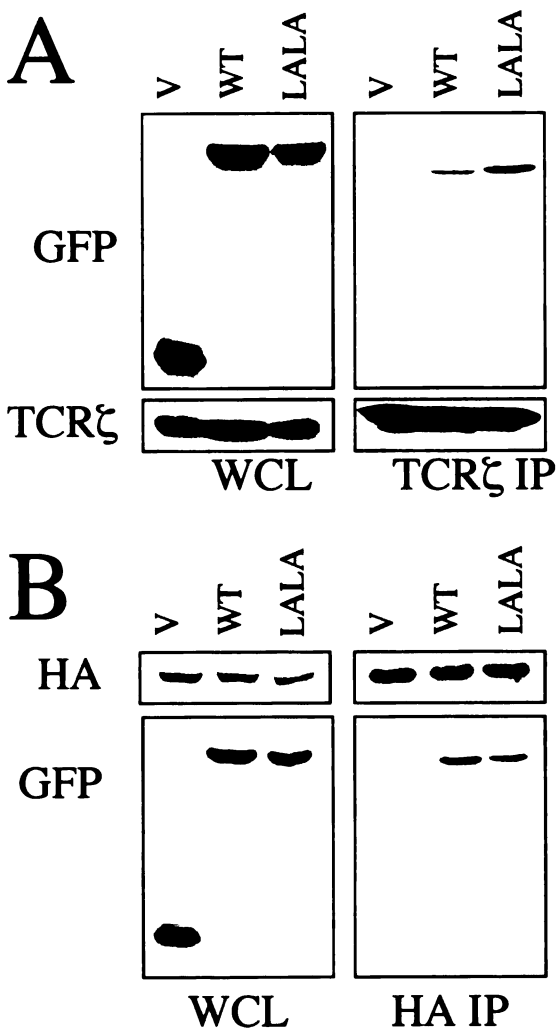

A
mSLAP
hSLAP
mSLAP2
hSLAP2
B
WT
L97A
L98A
LALA
GFP-
GFP+
CD3ε
C
CD3ε geoMFI (% GFP-)
100
75
50
25
0
WT
L97A
L98A
LALA
GFP-
GFP+
D
TCRζ geoMFI (% GFP-)
100
75
50
25
0
WT
L97A
L98A
LALA
GFP-
GFP+
E
WT
L97A
L98A
LALA
HA-cCbl
SLAP-GFP
α-tubulin

levels of SLAP-GFP or HA-tagged c-Cbl since expression of both SLAP-GFP and HA-c-Cbl was similar in all of the transfectants (Figure 2E).

The SH2 domain of SLAP is required for binding to phosphorylated TCRζ (Sosinowski et al., 2000; Tang et al., 1999) and is also required for TCR/CD3 downregulation following cotransfection with SLAP and c-Cbl. Since the dileucine-based motif is located within the SH2 domain of SLAP, it is possible that mutation of the dileucine motif disrupts the function of the SLAP SH2 domain. Therefore, we performed coimmunoprecipitations to test whether mutation of the dileucine-based motif prevented the interaction of SLAP with TCRζ. Lysates from Jurkat T cells that had been transiently transfected with GFP-tagged SLAP constructs were immunoprecipitated with anti-TCRζ antibodies, run on an SDS-PAGE gel and analyzed by Western Blot analysis. Mutation of the SLAP dileucine motif did not reduce the interaction between SLAP and TCRζ (Figure 3A). In contrast, the interaction between SLAP and TCRζ appeared to be enhanced in the presence of the dileucine mutation, suggesting that the dileucine motif may contribute to the release of TCRζ from SLAP.

SLAP and c-Cbl have previously been shown to interact via the C-terminus of SLAP (Tang et al., 1999). We have also observed an interaction between SLAP and c-Cbl. However, we found that the interaction between SLAP and c-Cbl involves regions present in both the N- and C-termini of SLAP. Since we were unable to identify a specific domain in the N-terminus of SLAP that interacts with c-Cbl, we tested whether the dileucine motif of SLAP was required. Lysates from Jurkat T cells cotransfected with

Figure 3: SLAP dileucine mutants bind TCRζ and c-Cbl. A) Western blot analysis of TCRζ immunoprecipitates from cells transfected with the indicated GFP-tagged constructs. The left panels show protein expression in whole cell lysates (WCL). The right panels show proteins present in α-TCRζ immunoprecipitates. B) Western blot analysis of HA immunoprecipitates from cells cotransfected with HA-tagged c-Cbl and the indicated GFP-tagged constructs. The left panels show protein expression in WCL. The right panels show proteins present in α-HA immunoprecipitates.


A
V
WT
LALA
V
WT
LALA
GFP
TCRζ
WCL
TCRζ IP
B
V
WT
LALA
V
WT
LALA
HA
GFP
WCL
HA IP

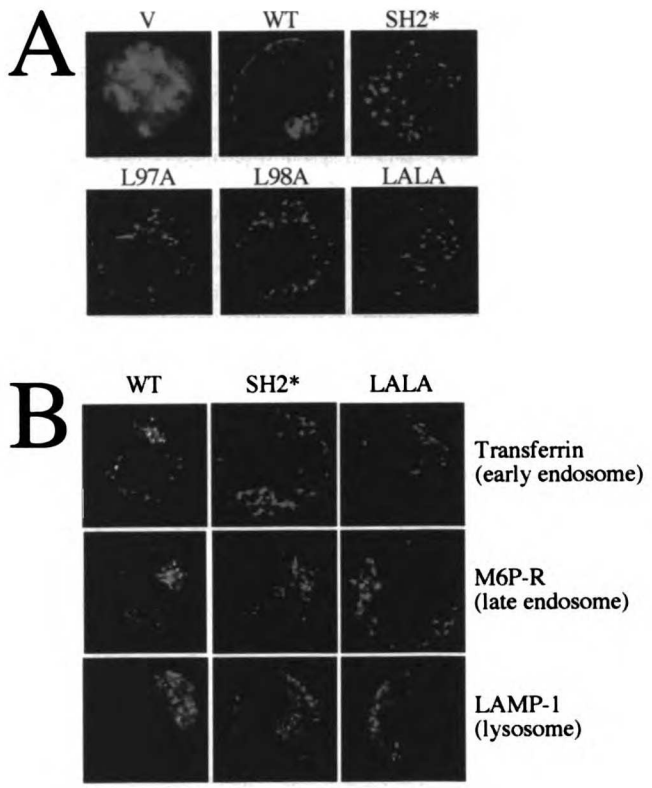
GFP-tagged SLAP constructs and HA-tagged c-Cbl, immunoprecipitated with anti-HA antibodies and immunoprecipitates were run on an SDS-PAGE gel. Western Blot analysis demonstrated that the interaction between SLAP and c-Cbl was unaffected by mutations within the dileucine-based motif of SLAP (Figure 3B).

Dileucine mutants of SLAP are mislocalized:

If the dileucine-based motif of SLAP functions as a lysosomal targeting motif, mutations within the dileucine should cause SLAP to be mislocalized. Therefore, Jurkat T cells were transiently transfected with GFP-tagged SLAP constructs and localization was determined by immunofluorescence microscopy. As previously observed, WT SLAP-GFP localized to a perinuclear vesicular compartment, whereas mutation of the SLAP SH2 domain caused a redistribution of SLAP-GFP from a perinuclear localization into punctate cytoplasmic vesicles (Figure 4A). Interestingly, mutations in the dileucine-based motif of SLAP also caused a redistribution of GFP-tagged SLAP similar to what is observed upon mutation of the SH2 domain. Furthermore, WT SLAP, but not the SH2 or dileucine point mutants of SLAP colocalized with the M6P-R (Figure 4B), demonstrating that the dileucine-based motif of SLAP is required for the subcellular localization of SLAP.

In addition to late endosomes, WT SLAP also colocalized with vesicles containing internalized transferrin, indicating that SLAP-GFP localizes to both early and late endosomes (Figure 4B). However, SLAP-GFP did not colocalize with the lysosomal resident protein, LAMP-1, suggesting that either SLAP is not targeted to lysosomes, or

Figure 4: The dileucine mutants of SLAP are mislocalized. A) Immunofluorescence microscopy of Jurkat T cells transiently transfected with the indicated GFP-tagged constructs. B) Immunofluorescence microscopy of Jurkat T cells transfected with the indicated GFP-tagged constructs and costained for early endosomes (Alexa647-transferrin), late endosomes (M6P-R) or lysosomes (LAMP-1), shown in red.


A
V
WT
SH2*
L97A
L98A
LALA
B
WT
SH2*
LALA
Transferrin
(early endosome)
M6P-R
(late endosome)
LAMP-1
(lysosome)

that SLAP present in lysosomes is rapidly degraded. In contrast, SLAP-GFP containing mutations in either the SH2 domain or the dileucine-based motif failed to colocalize either with internalized transferrin or LAMP-1. These data demonstrate that the dileucine motif in SLAP is required for targeting SLAP to both early and late endosomes. Furthermore, these data also provide strong evidence that the subcellular localization of SLAP may be important for TCR/CD3 downregulation and TCRζ degradation mediated by SLAP and c-Cbl.

Discussion:

We have identified a novel dileucine-based motif within the SH2 domain of SLAP that is required for SLAP mediated TCR/CD3 downregulation and TCRζ degradation. Despite the placement of the dileucine motif within the SLAP SH2 domain, mutation of the dileucine did not prevent SLAP from interacting with the TCRζ chain, suggesting that the phosphotyrosine binding function is unaffected by mutations in the dileucine motif. This is consistent with the dileucine motif being position near the N-terminal boundary of the SLAP SH2 domain. However, as previously observed for the SH2 mutant of SLAP, the dileucine mutants were mislocalized from a perinuclear vesicular location into punctate cytoplasmic vesicles that failed to colocalize with both early and late endosomes. Therefore, the placement of the dileucine-based motif within the SH2 domain of SLAP suggests that interactions via the SLAP SH2 domain may regulate the accessibility of the dileucine motif to protein sorting complexes.

The dileucine motif within SLAP does not conform to the previously described dileucine-based lysosomal targeting motifs in which the acidic residue is placed four amino acids upstream (-4) of the dileucine (Bonifacino and Traub, 2003). In contrast, the acidic residue in the SLAP dileucine motif is at the –5 position. However, dileucine-based motifs with the acidic amino acid residue at both -5 and -6 positions have previously been described (Bonifacino and Traub, 2003). In such cases, the residues present at both the –4 and –5 positions also appear to be involved in protein sorting and are often charged residues. Notably, the amino acid residues present at both the –4 and –6 positions of the SLAP dileucine-based motif are also charged residues (lysine and arginine, respectively),

which is in agreement with the precedent for charged residues to be located within this region.

Since the SH2 mutant of SLAP is mislocalized, we initially suspected that SLAP might bind a phosphoprotein present in late endosomes. Since SLAP interacts with TCRζ via its SH2 domain, we hypothesized that interactions with phosphorylated TCRζ could target SLAP to late endosomes via the dileucine-based motif present in CD3γ. Surprisingly, localization of SLAP-GFP was unaffected by mutation of either the CD3γ dileucine or the upstream PKC phosphorylation site, indicating that additional motif(s) must be required to target SLAP to late endosomes. In addition, downregulation of the TCR/CD3 complex still occurs despite mutations in the CD3γ chain, indicating that an intact dileucine-based motif in CD3γ is not required for SLAP/c-Cbl mediated downregulation of the TCR/CD3 complex.

We observed that downregulation of TCR/CD3 complexes containing mutant CD3γ molecules was less robust than the downregulation of complexes containing WT CD3γ chain. Previous studies have demonstrated that the CD3γ dileucine-based motif is required for constitutive internalization and recycling of the TCR/CD3 complex (Dietrich et al., 2002). In addition, transient transfection of SLAP has indicated that the majority of SLAP is present in intracellular vesicles and not at the plasma membrane (Sosinowski et al., 2000). Therefore, it is possible that the decreased TCR/CD3 downregulation observed in the absence of the dileucine-based motif is due to the failure of mutant TCR/CD3 complexes to be efficiently internalized from the plasma membrane and trafficked to

vesicles that contain SLAP. However, it is possible that the remaining downregulation that does occur is due to a small proportion of SLAP that traffics to the plasma membrane where it induces TCR/CD3 downregulation in the absence of constitutive TCR/CD3 internalization.

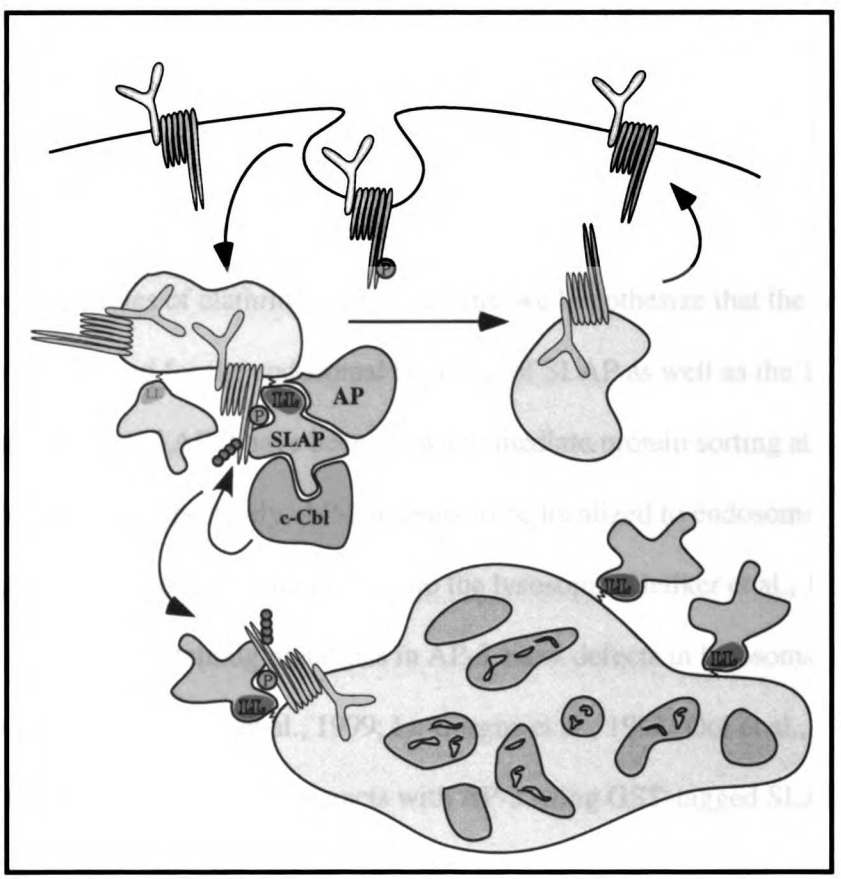
Similar to mutations in the CD3γ dileucine-based motif, TCR/CD3 downregulation following cotransfection of SLAP and c-Cbl is less robust if the serine upstream of the dileucine-based motif is mutated. While phosphorylation of this serine residue is not required for constitutive internalization of the TCR/CD3 complex (Dietrich et al., 2002), previous studies have demonstrated that phosphorylation of S126 increases the rate of TCR/CD3 internalization via the dileucine approximately ten-fold (Menne et al., 2002). In addition, TCR/CD3 complexes that are serine phosphorylated do not immediately recycle back to the plasma membrane; instead, the serine must be dephosphorylated prior to recycling (Dietrich et al., 1998). Therefore, it is possible that serine phosphorylation increases the time that the TCR/CD3 complex remains internalized, thus increasing the possibility that the TCR/CD3 complex will interact with SLAP. Alternately, TCR/CD3 complexes containing serine phosphorylated CD3γ may be preferentially sorted into vesicles that contain SLAP.

In contrast to downregulation of surface TCR/CD3 expression, degradation of TCRζ was unaffected in JGN T cells expressing either CD3γ.S126V or CD3γ.LLAA. These data suggest that while degradation of TCRζ proceeds efficiently in the absence of the CD3γ dileucine motif, retention and/or degradation of the remainder of the TCR/CD3 complex

may still at least partially require both the dileucine motif of CD3γ as well as the upstream serine residue. In contrast, JGN T cells reconstituted with WT CD3γ as well as the parental Jurkat T cell line displayed similar levels of TCR/CD3 downregulation and TCRζ degradation (compare Figures 4.1E-F, 4.2C-D). Previous studies have indicated that there may be some additional defect(s) in the parental CD3γ-/- JGN T cell line (von Essen et al., 2002), suggesting that the failure to efficiently downregulate TCR/CD3 expression in all of the JGN T cells may be due to an intrinsic defect in this mutagenized cell line.

Mutations in either the dileucine-based motif or the SH2 domain of SLAP caused SLAP to mislocalize from perinuclear cytoplasmic vesicles that colocalize with both transferrin and the M6P-R into punctate cytoplasmic vesicles that no longer colocalize with either of these molecules. This observation suggested that interactions via the SH2 domain might regulate the accessibility of the dileucine motif to clathrin adaptor proteins (Figure 5).

Figure 5: Model. TCR/CD3 complexes containing phosphorylated TCRζ are internalized and interact with SLAP via the SLAP SH2 domain. Binding to phospho-TCRζ causes the dileucine-based motif of SLAP to be exposed and bind to clathrin adaptor protein(s). Subsequently, the SLAP/TCR complex is targeted to the lysosome for degradation.


AP
SLAP
c-Cbl
ITIM
P

Therefore, we hypothesize that the dileucine motif in SLAP remains hidden and/or inaccessible until a phosphorylated binding partner, i.e. phospho-TCRζ, occupies the SH2 domain. In this model, TCR/CD3 complexes containing phosphorylated TCRζ would be internalized and transported to endosomes containing SLAP. Upon binding to phospho-TCRζ, the dileucine-based motif within the SH2 domain would be exposed and could bind to clathrin adaptor proteins. Subsequently, TCR/SLAP complexes would be recruited into clathrin-coated vesicles that target the TCR/SLAP complex to specific endosomal compartments.

We have not yet tested whether SLAP interacts with clathrin adaptor proteins. However, based on previous studies of clathrin adaptor proteins, we hypothesize that the AP-3 complex may be required for the endosomal targeting of SLAP as well as the TCR/CD3 complex. While AP-1 and AP-2 have been shown to mediate protein sorting at the golgi and plasma membrane, respectively, AP-3 appears to be localized to endosomes and be required, at least in part, for targeting proteins to the lysosome (Heilker et al., 1999). Furthermore, cell lines containing mutations in AP-3 show defects in lysosomal transport (Blumstein et al., 2001; Heilker et al., 1999; Le Borgne et al., 1998; Ooi et al., 1997). We are currently testing whether SLAP interacts with AP-3 using GST-tagged SLAP fusion proteins.

The model proposed in Figure 5 suggests that the dileucine motif within SLAP is sufficient for targeting the TCRζ chain to late endosomes for degradation. However, we have previously demonstrated that neither TCR/CD3 downregulation nor TCRζ

degradation can occur if SLAP is cotransfected with c-Cbl mutants that lack E3 ubiquitin ligase activity, indicating that ubiquitination of TCRζ is required for TCR/CD3 downregulation and TCRζ degradation. Therefore, we speculate that ubiquitination of TCRζ is not required for the initial targeting of the TCR/CD3 complexes to late endosomes. In contrast, TCRζ ubiquitination may be required for the subsequent sorting of molecules that are destined to be degraded away from molecules that are to be recycled back to the plasma membrane. The ESCRT-I complex has previously been demonstrated to mediate the sorting of ubiquitinated proteins into the degradative vesicles of late endosomes/multivesicular bodies. Furthermore, mutations in ESCRT-I cause proteins that would normally be degraded to recycle back to the plasma membrane (Babst, 2005). Therefore, SLAP appears to be poised within the endosomal system to regulate the expression of TCRζ via two separate mechanisms. First, SLAP functions as an adaptor for the E3 ubiquitin ligase c-Cbl, thereby targeting TCRζ for ubiquitination. In addition, SLAP may be required for the subsequent targeting of ubiquitinated TCRζ molecules to late endosomes. By performing these dual functions, SLAP may play a key role in targeting component(s) of the TCR/CD3 complex for lysosomal degradation.

Materials and methods:

Cell lines and plasmids: Jurkat T cells were maintained in RPMI supplemented with 10% FBS, 2 mM glutamine, penicillin and streptomycin. The CD3γ-/-Jurkat T cell derivative JGN as well as reconstituted cell lines (CD3γ-WT, CD3γ-S126V, CD3γ-LLAA) were provided by C. Geisler, and were maintained as described above (Dietrich et al., 1998; Geisler, 1992). Plasmids encoding GFP, and GFP-tagged SLAP (WT, SH2*, L97A, L98A, LALA) were constructed by PCR amplification of murine SLAP from pEF-BOS and ligation into pN1-GFP (Clontech; Palo Alto, CA) via EcoRI and BamHI sites and subcloned into pCDEF3 using EcoRI and NotI sites. HA-tagged c-Cbl was provided by H. Band and was subcloned from pAlterMAX into pCDEF3 via EcoRI and NotI sites.

Immunofluorescence microscopy: Jurkat or JGN T cells were transfected overnight with the indicated GFP-tagged constructs. For cells labeled with transferrin, transfected cells were washed and resuspended in serum free RPMI containing 50 μg/mL Alexa647-labeled transferrin (Molecular Probes; Eugene, OR) for 20 minutes at 37°C. After washing in PBS, cells were resuspended in PBS containing 1% BSA and 0.01% sodium azide and allowed to settle on poly-L-lysine coated slides for 5 minutes at room temperature. Samples were fixed in methanol and acetone (50:50) for 5 minutes at –20°C. After drying, samples were incubated in blocking buffer (PBS with 0.5% BSA, 0.5% milk, 1% normal goat serum) for 10 minutes, then in primary antibody (α-Mannose-6-Phosphate Receptor, Affinity BioReagents; Golden, CO at 1:100 or α-LAMP-1, Pharmingen; San Diego, CA at 1:20) in blocking buffer for 2 hours at 37°C. After washing for 10 minutes, samples were incubated in Alexa555-goat-α-mouse IgG

(Molecular Probes; Eugene, OR at 1:500) for 20 minutes. Samples were washed, coverslipped and visualized on a Marianas Turn-Key system (Intelligent Imaging; Denver, CO). Images were deconvolved using SlideBook software.

FACS staining: After washing in PBS, transfected cells were stained with APC-conjugated α-CD3ε (HIT3a, BD Biosciences; San Diego, CA) in FACS buffer (PBS with 1% BSA and 0.01% sodium azide) for 30 minutes at 4°C. For intracellular staining, cells were fixed in 4% paraformaldehyde for 20 minutes. Cells were washed, resuspended in permeabilization buffer (FACS buffer containing 0.5% saponin and 1% normal mouse serum) containing PE-conjugated α-TCRζ (6B10.2, Santa Cruz Biotechnology; Santa Cruz, CA at 1:50) for 1 hour. Samples were washed three times in FACS buffer prior to FACS analysis.

Immunoprecipitations and Western Blot Analysis: Jurkat or JGN T cells were transfected overnight with the indicated constructs. Cells were washed in PBS and lysed at 100 x 10^6/mL in RIPA lysis buffer supplemented with protease inhibitors (leupeptin, aprotinin, PMSF, pepstatin A) for 30 minutes on ice. Postnuclear supernatants were prepared by centrifuging samples (16,000 x g) for 30 minutes at 4°C. Postnuclear supernatants were mixed with SDS loading buffer and boiled for 5 minutes. Samples were electrophoresed in a 10% SDS-PAGE gel, transferred to Immobilon and blotted for HA (12CA5, Boehringer Mannheim; Indianapolis, IN), GFP (JL8, Clontech; Palo Alto, CA) and α-tubulin (B-5-1-2, Sigma; St. Louis, MO). Membranes were then incubated in

secondary antibodies coupled to horseradish peroxidase and detected using enhanced chemiluminescence (Amersham Biosciences; Uppsla, Sweden).

For coimmunoprecipitation experiments, Jurkat T cells transfected for 4.5 hours with the indicated constructs. Cells were washed in PBS and lysed at 50 $x10^6$/mL in RIPA lysis buffer and post nuclear supernatants were prepared as described above. Postnuclear supernatants were immunoprecipitated with either α-TCRζ (6B10.2) or α-HA (12CA5) and protein G (Amersham Biosciences; Uppsla, Sweden) for 30 minutes at 4°C. Immunoprecipitates were washed four times with lysis buffer, resuspended in SDS loading buffer and boiled for 5 minutes. Immunoprecipitates were electrophoresed, transferred and western blotted for GFP (JL8), TCRζ (8D3, Pharmingen; San Diego, CA) and HA (12CA5) as described above.

Chapter 5:

Implications and Future Directions

The studies presented here have provided a greater understanding of the role of Src-Like Adaptor Protein (SLAP) in regulating surface expression of the TCR/CD3 complex on DP thymocytes. In addition, we have demonstrated that SLAP and c-Cbl function together in a common pathway that ensures that developing thymocytes receive the proper developmental signals through the TCR/CD3 complex. By binding to TCRζ and c-Cbl, SLAP recruits the E3 ligase activity of c-Cbl to fully assembled TCR/CD3 complexes. Once recruited, c-Cbl ubiquitinates the TCRζ chain, targeting TCRζ for degradation. Degradation of TCRζ prevents fully assembled TCR/CD3 complexes from accumulating in the recycling pool, thus preventing TCR/CD3 re-expression at the cell surface due to recycling of the TCR/CD3 complex. While many of the initial steps in SLAP/c-Cbl mediated TCR/CD3 downregulation have been identified, a number of questions regarding the mechanism and function of TCR/CD3 downregulation in the thymus have yet to be addressed.

Structure/Function Analysis of SLAP and c-Cbl:

Mutational analysis of SLAP has demonstrated that TCR/CD3 downregulation requires multiple domains of SLAP. We and others have shown that the SH2 domain of SLAP is required to bind to phosphorylated TCRζ chains (Sosinowski et al., 2000; Tang et al., 1999). Interestingly, mice expressing a truncated TCRζ chain (which lacks the majority of the TCRζ cytoplasmic domain) display increased surface TCR/CD3 expression as well as an increased pool of recycling TCR/CD3 complexes in DP, but not SP thymocytes similar to what is observed in the absence of SLAP and/or c-Cbl. Furthermore, the TCRζ cytoplasmic domain is sufficient to mediate TCR/CD3 downregulation, since a CD8:ζ

chimera expressed in the absence of the remaining TCR/CD3 complex is downregulated following cotransfection of SLAP and c-Cbl. Therefore, these data support the model that the interaction between the SH2 domain of SLAP and the cytoplasmic domain of TCRζ is required for TCR/CD3 downregulation by SLAP and c-Cbl.

In addition to the SH2 domain, the myristolation site of SLAP is required to downregulate TCR/CD3 expression. Previous data, as well as our unpublished results have demonstrated that mutation of the myristolation site leads to cytoplasmic localization of SLAP (Manes et al., 2000). In contrast, WT SLAP localizes to a cluster of perinuclear vesicles which partially colocalize with both early and late endosomes (Manes et al., 2000; Sosinowski et al., 2000). GFP-tagged SLAP also colocalizes with TCRζ in this perinuclear compartment. Interestingly, mutation of the SLAP SH2 domain causes a redistribution of SLAP out of this perinuclear region into punctate cytoplasmic vesicles that no longer colocalize with either the early endosomal or the late endosomal markers used in this study. The identity of these punctate cytoplasmic vesicles is not clear. However, our studies suggest that targeting of SLAP to membranes, especially to a perinuclear vesicular compartment is required for SLAP function.

We have demonstrated that the C-terminus of SLAP is required to regulate TCR/CD3 expression. Previous studies have suggested that the C-terminus of SLAP is required to bind c-Cbl (Tang et al., 1999). We observed that the interaction between SLAP and c-Cbl is decreased, but not absent in the absence of the SLAP C-terminus. Surprisingly, the interaction is completely undetectable if the SLAP N-terminus is truncated. We have

been unable to identify a specific domain in the N-terminus of SLAP that is required for its interaction with c-Cbl. However, our studies indicate that the interaction between SLAP and c-Cbl is more complex that previously appreciated and may involve multiple sites of interaction and/or additional binding partners. Notably, the highly homologous Src kinase, Lck has been shown to interact with c-Cbl via both its SH3 and SH2 domains. Therefore, it is possible that SLAP interacts with c-Cbl via regions present both in the SH3 and/or SH2 domains as well as the C-terminus of SLAP. Regardless, it is unclear why cotransfection of SLAP and c-Cbl fails to downregulate TCR/CD3 expression in the absence of the SLAP C-terminus if the interaction between SLAP and c-Cbl is preserved. One possibility is that the C-terminus of SLAP may have unique binding partners that have yet to be identified. However, it is also possible that while the SH3 and/or SH2 domains of SLAP are capable of binding to c-Cbl, this interaction either prevents the additional interaction with TCRζ or that conformational constraints do not allow for ubiquitination of TCRζ to occur.

Within c-Cbl, only the highly conserved N-terminus containing the TKB and RF domains is required for TCR/CD3 downregulation and TCRζ chain degradation following cotransfection with SLAP. Moreover, the RF, but not the TKB domain is essential for the function of c-Cbl; mutation of the RF domain either by a partial deletion or by multiple point mutations within the RF completely inhibit TCR/CD3 downregulation following cotransfection with SLAP and c-Cbl. In contrast, only the TKB domain is required for the interaction between SLAP and c-Cbl (Tang et al., 1999). Interestingly, mutation of the partial SH2 domain of c-Cbl has no effect on the interaction between SLAP and c-Cbl,

indicating that region(s) present in either the 4H bundle and/or the EF hand of c-Cbl may participate in the interaction with SLAP. Additional studies will be required to fully characterize the interaction between SLAP and c-Cbl.

We have demonstrated that c-Cbl is able to mediate ubiquitination of the TCRζ chain, but only if SLAP is present, demonstrating that SLAP functions as an adaptor by linking the TCRζ chain to the E3 ligase activity of c-Cbl. Overexpression studies have indicated that ZAP-70 can directly interact with c-Cbl and, thus, might also function as an adaptor for c-Cbl to mediate TCR/CD3 downregulation (Wang et al., 2001). However, TCR/CD3 expression as well as the size of the CD3ε recycling pool is normal in ZAP-70 deficient thymocytes, indicating that ZAP-70 does not function to couple the TCR/CD3 complex to c-Cbl *in vivo*. In contrast, thymocytes lacking SLAP and/or c-Cbl have a profound alterations in TCR/CD3 expression, the size of the CD3ε recycling pool and TCRζ degradation, suggesting that SLAP and c-Cbl mediate TCRζ chain ubiquitination and degradation even when expressed at physiologic levels.

Multiple SLAP and Cbl Family Members:

The ability of SLAP to regulate TCR/CD3 expression on thymocytes and Jurkat T requires the E3 ligase activity of c-Cbl. Surprisingly, even the closely related Cbl-b fails to downregulate TCR/CD3 expression when cotransfected with SLAP despite the high degree of similarity between c-Cbl and Cbl-b, especially in the TKB and RF domains. These data suggest that the N-termini of Cbl family members may have a greater effect on substrate specificity than previously appreciated. However, it remains to be

determined whether SLAP can interact with Cbl-b. No alterations in steady state TCR/CD3 expression have been observed in the absence of Cbl-b (Bachmaier et al., 2000; Chiang et al., 2000). However, it is possible that SLAP, together with Cbl-b targets molecules not included in the TCR/CD3 complex for ubiquitination and degradation. In particular, Cbl-b has been shown to have E3 ligase activity for a number of signaling molecules downstream of the TCR/CD3 complex including Vav and the p85 subunit of PI-3 kinase (Fang and Liu, 2001; Fang et al., 2001; Miura-Shimura et al., 2003). In addition, c-Cbl has also been shown to target a variety of intracellular signaling molecules for ubiquitination (Ota et al., 2000; Rao et al., 2001; Rao et al., 2002b). Further studies will be required to determine whether SLAP, together with either c-Cbl or Cbl-b functions to regulate the expression of signaling molecules not present in the TCR/CD3 complex.

Both c-Cbl and Cbl-b are required for ligand-induced TCR/CD3 downregulation in peripheral T cells (Naramura et al., 2002). It is unclear how c-Cbl and Cbl-b downregulate TCR/CD3 expression in the periphery. Previous studies have indicated that SLAP is not expressed in resting T cells (Sosinowski et al., 2001). Therefore, it is unlikely that SLAP can adapt c-Cbl or Cbl-b to the activated TCR/CD3 complex in these short-term assays. However, it is possible that the SLAP family member, SLAP-2, which has been shown to be expressed in resting T cells could function as an adaptor for c-Cbl and/or Cbl-b in peripheral T cells (L.Dragone and A. Weiss, manuscript in preparation). SLAP-2 has previously been shown to interact with c-Cbl and may facilitate ligand-induced downregulation of the TCR/CD3 complex in Jurkat T cells (Loreto et al., 2002).

However, the function of SLAP-2 in thymocytes and peripheral T cells has yet to be determined. Ongoing studies in the lab are currently addressing a possible function for SLAP-2 during T cell development and in peripheral T cell function.

Multiple Functions for SLAP and/or c-Cbl:

In addition to increased TCR/CD3 expression, levels of CD5and CD69 and are also increased in the absence of SLAP and/or c-Cbl. SLAP and/or c-Cbl deficient thymocytes also display increases in positive selection. Together, these data indicate that TCR signaling is increased by the absence of SLAP or c-Cbl (Naramura et al., 1998; Sosinowski et al., 2001). Transient transfection of either SLAP or c-Cbl into Jurkat T cells can inhibit TCR signaling without altering expression of the TCR/CD3 complex (Rao et al., 2002a; Sosinowski et al., 2000). Therefore, SLAP and c-Cbl may have effects on TCR signaling that are distinct from its effects on TCR expression levels. Overexpression studies in cell lines have demonstrated that c-Cbl is a negative regulator of TCR signal transduction by regulating the expression of both Lck and ZAP-70 (Duan et al., 2004; Rao et al., 2002a). Importantly, these studies are consistent with the increased levels of Lck as well as increased tyrosine phosphorylation that is observed in c-Cbl deficient thymocytes, demonstrating that c-Cbl may inhibit signaling even when expressed at physiologic levels (Thien et al., 1999).

The increased TCR/CD3 expression likely contributes to the increased TCR signal transduction that is observed in *c-Cbl*$^{-/-}$ thymocytes. However, expression of Lck is also increased in the absence of c-Cbl. In contrast, despite the evidence that c-Cbl regulates

ZAP-70 expression in cell lines, thymocytes and peripheral T cells express normal levels of ZAP-70, suggesting that *in vivo*, Lck is more sensitive to regulation by c-Cbl. In WT mice, a small pool of the TCRζ chain is constitutively tyrosine phosphorylated and bound to ZAP-70 in DP thymocytes and LN T cells (Nakayama et al., 1989; Stefanova et al., 2002). However, ZAP-70 is not detectably phosphorylated, even when bound to TCRζ (van Oers et al., 1994). Overexpression studies in cell lines have demonstrated that c-Cbl regulates ZAP-70 via the reciprocal interaction between the TKB domain of c-Cbl and the negative regulatory tyrosine of ZAP-70 (Y292) when phosphorylated (Duan et al., 2004; Rao et al., 2002a). Therefore, since ZAP-70 is not phosphorylated, this may account for why c-Cbl is unable to affect expression of ZAP-70 in thymocytes and resting T cells. In contrast to ZAP-70, the interaction of c-Cbl with Lck is more complex and appears to occur independently of the c-Cbl TKB domain (Duan et al., 2004; Rao et al., 2002a). Notably, mice expressing mutations either in the TKB domain of c-Cbl (G304E) or in the analogous negative regulatory tyrosine residue of ZAP-70 (Y292F) express normal levels of both ZAP-70 and Lck in the thymus (Magnan et al., 2001; Thien et al., 2003). In addition, activation of ZAP-70 is not altered if the interaction between c-Cbl and ZAP-70 is disrupted. Therefore, the increased activity of ZAP-70 in *c-Cbl*$^{-/-}$ thymocytes is likely to be a secondary effect due to increased expression of Lck.

In contrast to *c-Cbl*$^{-/-}$ thymocytes, no increases in tyrosine phosphorylation have been observed in thymocytes deficient in SLAP (T. Sosinowski and A. Weiss, unpublished). Therefore, it is possible that the inhibition of TCR signaling by SLAP that is observed in Jurkat T cells is due to the use of an overexpression system. This hypothesis is supported

by studies in Jurkat T cells stably expressing a SLAP-GFP fusion protein. We have been unable to detect a change in either the kinetics or the overall α-CD3 induced tyrosine phosphorylation in the presence of SLAP-GFP as compared to a control cell line (data not shown). Expression of SLAP in stable transfectants is much higher than in DP thymocytes; however, SLAP has no detectable effect on α-CD3 induced tyrosine phosphorylation. Therefore, although SLAP is capable of functioning as a dominant negative when expressed at superphysiological levels, it is unlikely that this occurs *in vivo*. However, we cannot exclude the possibility that SLAP inhibits TCR signaling downstream of tyrosine phosphorylation by a mechanism that has yet to be described.

Lysosomal Targeting of the TCR/CD3 Complex:

We have demonstrated that both SLAP and c-Cbl are required for the ubiquitination and subsequent degradation of the TCRζ chain. Previous studies have indicated that degradation of TCRζ occurs in the lysosome (D'Oro et al., 1997; Liu et al., 2000; Valitutti et al., 1997; von Essen et al., 2004). However, the mechanism by which TCRζ is targeted for lysosomal degradation remains unclear. Previous studies have identified a dileucine-based motif present in CD3γ that mediates the constitutive internalization of the TCR/CD3 complex (Dietrich et al., 1994; Dietrich et al., 2002). In addition to targeting transmembrane proteins for internalization, dileucine-based motifs have also been shown to regulate lysosomal targeting (Bonifacino and Traub, 2003). Therefore, it is possible that the dileucine motif present in CD3γ can function as a lysosomal targeting motif for the TCR/CD3 complex. However, we have shown that the TCRζ cytoplasmic domain is both necessary and sufficient for SLAP/c-Cbl mediated TCR/CD3 downregulation and

TCRζ degradation, indicating that the dileucine motif in CD3γ is not required to target the TCR/CD3 complex for lysosomal degradation. Therefore, additional targeting motif(s) must be required.

Due to the similarity of tyrosine-based signaling motifs with tyrosine-based lysosomal targeting motifs, it is possible that signaling motif(s) present the CD3γδε and TCRζ cytoplasmic domains could function to target the TCR/CD3 complex to lysosomes. Alternatively, it is possible that SLAP itself targets the TCR/CD3 complex to the lysosome. SLAP contains a dileucine-based motif within its SH2 domain that is required for TCR/CD3 downregulation and TCRζ degradation. Interestingly, mutation of the dileucine motif causes the mislocalization of SLAP from perinuclear localization into punctate cytoplasmic vesicles similar to the mislocalization observed upon mutation of the SH2 domain. However, failure to downregulate the TCR/CD3 complex in the absence of the dileucine motif is not a consequence of disrupting the SH2 domain, since mutation of both leucines has no negative effect on the ability of SLAP to bind TCRζ. In contrast, the interaction between SLAP and TCRζ is appreciably increased by the dileucine mutation, suggesting that the dileucine motif may contribute to the release of TCRζ upon delivery to the lysosome.

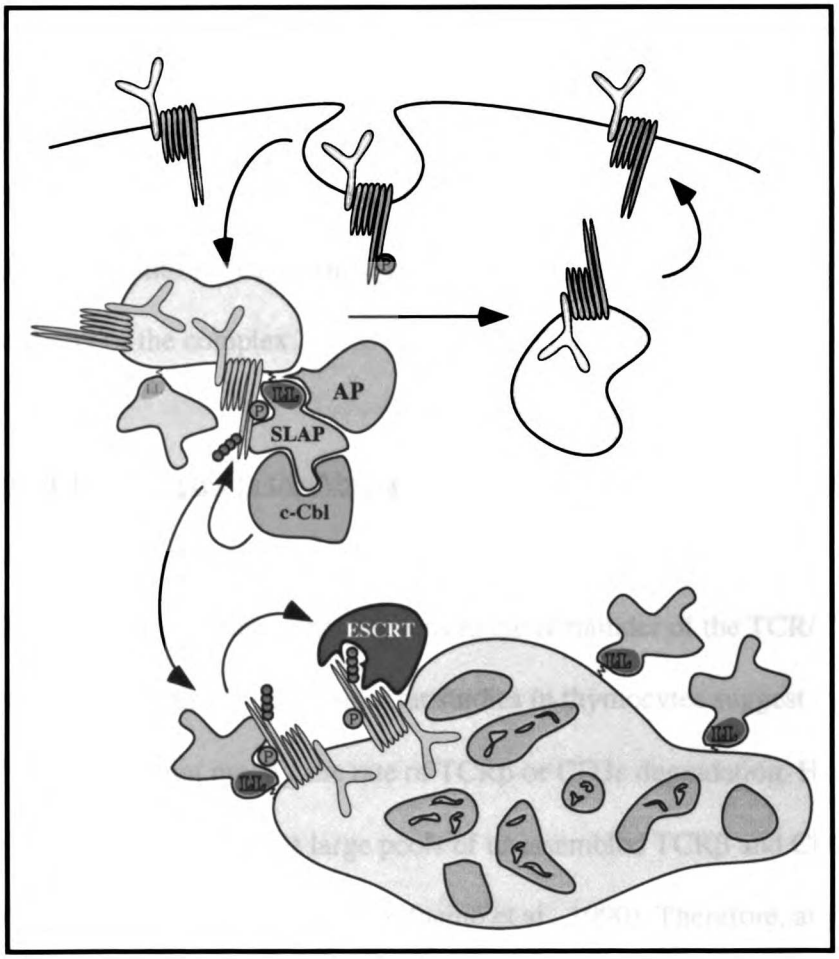
The initial discovery that the SH2 domain of SLAP is required for perinuclear localization suggested that SLAP localization is dependent on a phosphoprotein present in late endosomes. However, as mutation of the dileucine motif of SLAP results in a similar mislocalization as mutation of the SH2 domain, we hypothesized that binding of

phosphorylated TCRζ to the SH2 domain of SLAP may induce a conformational change of the SH2 domain that regulates the accessibility of the dileucine-based motif (Figure 1). Once exposed, the dileucine-based motif could be recognized by clathrin adaptor proteins that would sort SLAP/TCR complexes to late endosomes and/or lysosomes. The failure of the SLAP dileucine mutant to downregulate TCR/CD3 expression would therefore result from TCR/CD3 complexes recycling back to the plasma membrane instead of being targeted to the lysosome for degradation. The function of the SLAP dileucine-based motif in TCR/CD3 downregulation is currently under investigation (M. Myers and A. Weiss, manuscript in preparation).

Role of TCRζ ubiquitination:

Studies in yeast and mammalian cell lines have demonstrated that ubiquitin itself can function as a lysosomal targeting motif for a variety of transmembrane proteins (Bonifacino and Weissman, 1998; Haglund et al., 2003; Hicke, 2001; Pelham, 2004; Umebayashi, 2003). The model presented in Figure 1 suggests that the dileucine-based motif in SLAP is sufficient for lysosomal targeting of the TCR/CD3 complex. Why, then, is TCRζ ubiquitinated? Previous data has shown that endocytosis of certain transmembrane receptors is dependent on ubiquitination (Bonifacino and Weissman, 1998; Haglund et al., 2003). Therefore, it is possible that ubiquitination of TCRζ may facilitate the endocytosis of the TCR/CD3 complex. However, the TCR/CD3 complex is continually endocytosed and recycled even in the absence of stimulation (i.e. without ubiquitination). Therefore, it is unlikely that TCRζ ubiquitination is required during endocytosis of the TCR/CD3 complex. An intriguing possibility is that ubiquitination

Figure 1: Proposed mechanism by which the SLAP targets the TCR/CD3 complex to lysosomes. Upon binding of SLAP to phosphorylated TCRζ chains, the dileucine motif within the SH2 domain of SLAP is exposed, thus enabling the motif to interact with clathrin adaptor protein (AP) complexes. The AP complexes recruit clathrin, and allow for SLAP/TCR complexes to be targeted to the lysosome for degradation. In contrast, SLAP molecules that are not bound to TCRζ (SH2*, e.g.) or do not display the dileucine motif (LALA, e.g.) cannot be targeted to the lysosome. Once bound to TCRζ, SLAP recruits c-Cbl, leading to ubiquitination of the TCRζ chain. Ubiquitinated TCR/CD3 molecules are bound by the ESCRT-I complex, which sorts ubiquitinated proteins into the inner vesicles of multivesicular bodies where the TCR/CD3 complex is degraded.


AP
SLAP
c-Cbl
ESCRT

may be required for sorting of TCRζ into multivesicular bodies (MVBs). The MVB is formed by the invagination of the outer (limiting) membrane of the late endosome (Figure 1), thus forming internal vesicles that are subject to lysosomal proteolytic enzymes upon maturation of the late endosome and/or fusion with the lysosome. Previous data has demonstrated that the Endosomal Sorting Complex Required for Transport (ESCRT-I) is required for sorting of ubiquitinated receptors into the interior of budding MVBs (Babst, 2005; Bonifacino and Traub, 2003). In the absence of ubiquitination, receptors are not sorted into the MVB and are instead recycled back to the plasma membrane. Therefore, ubiquitination of the TCRζ chain may be required for sorting of the TCR/CD3 complex into the degradative compartment of late endosomes, thereby preventing recycling of the complex.

Fate of the TCR/CD3 Complex in the Absence of TCRζ:

Our studies clearly demonstrate that SLAP and c-Cbl together target TCRζ for degradation. However, it is unclear what happens to the remainder of the TCR/CD3 complex in the absence of the TCRζ chain. Our studies in thymocytes suggest that expression of SLAP does not modify the rate of TCRβ or CD3ε degradation. However, these data are difficult to interpret, as large pools of unassembled TCRβ and CD3ε chains are present in the ER of DP thymocytes (Bonifacino et al., 1990). Therefore, any differences in degradation of fully assembled TCRβ and CD3ε chains may not be detectable. Additional studies will be required to determine if the remainder of the TCR/CD3 complex is degraded by a SLAP-dependent mechanism. Notably, cotransfection of SLAP and c-Cbl into Jurkat T cells not only downregulates surface

expression of the TCR/CD3 complex, but total levels of both TCRαβ and CD3ε as well (data not shown). In contrast to DP thymocytes, TCR/CD3 assembly proceeds relatively efficiently in Jurkat T cells; consequently, the amount of unassembled TCR/CD3 chains is much lower in Jurkat T cells than in DP thymocytes. Therefore, it is possible that degradation of TCRβ and CD3ε by SLAP and c-Cbl has been unmasked in Jurkat T cells by the relative absence of unassembled receptor subunits.

Alternatively, the differences in TCR/CD3 degradation may be due to a fundamental difference between DP thymocytes and a more mature T cells. Separation of the TCR/CD3 complex has previously been observed after ligation of the TCR (Kishimoto et al., 1995; La Gruta et al., 2004). Furthermore, differential regulation of TCRβ and CD3ε has been observed on SP thymocytes and peripheral T cells expressing the TKB mutation of c-Cbl (G304E), indicating that expression of fully assembled TCR/CD3 complexes may be more complex than has previously been appreciated (Thien et al., 2003). Only fully assembled TCR/CD3 complexes are believed to be capable of leaving the ER prior to expression on the cell surface. However, previous studies have demonstrated that low levels of the CD3 chains are still expressed at the cell surface in the absence of TCRζ (Ono et al., 1995). These data suggest that the requirement for fully assembled complexes may not be absolute. Expression of CD3 in the absence of TCRζ is presumed to be due to escape of partially assembled complexes from the ER. In contrast, it is possible that non-TCR/CD3 subunits may substitute for TCRζ and allow for the expression of nontraditional TCR/CD3 complexes at the cell surface. In particular, it has previously been shown that a molecule homologous to TCRζ, FcεRI, can substitute for the absence

of TCRζ and restore expression of the TCR/CD3 complex (Rodewald et al., 1991). Notably, FcεRI has previously been shown to be expressed in T cells (Orloff et al., 1990). Therefore, it is possible that FcεRI could compensate for the loss of TCRζ if separated from the remainder of the TCR/CD3 complex. In addition, mRNA of another TCRζ homologue, DAP-12, has been shown to be upregulated in positively selected thymocytes (Mick et al., 2004). Future studies will be required to determine whether either FcεRI, DAP-12 or another unidentified protein can substitute for the absence of TCRζ.

SLAP Expression and Function in Peripheral Lymphocytes:

One of the most intriguing questions regarding SLAP function is whether the increased TCR/CD3 expression during thymocyte development affects peripheral T cell function in *SLAP*$^{-/-}$ mice. Analysis of peripheral lymphoid organs in *SLAP*$^{-/-}$ mice has not revealed an obvious defect in the peripheral lymphoid compartment (T. Sosinowski and A. Weiss, unpublished). *SLAP*$^{-/-}$ T cells appear to be present in normal numbers in the spleen and lymph node. Furthermore, no differences were observed in TCR/CD3, CD4, CD5 and CD69 expression on *SLAP*$^{-/-}$ peripheral T cells. As SLAP protein expression appears to be restricted predominantly to the thymus, SLAP may not regulate receptor expression in the periphery. However, the function of *SLAP*$^{-/-}$ T cells has yet to be tested. The current view of thymocyte development proposes that only thymocytes expressing TCRs with intermediate avidity for MHC will survive both positive and negative selection and exit into the peripheral lymphoid compartment (Love and Chan, 2003; Sebzda et al., 1999). Thymocytes expressing TCRs with low avidity do not receive positively selecting signals through the TCR and therefore die by neglect. In contrast, thymocytes expressing high

avidity TCRs generate signals through the TCR that induce deletion via apoptosis. TCR avidity depends not only on the intrinsic affinity of the TCR for MHC, but also depends on the number of TCRs that interact with peptide/MHC molecules. Therefore, increased TCR/CD3 expression on SLAP deficient thymocytes should increase the overall avidity of the TCR for MHC and therefore select for thymocytes with lower affinity for self-peptide/MHC complexes (Figure 2). However, the effect of SLAP on TCR affinity for self-peptides has not been tested.

In addition to its role in the thymus, SLAP may also function in peripheral immune cells as well. It has previously been shown that SLAP protein expression is upregulated by lymph node T cells after several days of stimulation (Sosinowski et al., 2001). Therefore, SLAP may function in T cells during the generation and/or termination of an immune response. Moreover, recent work has indicated that SLAP is expressed during B cell development and in peripheral B cells (L. Dragone and A. Weiss. manuscript in preparation). Therefore, SLAP may also regulate B cell development and function. Ongoing studies in the lab are directed at studying the effect of SLAP deficiency on peripheral lymphoid function as well as during B cell development.

Conclusions:

We have provided a mechanistic description of how SLAP, together with c-Cbl regulates TCR/CD3 expression on DP thymocytes by targeting TCRζ chains present in fully assembled TCR/CD3 complexes for degradation. Despite the significant advances that have been made in this field, many interesting questions still remain. The identification of

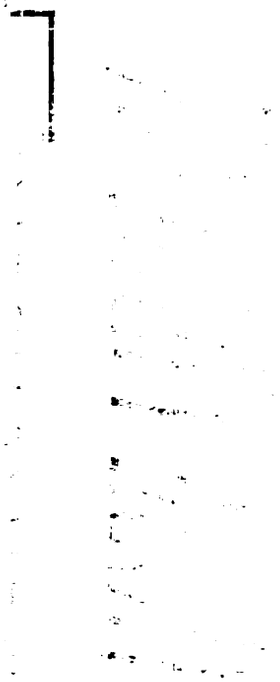
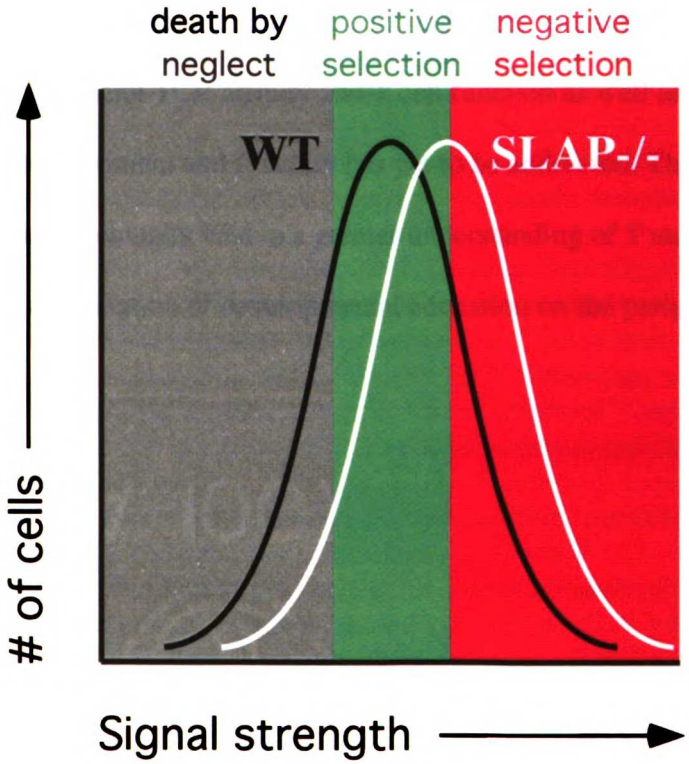


Figure 2: Proposed mechanism by which SLAP regulates thymocyte development. In the absence of SLAP, increased TCR/CD3 expression increases signals received through the TCR. Consequently, thymocytes with low affinity TCRs that would normally fail positive selection instead develop into mature T cells. In contrast, thymocytes with intermediate affinity TCRs fail negative selection and are deleted from the thymus. Consequently, SLAP-/- peripheral T cells may interact only weakly with peptide/MHC, and may be ineffective during an immune response.


death by neglect
positive selection
negative selection
WT
SLAP-/-
# of cells
Signal strength

additional SLAP targets and effector proteins may further to our understanding of the mechanism by which SLAP regulates TCR/CD3 expression. In particular, studies directed at the dileucine-based motif of SLAP as well as the possible involvement of the ESCRT-I complex in SLAP/c-Cbl mediated TCR/CD3 downregulation may provide further insight into the how TCRζ (and possibly the remainder of the TCR/CD3 complex) is targeted to the lysosome for degradation. A comprehensive description of SLAP function may also provide greater insight into the general mechanism(s) required for internalization, recycling and degradation of antigen receptors. Finally, the outcome of SLAP deficiency on peripheral TCR affinity and T cell function as well as the role of SLAP during B cell development and function has yet to be addressed. Therefore, future studies in this field may eventually lead to a greater understanding of T and B cell development and the contribution of developmental education on the peripheral adaptive immune system.

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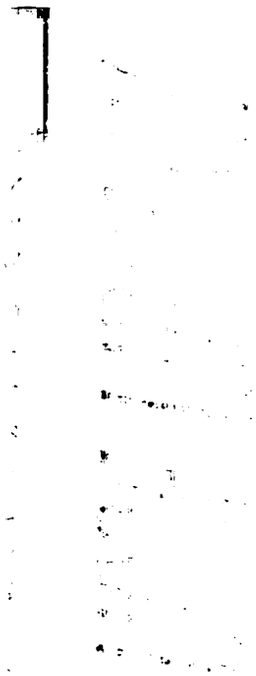

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