Title
The role of LAT and CD148 in T cell signaling

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The Role of LAT and CD148 in T Cell Signaling

by

Joseph Lin

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in

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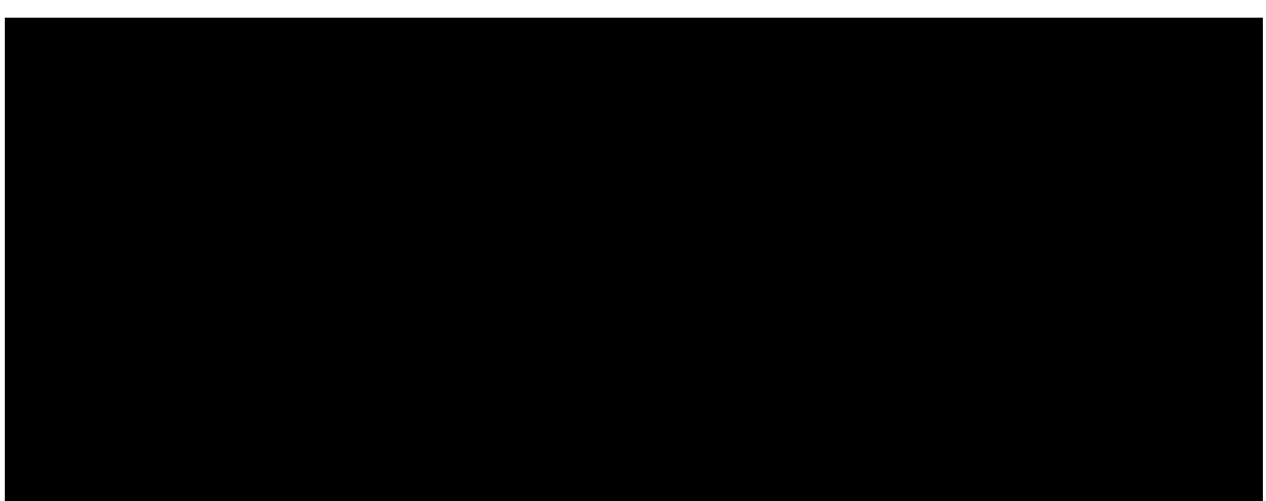
UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



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Preface

Many people deserve a great deal of thanks for helping me get to where I am today, which is sitting here writing the preface to my thesis. First of all, the MUNI bus driver..., just kidding. I should start off by first thanking my family, especially my parents, for their continued encouragement and support throughout the many years of schooling to get me to this point. I'd also like to thank my siblings for the many motivational words such as, "Wow, you've been in school a long time", "You only get paid how much?", and "How much longer until you're done?"

Of course I want to thank Art for everything he has done for me. I really appreciate all the effort he has put into helping me grow as a scientist. He has mentored me with the perfect balance of direct guidance, yet still allowing me to struggle with my own crazy ideas. I have truly valued the advice and freedom he has given me scientifically. Other members of my thesis committee, Lewis Lanier and Nigel Killeen also deserve thanks for giving me support and always having their doors open.

The entire Weiss lab, past and present, has been the single most enjoyable part of graduate school in the past five years. They have provided me with a plethora of scientific knowledge, suggestions, and ideas as well as endless amounts of entertainment. My roomies in U308 deserve special acknowledgement for the many stimulating conversations about relationships and other topics such as, "Is fire alive?" I especially want to thank Mike and Larry for all their scientific help and advice throughout the years. Thanks also goes to Michelle H. for teaching important mouse handling techniques such as probing mice for plugs at 8:00am and getting mice to pee on a stick. Of course, I want

to thank Tomek who taught me many things, both science and non-science (fishing). Finally, many thanks to Marianne who was like a mom away from home. Thanks for looking out for me and getting me all those cookies. I will miss everyone tremendously.

Weiss Lab Members During My Tenure

Art Weiss, Jeanne Baker, Tomas Brdicka*, Jeff Critchfield, Lenny Dragone, Tim Finco*, Mary Anne Ford, Stan Grell, Vikas Gupta, Michelle Hermiston, Terri Kadlecek*, Larry Kane, Greg Ku*, Michelle Kuhne, Susan Levin*, Jen Liou*, Kristen Lynch, Gloria Machado, Ravi Majeti, Marianne Mollenauer, Margo Myers, Ajay Nirula, Emil Palacios, Hyewon Phee*, Jeroen Roose, Al Roque, Ginny Shapiro, Tomek Sosinowski, Allison Tan, Mike Tomlinson*, Carmen White, Zheng Xu, Debbie Yablonski*, and Jing Zhu.

*Members of team U308

Work in this thesis was carried out under the guidance and in the laboratory of Dr. Arthur Weiss. Chapter 1 is adapted from the following:

Lin, J., and Weiss, A. (2001). T cell receptor signalling. J Cell Sci *114*, 243-244.

Data presented in Chapter 2 was done under the supervision of both Timothy Finco and Arthur Weiss. This work appears in the following:

Lin, J., Weiss, A., and Finco, T. S. (1999). Localization of LAT in glycolipid-enriched microdomains is required for T cell activation. J Biol Chem *274*, 28861-28864.

Data presented in Chapter 3 appear in the following:

Lin, J., and Weiss, A. (2001). Identification of the minimal tyrosine residues required for linker for activation of T cell function. J Biol Chem *276*, 29588-29595.

Data presented in Chapter 4 was generated in collaboration with Jeanne Baker and Jing Zhu. Data presented in Chapter 5 appear in the following:

Lin, J., and Weiss, A. (2003). The tyrosine phosphatase CD148 is excluded from the immunologic synapse and down-regulates prolonged T cell signaling. J Cell Biol 162, 673-682.

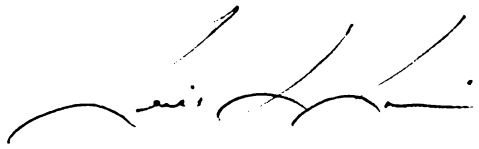
Abstract

The Role of LAT and CD148 in T Cell Signaling

Joseph Lin

TCR receptor signaling involves the complex interplay of multiple proteins leading to proliferation, differentiation and the gain of effector functions of the T cell. One protein central to T cell activation is the transmembrane adaptor molecule linker for the activation of T cells (LAT). LAT contains two cysteine residues, proximal to the transmembrane region, which are sites of palmitoylation. Using site directed mutagenesis we show that these two cysteines are required for LAT recruitment into lipid rafts and that this recruitment is essential to reconstitute TCR signaling in a LAT deficient cell. LAT also contains multiple tyrosine residues that (when phosphorylated) act as docking sites for SH2 domain containing molecules. Taking a structure-function approach, we showed that three tyrosine residues 132, 171, and 191 are sufficient for Ca2+ mobilization in response to TCR stimulation. However, for complete reconstitution of Erk phosphorylation and NFAT activation, tyrosine residues 110 and 226, in addition to 132, 171, and 191, are required. Since LAT has no intrinsic enzymatic activity, a method the cell uses to control LAT function is by regulating its phosphorylation state. One phosphatase, that has been demonstrated to have an effect on LAT phosphorylation, is the receptor-like tyrosine phosphatase CD148. CD148 is only expressed at low levels

in resting T cells, however it is upregulated following stimulation presumably to downregulate prolonged signaling events. We show, using immunofluorescence, that CD148 is excluded from the immunologic synapse, potentially preventing access to dephosphorylate LAT. This exclusion may be a mechanism whereby a cell can regulate a phosphatase by sequestering it away from substrates within the synapse.



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Chapter 1

Introduction to T Cell Signaling

Pathways in T Cell Receptor Signaling

T cell receptor (TCR) signaling has been an area of intense study for many years. New proteins are being continually discovered each year, making the task of understanding the various pathways evermore challenging. Several reviews have been written summarizing many of the molecules and their pathways (Kane et al., 2000; Tomlinson et al., 2000; Wange and Samelson, 1996; Weiss and Littman, 1994). The following adapted from Lin et al., is only an introduction to the many TCR-responsive proteins and the mechanisms by which they lead to production of the cytokine interleukin-2 (Figure 1.1) (Lin and Weiss, 2001b).

Activation of a T cell is initiated by engagement of the TCR to antigen presented on a major histocompatibility complex (MHC) molecule on an antigen presenting cell (APC). This TCR interaction also induces the association of CD4 or CD8 to the MHC molecule. Since Lck can bind to both CD4 and CD8, association of CD4 or CD8 to the MHC molecule induces the recruitment of the Src family kinase Lck to the TCR. There, it becomes activated and proceeds to phosphorylate immunoreceptor tyrosine-based activation motifs (ITAMs) in the CDε,δ, and γ and ζ subunits of the TCR. A functional ITAM consists of a YxxL/I $x_{(7-8)}$YxxL/I preceded by one or more charged amino acids. Phosphorylated tyrosine residues within ITAMs promote the recruitment and subsequent activation of another tyrosine kinase ZAP-70 (Weiss and Littman, 1994). One known substrate of ZAP-70 is the adapter molecule Linker for the Activation of T cells (LAT). LAT is a transmembrane protein containing nine tyrosine residues conserved between human, mouse, and rat. A ClustalW alignment of LAT from the three species is shown in

Figure 1.1 Pathways involved in IL-2 production. This model summarizes findings from multiple studies investigating the role of several signaling molecules in mediating the pathways from TCR engagement to the production of IL-2.

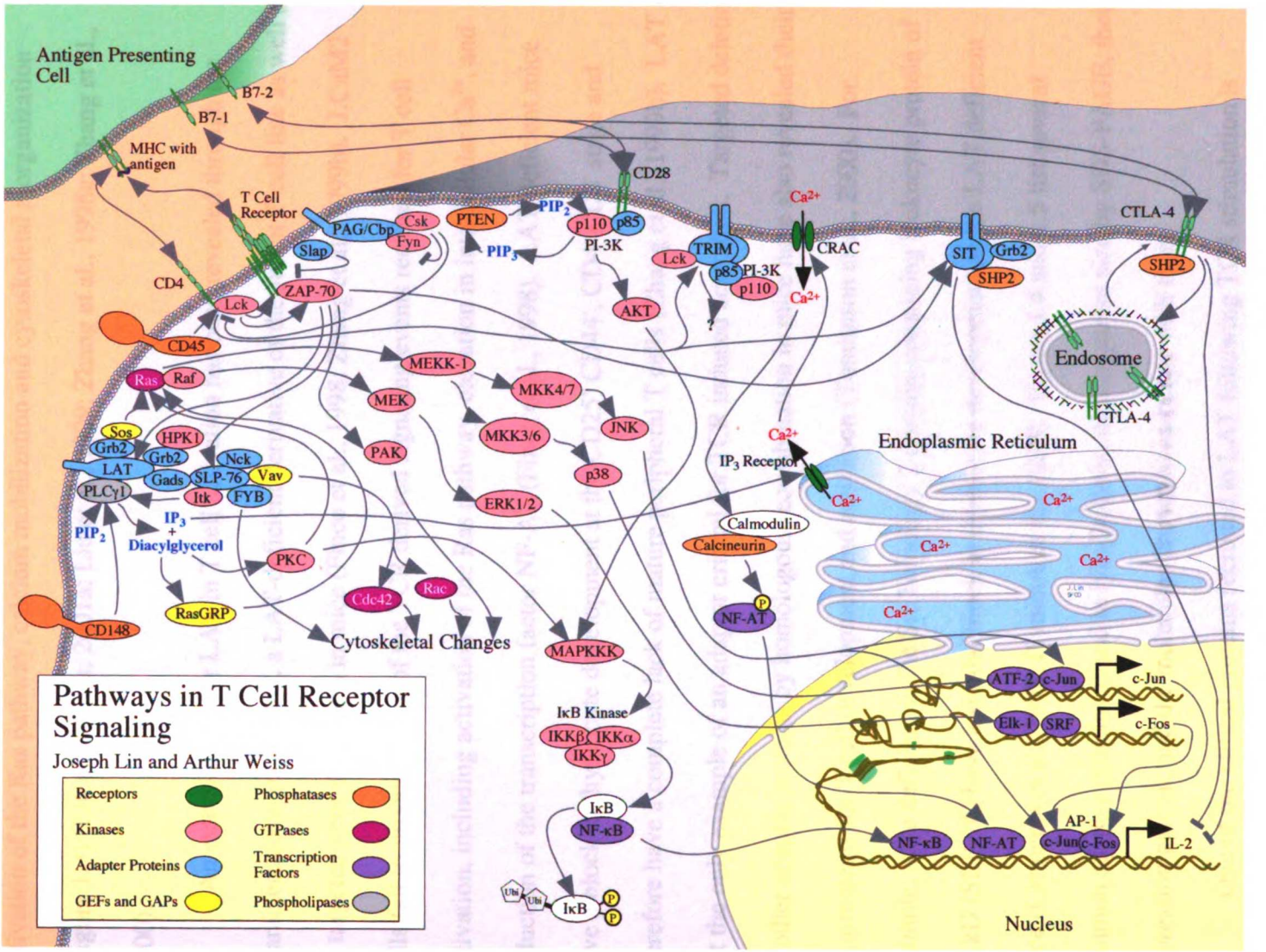

Antigen Presenting Cell
B7-2
B7-1
MHC with antigen
T Cell Receptor
CD28
CD4
CD45
CD148
PAG/Cbp
Csk
Fyn
Slap
ZAP-70
Lck
PTEN
PIP2
PIP3
p110
p85
PI-3K
AKT
TRIM
Lck
p85 PI-3K
p110
Ca2+
CRAC
SIT
Grb2
SHP2
CTLA-4
SHP2
Endosome
CTLA-4
Ras
Raf
MEKK-1
MEK
MKK4/7
MKK3/6
JNK
p38
PAK
ERK1/2
Sos
HPK1
Grb2
Grb2
Nck
LAT
Gads
SLP-76
Vav
PLCγ1
Itk
FYB
PIP2
IP3 + Diacylglycerol
PKC
RasGRP
Cdc42
Rac
Cytoskeletal Changes
IP3 Receptor
Endoplasmic Reticulum
Calmodulin
Calcineurin
NF-AT
MAPKKK
IκB Kinase
IKKβ IKKα
IKKγ
IκB
NF-κB
Ubi
Ubi
IκB
P
P
ATF-2
c-Jun
c-Jun
Elk-1
SRF
c-Fos
AP-1
NF-κB
NF-AT
c-Jun
c-Fos
IL-2
Nucleus
Pathways in T Cell Receptor Signaling
Joseph Lin and Arthur Weiss
Receptors
Kinases
Adapter Proteins
GEFs and GAPs
Phosphatases
GTPases
Transcription Factors
Phospholipases

Figure1.2 (Thompson et al., 1994). Phosphorylation of tyrosine residues on LAT results in the generation of binding sites for the recruitment of other proteins involved in activation of the Ras pathway, calcium mobilization and cytoskeletal reorganization (Figure 1.3) (Lin and Weiss, 2001a; Liu et al., 1999; Zhang et al., 1998a; Zhang et al., 2000).

An essential role for LAT in T cell activation has been revealed through the characterization of J.CaM2, a LAT-deficient derivative of the Jurkat T cell line as well as by targeted gene disruption in mice (Finco et al., 1998; Zhang et al., 1999b). J.CaM2 cells fail to produce many of the TCR derived signaling events required for T cell activation, including activation of the Ras pathway, elevation in intracellular Ca^{2+}, and induction of the transcription factor NF-AT (Finco et al., 1998). LAT-deficient mice have a block in thymocyte development at the $CD25^{+}$ $CD44^{-}$, $CD4^{-}$ $CD8^{-}$ stage and therefore have a complete lack of mature peripheral T cells (Zhang et al., 1999b). LAT is not the only example of an adaptor critical for TCR induced activation. Targeted deletion of other adaptor proteins by homologous recombination in mice have also revealed their requirements for T cell development and activation (Tomlinson et al., 2000). For example, mice deficient in the Src homology 2 domain-containing leukocyte protein of 76 kD (SLP-76) have a similar block in thymocyte development as the LAT deficient mice (Clements et al., 1998; Pivniouk et al., 1998). Figure 1.4 and 1.5 list several common adaptor proteins found in lymphocytes, their apparent size by SDS-PAGE, their expression pattern, and the molecules known to associate with them.

One of the critical proteins recruited to LAT following TCR stimulation is phospholipase C-γ1 (PLC-γ1) (DeBell et al., 1999; Takata et al., 1995; Zhang et al.,

Figure 1.2 Amino acid alignment of LAT from human, mouse, and rat. An alignment of human, mouse, and rat LAT using the ClustalW algorithm is shown where red dots indicate the conserved cysteine residues that can be palmitoylated and yellow dots indicate the conserved tyrosine residues.

ClustalW Formatted Alignments

Figure 1.3 Model of proximal T cell receptor signaling. (A) The left panel depicts an unstimulated TCR. Upon TCR and CD4/8 engagement with the appropriate MHC molecule loaded with peptide, the CD4/8 associated Lck phosphorylates the tyrosine residues within the ITAMs of the TCR allowing for the binding and activation of ZAP-70. (B) Activated ZAP-70 phosphorylates the adaptor protein LAT, which requires localization to lipid rafts for proper function. (C) Phosphorylated LAT can then recruit other adaptor proteins such as Gads and SLP-76. (D) This complex then serves as a docking site at the plasma membrane allowing for the recruitment of other signaling molecules to the membrane to potentiate the signaling cascade.

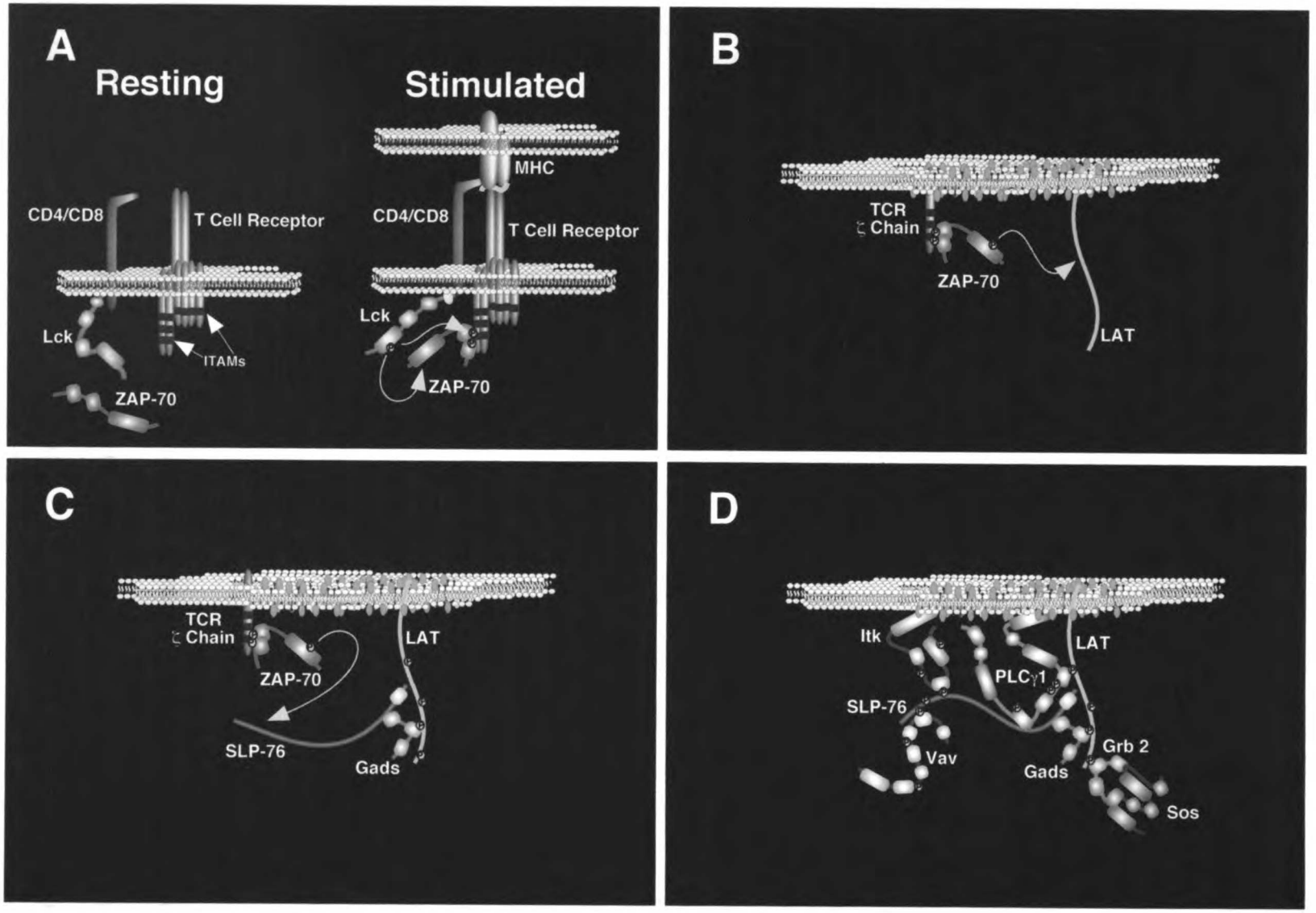
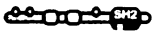
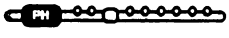
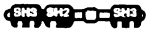
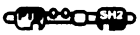
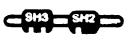
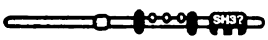
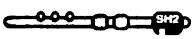

A
Resting
Stimulated
CD4/CD8
T Cell Receptor
Lck
ITAMs
ZAP-70
MHC
CD4/CD8
T Cell Receptor
Lck
ZAP-70
B
TCR
ζ Chain
ZAP-70
LAT
C
TCR
ζ Chain
ZAP-70
LAT
SLP-76
Gads
D
Itk
LAT
PLCγ1
SLP-76
Vav
Gads
Grb 2
Sos

Figure 1.4 List of common cytoplasmic adaptor molecules. Common cytoplasmic adaptor proteins with a potential role in antigen receptor signaling. The domain structure of each adaptor is shown in diagrammatic form, followed by size (kD) on a SDS PAGE under reducing conditions, expression pattern, and associated molecules.

Cytoplasmic Adapters

Name	Structure	Size (kD)	Expression	Associated Molecules
BLNK		65	B cells and Macrophages	PLCγ-2, Btk, Nck, Vav, Grb2
Cbl family		120	Ubiquitous	Grb2, p85, Crk, SLAP, Syk, ZAP-70, BLNK
CLNK		54	T, NK, and Mast cells	p92
Crk family		28,40,42	Ubiquitous	Cbl, C3G, Paxillin, Cas
Dok family		56, 62	Leukocytes	SHIP, RasGAP, LAT SLP-76
GAB family		97, 115	Ubiquitous	SHP2, Grb2, CrkL p85
Gads		40	T, NK, Macrophages, Mast cells, Platelets	LAT, SLP-76, Shc
Grap		28	Lymphocytes	LAT, Shc, Sos, Sam68
Grb2		28	Ubiquitous	Cbl, LAT, Sos, HPK1, SLP-76, Shc, SHP2
Nck		47	Ubiquitous	PAK, SLP-76, Sos, Cbl, WASP, IRS-1, NIK
RIBP		45	T and NK cells	Txk, Itk, Lck
Sap		15	T and NK cells	2B4, SLAM
Shb		55, 66	Ubiquitous	LAT, p85, Src, Eps8, Grb2, CD3ζ, PLCγ-1
Shc		46, 52, 66	Ubiquitous	SHIP, Grb2, RasGAP, ZAP-70, CD3ζ, Igα/β
SKAP55		55	T cells	SLAP-130, Fyn
SLAP		34	Lymphocytes	ZAP-70, SLP-76, Cbl, Vav, CD3ζ
SLAP-130/Fyb		130	T and Myeloid cells	Fyn, SKAP55, SLP-76
SLP-76		76	T, NK, Macrophages, Mast cells, Platelets	Gads, Vav, Itk, LAT, SLAP-130, Nck, Grb-2
3BP2		80	Lymphocytes	LAT, Cbl, ZAP-70 Grb2, PLCγ-1, Syk

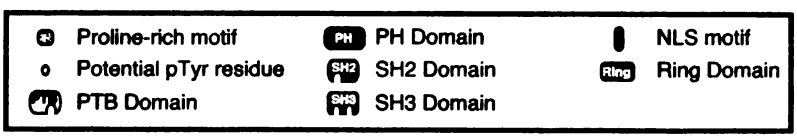
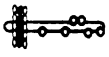
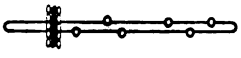
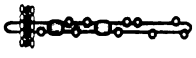
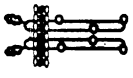
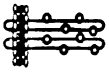
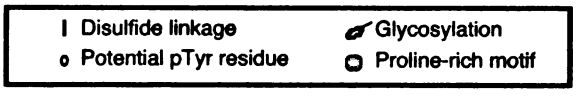


Figure 1.5 List of transmembrane adaptor molecules. Transmembrane adaptor proteins with a potential role in antigen receptor signaling. The domain structure of each adaptor is shown in diagrammatic form, followed by size (kD) on a SDS PAGE under reducing conditions, expression pattern, and associated molecules.

Transmembrane Adapters

Name	Structure	Size (kD)	Expression	Associated Molecules
LAB/NTAL		30	B, Mast, NK cells, and monocytes	Grb2, Sos, Gab-1, cCbl
LAT		36-38	T, NK, Mast cells, Platelets	SLP-76, Grb2, Shb, Gads, PLCγ-1, cCbl, Vav, Itk, p85
LAX		70	T, B, and NK cells	Grb2, Gads, p85 of PI3K
PAG/Cbp		75-85	Ubiquitous	Csk, Fyn
SIT		30-40	Lymphocytes	SHP2
TRIM		29	T and NK cells	p85 of PI3K



2000). Activated PLC-γ1 is responsible for the production of the second messengers diacylglycerol (DAG) and inositol 1,4,5 triphosphate (IP_3) by cleaving phosphatidylinositol 4,5 bisphosphate (PIP_2) at the plasma membrane. These second messengers are essential for T cell activation. DAG activates a number of proteins such as various isoforms of protein kinase C (PKC) and Ras guanyl nucleotide releasing protein (RasGRP), while IP_3 binds to IP_3 receptors on the surface of the endoplasmic reticulum (ER) (Hogquist, 2001; Isakov and Altman, 2002). Upon IP_3 receptor binding, Ca^{2+} stores in the ER are released into the cytoplasm. Emptying of the ER of Ca^{2+} triggers the opening of Ca^{2+} release-activated Ca^{2+} (CRAC) channels at the plasma membrane allowing for an influx of extracellular Ca^{2+} (Broad et al., 2001). The increased Ca^{2+} levels then activate the protein phosphatase calcineurin by disrupting the inhibitory effects of calmodulin. Calcineurin activation leads to the dephosphorylation of the transcription factor Nuclear Factor of Activated T cells (NFAT), allowing it to enter the nucleus where it cooperates with other transcription factors to bind promoters (Crabtree and Olson, 2002).

Activation of the GTPase Ras occurs as a result of recruitment of its exchange factors Sos and RasGRP to the membrane (Dower et al., 2000; Ebinu et al., 2000; Kim et al., 2000). Sos is recruited to the membrane by the adaptor molecule Grb2, which through its SH2 domain, binds phosphorylated tyrosines on various transmembrane molecules. In contrast, RasGRP contains EF hands and a C1 domain, which binds to Ca^{2+} and DAG respectively. Other proteins, such as the many isoforms of PKC, may also play a role in Ras activation. GTP bound Ras leads to the activation of a number of serine/threonine kinases and dual specificity kinases, which are responsible for the

eventual activation of the mitogen-activated protein (MAP) kinases Erk1/2, JNK and p38. These MAP kinases directly phosphorylate transcription factors involved in the formation of the heterodimeric transcription factor AP-1.

Another family of transcription factors important in the generation of IL-2 is the NF-kB family. Activation of the NF-kB family is dependent on stimulation of the TCR and co-stimulation via CD28. For a more comprehensive review, see Kane et al (Kane et al., 2002). The serine/threonine kinase Akt and the MAPKKKs participate in activation of the heterotrimeric IkB kinase complex. The IkB kinase (IKK) complex regulates NF-kB activity by phosphorylating IkB, leading to IkB ubiquitination and subsequent degradation. Freed from its association with IkB, NF-kB proteins are free to move into the nucleus and activate transcription.

Many recent studies have focused on functional and physical interactions between the TCR and cytoskeleton. TCR clustering at the site of antigen presenting cell (APC) contact and re-orientation of the microtubule organizing center (MTOC) are just some examples. Although proteins such as Vav, Cdc42, Rac and Fyb have been implicated in these events, the exact pathways still remain unclear. The cytoskeleton may also play a role in downregulating IL-2 production by promoting the trafficking of CTLA-4 to the plasma membrane from endosomes. At the plasma membrane, CTLA-4 can then bind its ligand B7-1 or B7-2 causing downregulation of signaling. Though SHP2 and regulation of TCR zeta chain accumulation in lipid rafts have been implicated in mediating CTLA-4 function, the exact mechanism remains unclear (Chikuma et al., 2003; Schneider and Rudd, 2000).

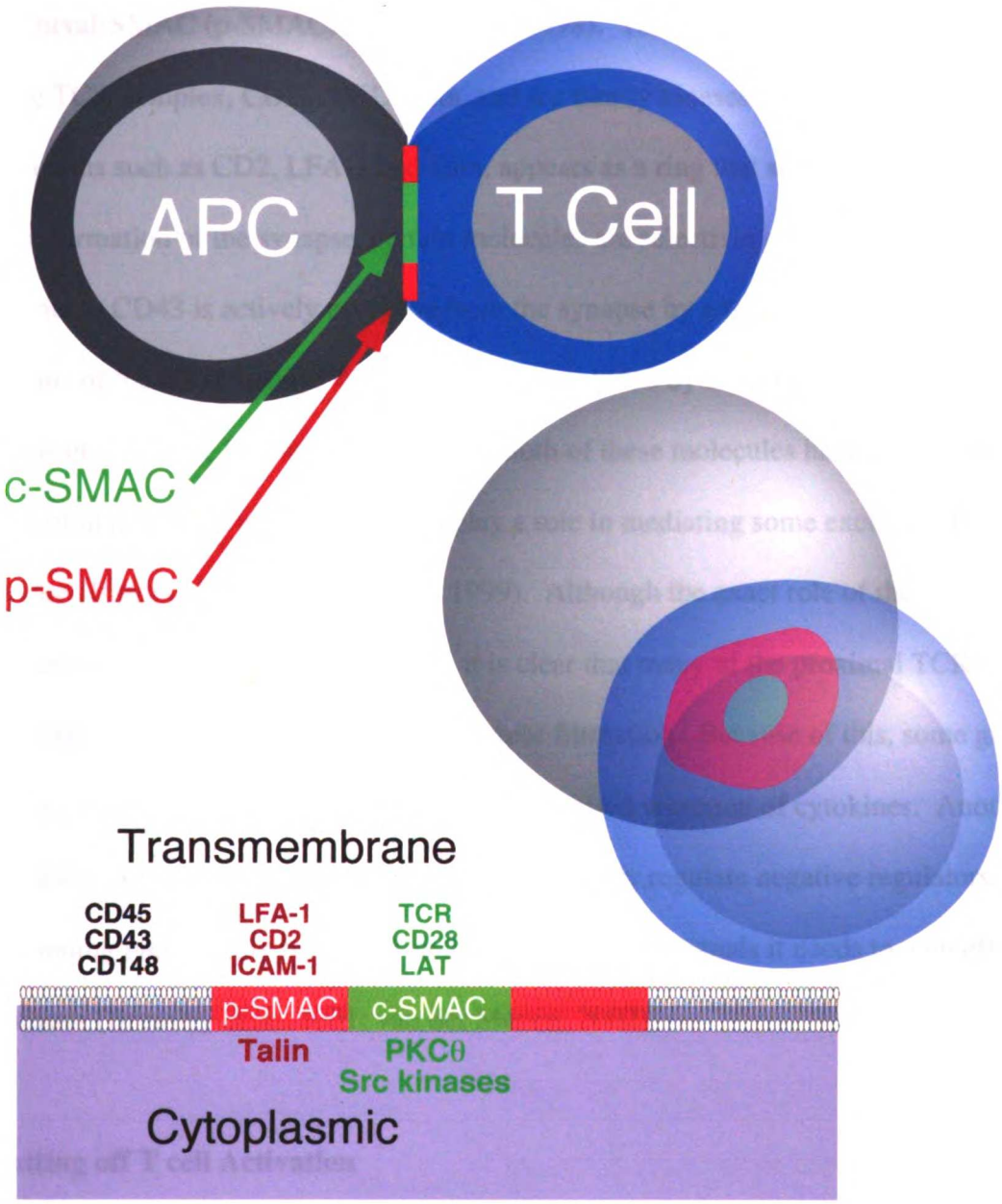
Compartmentalization of T Cell Signaling Molecules

The complex interplay between the signaling molecules involved in T cell activation described above is regulated at multiple levels. Protein levels, phosphorylation state, intrinsic activity regulated by conformation, inhibitory binding partners, and localization are only a few examples of possible modes of regulation. More recently, the idea that localization into specialized structures or regions of a cell has attracted the attention of many labs.

In the late 1990s, the concept of membrane microdomains became a hot topic. Originally defined as a biochemical membrane solubility property, lipid rafts, also known as DRMs (detergent resistant membranes), DIGs (detergent insoluble glycolipid-enriched complexes), or GEMs (glycolipid-enriched microdomains), were described as the insoluble fraction of cells lysed in Triton X-100 at 4°C that could be recovered in the low-density fraction of a sucrose gradient (Simons and Ikonen, 1997; Xavier et al., 1998). This fraction was enriched in cholesterol and sphingolipids as well as some lipid-modified proteins involved in signal transduction. Though controversy still exists around the importance, existence and function of these microdomains on the plasma membrane, it has become clear that localization of some proteins to these detergent insoluble fractions is essential for their ability to function.

At about the same time as when the idea of lipid rafts was popularized, Monks and colleagues published a paper describing an organized structure at the site of T cell-APC contact calling it the supramolecular activation cluster (SMAC) (Monks et al., 1998). This structure is also referred to as immunologic synapse (Figure 1.6). Since then, many more papers describing this structure have been published attempting to

Figure 1.6 Model of the immunologic synapse. When a T cell engages an antigen presenting cell (APC) containing the appropriate peptide loaded on the MHC molecule, the two cells form a conjugate and generate what is known as an immunologic synapse at the site of contact. Over the course of a few minutes, the synapse develops into two distinct regions. The central supramolecular activation cluster (c-SMAC) in the middle is shown in green, surrounded by the peripheral-SMAC (p-SMAC) shown in red. Some molecules that reside in the immunologic synapse are listed as well as a few molecules excluded from the synapse.

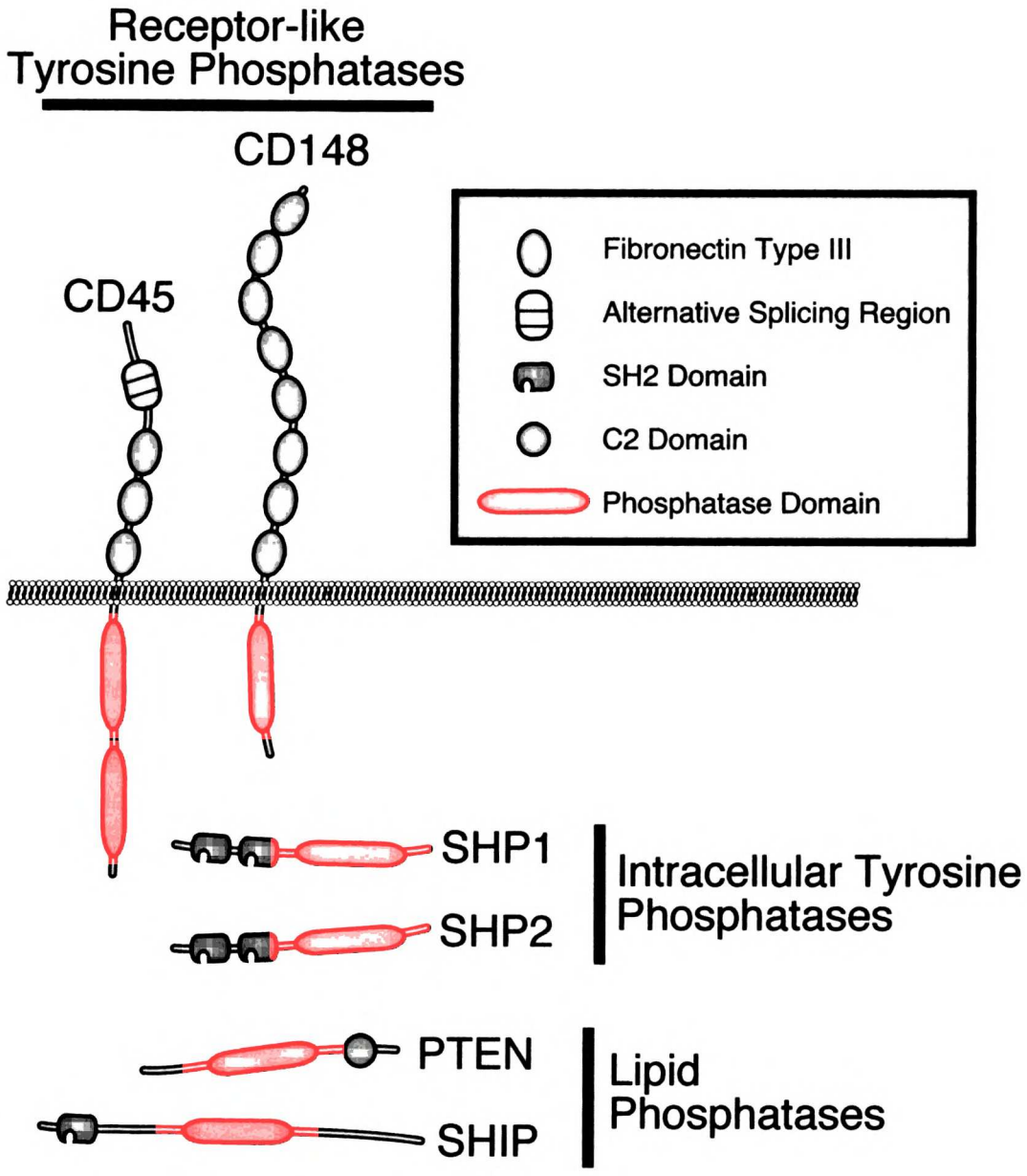

APC
T Cell
c-SMAC
p-SMAC
Transmembrane
CD45
CD43
CD148
LFA-1
CD2
ICAM-1
TCR
CD28
LAT
p-SMAC
c-SMAC
Talin
PKCθ
Src kinases
Cytoplasmic

ascribe a function to the synapse (Bromley et al., 2001; van der Merwe, 2002). The immunologic synapse is composed of two parts. The central-SMAC (c-SMAC) and the peripheral-SMAC (p-SMAC) (Monks et al., 1998). The c–SMAC contains proteins such as the TCR complex, CD28, PKC-theta, and src family kinases. The p-SMAC, consisting of proteins such as CD2, LFA-1 and talin, appears as a ring that surrounds the c-SMAC. Upon formation of the synapse, certain molecules are selectively excluded from the site of contact. CD43 is actively excluded from the synapse by a mechanism involving proteins of the ERM family, whereas CD45 is excluded by an unknown mechanism (Delon et al., 2001; Johnson et al., 2000). Both of these molecules have a large bulky extracellular domain which could also play a role in mediating some exclusion from the synapse (Irles et al., 2002; Wild et al., 1999). Although the exact role of the immunologic synapse is still unknown, it is clear that many of the proximal TCR signaling events occur independent of synapse formation. Because of this, some groups propose that the role of the synapse is for the targeted secretion of cytokines. Another possibility is that the role of the synapse is to spatially regulate negative regulators from functioning prematurely before a T cell can generate the signals it needs to gain effector functions (Bromley et al., 2001; van der Merwe, 2002).

Shutting off T cell Activation

Considerably more is known about how T cells become activated, but equally important is how these cells shut down their response (Cooper et al., 1983; Pawson, 1994; Pawson and Scott, 1997). One candidate group of proteins is phosphatases (Figure 1.7). Multiple types of phosphatases play a role in lymphocyte signaling, including both

Figure 1.7 Common phosphatases with a role in lymphocyte signaling. Three categories of phosphatases that play a role in lymphocyte signaling are shown: 1) Receptor-like PTPs (RPTPs) 2) Intracellular PTPs 3)Lipid phosphatases.


Receptor-like
Tyrosine Phosphatases
CD148
CD45
Fibronectin Type III
Alternative Splicing Region
SH2 Domain
C2 Domain
Phosphatase Domain
SHP1
SHP2
Intracellular Tyrosine
Phosphatases
PTEN
SHIP
Lipid
Phosphatases

protein and lipid phosphatases, but most studies in T cells have focused on protein tyrosine phosphatases (Li and Dixon, 2000). Though some protein tyrosine phosphatases (PTPs) have positive regulatory roles in T cell activation such as CD45, most PTPs have been implicated in having negative regulatory effects. PTPs fall into two major types: 1) Receptor-like PTPs (RPTPs) or 2) Intracellular PTPs. Regardless of type, all PTPs contain the conserved (I/V)HCxxGxxR(S/T) sequence responsible for the catalytic activity in the phosphatase domain. Through loss of function studies it has become clear that without some of these PTPs disease pathogenesis can be very rapid and severe (Li and Dixon, 2000).

One phosphatase of particular interest in T cell receptor mediated signaling is CD148. Initially cloned by multiple groups, most studies have described a role for CD148 (also known as DEP-1, PTP-eta, PTPRJ, and Byp) in cell cycle and growth control (Gaya et al., 1999). Targeted disruption of the catalytic activity of CD148 in the germline of mice results in an early embryonic lethality due to defects in angiogenesis (Takahashi et al., 2003). Previous studies in T cells have shown a profound effect on the phosphorylation state of LAT in cells expressing CD148, but much is still not known about the role of CD148 in regulating other aspects of the immune response (Baker et al., 2001; Tangye et al., 1998c).

Topics to be Covered

The following four chapters will present studies of the roles of the adaptor protein LAT and the receptor-like protein tyrosine phosphatase CD148 in TCR induced signaling. Chapter 2 will use mutational and biochemical approaches to address the role

of the two palmitoylation sites in LAT for downstream signaling. Chapter 3 describes structure-function analyses of the ten tyrosine residues in human LAT to determine which residues are required for signaling as well as which are the minimal sites required for LAT function. Chapter 4 will survey the expression pattern of CD148 and assess which signals are required for CD148 upregulation in T cells. Chapter 5 will examine the role of CD148 in TCR induced signaling and hypothesize a possible function for its localization in T cell-APC conjugates. Finally, Chapter 6 will discuss the implications of the findings presented here and propose future directions.

Chapter 2
Localization of LAT in Glycolipid-enriched Microdomains (GEMs) is Required for T cell Activation

Summary

LAT, a transmembrane adapter protein found in glycolipid-enriched microdomains (GEMs), is essential for T cell activation. In this study, we have utilized a LAT-deficient mutant of the Jurkat T cell line, J.CaM2, to explore various requirements for LAT function. First, we demonstrate that LAT must be present in GEMs for coupling TCR engagement to activation of the Ras signaling pathway, increases in intracellular Ca^{2+}, and induction of the transcription factor NF-AT. Second, we show that the extracellular and transmembrane domains of LAT are dispensable for these TCR-mediated events once LAT has localized to GEMs. These results provide important insights into both the structural domains of LAT and its subcellular localization that are required for effective TCR signaling.

Introduction

Engagement of the T cell receptor (TCR) with either antigen or antibodies that bind TCR subunits results in the initiation of an intracellular signaling cascade, a complex series of biochemical events that culminate in T cell proliferation, differentiation, and gain of effector functions (Wange and Samelson, 1996; Weiss and Littman, 1994). The earliest of the signal transduction processes that occur following TCR stimulation include the activation of Src (Lck, Fyn) and Syk (ZAP-70, Syk) families of tyrosine kinases. These kinases subsequently phosphorylate downstream substrates allowing for a continuation of the signaling cascade. Phosphorylation of substrates by Src and Syk kinases can mediate the induction of enzymatic activity, for example with Vav and phospholipase Cγ–1 (PLCγ–1) (Crespo et al., 1997; Han et al., 1997; Secrist et al., 1991; Weiss et al., 1991), and may also facilitate protein-protein interactions, such as with SLP-76 and Cbl (Buday et al., 1996; Fournel et al., 1996; Fukazawa et al., 1995; Reedquist et al., 1996; Tuosto et al., 1996; Wu et al., 1996). Both of these outcomes of tyrosine phosphorylation are critical for effective T cell activation.

One molecule that becomes heavily tyrosine phosphorylated in response to TCR engagement is LAT (Linker for Activation of T Cells), a transmembrane protein whose expression is limited to T, NK, and mast cells. Although LAT lacks intrinsic enzymatic activity, tyrosine phosphorylation at multiple tyrosine residues within LAT facilitates its association with a number of signaling molecules that may contribute to T cell activation, including Grb2, GADs, the p85 subunit of PI3-kinase, PLCγ–1, Vav, SLP-76, and Cbl (Buday et al., 1994; Gilliland et al., 1992; Sieh et al., 1994; Trub et al., 1997; Weber et al., 1998; Zhang et al., 1998a). An essential role for LAT in T cell activation has been revealed through the characterization of J.CaM2, a LAT-deficient derivative of the Jurkat T cell line (Finco et al., 1998). J.CaM2 cells fail to produce many of the TCR derived signaling events required for T cell activation, including activation of the Ras pathway,

elevation in intracellular Ca^{2+}, and induction of the transcription factor NF-AT. Importantly, re-expression of wild type LAT in J.CaM2 restores all of these processes (Finco et al., 1998). More recently, the analysis of mice that lack LAT expression has also revealed a necessary role for this molecule in T cell development (Zhang et al., 1999b).

LAT contains a very short extracellular region, a transmembrane domain, and a tyrosine-rich cytoplasmic tail. Consistent with these structural features, LAT is found predominantly in the plasma membrane of T cells (Buday et al., 1994; Sieh et al., 1994; Zhang et al., 1998a). Furthermore, LAT is palmitoylated on two conserved cysteines (amino acids 26 and 29) and this modification localizes LAT to glycolipid-enriched microdomains (GEMs) within the plasma membrane (Zhang et al., 1998b). GEMs, sometimes referred to as detergent-insoluble lipid rafts, have been proposed to function as platforms for the formation of multi-component signaling complexes (Simons and Ikonen, 1997). Interestingly, other molecules in addition to LAT, including Lck, Vav, Grb2, PLCγ–1, and Ras, are also either constitutively associated with GEMs or redistribute into GEMs following TCR engagement (Brdicka et al., 1998; Montixi et al., 1998; Xavier et al., 1998). However, it remains unclear whether the localization of specific molecules to GEMs is actually necessary for their function in T cell signaling.

In this report, we demonstrate that LAT must be present in GEMs for the activation of the Ras pathway, an increase in intracellular Ca^{+2}, and the stimulation of NF-AT-dependent transcriptional activity in response to TCR engagement. Also, we show that the extracellular and transmembrane domains of LAT are not required for these processes once LAT has localized to GEMs.

Results

LAT localization to GEMs is required for function

Although palmitoylation of LAT on cysteines 26 and 29 is not necessary for LAT localization to the plasma membrane, it is essential for its distribution into GEMs (Zhang et al., 1998b). To determine whether the presence of LAT within GEMs is required for coupling TCR engagement to downstream signaling events, an expression vector was created encoding a form of LAT containing serines, instead of cysteines, at these palmitoylation sites (C26/29S-LAT). Although C26/29S-LAT was expressed at levels similar to wild-type LAT (wt-LAT) following transfection of vectors encoding either protein into LAT-deficient J.CaM2 T cells (Figure 2.1A), C26/29S-LAT was completely absent in GEM fractions and, instead, was found exclusively in the Triton-soluble fraction (Figure 2.1B). Conversely, a majority of transfected wt-LAT was found in GEMs with little detected in the Triton-soluble fraction (Figure 2.1B). To ensure that our method of GEM isolation yielded fractions devoid of cross-contamination, we also assayed for the presence of GEM and Triton-soluble specific markers, namely the ganglioside GM1 (using cholera toxin) and ZAP-70, respectively. Consistent with other studies (Brdicka et al., 1998; Simons and Ikonen, 1997; Zhang et al., 1998b), we detected the ganglioside GM1 only in the GEM fraction and ZAP-70 only in the Triton-soluble fraction of unstimulated cells (data not shown).

Following TCR engagement, LAT becomes heavily tyrosine phosphorylated. To determine whether LAT localization to GEMs is required for its inducible tyrosine phosphorylation, J.CaM2 cells were transfected with vectors encoding either wt-LAT or C26/29S-LAT and subsequently stimulated through the TCR followed by the analysis of LAT phosphorylation. Surprisingly, C26/29S-LAT was constitutively phosphorylated on tyrosine residues in the basal state (Figure 2.2). However, consistent with previous results, the inability of LAT to localize to GEMs correlated in its lack of inducible

Figure 2.1 Localization of LAT variants to lipid rafts. J.CaM2 cells were transfected with the various myc-tagged LAT constructs and 20 hrs later cells lysed. (A) Half of the cells were lysed in 1%NP-40, separated by SDS-PAGE, and analyzed by western blot with an anti-myc MAb to detect LAT. (B) The remaining cells were lysed in 0.5% Triton and subjected to ultracentrifugation in a sucrose gradient to purify GEMs. The GEM fraction (fraction 3) and a Triton-soluble fraction (fraction 11) were separated by SDS-PAGE and analyzed by western blot with an anti-myc MAb to detect LAT (the slightly faster mobility band detected in empty-vector-transfected cells is due to non-specific cross-reactivity). Blots of fraction 3 and 11 were also probed with anti-Lck and anti-ZAP-70 antibodies respectively to verify equal loading of samples.

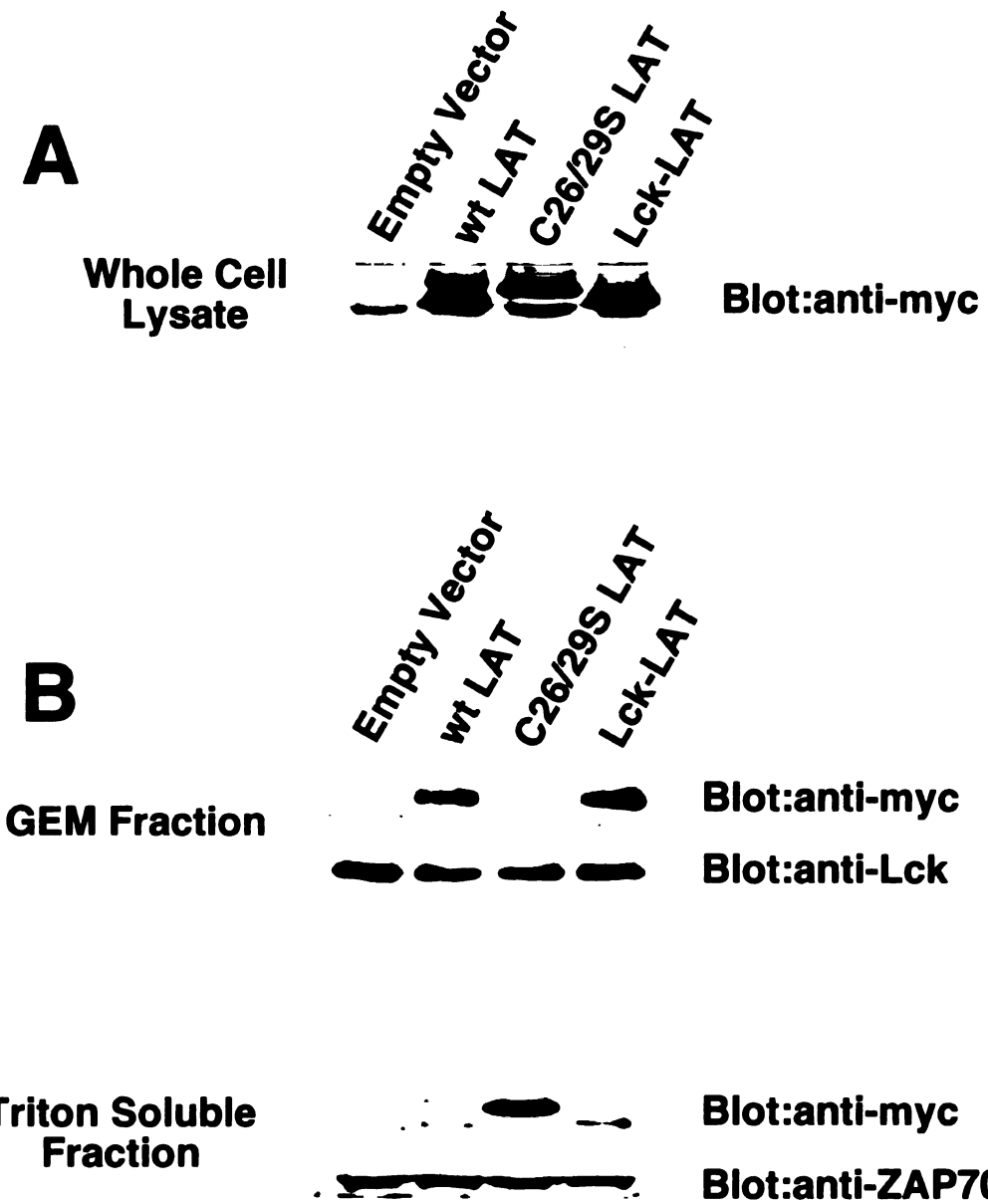
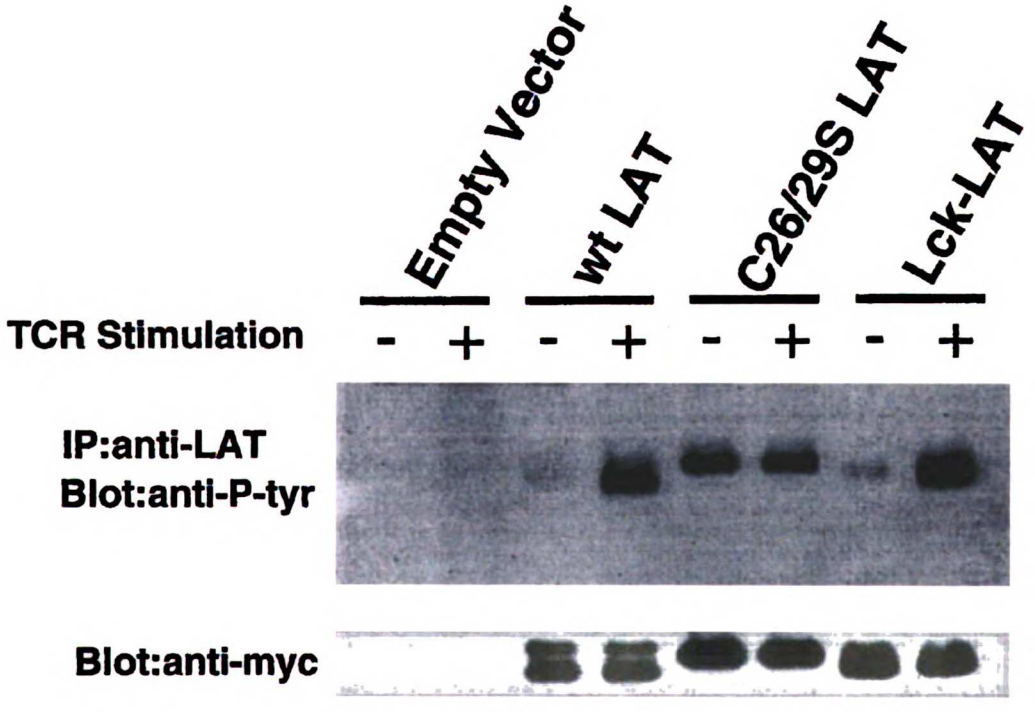

A
Empty Vector
wt LAT
C26/29S LAT
Lck-LAT
Whole Cell Lysate
Blot:anti-myc
B
Empty Vector
wt LAT
C26/29S LAT
Lck-LAT
GEM Fraction
Blot:anti-myc
Blot:anti-Lck
Triton Soluble Fraction
Blot:anti-myc
Blot:anti-ZAP70

Figure 2.2 Tyrosine phosphorylation of LAT variants in response to TCR stimulation. J.CaM2 cells were transfected with the myc-tagged LAT constructs and 20 hrs later stimulated for 2 min with C305 or a PBS control. Cells were subsequently lysed, LAT immunoprecipitated with an anti-LAT antibody, and immunoprecipitated proteins separated by SDS-PAGE. Proteins were transferred to membrane and analyzed by western blotting with an anti-myc MAb to detect LAT. Blots were subsequently stripped and re-probed with an anti-phospho-tyrosine antibody.

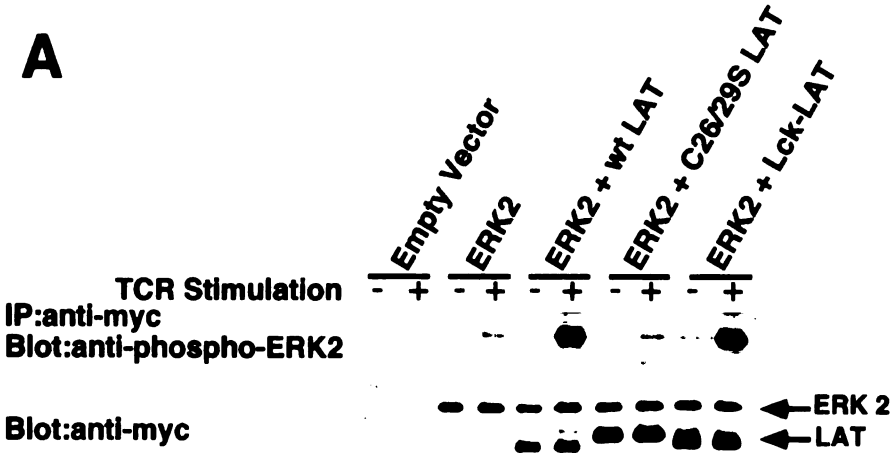

Empty Vector
wt LAT
C26/29S LAT
Lck-LAT
TCR Stimulation
- + - + - + - +
IP:anti-LAT
Blot:anti-P-tyr
Blot:anti-myc

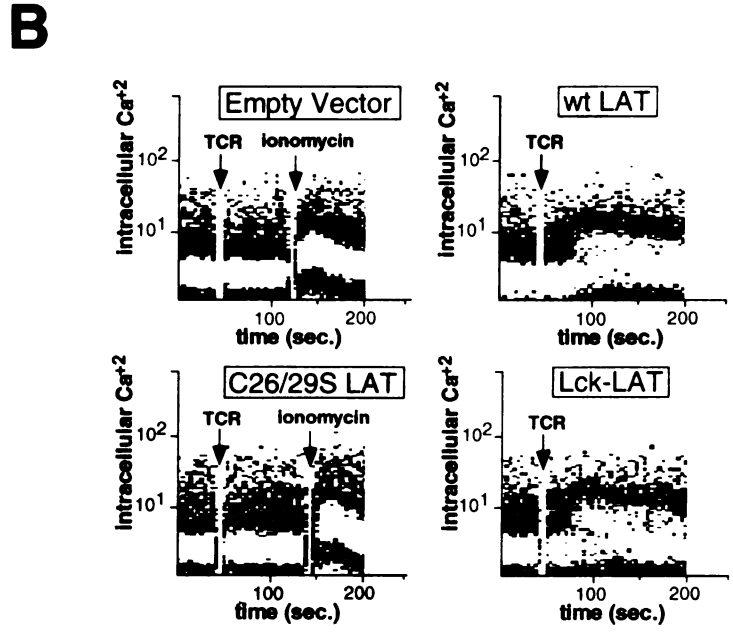
tyrosine phosphorylation following TCR stimulation (Figure 2.2) (Zhang et al., 1998b). As expected, tyrosine phosphorylation of transfected wt-LAT was not appreciable in the basal state, but increased dramatically following TCR engagement (Figure 2.2). Thus, mutation of cysteines 26 and 29 to serines in LAT abrogated its localization to GEMs and resulted in its constitutive tyrosine phosphorylation. In addition, C26/29S-LAT also failed to display enhanced tyrosine phosphorylation following TCR engagement.

In contrast to the response seen in wild type Jurkat T cells following TCR engagement, LAT-deficient J.CaM2 cells fail to display an activation of the Ras signaling pathway, an increase in intracellular Ca^{2+}, or an induction of the transcription factor NF-AT. Importantly, re-expression of wild type LAT in J.CaM2 restores all of these processes (Finco et al., 1998). Since C26/29S-LAT exhibited a significant constitutive level of tyrosine phosphorylation in J.CaM2, it was conceivable that it may be able to function in TCR-mediated signaling despite its inability to associate with GEMs or display inducible tyrosine phosphorylation. To examine this possibility, C26/29S-LAT was transfected into J.CaM2 and cells were subsequently stimulated through the TCR receptor followed by analysis for Ras activation, intracellular Ca^{2+} increases, and NF-AT induction. Control experiments were performed in tandem by transfecting J.CaM2 with either empty vector or a vector encoding wt-LAT.

To examine the Ras signaling pathway, we focused on the phosphorylation of Erk2, which occurs as a consequence of Ras activation in T cells following TCR engagement (Izquierdo et al., 1993). Consistent with previous results (Finco et al., 1998), J.CaM2 cells receiving wt-LAT, but not empty vector, demonstrated Erk2 phosphorylation following TCR engagement (Figure 2.3A). Interestingly, cells expressing C26/29S-LAT failed to exhibit Erk2 phosphorylation in response to TCR stimulation (Figure 2.3B). A similar pattern of results was seen when the levels of intracellular Ca^{2+} and induction of NF-AT were analyzed; cells receiving wt-LAT, but not

Figure 2.3 TCR-mediated Erk2 phosphorylation and intracellular Ca2+ levels in the presence of LAT variants. (A) J.CaM2 cells were transfected with 2.5-10 μg the myc-tagged LAT constructs (to yield equal protein expression) and 7.5 μg of myc-tagged Erk2. 20 hrs after transfection, cells were either stimulated with C305 or a PBS control for 2 min, then lysed and anti-myc immunoprecipitations performed. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to membrane, and blotted with anti-myc MAb. Blots were then stripped and then re-probed with an anti-phospho-ERK2 Ab. (B) Cells were transfected with 1-4 μg of the indicated LAT constructs 20 hours prior to staining with anti-CD8 MAb conjugated to PE and loading of the calcium indicator Fluro-3-AM. Cells were subsequently analyzed by flow cytometry to determine intracellular Ca^{2+} levels. Addition of C305 (1:500) or ionomycin (final concentration = 1 μM) is indicated. Intracellular Ca^{+2} levels shown are for cells staining positive for transfected CD8.


A
Empty Vector
ERK2
ERK2 + wt LAT
ERK2 + C26/29S LAT
ERK2 + Lck-LAT
TCR Stimulation
- + - + - + - + - +
IP:anti-myc
Blot:anti-phospho-ERK2
Blot:anti-myc
ERK 2
LAT


B
Empty Vector
TCR
Ionomycin
wt LAT
C26/29S LAT
Lck-LAT
Intracellular Ca+2
time (sec.)

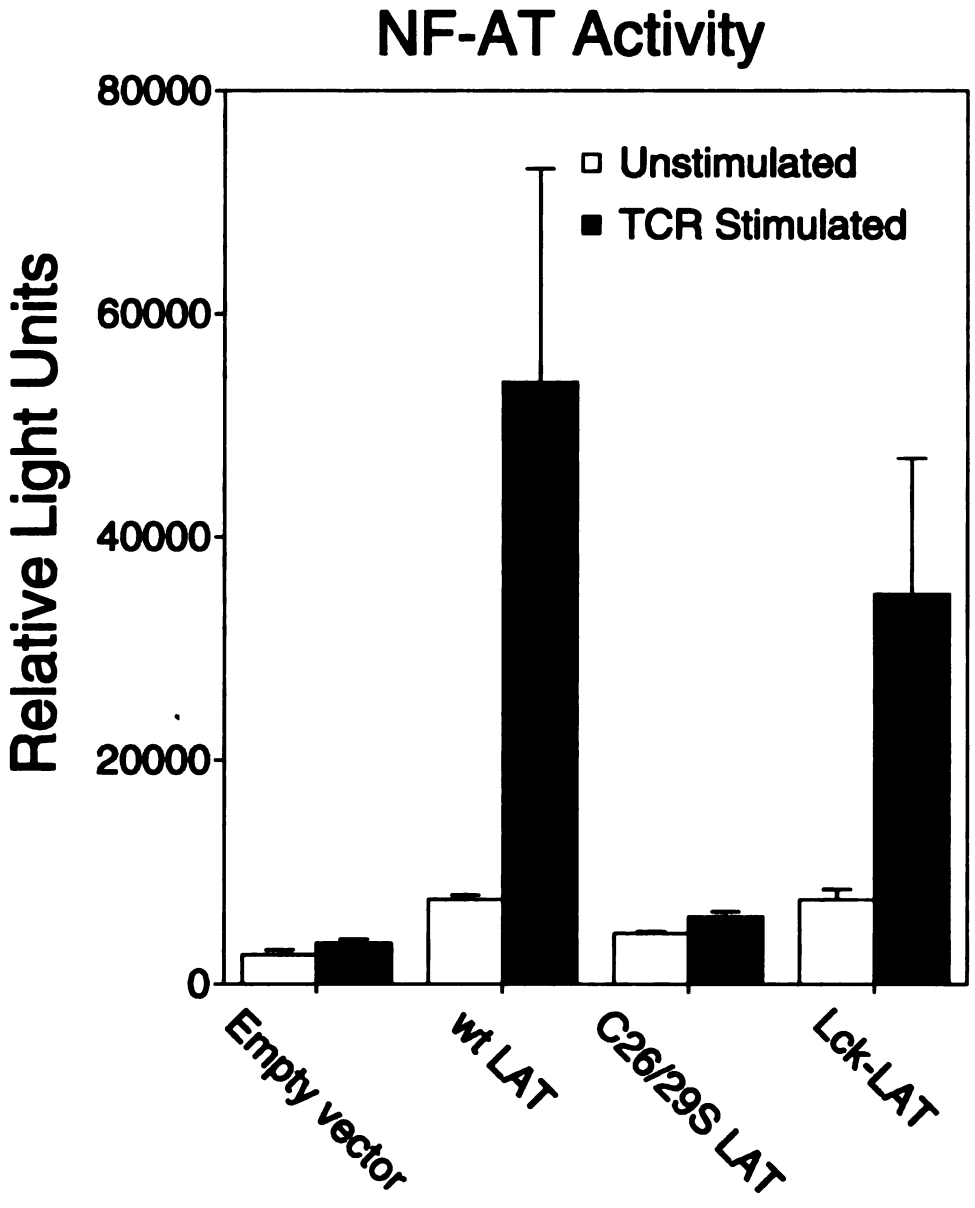
C26/29S-LAT, restored intracellular Ca^{2+} mobilization (Figure 2.3B) and activation of NF-AT (Figure 2.4) in response to TCR engagement. Together these results indicate that LAT localization to GEMs is required for coupling TCR stimulation with downstream signaling events.

The extracellular and transmembrane domains of LAT are dispensable for function.

As an initial step toward identifying regions of LAT required for function, we focused on its extracellular and transmembrane domains. Since our results suggested that LAT must localize to GEMs in order to facilitate activation of downstream signaling pathways, we studied the role of these domains in the context of GEMs. A vector was created that encoded a form of LAT where the extracellular and transmembrane domains were replaced with the first 10 amino acids from the N-terminus of Lck (Lck-LAT). This Lck motif provides the necessary signals for myristoylation and palmitoylation and when attached to a heterologous protein can target it to GEMs (Zhang et al., 1998b). Thus, Lck-LAT should localize to GEMs but will lack both the extracellular and transmembrane domains of LAT. As shown in Figure 2.1A and B, respectively, Lck-LAT was expressed at levels comparable to wt-LAT and localized primarily to the GEM fraction of cell lysates.

We next analyzed Lck-LAT phosphorylation, and in a manner similar to wt-LAT, Lck-LAT became heavily tyrosine phosphorylated after engagement of the TCR (Figure 2.2). Furthermore, cells expressing Lck-LAT demonstrated TCR-mediated activation of the Ras signaling pathway (Figure 2.3A), mobilization of intracellular Ca^{2+} (Figure 2.3B), and induction of NF-AT (Figure 2.4). Taken together, the results obtained with Lck-LAT demonstrated that once LAT localizes to GEMs, both its extracellular and transmembrane domains were dispensable for coupling TCR engagement to the activation of downstream signaling pathways.

Figure 2.4 TCR-mediated NF-AT activity in the presence of various LAT variants. J.CaM2 cells were transfected with 10-40 ng of the various myc-tagged LAT vectors to obtain equal protein expression and 15 μg of the NF-AT-luciferase reporter construct. 19 hours later, equal numbers of cells were either left untreated (-) or stimulated (+) with immobilized anti-TCR MAb for 6 hrs, lysed and the level of luciferase activity measured. Shown is the average and standard error in relative light units of three independent experiments.


NF-AT Activity
Relative Light Units
80000
60000
40000
20000
0
Unstimulated
TCR Stimulated
Empty vector
wt LAT
C26/29S LAT
Lck-LAT

Discussion

Glycolipid-enriched microdomains (GEMs) have increasingly been implicated as critical components of intracellular signaling cascades. In T cells for instance, a number of important signaling molecules have been shown to localize to GEMs, either constitutively or following TCR stimulation. In addition, treatment of T cells with various agents that disrupt GEM structure can inhibit effective TCR signaling (Xavier et al., 1998). Thus, the formation of GEM-dependent, higher order signaling complexes may play a central role in T cell activation.

The contribution of LAT to T cell activation likely derives from its TCR-mediated tyrosine phosphorylation, a process which facilitates LAT association with multiple signaling proteins, including Grb2, GADs, SLP-76, Vav, PLCγ-1, Cbl, and the p85 subunit of PI-3 kinase. The interaction of these molecules with LAT may result in their activation and/or may promote protein-protein interactions that are crucial for downstream signaling events. Here we demonstrate that a form of the adaptor molecule LAT (C26/29S-LAT) that fails to localize to GEMs cannot support TCR-mediated signal transduction. These results are consistent with other studies that have suggested that GEMs play an important role in T cell activation. More importantly however, our results strongly suggest that LAT function may depend on its ability to recruit multiple signaling proteins to GEMs, where proper protein interactions can form and/or where essential substrates may be located. Relevant to this idea is the observation that C26/29S-LAT displayed a high level of basal tyrosine phosphorylation, yet could not support productive TCR signaling. One conceivable explanation for this result is that although C26/29S-LAT binds critical signaling molecules, it is unable to couple TCR engagement to downstream signaling processes due to its failure to localize into GEMs. Alternatively, since LAT contains 9 sites of potential tyrosine phosphorylation, it is possible that the constitutive sites of tyrosine phosphorylation in C26/29S-LAT are not those that mediate

the recruitment of appropriate signaling molecules. Of note, previous analysis of a GEM-excluded LAT variant containing alanines in place of cysteines at residues 26 and 29 did not reveal a high constitutive level of LAT tyrosine phosphorylation (Zhang et al., 1998b). Although an explanation for this discrepancy is presenting lacking, variations in results may reflect the different cell types utilized (Jurkat versus J.CaM2) or experimental approaches employed (transient versus stable transfection of LAT variants).

In this study, we also addressed which domains of LAT are critical for function once the protein has localized to GEMs. In particular, we focused on the potential role of the extracellular and transmembrane domains. A comparison of LAT protein sequences from mouse, human, and rat revealed the presence of a conserved, alpha-helix disrupting proline within the transmembrane domain which could potentially destabilize LAT insertion into lipid bilayers (Weber et al., 1998; Zhang et al., 1998a). Often proteins which contain such atypical amino acids within the transmembrane region are stabilized through the interaction of this domain with other molecules. It was therefore possible that LAT interacted with another protein via its transmembrane domain and that this interaction may be critical for LAT function. It was also possible that the extracellular and transmembrane domains were required for LAT function by some other mechanism. However, our results clearly show that both the extracellular and transmembrane domains are dispensable for T cell activation. It is possible that these domains are required for other functions of LAT. For example, the transmembrane region may initially localize LAT to the plasma membrane, permitting palmitoylation of LAT on cysteine residues 26 and 29 and thereby resulting in subsequent LAT insertion into GEMs. Alternatively, the extracellular and transmembrane domains may be required for coupling TCR engagement to downstream pathways that were not examined in the present study or that are required for functions independent of signaling. Future studies will hopefully address these alternative possibilities.

Experimental Procedures

Cell Lines and Reagents

The LAT-deficient Jurkat T cell derivative J.CaM2 (Goldsmith et al., 1988) was maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2mM glutamine, penicillin, and streptomycin. Cells were stimulated with C305, an anti-Jurkat Ti β chain MAb (Weiss and Stobo, 1984). The anti-phosphotyrosine MAb, 4G10, and the anti-phospho-ERK2 antibody were obtained from Upstate Biotechnology, Inc. and New England Biolabs respectively. Anti-myc MAb was derived from the 9E10 hybridoma. Antibodies for LAT (Zhang et al., 1998a), Lck (Burkhardt et al., 1994), and ZAP-70 (Finco et al., 1998) have been previously described. For use in transfections, a myc-tagged form of LAT was excised from pBluescript with EcoRI and XbaI and inserted into the expression vector, pcdef3. C26/29S-LAT, also in pcdef3, was created with the QuickChange site-directed mutagenesis kit (Stratagene). The Lck-LAT chimera was made by digesting pcdef3-LAT with EcoRI and ApaLI to remove the extracellular and transmembrane domains of LAT and replacing this region, in frame, with an oligo encoding the N-terminus 10 amino acids of Lck. Erk2 and CD8 were expressed from pEF-BOS. The NF-AT-luciferase reporter plasmid has been previously described (Shapiro et al., 1996).

Purification of GEM Fractions

$2x10^7$ J.CaM2 cells were transfected and 20 hrs later lysed on ice in 1 ml 0.5% Triton X-100 in TNE buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 5mM EDTA) with a combination of protease and phosphatase inhibitors. All subsequent steps were performed at 4°C. Lysates were mixed with 80% sucrose in TNE, transferred to centrifuge tubes, and overlaid with 2 ml of 30% sucrose in TNE and 1ml of 5% sucrose

in TNE. Samples were centrifuged for 20-22 hours at 45,000 rpm in a Beckman SW55Ti at 4°C and twelve 400 µl fractions collected from the top of each gradient.

Transfection, Stimulation, and Luciferase Assay

For transient transfections, $2x10^7$ J.CaM2 cells in 400 µl of RPMI 1640 were electroporated at 250V, 960 µF with 30-50 µg of DNA total. Unless otherwise noted, 2.5-10 µg of the various LAT vectors were used to obtain equal protein expression. TCR stimulation for analysis of LAT and ERK2 phosphorylation involved incubating PBS-washed cells ($8x10^7$cells/ml) at 37 °C for 15 min followed by addition of anti-TCR MAb (1:500) or PBS as a control for 2 min. Cells were immediately pelleted and lysed. For TCR stimulation in luciferase assays, cells were either left untreated or were incubated with immobilized anti-TCR MAb or PMA (25 ng/ml) and ionomycin (1 µM) for 6 hours. Cells were then harvested, lysed, and assayed for luciferase activity (Shapiro et al., 1996).

Preparation of Lysates, Immunoprecipitation, and Western Blotting

Cells were lysed at 10^7 cells/150 µl in lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl pH 7.6, 2 mM EDTA, and protease and phosphatase inhibitors). After a 10 min incubation on ice, samples were clarified by centrifugation at 14,000 rpm for 10 min. For immunoprecipitations, lysates were incubated with primary antibody for at least 1 hour and then Protein G-sepharose beads for one hour. Beads were then washed 3X in 500 µl lysis buffer. Samples were resuspended in reducing SDS sample buffer, heated at 95 °C for 5 min, separated by SDS-PAGE and proteins transferred to Immobilon-P (Millipore) for analysis by western blotting. Membranes were blocked with 3% BSA, incubated with the indicated primary antibodies followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Reactive proteins were subsequently visualized by enhanced chemiluminescence (Amersham).

Measurement of Intracellular Ca^{2+} Levels

J.CaM2 cells were transfected with the various LAT constructs and 20 μg of vector encoding CD8. 20 hrs after transfection, cells were incubated with anti-CD8 MAb conjugated to PE (Becton Dickinson), then washed and incubated with the Ca^{2+} indicator Fluro-3-AM (Molecular Probes) at a final concentration of 2.5 μM. After a 30 min incubation samples were again washed, resuspended in 1 ml, and subsequently warmed to 37 °C for 5 min prior to analysis by flow cytometry (FACScan, Becton Dickinson).

Chapter 3

Identification of the Minimal Tyrosine Residues Required for LAT Function

Summary

The linker for activation of T cells (LAT) is essential for signaling through the T cell receptor (TCR). Following TCR stimulation, LAT becomes tyrosine phosphorylated, creating docking sites for other signaling proteins such as phospholipase C-γ1 (PLC-γ1), Grb2, and Gads. In this study, we have attempted to identify the critical tyrosine residues in LAT that mediate TCR activation-induced mobilization of intracellular Ca^{2+} and activation of the MAP kinases Erk-1 and -2. Using the LAT deficient Jurkat derivative, J.CaM2, stable cell lines were established expressing various tyrosine mutants of LAT. We show that three specific tyrosine residues (132,171,191) are necessary and sufficient to achieve a Ca^{2+} flux following TCR stimulation. These tyrosine residues function by reconstituting PLC-γ1 phosphorylation and recruitment to LAT. However, these same tyrosines can only partially reconstitute Erk activation. Full reconstitution of Erk requires two additional tyrosine residues (110,226), both of which have the Grb2 binding motif YxN. This reconstitution requires that the critical tyrosine residues for Erk activation be on the same molecule of LAT, demonstrating that a single LAT molecule may nucleate multiple protein-protein interactions involving multiple binding domains.

Introduction

Engagement of the T cell receptor (TCR) triggers a complex cascade of events culminating in T cell proliferation, differentiation, and increased gene transcription (Wange and Samelson, 1996; Weiss and Littman, 1994). The initial steps of this process are carried out by the Src and Syk families of tyrosine kinases (Kane et al., 2000). Targets of the Syk family of tyrosine kinases includes an emerging class of proteins known as adaptors. Although these proteins lack intrinsic enzymatic activity, they function to promote intermolecular interactions utilizing multiple protein-protein interaction domains or motifs (Tomlinson et al., 2000). Examples of interaction domains include Src homology (SH) 2 domains, which bind phosphotyrosine residues, and SH3 domains, which bind proline-rich regions.

One adaptor protein essential for T cell activation is the transmembrane adaptor protein, linker for activation of T cells (LAT) (Finco et al., 1998; Zhang et al., 1998a; Zhang et al., 1999b). Following TCR engagement, LAT becomes phosphorylated on multiple tyrosine residues thereby allowing other proteins important for T cell activation to be recruited by SH2-phosphotyrosine interactions. Multiple proteins have been demonstrated by coimmunoprecipitation experiments to be recruited to LAT, either directly or indirectly, such as phospholipase C-γ1 (PLC-γ1), Grb2, Sos, Gads, SLP-76, Vav, Cbl, Itk, and the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (Ching et al., 2000; Liu et al., 1999; Shan and Wange, 1999; Zhang et al., 2000). In addition to the many tyrosine residues, LAT also contains two cysteine residues proximal to the transmembrane domain. These two cysteine residues are palmitoylated resulting in localization of LAT into glycolipid-enriched microdomains (GEMs) within the plasma

membrane (Zhang et al., 1998b). Without correct localization into GEMs, LAT cannot mediate downstream signaling events induced by TCR stimulation (Lin et al., 1999; Zhang et al., 1999a). Since LAT recruits so many proteins to one location, it appears likely that LAT, with the help of other adaptor proteins, functions as a platform where many signal transduction proteins co-localize to propagate and integrate the signals initiated by TCR stimulation.

The essential role of LAT in T cell development has been demonstrated using targeted gene disruption in mice. LAT-deficient mice have a block in thymocyte development at the immature $CD25^+$ $CD44^-$, $CD4^-$ $CD8^-$ stage and a complete lack of mature peripheral T cells (Zhang et al., 1999b). Blocks at this early stage of thymocyte development are similar to those observed in other mice lacking proteins, such as the Src kinases Lck and Fyn, and the Syk kinases ZAP-70 and Syk, that mediate pre-TCR signaling events (van Oers, 1999).

The LAT-deficient Jurkat derivative, J.CaM2, has provided considerable insight into the mechanism by which LAT mediates TCR signaling events. This cell line is deficient in many pathways activated by TCR stimulation, such as Ca^{2+} mobilization and Ras/MAPK activation (Finco et al., 1998; Goldsmith et al., 1988). These defects are, in part, due to an inability to recruit PLC-γ1 to the membrane where it is phosphorylated and becomes activated. PLC-γ1 activation is essential for the generation of the second messengers inositol-1, 4,5-triphosphate (IP_3) and diacylglycerol (DAG) from phosphatidylinositol-4, 5-bisphosphate (PIP_2). IP_3 binds receptors that regulate the release of stored Ca^{2+}, whereas DAG is required to activate proteins such as protein kinase C (PKC) and Ras guanyl nucleotide releasing protein (RasGRP) (Downward et al.,

1990; Ebinu et al., 2000).The absence of LAT may also result in the loss of Ras activation through the inability to recruit the Ras exchange factor Sos to the membrane via the adaptor Grb2.

Analysis of the various coimmunoprecipitation studies involving LAT has led to a model where a complex of adaptors involving LAT, Grb2, Gads and SLP-76 are localized to GEMs within the plasma membrane in order to recruit other signaling proteins (Liu et al., 1999; Zhang et al., 1998a; Zhang et al., 2000). Grb2 is an adaptor protein with a central SH2 domain flanked on both sides with SH3 domains, which bind Sos. Human LAT contains four tyrosine residues that fit the Grb2-SH2 binding motif of YxN that are conserved between human, mouse and rat ($Tyr^{110,171,191,226}$). Three of these tyrosines ($Tyr^{171,191,226}$), when mutated, have been shown to disrupt Grb2 recruitment to LAT. A Grb2-like molecule, known as Gads, also binds directly to LAT at only two of these sites ($Tyr^{171,191}$). Like Grb2, Gads contains two SH3 domains flanking a single SH2 domain. However, Gads also contains a proline-rich region between the SH2 domain and the C-terminal SH3 domain. Studies by Liu and colleagues have demonstrated that Gads is responsible for mediating the recruitment of SLP-76 to LAT (Liu et al., 1999).

The SLP-76 adaptor protein contains proline-rich regions, a C-terminal SH2 domain and three N-terminal phosphotyrosine residues, all of which have been demonstrated to recruit other signaling proteins. The C-terminal SH3 domain of Gads binds to proline-rich sequences within SLP-76 for its recruitment to LAT (Liu et al., 1999). SLAP-130/Fyb and HPK1 both contain phosphotyrosine residues that can bind the SH2 domain of SLP-76 (Musci et al., 1997). Proteins such as Itk, Rlk and Vav are thought to bind directly to phosphotyrosine residues at the N-terminus of SLP-76,

explaining why they are detected in LAT immunoprecipitations (Su et al., 1999; Tuosto et al., 1996; Wu et al., 1996).

Mice with targeted disruption of SLP-76 have a phenotype very similar to that of LAT-deficient mice, a profound block in thymic development with the absence of peripheral T cells (Clements et al., 1998; Pivniouk et al., 1998). Not suprisingly, the recently described Gads-deficient mice also have a similar, although less severe, phenotype (Yoder et al., 2001). This is most likely due to a partial compensation by Grb2 in the absence of Gads. A Jurkat mutant cell line lacking SLP-76 displays a phenotype similar to J.CaM2 cells, yet somewhat less drastic. These cells, named J14, have decreases in Ca^{2+} flux and Ras activation in response to TCR stimulation (Yablonski et al., 1998). These findings suggest that SLP-76 plays a critical role in LAT-dependent signal transduction. Indeed, overexpression of a LAT SLP-76 chimera was recently shown to substitute for LAT function (Boerth et al., 2000).

The multiple protein interactions that have been demonstrated with LAT led us to further examine the role of different tyrosine residues and the activities that they mediate. Human LAT has ten total tyrosines, nine of which are conserved between human, mouse and rat. Previous studies have shown the importance of some of these residues for recruitment of PLC-γ1, Grb2 and Gads (Liu et al., 1999; Zhang et al., 2000). In this study, we expand on the analysis of these and other tyrosine residues by identifying the minimal tyrosine residues required for LAT function. Utilizing the LAT deficient J.CaM2 cell line, multiple stable lines expressing various tyrosine mutants of LAT were generated and their abilities to mediate activation of downstream pathways were studied. Ca^{2+} mobilization is dependent on three of the tyrosines ($Tyr^{132,171,191}$), while full Ras

pathway activation requires two additional tyrosine residues ($Tyr^{110,226}$). These two additional tyrosines contribute to the recruitment of Grb2 to the LAT complex. In addition, the critical tyrosine residues must also be on the same molecule of LAT for reconstitution of TCR mediated signaling events. Therefore, these data provide evidence for the important function of an assembly of proteins on a single LAT molecule. Moreover, these studies show that the tyrosine residues have different roles in mediating events downstream of LAT.

Results

Generation of Stable LAT Mutant Lines from LAT-deficient J.CaM2 Cells

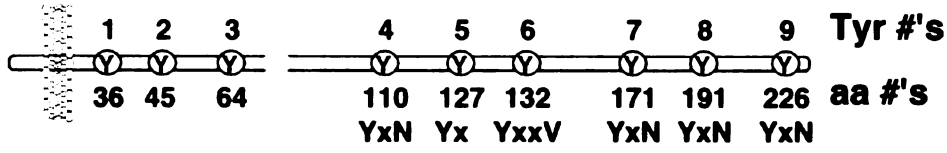
The LAT-deficient Jurkat-derived cell line J.CaM2 was used to produce cell lines stably expressing various tyrosine mutants of LAT. To aid in the description of the various tyrosine mutants of LAT, each of the ten tyrosines was given a number. Figure 3.1A depicts the location of the tyrosine residues in human LAT by amino acid numbers (a.a.#'s). The fourth tyrosine in human LAT was designated site #3.5 since it is not conserved between species. Throughout this paper, Y-F denotes mutant forms of LAT where tyrosine residues were changed to phenylalanine, whereas F-Y denotes mutants in which all tyrosines were initially changed to phenylalanine and then the specified residue mutated back to tyrosine. For example, F-Y#6 describes a mutant of LAT that has only one tyrosine at position 132.

Previous studies have identified multiple tyrosines within LAT that are phosphorylated and function as SH2 binding sites. Site #6 (YLVV) was shown to be important in the recruitment of PLC-γ1 to LAT (Zhang et al., 2000), while site #7 and site #8 are identical motifs (YVNV) to which either of the adaptor proteins Gads or Grb2 can bind and thereby recruit SLP-76 or Sos, respectively (Liu et al., 1999; Zhang et al., 1998a). Also conserved between human, mouse and rat are two more YxN motifs found at site #4 and site #9 that could play roles in Grb2 recruitment.

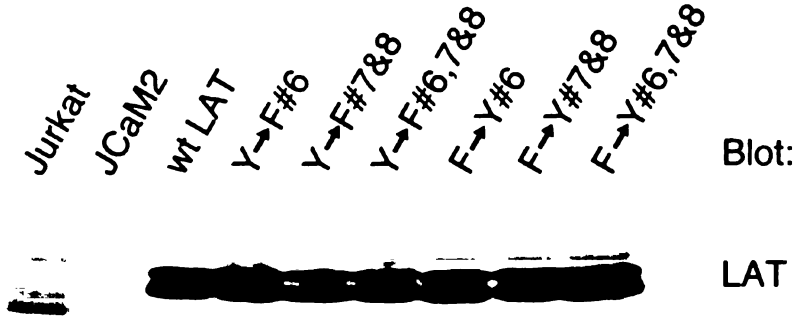
All of the tyrosine mutants of LAT contain a C-terminal myc-tag and were expressed using a vector containing the hEF-1a promoter. The cDNA constructs were electroporated into J.CaM2 cells and clones with stable integration were selected for in G418. For each mutant, multiple clones were tested for LAT expression levels by

Figure 3.1 LAT expression in cell lines with various tyrosine mutants. (A) Schematic representation of LAT depicting the location of the ten total tyrosine residues of which only nine are conserved between human and mouse. The tyrosine numbers (Tyr #'s) are used throughout this paper to indicate which residues have been mutated. Tyrosines that fit consensus SH2 binding motifs are shown. (B) Stable clones were generated by electroporation followed by selection for plasmid integration with G418. LAT expression was analyzed by western blotting of $1x10^6$ cells lysed in 1% NP-40. The mobility shift between endogenous LAT in Jurkat and transfected LAT is due to a C-terminal myc tag fused to LAT.

A


Human LAT
1 2 3 4 5 6 7 8 9
Tyr #'s
36 45 64 110 127 132 171 191 226
aa #'s
YxN Yx YxxV YxN YxN YxN

B

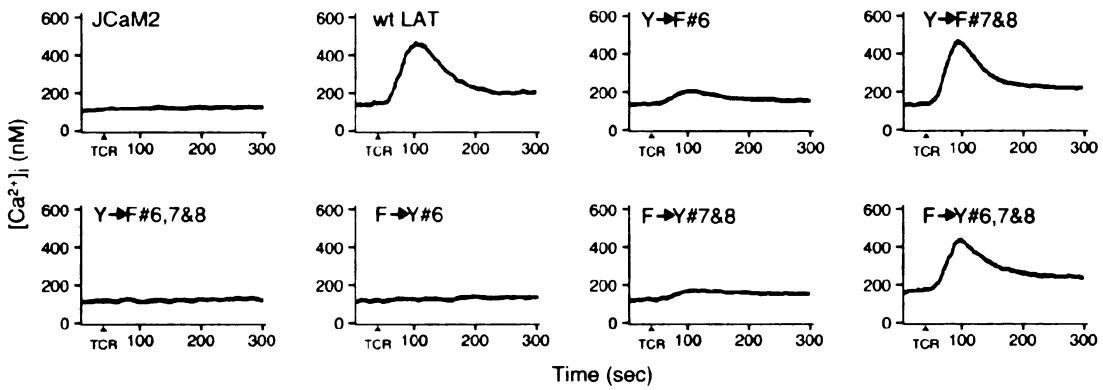

Jurkat
JCaM2
wt LAT
Y→F#6
Y→F#7&8
Y→F#6,7&8
F→Y#6
F→Y#7&8
F→Y#6,7&8
Blot:
LAT

western blotting. Representative clones are presented here. Figure 3.1B shows that the clones presented in these studies had equivalent LAT expression. The levels of LAT in the transfectants were slightly higher than that of endogenous LAT in Jurkat. Uniform expression of the TCR was documented by staining for CD3 followed by analysis using flow cytometry (data not shown).

Ca^{2+} Mobilization in Various Tyrosine Mutants of LAT

Previous studies have demonstrated that the inability of J.CaM2 cells to flux Ca^{2+} is due to its deficiency in LAT expression (Finco et al., 1998). To determine which tyrosine residues are required for a Ca^{2+} flux, cell lines expressing the tyrosine mutants of LAT were stimulated via the TCR and the subsequent Ca^{2+} flux measured on a fluorescence spectrophotometer. The Y-F#6 cell line had a markedly decreased Ca^{2+} flux while Y-F#7&8 had a normal response when compared to wt LAT (Figure 3.2A). However, Y-F#6,7&8 had a complete loss in its ability to flux Ca^{2+} in response to TCR stimulation. These results implicate sites #6,7&8 in mediating the Ca^{2+} response. To further extend this analysis, cells expressing forms of LAT containing only certain tyrosine residues on an all phenylalanine background were tested in the same manner. The F-Y#6 version of LAT did not reconstitute Ca^{2+} signaling and the F-Y#7&8 version of LAT only restored a slight, almost undetectable flux. However, the F-Y#6,7&8 form of LAT reconstituted the Ca^{2+} flux following TCR stimulation to the same extent as wt LAT (Figure 3.2A). For these experiments, multiple clones of all mutants gave similar results. These data demonstrate that sites #6,7&8 are necessary and sufficient for Ca^{2+} mobilization in the LAT deficient J.CaM2 cell line. A potential explanation could come

Figure 3.2 Ca^{2+} flux in response to TCR stimulation in various mutants of LAT. The indicated stable cell lines were loaded with Indo-1 and stimulated with anti-TCR mAb after 30 sec. Fluorescence at 400- and 500-nm wavelengths was measured after excitation at 350-nm to determine the intracellular free calcium concentration. Ca^{2+} flux data are representative of multiple experiments using various clones with the same mutations.

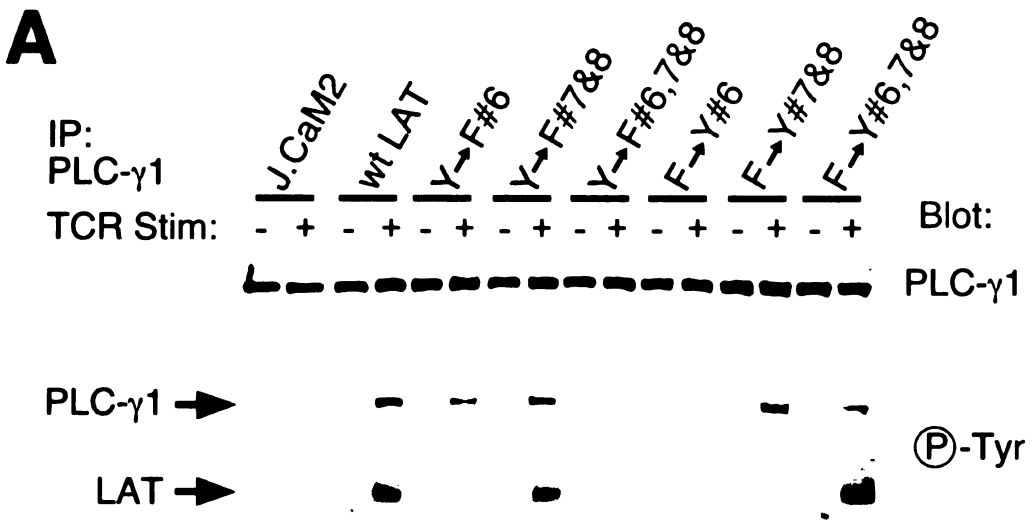

JCaM2
wt LAT
Y→F#6
Y→F#7&8
Y→F#6,7&8
F→Y#6
F→Y#7&8
F→Y#6,7&8
600
400
200
0
TCR
100
200
300
[Ca2+]i (nM)
Time (sec)

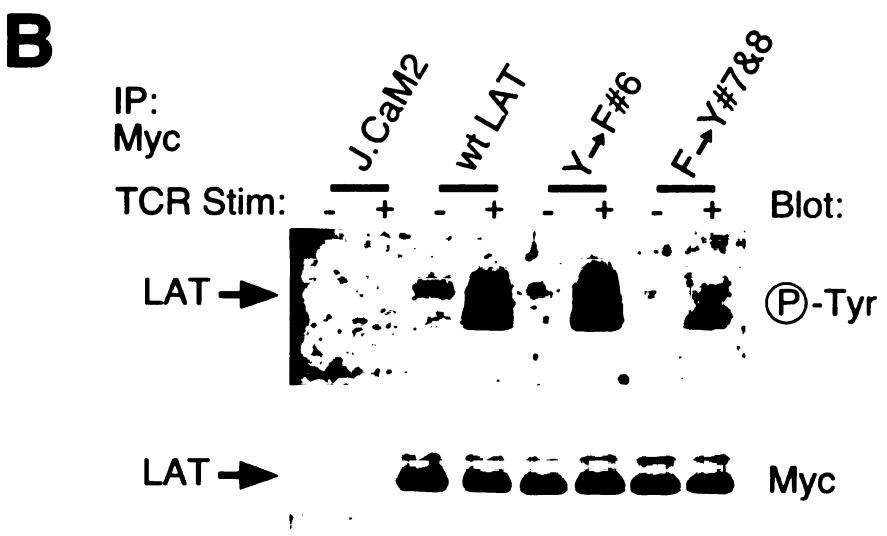
from previous studies that have shown that site #6 binds to PLC-γ1 and sites #7&8 bind Gads, thereby indirectly recruiting SLP-76 to LAT. This suggests that a LAT, PLC-γ1, Gads, SLP-76 complex may be necessary and sufficient for Ca^{2+} mobilization.

PLC-γ1 phosphorylation and recruitment to LAT

Since defective Ca^{2+} mobilization was observed with Y-F#6, F-Y#6 and F-Y#7&8, we decided to investigate PLC-γ1 phosphorylation in these cell lines, since PLC-γ1 is responsible for generating the IP_3 required for Ca^{2+} mobilization. Cells were stimulated with anti-TCR and PLC-γ1 immunoprecipitations were performed from cellular lysates and the samples separated by SDS PAGE. Western blotting with an anti-phosphotyrosine antibody indicated that PLC-γ1 was phosphorylated in J.CaM2 cells reconstituted with wt LAT, Y-F#6, Y-F#7&8, F-Y#7&8 and F-Y#6,7&8 (Figure 3.3A middle panel). Thus, the phosphorylation state of PLC-γ1 does not exactly correlate with the mobilization of intracellular Ca^{2+}. In support of this observation, phosphorylation-independent mechanisms of PLC-γ1 activation have been noted previously in the literature (Sekiya et al., 1999). Moreover, our analysis fails to distinguish among the various reported sites of PLC-γ1 tyrosine phosphorylation (Kim et al., 2000). When the association of PLC-γ1 with phospho-LAT was examined, a direct correlation was observed between association and Ca^{2+} flux in response to TCR stimulation (Figure 3.3A lower panel). Due to the poor ability of the LAT antibody to recognize phosphorylated LAT, we were not able to show inducible association of LAT with PLC-γ1. However, a phosphorylated band that co-migrates with LAT can be detected by phosphotyrosine blotting. Samples were also blotted with an anti-PLC-γ1 antibody to show equal protein

Figure 3.3 Association of the various LAT mutants with PLC-γ1. (A) $2x10^7$ of the indicated stable cell lines were either left unstimulated or stimulated with anti-TCR mAb for 2 min. Cells were lysed in 1% Brij and PLC-γ1 immunoprecipitated (IP) from lysates. Samples were separated by SDS PAGE and bands visualized by western blotting with anti-PLC-γ1 and anti-phosphotyrosine antibodies. (B) The phosphorylation state of wt LAT, Y-F6, and F-Y7,8 were studied by stimulating cells with anti-TCR mAb or phosphate-buffered saline as a control for 2 min. Samples were lysed, IP with anti-myc, and then separated via SDS-PAGE. Phosphorylated LAT was detected by blotting with an anti-phosphotyrosine mAb. Samples were also blotted with anti-Myc to show equal protein expression.


A
IP:
PLC-γ1
J.CaM2
wt LAT
Y→F#6
Y→F#7&8
Y→F#6,7&8
F→Y#6
F→Y#7&8
F→Y#6,7&8
TCR Stim: - + - + - + - + - + - + - + - +
Blot:
PLC-γ1
PLC-γ1
LAT
Ⓟ-Tyr


B
IP:
Myc
J.CaM2
wt LAT
Y→F#6
F→Y#7&8
TCR Stim: - + - + - + - +
Blot:
LAT
Ⓟ-Tyr
LAT
Myc

levels in the immunoprecipitates (Figure 3.3A upper panel). To ensure that the lack of a phospho-LAT band in the PLC-γ1 immunoprecipitates is not due to an inability of these mutants of LAT to become phosphorylated, an additional study was done. Anti-myc immunoprecipitates were performed on the LAT mutant lines in which an interaction of PLC-γ1 with phospho-LAT could not be detected, but still exhibited PLC-γ1 phosphorylation upon stimulation. Immunoprecipitates from unstimulated or TCR-stimulated cells reconstituted with wt LAT, Y-F#6 and F-Y#7&8 were separated by SDS PAGE followed by blotting with an anti-phosphotyrosine antibody. Figure 3.3B shows that the phosphorylation state of the Y-F#6 mutant was very similar to that of wt LAT and that the F-Y#7&8 mutant was still phosphorylated, though at a reduced level. These data suggest that phosphorylation of PLC-γ1 alone is not sufficient to elicit a Ca^{2+} flux. Mobilization of intracellular Ca^{2+} following TCR stimulation also requires PLC-γ1 recruitment to LAT.

Tyrosines in LAT Required for Reconstitution of Erk Phosphorylation

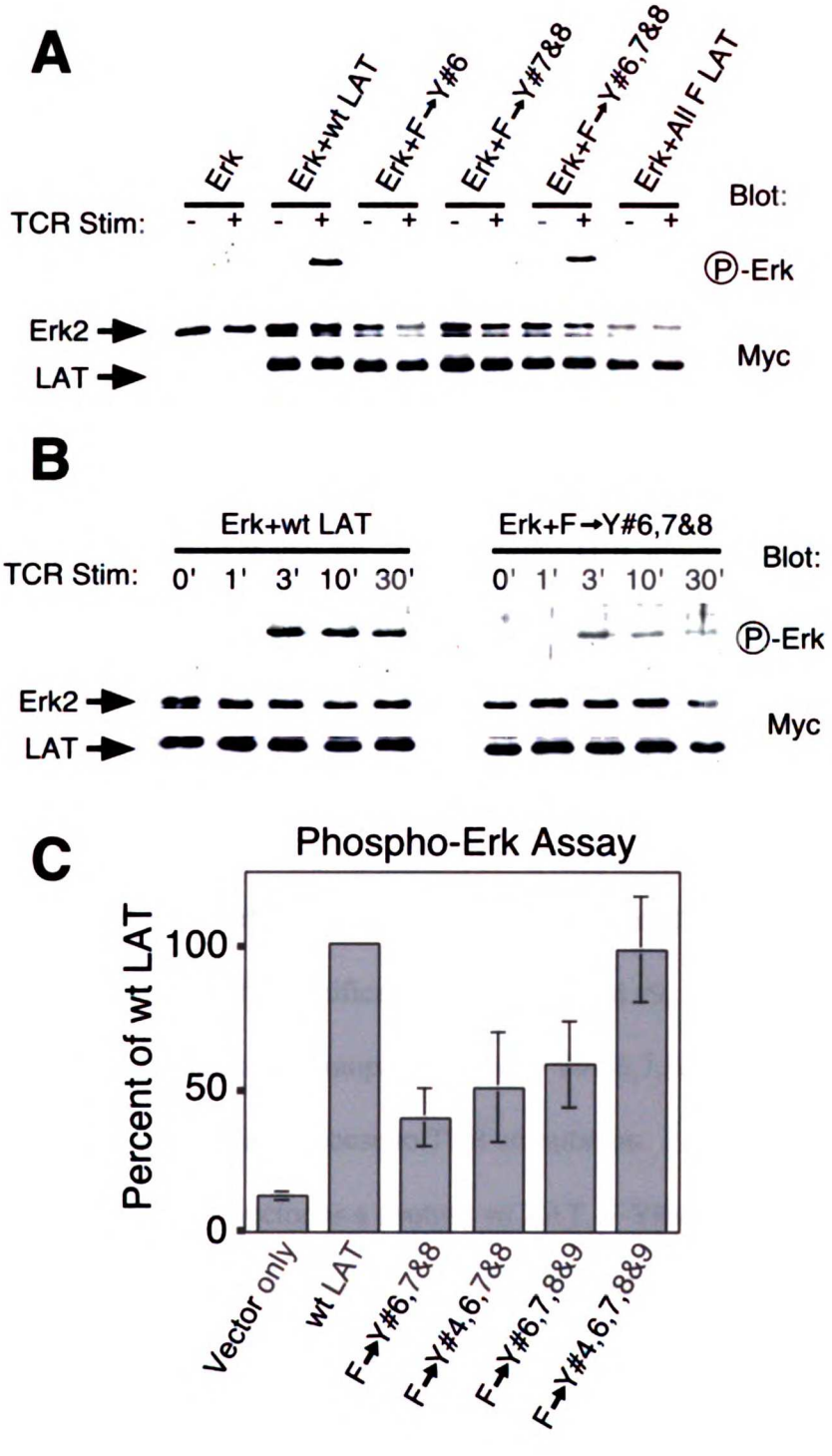
Since the F-Y#6,7&8 form of LAT was sufficient to reconstitute the mobilization of intracellular Ca^{2+} following stimulation through the TCR, we were interested to determine if any or all of these three sites could reconstitute other signaling pathways in J.CaM2. Previous studies performed with J.CaM2 have demonstrated a deficiency in the activation of the Ras pathway. One downstream indicator of this pathway is activation of the Erk MAP kinase. To investigate which of the tyrosines in LAT are sufficient for the activation of Erk, J.CaM2 cells were cotransfected with a myc-tagged Erk2 construct and various LAT constructs that had sites #6, #7&8 and #6,7&8 restored to tyrosines from an

all phenylalanine background. As controls, wt LAT and LAT with no tyrosines ("all F") were used. Cells were then stimulated through the TCR followed by immunoprecipitation with an anti-myc antibody to isolate the transfected proteins. An indicator of Erk kinase activity is phosphorylation on Thr #202 and Tyr #204, which can be followed with phospho-specific Erk western blots.

When J.CaM2 cells were transfected with Erk and a vector control or the "all F" LAT that has no tyrosine residues, Erk did not become activated following TCR stimulation (Figure 3.4A). If the cells were cotransfected with a wt LAT construct, Erk was phosphorylated following TCR stimulation. Neither the F-Y#6 mutant nor the F-Y#7&8 mutant form of LAT could reconstitute Erk phosphorylation in J.CaM2 cells. However, an F-Y#6,7&8 form of LAT could partially reconstitute the defective signaling pathway, demonstrating the importance of all three of these sites (Figure 3.4A). For reasons unknown, mutants of LAT that have most or all of the tyrosine residues changed to phenylalanine reproducibly have a slightly faster mobility. One possible explanation is that the lost hydroxyl groups on the tyrosine residues creates a protein that is now more hydrophobic, resulting in a change in mobility.

A time course of Erk activation was then performed to determine if the partial response at 3 min, is due to a shift in kinetics or a decrease in the maximum response. J.CaM2 cells cotransfected with Erk2 and either wt LAT or F-Y#6,7&8 were stimulated with anti-TCR mAb for 0, 1, 3, 10 or 30 min. The phosphorylation states of Erk at the different time points were compared. The results show that the partial reconstitution of the Erk pathway is due to a decreased magnitude in the response, rather than a shift in kinetics (Figure 3.4B).

Figure 3.4 Reconstitution of the TCR-mediated Erk2 phosphorylation. (A) $4x10^7$ J.CaM2 cells were transfected with 20 μg myc-tagged Erk2 along with 10 μg total of the indicated LAT mutants. 24 h after transfection, cells were either stimulated with anti-TCR mAb or left unstimulated for 3 min. and then lysed. Immunoprecipitations with anti-myc were done on lysates and phosphorylated Erk2 was detected by western blotting. (B) Stimulation time course was performed on transfected J.CaM2 cells. Cells were either left unstimulated or stimulated for 1, 3, 10, and 30 min. Phosphorylation status of Erk2 was detected by western blotting with a phospho-Erk specific antibody. Samples were blotted with anti-myc to show equal protein expression. (C) J.CaM2 cells were transfected with LAT constructs with the indicated tyrosines added back. Net intensity of the phosphorylated Erk2 band from western blots was quantitated with a Kodak imaging station for the various transfections. The data are plotted as the percent activation of phospho-Erk compared to wt LAT. Shown is the average and standard error of three independent experiments.

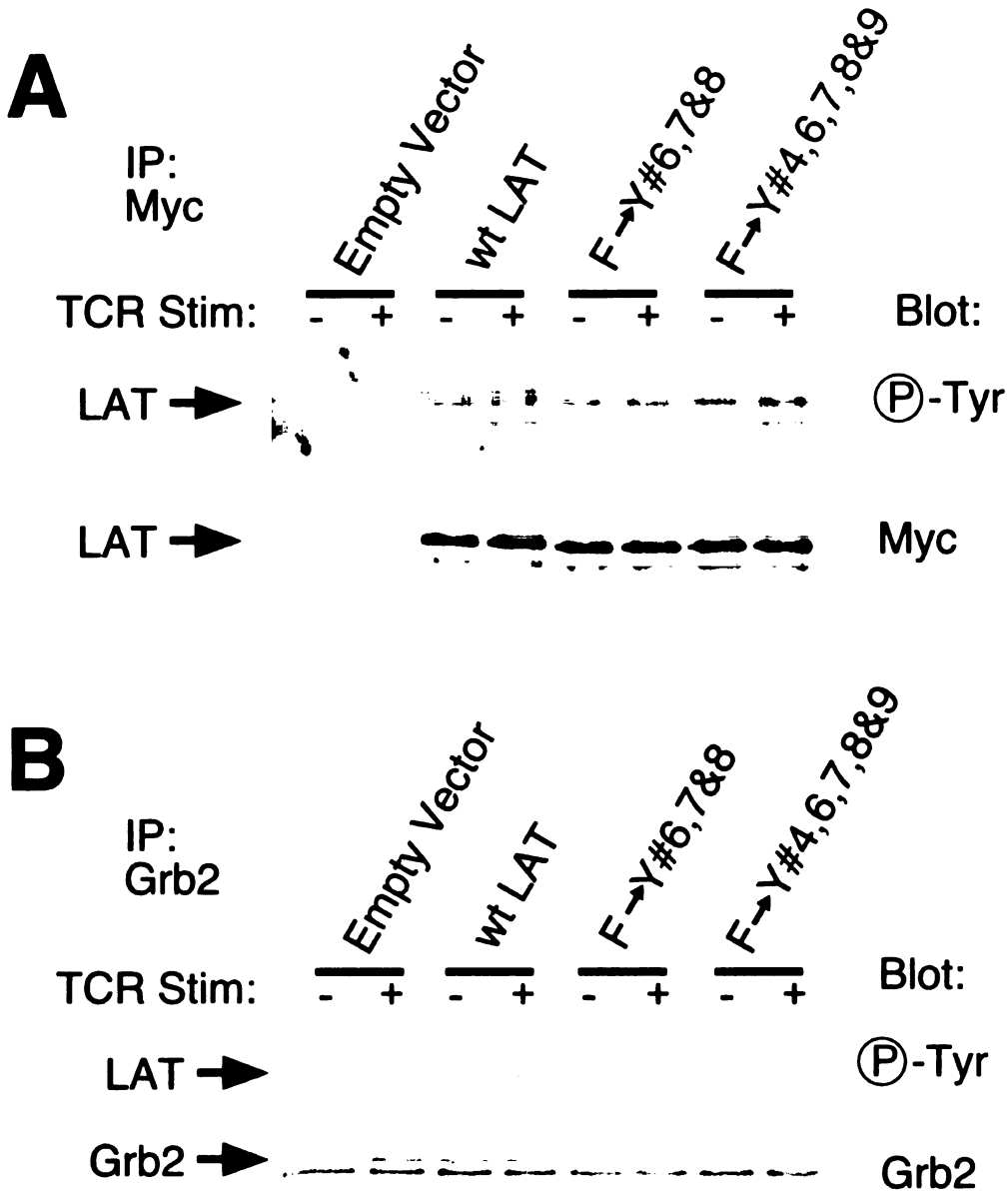

A
Erk
Erk+wt LAT
Erk+F→Y#6
Erk+F→Y#7&8
Erk+F→Y#6,7&8
Erk+All F LAT
TCR Stim:
- + - + - + - + - + - +
Blot:
Ⓟ-Erk
Erk2
LAT
Myc
B
Erk+wt LAT
Erk+F→Y#6,7&8
TCR Stim:
0' 1' 3' 10' 30'
0' 1' 3' 10' 30'
Blot:
Ⓟ-Erk
Erk2
LAT
Myc
C
Phospho-Erk Assay
Percent of wt LAT
100
50
0
Vector only
wt LAT
F→Y#6,7&8
F→Y#4,6,7&8
F→Y#6,7,8&9
F→Y#4,6,7,8&9

To further identify tyrosine residues that may play a role in Erk pathway activation, other forms of LAT that contained additional tyrosines added back were tested. From the amino acid sequence, there are 2 more potential Grb2 binding motifs, sites #4 and #9, that are fully conserved between human, mouse and rat. The LAT/Grb2 interaction could function to recruit Sos to the membrane and therefore also contribute to Ras activation, which is upstream of the MAP kinases. Tyrosine at sites #4 and #9 were added alone or together with sites #6,7&8 to determine if they contribute to activation of the Erk pathway. Addition of either site #4 or #9 to F-Y#6,7&8 only slightly increased the phosphorylation of Erk over sites #6,7&8 following TCR stimulation (Figure 3.4C). However, F-Y#4,6,7,8&9 could fully reconstitute the Erk deficiency found in J.CaM2, demonstrating that the additional potential Grb2 binding sites are important for the activation of Erk.

Phosphorylation and Association of LAT Mutants with Grb2

To further investigate the significance of sites #4 and #9, the phosphorylation state of the F-Y#6,7&8 mutant was compared to the F-Y#4,6,7,8&9 mutant to determine if the sites are phosphorylated in response to TCR stimulation. J.CaM2 cells were transfected with either empty vector as a control, wt LAT, F-Y#6,7&8 or F-Y#4,6,7,8&9. Cells were then stimulated with anti-TCR mAb and the transfected proteins immunoprecipitated with anti-myc. As demonstrated in Figure 3.5A, the F-Y#4,6,7,8&9 mutant is phosphorylated to a greater extent when compared to the F-Y#6,7&8 mutant, indicating that either sites #4 or #9 or both are indeed phosphorylated upon TCR stimulation.

Figure 3.5 Phosphorylation and association of LAT mutants with Grb2. (A) $4x10^7$ J.CaM2 cells were transfected with 10 μg of the indicated LAT mutants. 24 h after transfection, cells were either stimulated with anti-TCR mAb or left unstimulated for 2 min. and then lysed. Immunoprecipitations with anti-myc were done on lysates and phosphorylated LAT was detected by western blotting. Samples were also blotted with anti-Myc to show equal expression. (B) 10^8 J.CaM2 cells were transfected with 25 μg of the indicated LAT constructs. Anti-Grb2 IPs were performed on the lysates. The phosphorylated LAT that co-IPed with Grb2 was detected by western blotting. Samples were also blotted with anti-Grb2 to show equal protein loading. The slightly faster mobility band on the Grb2 blot is light chain as determined with a blocking peptide to the Grb2 antibody.


A
IP:
Myc
Empty Vector
wt LAT
F→Y#6,7&8
F→Y#4,6,7,8&9
TCR Stim:
- + - + - + - +
Blot:
LAT
Ⓟ-Tyr
LAT
Myc
B
IP:
Grb2
Empty Vector
wt LAT
F→Y#6,7&8
F→Y#4,6,7,8&9
TCR Stim:
- + - + - + - +
Blot:
LAT
Ⓟ-Tyr
Grb2
Grb2

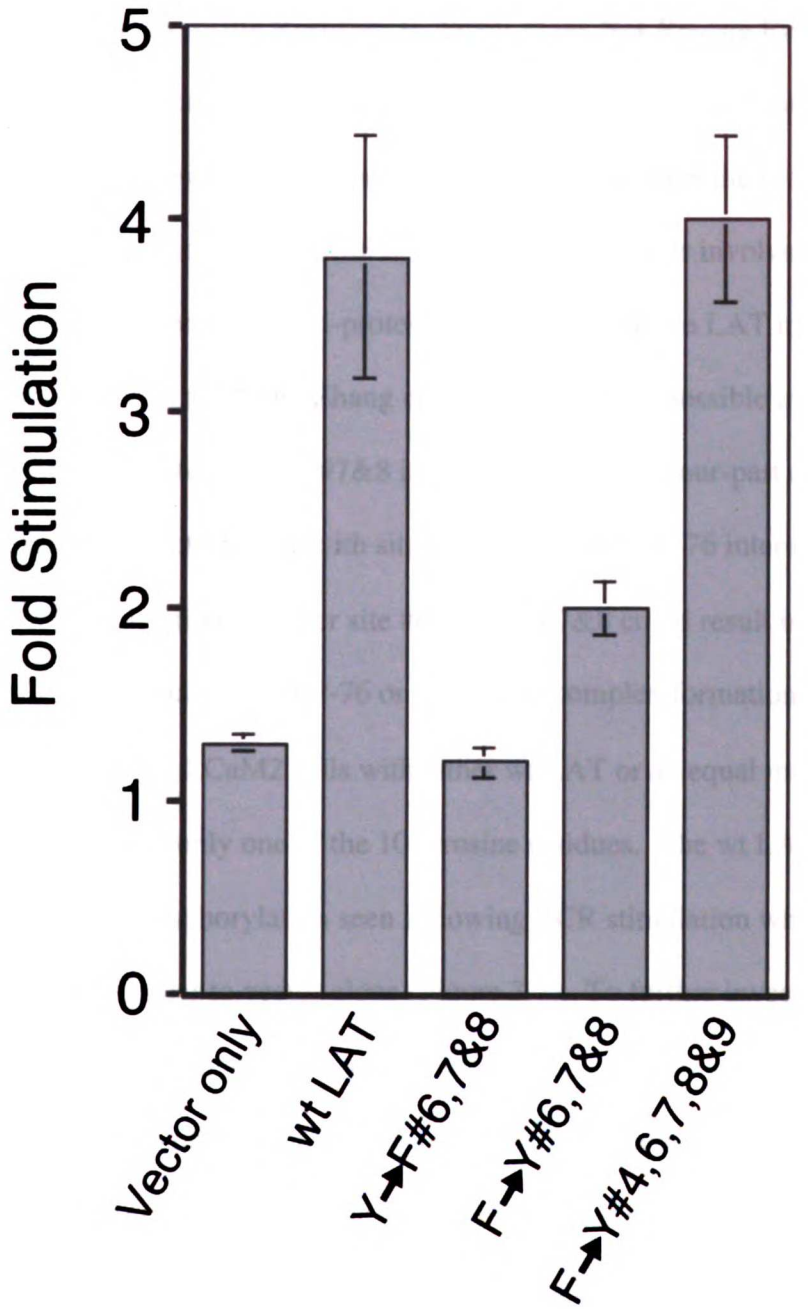
To explore the possible mechanism by which site #4 and #9 function to increase Erk phosphorylation, coimmunoprecipitation studies were carried out. Since both sites #4 and #9 are Grb2 consensus binding motifs (YxN), Grb2 immunoprecipitates were performed to determine if the addition of sites #4 and #9 could enhance phospho-LAT recruitment to Grb2. Grb2 immunoprecipitates from cells transfected with wt LAT resulted in phospho-LAT interaction with Grb2, whereas cells transfected with the F-Y#6,7&8 mutant resulted in a reduced interaction (Figure 3.5B). It is, however, important to keep in mind that the phosphorylation state of the F-Y#6,7&8 mutant is lower than that of wt LAT. When sites #4 and #9 are added to the F-Y#6,7&8 mutant, a greater amount of phospho-LAT is seen coimmunoprecipitating with Grb2. These data demonstrate that sites #4 and #9 function to enhance the recruitment of Grb2 to LAT.

Sites #4,6,7,8&9 Reconstitutes NF-AT Activation to wt LAT Levels

As a functional readout for TCR stimulation, transcriptional reporter studies were performed with a construct that contains composite binding sites for AP-1 and the nuclear factor of activated T cells (NF-AT). A 3x NF-AT-luciferase reporter was co-transfected with the various LAT mutants into J.CaM2 cells. Reconstitution of J.CaM2 with wt LAT resulted in approximately a 4-fold activation of NF-AT over unstimulated cells, whereas the Y-F#6,7&8 mutant was equivalent to that of a vector control (Figure 3.6). The F-Y#6,7&8 only resulted in a partial reconstitution, whereas the F-Y#4,6,7,8&9 restored the response comparably to that of wt LAT. Since the reporter is composed of both AP-1 and NF-AT sites, a deficiency in the function of either transcription factor would lead to failure to induce luciferase expression. Thus, the NF-AT results are similar to those seen

Figure 3.6 NFAT activation in cells expressing mutants of LAT. 20 μg NF-AT-luciferase reporter construct was co-transfected with 1 μg of the various LAT mutants into J.CaM2 cells. $1x10^5$ cells were either unstimulated or stimulated with plate bound (anti-TCR mAb (C305). 8 hrs later, cells were lysed and assayed for luciferase activity. The graph represents the fold activation induced by TCR stimulation over unstimulated. Data are derived from three independent experiments each done in duplicate.

NF-AT Luciferase Assay

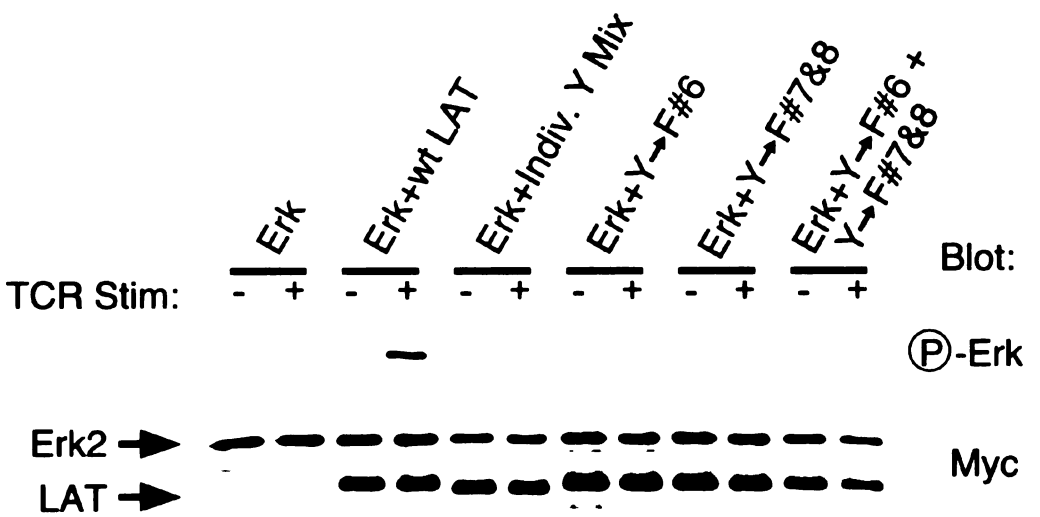


in Erk activation where the F-Y#6,7&8 provides only partial rescue whereas the F-Y#4,6,7,8&9 essentially reconstitutes wt LAT function.

Reconstitution of the Tyrosine Residues in Trans Does Not Rescue Erk Signaling

Since LAT can recruit several molecules to GEMs within the plasma membrane, an interesting question is whether this recruitment simply localizes the proteins to a discrete location within the plasma membrane, or whether LAT is involved in the formation and coordination of a multi-protein complex on a single LAT molecule at the plasma membrane (Lin et al., 1999; Zhang et al., 1998b)? One possible interpretation of the data concerning site #6 and sites #7&8 is the formation of a four-part complex including the interaction of PLC-γ1 with site #6 and a Gads/SLP-76 interaction with sites #7 or #8. Therefore, deletion of either site #6 or sites #7&8 could result in less efficient recruitment of PLC-γ1 and Gads/SLP-76 or less stable complex formation. To test this hypothesis, we transected J.CaM2 cells with either wt LAT or an equal mix of LAT constructs that each have only one of the 10 tyrosine residues. The wt LAT construct reconstituted the Erk phosphorylation seen following TCR stimulation while the mix of LAT constructs was similar to vector alone (Figure 3.7). To further investigate the potential cooperative binding between site #6 and #7&8, site #6 and site #7&8 were mutated from tyrosine to phenylalanine and were transfected alone or together in equal amounts. Similar to previous results, the Y-F#6 and the Y-F#7&8 did not reconstitute the Erk pathway (Zhang et al., 2000). Interestingly, an equal mix of the two constructs also did not substantially reconstitute Erk phosphorylation when compared to wt LAT. These data demonstrate that at least sites #6,7&8 must be on the same molecule of LAT for

Figure 3.7 Reconstitution of Erk2 phosphorylation in J.CaM2 does not work in trans. $4x10^7$ J.CaM2 cells were transfected with 20 μg Myc-tagged Erk2 along with 10 μg total of the indicated LAT mutants. The individual tyrosine mix indicates 1 μg of ten constructs each containing only one of the ten total tyrosine residues and the Y-F#6 + Y-F#7&8 mixture contains 5 μg of each construct. Cells were either left unstimulated or stimulated for 3 min. followed by myc immunoprecipitations of the lysate. Phosphorylation status of Erk2 was detected by western blotting and myc western blots show equal protein expression.


Erk
Erk+wt LAT
Erk+Indiv. Y Mix
Erk+Y→F#6
Erk+Y→F#7&8
Erk+Y→F#6 + Y→F#7&8
TCR Stim:
- + - + - + - + - + - +
Blot:
Ⓟ-Erk
Erk2
LAT
Myc

reconstitution of function. The results support the idea that multiple domain interactions are required for the LAT complex to reconstitute the Erk signaling pathway.

Discussion

The critical role of the transmembrane adaptor protein LAT in mediating signals generated by TCR engagement has been demonstrated by many approaches. Experiments involving targeted gene disruption of LAT and characterization of Jurkat T cell mutants, which lack expression of LAT, have shed considerable light on LAT function in signaling (Finco et al., 1998; Zhang et al., 1998a; Zhang et al., 1999b). Following TCR stimulation, LAT rapidly becomes tyrosine phosphorylated at multiple residues, allowing for the recruitment of multiple signaling proteins to the plasma membrane. Localization of LAT to cholesterol-rich lipid microdomains within the plasma membrane, otherwise known as GEMs, has been demonstrated to be critical for function (Lin et al., 1999; Zhang et al., 1998b). However, the minimal tyrosine residues required for LAT function were unclear. Moreover, it was not determined whether a single LAT molecule can form a discrete signaling complex or whether many LAT molecules interacting with distinct complexes can function in trans.

To address these issues, we performed a structure/function analysis of LAT, with a focus on the different tyrosine residues. Our analysis of the tyrosine residues in LAT has led to some interesting findings. In the Y-F#6 and F-Y#7&8 reconstituted cells, PLC-γ1 phosphorylation following TCR stimulation was restored, yet a defect in Ca^{2+} flux was still detected. It is believed that Syk and Tec family kinases Itk and Rlk are involved in the phosphorylation of PLC-γ1 in T cells. Since Itk has been previously demonstrated to associate with the LAT complex, a simple model of activation of PLC-γ1 activity would involve recruitment of PLC-γ1 to LAT where Itk and Rlk are also recruited, possibly via a Gads/SLP-76 interaction (Ching et al., 2000; Shan and Wange,

1999; Su et al., 1999). It may also be possible that Tec family members could bind LAT directly since they contain SH2 domains. Alternatively, since Itk and PLC-γ1 both contain PH domains, which are important in membrane localization, PLC-γ1 could potentially become phosphorylated without recruitment to LAT. However, to activate PLC-γ1 enzymatic activity, perhaps localization to GEMs via an association to the LAT complex is required. Data presented in this study support this model since mutation of site #6 only partially reduced the association of PLC-γ1 with LAT and only a reduction of Ca^{2+} mobilization was observed. Our data differ with previously published data showing that mutation at site #6 resulted in the loss of PLC-γ1 phosphorylation and an increased magnitude, but more transient, Ca^{2+} mobilization following TCR stimulation (Zhang et al., 2000). Though it is difficult to reconcile these differences, in both cases, multiple clones of the same mutation were tested. One potential difference is the method of stimulation. In the studies reported here, the TCR was stimulated with relatively high concentrations of purified anti-TCR mAb (C305) whereas previous studies used relatively low concentrations of C305 mAb for biochemical studies and the anti-CD3 mAb OKT3 for Ca^{2+} flux studies. The decreased Ca^{2+} flux in response to C305 presented in this paper was also observed on a flow cytometer (FACS Calibur) with cells loaded with the Ca^{2+} indicator Fluo-3 (data not shown).

Our studies suggest a link, but not complete overlap, in the requirements for PLC-γ1 and Erk activation. Erk activation is likely to reflect Ras activation in T cells. Previous studies using transformed chicken B cells have suggested that PLC-γ2 plays a role in activating the Ras pathway. Loss of PLC-γ2 expression or mutations that abrogate PLC-γ2 activity prevent Ras activation (Takata et al., 1995). Recent studies have

demonstrated that overexpression of another exchange factor for Ras, RasGRP, can enhance TCR-Ras-Erk signaling and increase IL-2 production in Jurkat cells (Ebinu et al., 2000). Some of the most convincing data come from targeted gene disruption of RasGRP in mice. Thymocytes from these mice fail to activate the Ras pathway following CD3 stimulation (Dower et al., 2000). Since RasGRP has both Ca^{2+} binding EF hands and a DAG-binding C1 domain, it is believed that RasGRP is dependent on PLC-γ1 activity, since PLC-γ1 is responsible for Ca^{2+} release and DAG production. Indeed, pharmacologic inhibitors of PLC-γ1 diminished activation of Ras following TCR stimulation (Ebinu et al., 2000). Another possible mechanism for PLC-γ1 regulation of Ras activation is for PLC-γ1 to directly recruit Sos to the LAT complex, independent of Grb2. Such an interaction has been reported to be constitutive and dependent on the SH3 domain of PLC-γ1 (Kim et al., 2000; Scholler et al., 2000). Kim and colleagues also showed that expression of a lipase-inactive PLC-γ1 could potentiate Ras activation; however, these studies were carried out in non-lymphoid cells (Kim et al., 2000).

Full reconstitution of Erk is dependent on sites #4,6,7,8&9 within LAT. Only partial reconstitution of the Erk activation was seen with F-Y#6,7&8. Our calcium mobilization data suggest that sites #6,7&8 are required and sufficient for full PLC-γ1 activation. The addition of tyrosines at sites #4 and #9 functions to recruit more molecules of Grb2 to the membrane. Since Grb2 is known to be coupled with the exchange factor Sos, this recruitment to the membrane may result in further activation of Ras. Sites #7&8 have been shown to not only bind Gads, but Grb2 as well. This could explain the partial Erk activation seen in the F-Y#6,7&8. These data demonstrate that sites #6,7&8, which are sufficient for PLC-γ1 activation, are not sufficient for full Erk

activation. Presumably, an additive effect is needed, requiring all of the conserved Grb2 consensus sites for full Erk activation.

The importance of having sites #6 and #7&8 on the same molecule of LAT supports the idea that LAT is involved in nucleating a multi-molecular signaling complex (Figure 3.7). Potential interactions among multiple domains may increase the stability of the complex. Data supporting a direct PLC-γ1 interaction with SLP-76 has recently been obtained (Tomlinson et al., 2000). In that study, mutants of SLP-76 were used to reconstitute the SLP-76 deficient Jurkat derivative J14. A proline rich region of SLP-76 is required for reconstitution of NF-AT activation and was shown to bind to the SH3 domain of PLC-γ1. Furthermore, a GST-SH3 domain of PLC-γ1 could directly interact with SLP-76. Interestingly, tyrosine to phenylalanine mutations at site #7&8 decreased PLC-γ1 association in one study even though subsequent experiments have suggested that site #6 is primarily responsible for PLC-γ1 association (Zhang et al., 1998a; Zhang et al., 2000).

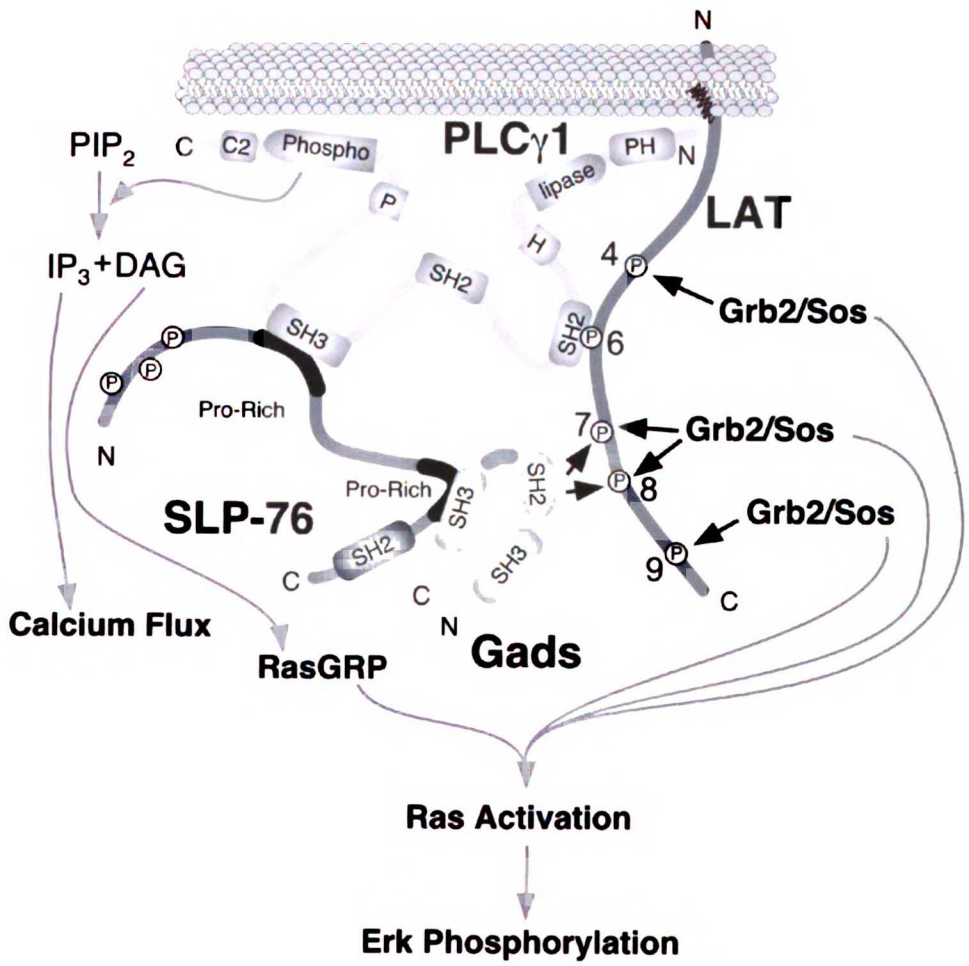
Other recent evidence also supports the notion that there is a direct interaction between PLC-γ1 and SLP-76. Boerth and colleagues demonstrated that targeting of SLP-76 to the plasma membrane was sufficient to reconstitute LAT deficient J.CaM2 cells (Boerth et al., 2000). In this report, a chimeric molecule was constructed containing the transmembrane domain of LAT attached to SLP-76 in order to localize it to the GEMs. When this chimeric protein was expressed in J.CaM2 cells, PLC-γ1 became phosphorylated following TCR stimulation. Though the phosphorylation state does not necessarily demonstrate activation of PLC-γ1's enzymatic activity, this chimeric molecule was also able to rescue NF-AT activation, implying that mobilization of

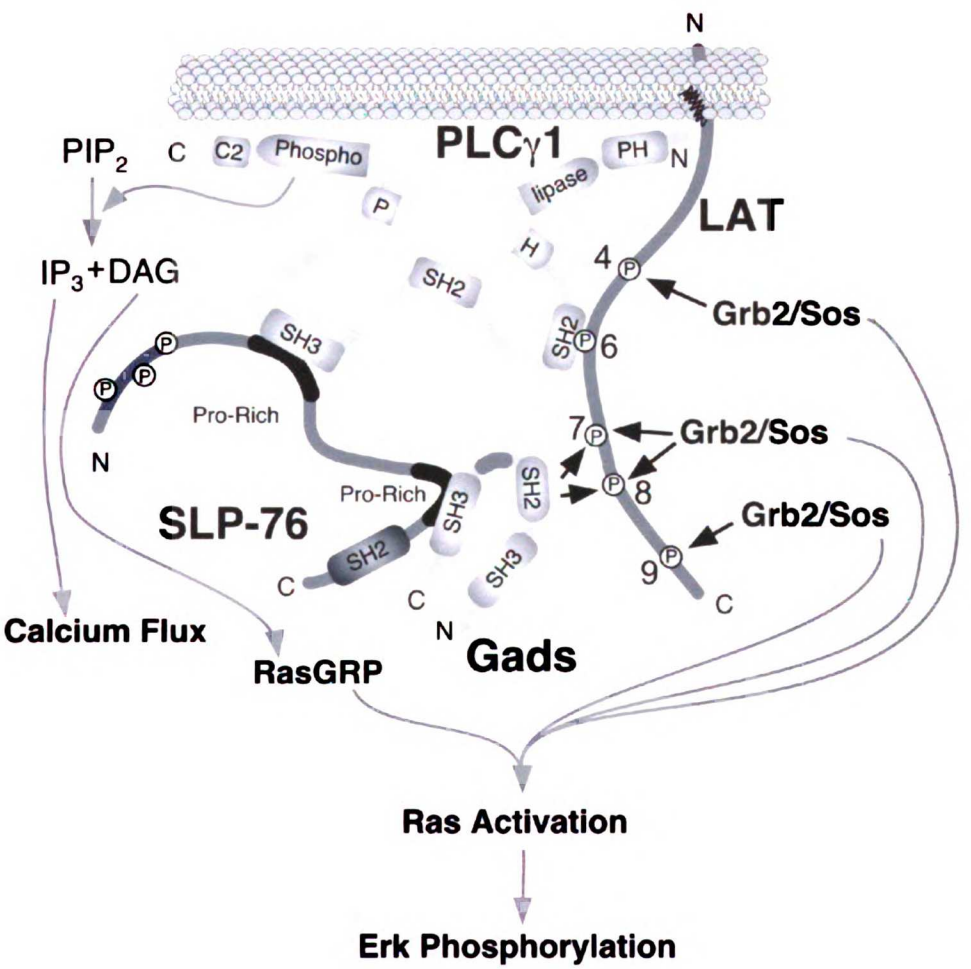
intracellular Ca^{2+} took place. One possible interpretation of this observation is that SLP-76 recruited PLC-γ1 to GEMs independent of LAT's tyrosine residues in this chimeric molecular system. Collectively, the data here and those of other studies suggest that PLC-γ1 simultaneously interacts with SLP-76 and LAT, which stabilizes a LAT/Gads/SLP-76/PLC-γ1 signaling complex (Figure 3.8).

The adaptor protein Gads, which links SLP-76 to LAT, is also important in generating a Ca^{2+} flux in response to receptor stimulation. Ishiai and colleagues examined the role of T cell-specific adaptor proteins in a B cell system (Ishiai et al., 2000). The adaptor protein B cell linker (BLNK) is thought to perform the functions of both SLP-76 and LAT in B cells because it has been demonstrated to recruit PLC-γ2, along with members of the Tec family, to the plasma membrane (Fu et al., 1998). BLNK contains multiple sites of tyrosine phosphorylation similar to LAT, yet it also possesses a C-terminal SH2 domain similar to SLP-76. When BLNK-deficient cells were reconstituted with LAT, SLP-76 or both, the cells still failed to mobilize Ca^{2+}. However, when Gads was expressed with both LAT and SLP-76, this fully reconstituted the Ca^{2+} flux in response to BCR stimulation (Ishiai et al., 2000). Although these studies involve PLC-γ2, previous data from DeBell had demonstrated that PLC-γ1 could functionally reconstitute a PLC-γ2 deficient DT40 B cell line (DeBell et al., 1999).

The data presented here show that distinct tyrosine residues in LAT are involved in different pathways. We have shown that three specific tyrosine residues ($Tyr^{132,171,191}$) are necessary and sufficient to achieve a Ca^{2+} flux following TCR stimulation. However, these same tyrosines can only partially reconstitute Erk activation. Complete reconstitution of Erk requires two additional tyrosine residues ($Tyr^{110,226}$), both of which

Figure 3.8 A model for how LAT can function to promote Ca^{2+} flux and Ras pathway activation. LAT is known to recruit both PLC-γ1 and the SLP-76/Gads complex. This model depicts a 4-part complex resulting in an increased avidity. In LAT mutants that do not allow recruitment of both PLC-γ1 and SLP-76/Gads simultaneously, PLC-γ1 cannot associate as tightly with LAT and therefore not lead to optimal activation. PLC-γ1 activity leads to the production of IP_3 and DAG. IP_3 functions to promote the release of Ca^{2+} from internal stores while DAG is known to activate an exchange factor for Ras called RasGRP. This in cooperation with the recruitment of another exchange factor Sos, by Grb2, leads to full activation of the Ras pathway.


N
PIP2
C
C2
Phospho
PLCγ1
PH
N
lipase
P
H
LAT
IP3+DAG
SH2
4
Grb2/Sos
SH3
SH2
6
Pro-Rich
7
Grb2/Sos
N
Pro-Rich
SH3
SH2
8
SLP-76
Grb2/Sos
SH2
SH3
9
C
C
C
N
Gads
Calcium Flux
RasGRP
Ras Activation
Erk Phosphorylation


N
PIP2
C
C2
Phospho
PLCγ1
PH
N
lipase
P
H
LAT
IP3+DAG
SH2
4
Grb2/Sos
SH3
SH2
6
Pro-Rich
7
Grb2/Sos
N
Pro-Rich
SH3
SH2
8
SLP-76
Grb2/Sos
SH2
SH3
9
C
C
N
Gads
Calcium Flux
RasGRP
Ras Activation
Erk Phosphorylation

have the Grb2-binding motif YxN. The addition of these two sites, ($Tyr^{110,226}$), results in increased association of phospho-LAT with Grb2. Our study also suggests that LAT plays a key role in coordinating the interaction of multiple molecules within a single complex. Evidence in support of a LAT/Gads/SLP-76/PLC-γ1 signaling complex is suggested by the data demonstrating that sites #6,7&8 must be on the same molecule of LAT. However, formal demonstration of this multi-protein complex awaits further investigation.

Experimental Procedures

Cell Lines, Transfections and Plasmids

The LAT-deficient Jurkat mutant J.CaM2 (Finco et al., 1998; Goldsmith et al., 1988) and subsequent stable lines were maintained in RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin and streptomycin. For stable and transient transfections, $2x10^7$ J.CaM2 cells were resuspended in 400 µl of RPMI 1640 and electroporated at 250 V, 960 µF using a Gene Pulsar electroporator (BioRad, Hercules, CA). For the generation of stable lines, transfected cells were plated 48 hrs after electroporation in media containing 2 µg/ml of G418. For transient expression assays, cells were utilized 24 hrs after transfection. Myc-tagged Erk2 was expressed using the pEF-BOS expression plasmid. Myc-tagged LAT was expressed using the pCDEF3 expression plasmid, which also encodes a neomycin resistance gene for the production of stable lines. Point mutants of LAT were generated with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Clones evaluated are as follows: wt LAT (clone 70), Y-F#6 (clone 107), Y-F#7&8 (clone 73), Y-F#6,7&8 (clone 55), F-Y#6 (clone 35), F-Y#7&8 (clone 15), F-Y#6,7&8 (clone 40.8).

Antibodies

TCR stimulation was performed with the anti-Jurkat TCR β-chain monoclonal antibody (mAb) C305 (Weiss and Stobo, 1984). The polyclonal anti-LAT, anti-PLC-γ1 mixed mAb and anti-phosphotyrosine 4G10 mAb were from Upstate Biotechnology Inc. (Lake Placid, NY). The polyclonal anti-Grb2 serum was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-phospho Erk (rabbit polyclonal) was from Cell Signaling

Technology Inc. (Beverly, MA) and the anti-myc mAb was derived from the 9E10 hybridoma.

Measurement of Intracellular Ca^{2+} Mobilization

To analyze intracellular Ca^{2+} mobilization, $3x10^6$ cells were resuspended in 1 ml of RPMI 1640 with 10% FBS and labeled in 3 μM of the fluorescent Ca^{2+} indicator dye Indo-1 AM (Molecular Probes, Eugene, OR) for 1 hr at 37°C. Cells were washed 3 times in Ca^{2+} buffer (0.1 % glucose w/v, 25 mM HEPES, 125 mM NaCl, 5 mM KCl, 1 mM Na_2HPO_4, 0.1% BSA w/v, 50 mM $MgCl_2$, 100 mM $CaCl_2$, pH 7.4) and the cell pellet was kept on ice. Prior to the assay, cells were resuspended in Ca^{2+} buffer and warmed to 37°C. 10^6 cells were stimulated with anti-TCR mAb C305, followed by ionomycin (1 μM). The fluorescence emission at 400- and 500-nm wavelengths was measured with an excitation at 355-nm using a Hitachi F-4500 fluorescence spectrophotometer, and the intracellular Ca^{2+} concentration was calculated based on the ratio of the fluorescence at 400- and 500-nm.

TCR Stimulation, Preparation of Lysates, Immunoprecipitations and Western Blotting

Cells ($2.5x10^7$cells/ml) were first rested at 37 °C for 20 min. and then stimulated with 1:250 dilution of purified anti-TCR mAb (1 mg/ml stock solution, C305) in PBS. Cells were lysed at 10^8 cells/ml in lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl pH 7.6). For coimmunoprecipitation assays, 1% Brij in 150 mM NaCl and 10 mM Tris pH 7.6 was used. Both lysis buffers contained 2 mM EDTA and a combination of

protease and phosphatase inhibitors. For immunoprecipitations, lysates were incubated with primary antibody for 45 min, followed by protein G-sepharose beads for 45 min, and were then washed 3 times with lysis buffer. Samples were separated by SDS-PAGE and proteins were analyzed by western blotting. Membranes were incubated with the indicated primary antibodies, followed by the appropriate secondary antibody conjugated to horseradish peroxidase. Reactive proteins were visualized by Renaissance, a chemiluminescence reagent (New England Nuclear, Boston, MA). For quantitation of bands, chemiluminescence was assessed on a Kodak Imaging Station by using Kodak 1D image analysis software version 3.5 (Rochester, NY).

NF-AT Luciferase Assays

J.CaM2 cells were transfected as described above with 20 μg of a 3x NF-AT-luciferase reporter construct and 1 μg of the indicated LAT construct in pCDEF3. The TCR was stimulated with immobilized anti-TCR mAb (C305) for 8 hrs. Cells were harvested, lysed and assayed for luciferase activity (Shapiro et al., 1996). Lysates were also blotted for LAT expression.

Chapter 4

Expression of the Receptor-like Tyrosine Phosphatase CD148

Summary

The importance of LAT tyrosine phosphorylation is clearly demonstrated in the previous chapters of this thesis, yet the phosphatase(s) involved in the dephosphorylation of LAT remains undefined. Recent studies with the receptor-like tyrosine phosphatase CD148 have demonstrated a specific dephosphorylation of LAT in cells expressing CD148 leading us to hypothesize that CD148 may control the state of LAT phosphorylation. To study the expression and role of CD148 in lymphocyte signaling, an antibody that recognizes the native form of the murine CD148 extracellular domain was generated[1]. CD148 is expressed at all stages of B cell development in the bone marrow, as well as on peripheral mature B cells. In contrast, only low CD148 expression is observed in developing thymocytes and mature T cells, with no difference between CD4+ and CD8+ cells. Following stimulation of peripheral B cells with anti-IgM and anti-CD40 antibodies or LPS, CD148 expression levels did not change substantially (5-fold), whereas stimulation of peripheral T cells with anti-CD3 and anti-CD28 antibodies or PMA and ionomycin leads to about a 40-fold increase of CD148 expression. This upregulation on T cells can be inhibited to some extent by PP2, FK506, U0126, and Ly294002, reagents that block the activity of src kinases, calcineurin, MEK, and phosphatidylinositol-3 kinase (PI3K), respectively. CD148 levels were also high on lymphocytes isolated from MRL *lpr/lpr* mice that develop lymphoproliferation due to a defect in Fas. Together, these data suggest that CD148 may play a basal regulatory role in B cells, but in T cells CD148 may help to terminate immune responses.

[1] The mAb (8A-1) and data from figure 4.1 were generated by Jeanne Baker. Jing Zhu and I were involved in characterizing the expression pattern of CD148 in the mouse.

Introduction

To respond to various stimuli, cells utilize complex signaling pathways to generate effector functions. One method cells utilize to transmit signals from one molecule to another is phosphorylation on tyrosine residues (Cooper et al., 1983; Pawson, 1994; Pawson and Scott, 1997). The state of phosphorylation is regulated by a balance of kinases and phosphatases (Li and Dixon, 2000; Pawson, 1994). Significantly less is known about the role phosphatases play in regulating the immune response when compared to kinases.

Hematopoietic cells express a broad range of protein tyrosine phosphatases (PTPs) (Li and Dixon, 2000). The PTPs fall into two major types: 1) receptor-like PTPs (RPTPs) or 2) intracellular PTPs. One of the most well studied RPTP is CD45. However, many labs have now begun to focus on the role of other RPTPs in signal transduction in hematopoietic cells. One such RPTP is CD148.

Initially cloned by multiple groups, most studies have described a role for CD148 (also known as DEP-1, PTP-eta, PTPRJ, and Byp) in cell cycle and growth control (Borges et al., 1996; Honda et al., 1994; Kuramochi et al., 1996; Ostman et al., 1994). Ostman and colleagues isolated the cDNA as a gene upregulated in densely culture fibroblasts (Ostman et al., 1994). Other groups have subsequently shown that malignant tumors and cell lines have reduced CD148 levels (Keane et al., 1996; Zhang et al., 1997). Also, overexpression of CD148 inhibits tumor cell growth by inhibiting MAP kinase activation and increasing the stability of the cell cycle regulator $p27^{Kip1}$ (Trapasso et al., 2000). Targeted disruption of the catalytic activity of CD148 in the germline results in an early embryonic lethality due to defects in angiogenesis (Takahashi et al., 2003).

A few studies examining the expression of CD148 in human lymphocytes have been published. Tangye and colleagues showed that CD148 is expressed at low levels on resting T cells, but is upregulated following in vitro activation (Tangye et al., 1998b). Co-crosslinking of CD148 with anti-CD3 mAbs augmented T cell proliferation over CD3 alone in an IL-2-dependent, cyclosporin A-sensitive manner. In human B cells, high expression of CD148 along with CD27, was shown to be a marker for memory B cells (Tangye et al., 1998a). As yet, much is still not known about the role of CD148 in regulating the immune response.

To further characterize the role of CD148 in immune responses, we have generated a mAb to murine CD148 and investigated its expression pattern on freshly isolated cells. Also, we have assayed changes of CD148 expression induced upon antigenic stimulation in mature B and T lymphocytes. B cells express CD148 throughout development and in the periphery. Murine T lymphocytes ónly express low levels through development, but express CD148 at higher levels following antigen receptor stimulation. Pharmacologic inhibitors of T cell activation inhibit the upregulation of CD148. Chronically active T cells from animal models of autoimmunity, such as MRL *lpr/lpr* mice and the c-cbl/cbl-b double knockout mice, constitutively express CD148. These findings suggest that the CD148 is involved in regulating T cell responses to immune challenges.

Results

Characterization of the anti-mCD148 mAb (8A-1)

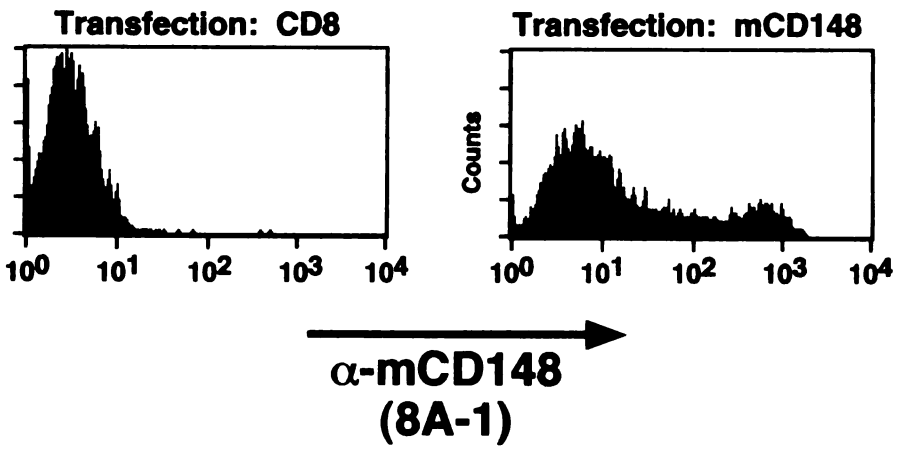
Anti-murine CD148 mAb (8A-1) was generated by immunizing Syrian hamsters in the foot pad with 293T cells expressing murine CD148. Cells from the draining lymph node were then fused and clones secreting anti-mCD148 antibody screened by flow cytometry. Figure 4.1A shows that only 293T cells transfected with mouse CD148, and not mouse CD8 as a control, stain positively with the 8A-1 mAb as analyzed by flow cytometry. To determine if 8A-1 could be used to immunoprecipitate CD148, lysates were made from cells isolated from thymus and spleen. Immunoprecipitations were then performed using a control Ig or 8A-1. Proteins were visualized by SDS-PAGE followed by immunoblotting with a polyclonal rabbit anti-sera (anti-Byp) generated to a peptide in the phosphatase domain of CD148. A band of the approximate size of CD148 (reducing gel 240-260 kD) appears in only the lanes loaded with immunoprecipitations prepared with 8A-1 and not the control Ig.

Expression on developing and mature B lymphocytes

Murine immature B cells were isolated from the bone marrow of C57/BL6 mice and stained with anti-CD148 along with CD25, CD43, IgM, IgD, and B220 as markers for B cell development. As shown in Figure 4.2, pro- (B220 lo, CD43+, CD25-), pre- (B220 +, CD43-, CD25+), immature (B220+, IgM+, IgD-), and mature B cells (B220+, IgM+, IgD+) in the bone marrow all express CD148. Peripheral B cells (CD19+) isolated from lymph nodes also expressed similar levels of CD148 (data not shown).

Figure 4.1 Specificity of anti-mCD148 mAb (8A-1). (A) 293T cells were transfected with either CD8 or CD148. Cells were then stained with 8A-1 mAb and expression analyzed by flow cytometry. (B) Lysates were made from cells isolated from thymus and spleen. Immunoprecipitations were then performed using a control Ig or 8A-1 mAb and proteins separated by SDS-PAGE, followed by immunoblotting with a polyclonal rabbit sera (anti-Byp) generated to the phosphatase domain of CD148.

A.


Transfected 293T Cells
Transfection: CD8
Transfection: mCD148
Counts
10^0 10^1 10^2 10^3 10^4
10^0 10^1 10^2 10^3 10^4
α-mCD148
(8A-1)

B.

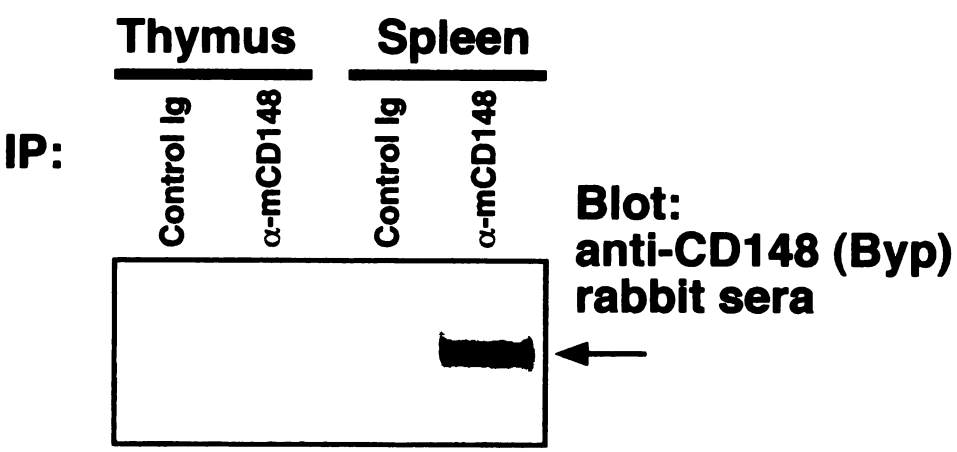
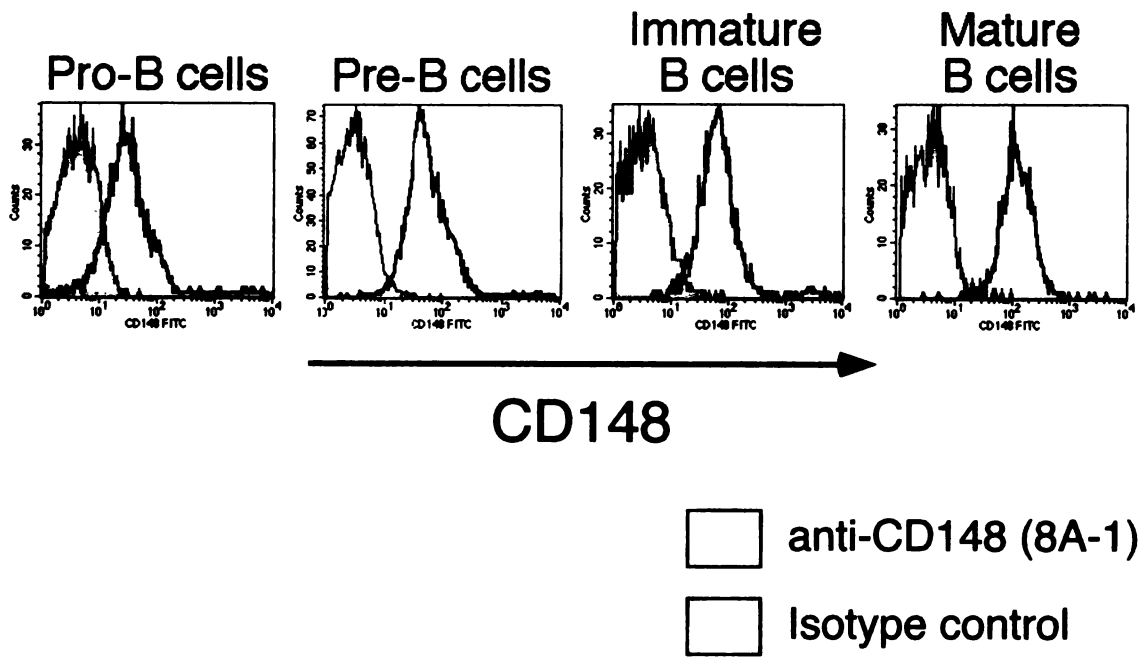

Thymus
Spleen
IP:
Control Ig
α-mCD148
Control Ig
α-mCD148
Blot:
anti-CD148 (Byp)
rabbit sera

Figure 4.2 Expression of CD148 in developing B cells in the bone marrow. Bone marrow cells were isolated from C57/BL6 mice and stained with the appropriate antibodies to determine the stage of B cell development. pro- (B220 lo, CD43+, CD25-), pre- (B220+, CD43-, CD25+), immature (B220+, IgM+, IgD-), and mature B cells (B220+, IgM+, IgD+). These cells were evaluated for CD148 expression by staining with anti-CD148 mAb (8A-1). Shaded histograms represent isotype matched Ig controls.


Pro-B cells
Pre-B cells
Immature B cells
Mature B cells
CD148
anti-CD148 (8A-1)
Isotype control

To study the effects of B cell activation on CD148 expression, splenic B cells were purified from wildtype mice. The B cells were stimulated for various amounts of time with anti-CD40 and anti-IgM antibodies or LPS (Figure 4.3). B cells did not substantially change their level of CD148 expression following stimulation with either anti-CD40 and anti-IgM antibodies or LPS. As a stimulation control, the activation marker CD69 was examined. Following either stimulation, CD69 was upregulated rapidly demonstrating that cells indeed responded to the stimuli (data not shown).

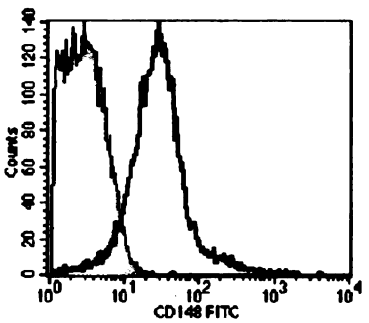
Expression on developing and mature T lymphocytes

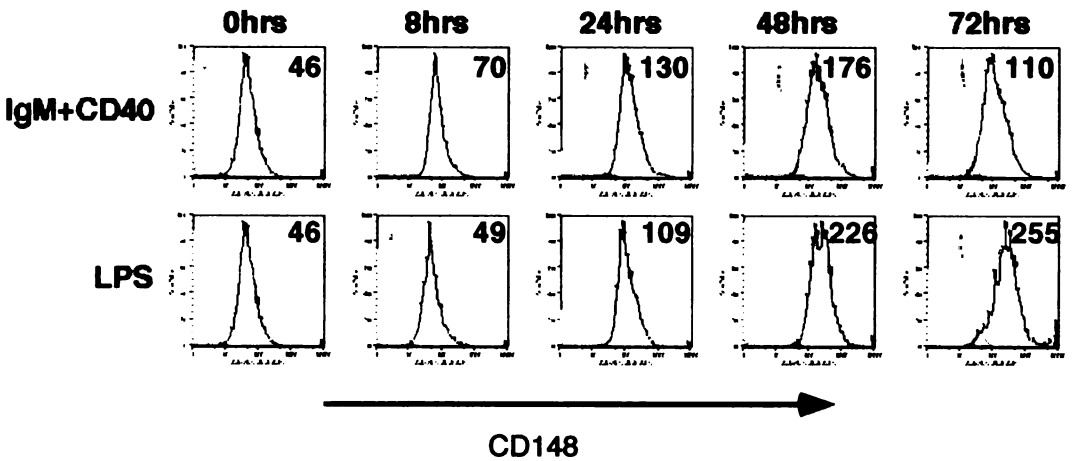
To examine the expression of CD148 on thymocytes, cells were stained with mAbs specific for CD4, CD8, and CD148. The highest level of CD148 expression was observed in the CD4-, CD8- double negative (DN) population of thymocytes; however, staining was much lower than that seen in developing B cells (Figure 4.4). CD4, CD8 double positive (DP) and both CD4 and CD8 single positive (SP) thymocytes express very low levels of CD148. Similarly, expression of CD148 on both CD4+ and CD8+ T cells in the periphery isolated from lymph nodes was also low.

To study the effects of T cell activation on CD148 expression, lymph nodes were isolated and T cells purified from wildtype mice. The T cells were stimulated for 0, 8, 24, 48, and 72 hrs with anti-CD3 and anti-CD28 antibodies or PMA and Ionomycin (Figure 4.5). In striking contrast to B cells, stimulated T cells showed a rapid and substantial increase of CD148 expression on the cell surface, peaking between 24 and 48 hours.

Figure 4.3 Expression of CD148 following stimulation of mature peripheral B cells. Mature peripheral B cells were isolated from spleens and purified by negative selection with anti-CD11b and anti-CD43 mAbs. Purified cells were stimulated with anti-IgM + anti-CD40 antibodies or LPS for the indicated times. Cells were then stained with anti-CD148 mAb and analyzed by flow cytometry. Numbers within histogram boxes indicate the geometric mean of the fluorescence intensity.

Peripheral B Cells





anti-CD148 (8A-1)

Isotype control

Figure 4.4 Expression of CD148 on thymocyte sub-populations. Thymocytes were isolated from C57/BL6 mice and stained with the indicated antibodies to determine the stage of T cell development. These cells were evaluated for expression of CD148 by staining with 8A-1 mAb. Shaded histograms represent isotype controls.

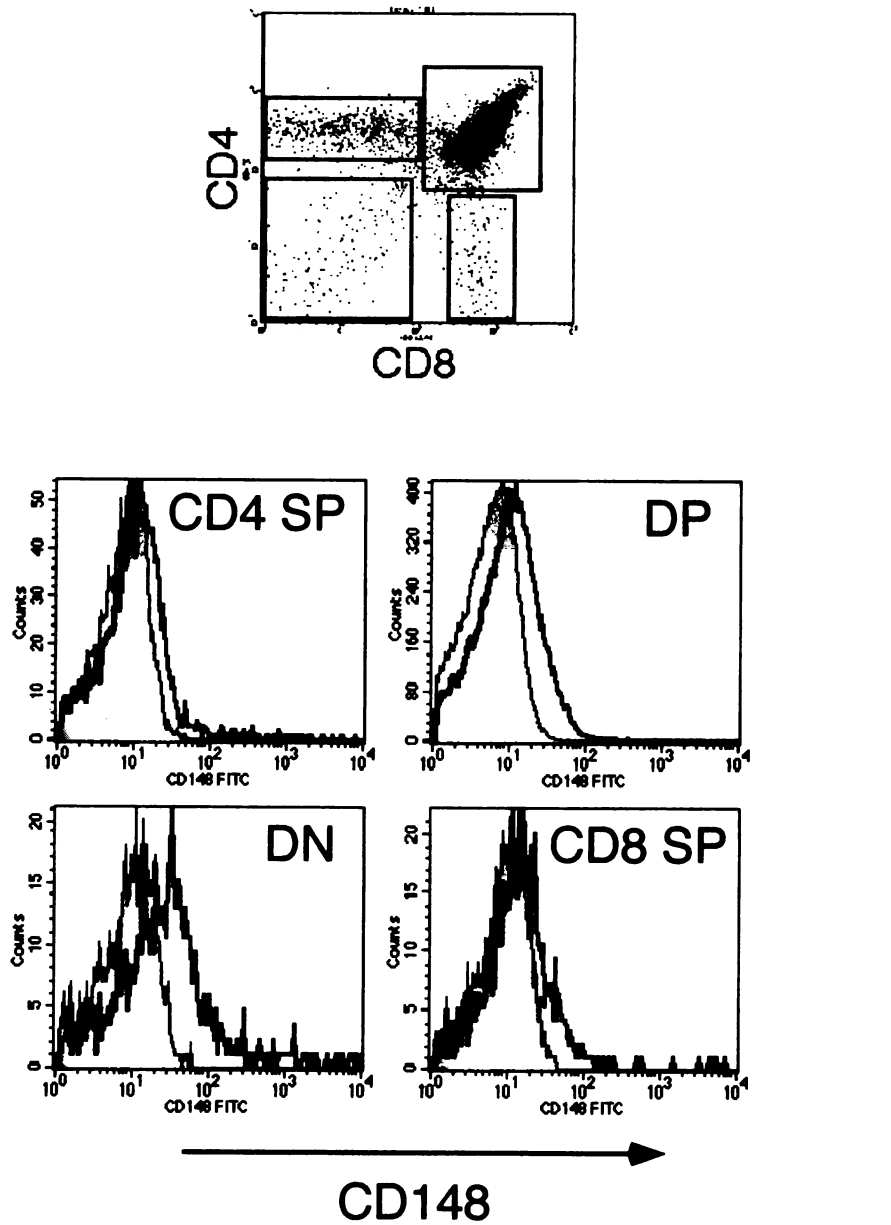
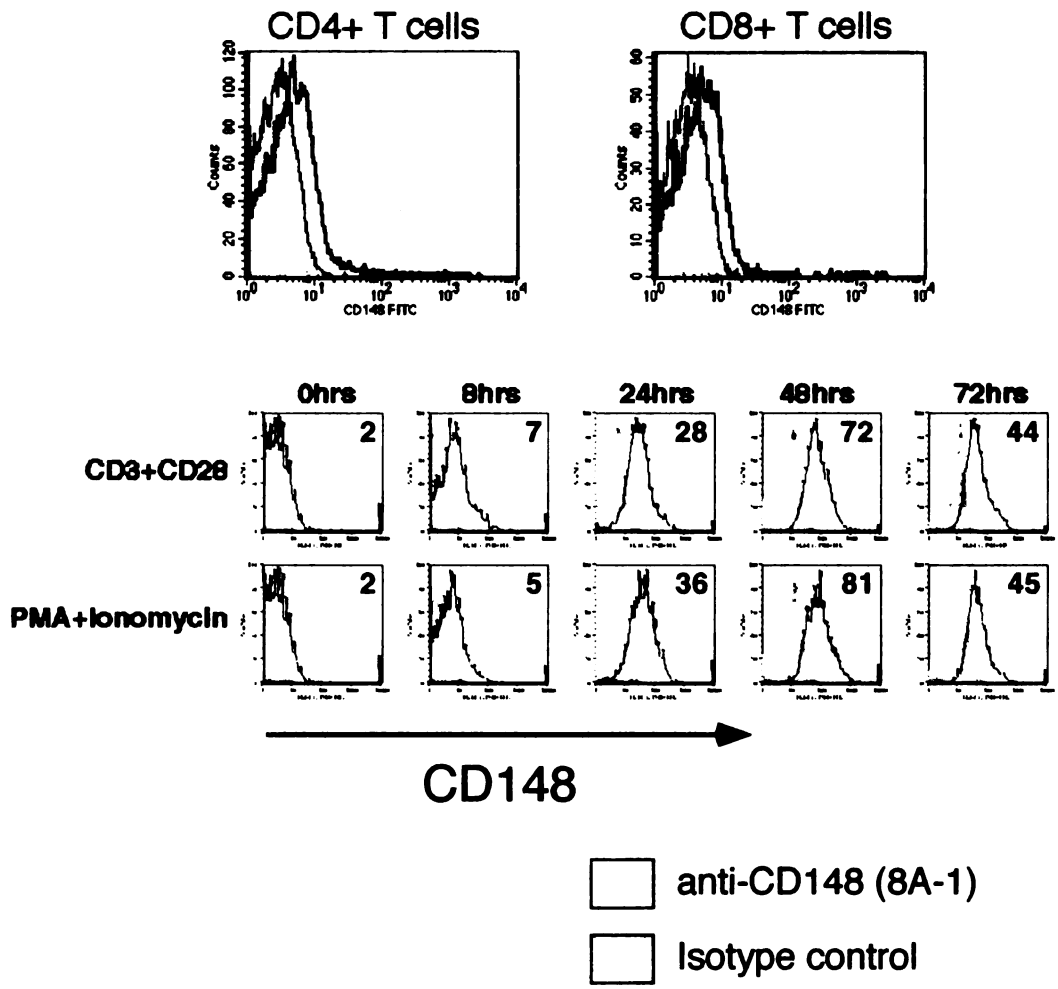

CD4
CD8
CD4 SP
DP
DN
CD8 SP
Counts
CD148 FITC
CD148
anti-CD148 (8A-1)
Isotype control

Figure 4.5 Expression of CD148 following stimulation of mature peripheral T cells. Mature peripheral T cells were isolated from spleens and purified by negative selection with anti-MHC II and anti-CD11b mAbs. Purified cells were stimulated with anti-CD3 + anti-CD28 mAbs or PMA + Ionomycin for the indicated time points. Cells were then stained with anti-CD148 mAbs and analyzed by flow cytometry. Numbers within histogram boxes indicate the geometric mean of the fluorescence intensity.


CD4+ T cells
CD8+ T cells
Counts
CD148 FITC
0hrs
8hrs
24hrs
48hrs
72hrs
CD3+CD28
PMA+Ionomycin
2
7
28
72
44
2
5
36
81
45
CD148
anti-CD148 (8A-1)
Isotype control

Inhibitors of CD148 upregulation upon T cell stimulation

To further investigate the signals required to upregulate CD148 on T cells, we utilized various pharmacologic inhibitors to block specific pathways required for T cell activation. To block the most proximal events of TCR induced signal transduction, we treated the cells with the src family inhibitor PP2. As expected, PP2 treatment potently blocked CD148 upregulation in response to anti-CD3 and anti-CD28 antibody stimulation (Figure 4.6).

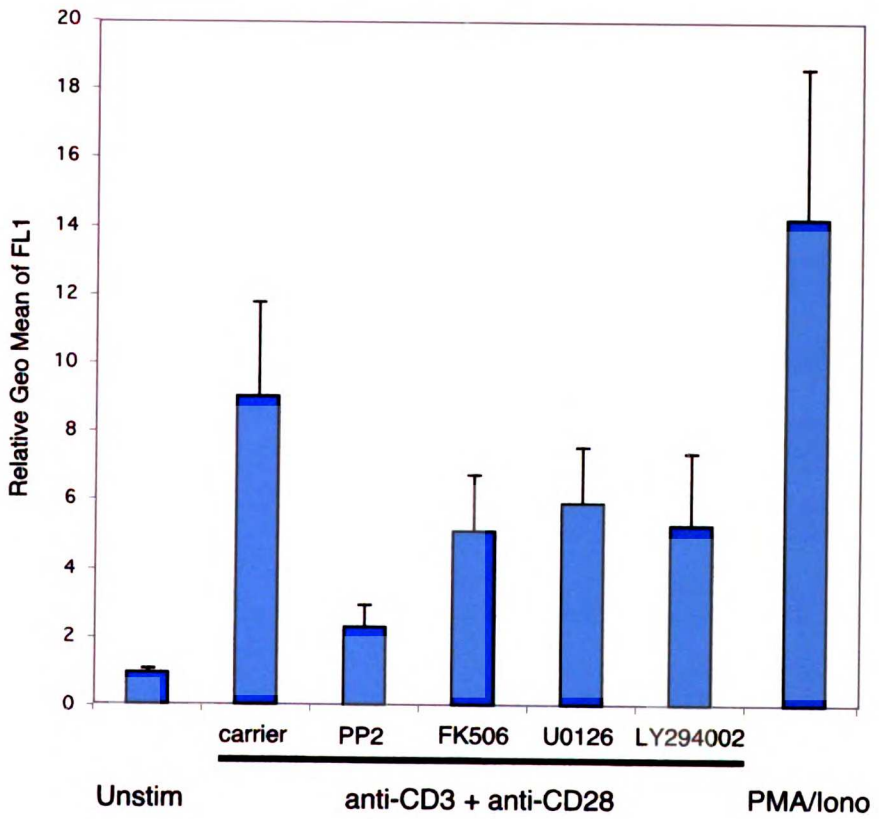
To inhibit molecules further downstream of TCR stimulation in the signaling cascade, the inhibitor FK506, which blocks the activity of the protein phosphatase calcineurin, and the inhibitor U0126 which blocks MEK activation, were used. Both of these pharmacologic agents inhibited CD148 upregulation less than the proximal inhibitor PP2, but considerably more than a vehicle control (DMSO) (Figure 4.6).

Stimulation with anti-CD3 mAb alone is much less effective at inducing CD148 upregulation in purified T cells when compared to anti-CD3 and anti-CD28 mAb stimulation (data not shown). Since CD28 has a consensus YxxM motif in its cytoplasmic tail that has been demonstrated to recruit PI3K, we used a specific inhibitor of PI3K, LY294002, to assess whether PI3K is required for the upregulation of CD148. Treatment of cells with Ly294002 also potentially inhibited the upregulation of CD148 in anti-CD3 and anti-CD28 mAb stimulated cells (Figure 4.6).

Autoimmunity models

To determine if CD148 levels are elevated on T cells that are characterized to have autoimmune phenotypes, cells from MRL *lpr/lpr* mice were studied. The *lpr* defect

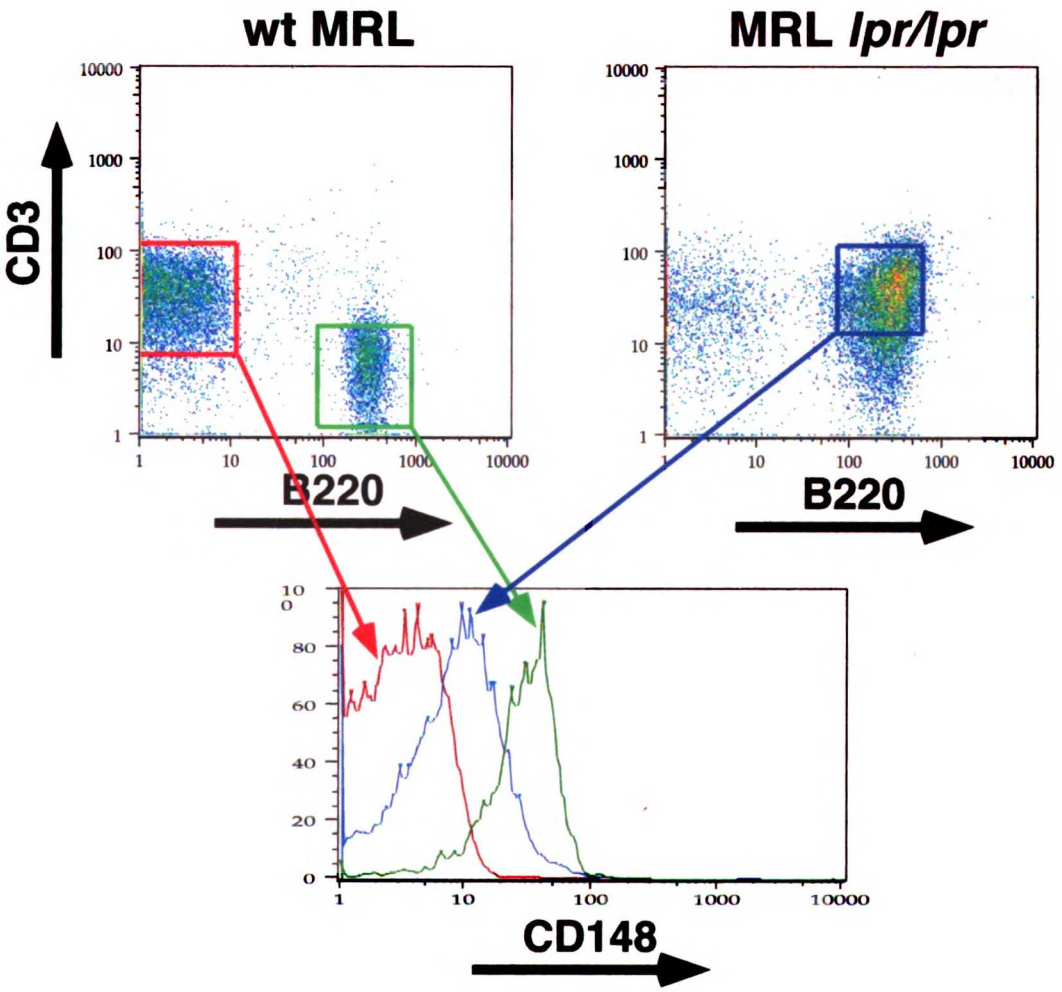
Figure 4.6 Inhibitors of T cell activation block CD148 upregulation. Primary murine lymph node cells were isolated and stimulated with anti-CD3 and anti-CD28 for 48 hrs in the presence of the indicated pharmacologic inhibitors; PP2 (20 μM), FK506 (100 ng/ml), LY294002 (5 μM), U0126 (10 μM). TCR-β+, 7AAD- cells were gated and the expression of CD148 analyzed. The graph represents the average geometric mean of Alexa 488 conjugated anti-CD148 from at least 3 experiments (Error bars represent the standard error of the mean).


Relative Geo Mean of FL1
20
18
16
14
12
10
8
6
4
2
0
carrier
PP2
FK506
U0126
LY294002
Unstim
anti-CD3 + anti-CD28
PMA/Iono

in mice causes a profound lymphoproliferative disorder on the MRL background due to a defect in Fas (Cohen and Eisenberg, 1991). Sera from these mice contain anti-nuclear antibodies, a hallmark of autoimmune disease. The majority of the T cells populating the MRL *lpr/lpr* lymph nodes have an unusual phenotype, CD4-, CD8-, CD3+, B200+ (Cohen and Eisenberg, 1991). When these cells were stained for CD148, they expressed higher basal levels of CD148 compared to that of CD3+ cells from a wildtype MRL mouse (Figure 4.7).

Figure 4.7 Expression of CD148 on lymph node cells from MRL *lpr/lpr* mice.

Lymph nodes were isolated from wt MRL and MRL *lpr/lpr* mice and analyzed for CD148 expression by flow cytometry. Staining for CD3 vs. B220 was done to determine gates for cell lineage. CD148 expression levels for the various lineages were then overlayed. Wt T cells are shown in red, wt B cells in green, and MRL *lpr/lpr* cells in blue.


wt MRL
MRL lpr/lpr
CD3
B220
B220
CD148
10000
1000
100
10
1
1
10
100
1000
10000
80
60
40
20
0

Discussion

To characterize the role of CD148 in the immune response, a mAb to murine CD148 was generated to investigate the expression pattern of CD148 on resting and stimulated primary lymphocytes. B cells express CD148 throughout development and in the periphery, whereas T lymphocytes only express low levels through development. Not until their TCRs are stimulated, do T cells in the periphery express high levels of CD148. Reagents that mimic downstream TCR signals, such as PMA and ionomycin, also upregulate CD148 in T cells. The expression patterns observed in murine lymphocytes, shown in this study, is similar to published results examining human lymphocytes in that B cells constitutively express CD148, whereas T cells express low levels of CD148 until activated (Gaya et al., 1999; Tangye et al., 1998b).

Interestingly, in human B cells, CD148 has a bi-modal distribution in which the cells expressing higher levels are thought to be memory B cells. This bi-modal distribution was not seen in the mouse (data not shown). Various mouse strains, such as C57/Bl6, MRL, BALB-c, and Black Swiss, were also examined to look at differential expression of CD148, but substantial differences were not observed. By RT-PCR, a few polymorphisms within the extracellular domain of CD148 were noted, but the significance of this has yet to be determined. Pharmacologic inhibitors of TCR-derived signals also inhibited the upregulation of CD148 on T cells. Chronically active T cells from at least one animal models of autoimmunity, the MRL *lpr/lpr* mouse, have constitutive expression of CD148. These findings suggest that the CD148 is involved in regulating T cell responses to immune challenges.

Most studies of CD148 have focused on its role in cell cycle (Ostman et al., 1994; Trapasso et al., 2000). Since CD148 expression does not change in response to mitogenic B cell stimulation, it is unlikely that it plays a role in cell cycle control in B cells. It is unclear if CD148 affects cell cycle control in thymocytes or mature T cells. Since levels of CD148 are low in the thymus and do not change markedly during thymocyte development, CD148 does not seem to be involved in thymic proliferation during TCR repertoire selection. In peripheral T cells, CD148 upregulation begins by eight hours after TCR stimulation, whereas stimulated T cells do not divide until about 30 hours later. In T cells, CD148 expression could either be terminating the signals emanating from the TCR, or CD148 could be acting on downstream events involved in cell cycle control.

The observation that CD148 expression is elevated in cells from the MRL *lpr/lpr* mouse suggests that CD148 may be upregulated as part of an inhibitory feedback loop. In one other model of autoimmune disease, activated T cells that expand in mice deficient in both c-cbl and cbl-b were noted to have higher levels of CD148 on T cells in the periphery (Naramura et al., 2002). These T cells appear chronically activated and contribute to an autoimmune phenotype since these mice also have higher levels of anti-dsDNA antibodies. Interestingly, the T cells from these mice exhibit a selective decrease in LAT and PLC-γ1 phosphorylation following TCR stimulation, a phenotype similar to that seen in Jurkat T cells stably expressing CD148 (Baker et al., 2001). These cells have a decrease in Ca^{2+} mobilization, Erk activation, and subsequent NFAT activation in response to TCR stimulation. A similar defect in TCR-mediated signal transduction has been reported for MRL *lpr/lpr* T cells (Cohen and Eisenberg, 1991). Data such as these

would lead to the prediction that mice with a targeted deletion of CD148 might develop hyper-reactive T cell responses or even frank autoimmunity. Unfortunately, mice deficient in CD148 die during embryogenesis due to angiogenic defects. A conditional knockout of CD148 in only the T cell compartment would be the best way to test this hypothesis.

The role of CD148 in B cells has not been thoroughly examined. One study has implied that CD148 is a good maker for memory cells on human splenic B cells along with CD27; however, functional data in human or murine B cells are lacking (Dong et al., 2002; Tangye et al., 1998a). If we assume that the substrates of CD148 in T cells are similar to that of B cells, one would predict that potential B cell substrates could be molecules such as LAB/NTAL, BLNK, or PLC-γ2 (Brdicka et al., 2002; Ishiai et al., 2000; Janssen et al., 2003). The expression of CD148 in B cells, however, is constitutive and does not change substantially following stimulation, suggesting that the function or regulatory mechanism in the respective cell types may be different. Similar to T cells, a conditional knockout in the B cell lineage would be the best way to address the role of CD148 in B cells.

Experimental Procedures

Antibodies and reagents

Anti-murine CD148 mAb (8A-1) was generated by footpad immunization of Syrian hamsters with 293T cells expressing murine CD148. Cells from the draining lymph nodes were then fused to a myeloma cell line using polyethylene glycol and plated to isolate single clones. Clones were selected for secretion of antibodies staining cells transfected with murine CD148, but not untransfected cells, as determined by flow cytometry. Positive clones were sub-cloned and assayed again. Anti-CD148 (Byp) polyclonal rabbit sera was generated using standard immunizations with the peptide IKPKKSKLIRVENF from the cytoplasmic domain of CD148 (conserved in human and mouse). Anti-CD3ε (2C11), anti-CD69, 7-Amino Actinomycin D (7AAD), and anti-TCR β (H57) were from BD Pharmingen (San Diego, CA). Anti-CD28 ascites was generated using hybridoma 37.51. Primary mouse lymphocytes were cultured in D-MEM with 10% FBS, 50 μM β-mercaptoethanol, 2 mM glutamine, non-essential amino acids, penicillin, and streptomycin.

In vitro cell stimulation

For B cell purification, splenocytes were labeled with anti-CD11b and anti-CD43 mAb-conjugated MACS beads (Miltenyi Biotec, Auburn, CA) and passed through a MACS LS column (Miltenyi Biotec) to deplete non-B cells. B cells were stimulated for the indicated times with anti-CD40 mAb (HM40-3) (1 μg/ml; Pharmingen) and goat anti-mouse IgM $F(ab)'_2$ (10 μg/ml; Jackson ImmunoResearch) or LPS (10 μg/ml; Sigma). For T cell purification, lymph node cells were passed through an R&D T cell enrichment

column (R&D Systems Inc., Minneapolis, MN) and negatively selected using the manufacturer's protocol. T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1:1000 ascites) or PMA (2 ng/ml) and Ionomycin (0.5 μM).

Inhibition of CD148 upregulation

Lymph node cells were cultured in a 24 well plate (Costar, Corning, NY) at $4x10^6$ cells/well in 1 ml of the media as described above. Inhibitors dissolved in DMSO or DMSO only were added for 30 min prior to stimulation with anti-CD3 and anti-CD28 mAbs. Cells were harvested at 40-48 hours following stimulation and stained with anti-CD69 mAb, anti-TCR β mAb, and 7AAD. Inhibitors were from Calbiochem (San Diego, CA) and the following final concentrations used; PP2 (20 μM), FK506 (100 ng/ml), LY294002 (5 μM), U0126 (10 μM).

Chapter 5

The Tyrosine Phosphatase CD148 is Excluded from the Immunologic Synapse and Downregulates Prolonged T Cell Signaling

Abstract

CD148 is a receptor-like protein tyrosine phosphatase upregulated on T cells following T cell receptor (TCR) stimulation. To examine the physiologic role of CD148 in TCR signaling, we utilized an inducible CD148 expressing Jurkat T cell clone. Expression of CD148 inhibits NFAT activation induced by soluble anti-TCR antibody, but not by antigen presenting cells (APCs) loaded with staphylococcal enterotoxin superantigen (SAg) or immobilized anti-TCR antibody. Immunofluorescence microscopy revealed that the extracellular domain of CD148 mediates its exclusion from the immunologic synapse, sequestering it from potential substrates. Targeting of the CD148 phosphatase domain to the immunologic synapse potently inhibited NFAT activation by all means of triggering through the TCR. These data lead us to propose a model where CD148 function is regulated in part by exclusion from substrates in the immunologic synapse. Upon T cell-APC disengagement, CD148 can then access and dephosphorylate substrates to downregulate prolongation of signaling.

Introduction

The activation of a T cell requires the complex interactions of a vast number of signaling molecules and eventually results in proliferation, differentiation, and secretion of cytokines such as interleukin 2 (IL-2). The initial triggering events are regulated by the Src and Syk families of protein tyrosine kinases (Kane et al., 2000; Lin and Weiss, 2001b). These kinases phosphorylate a large number of substrates leading to the further activation of other enzymes and allow for molecules with no intrinsic enzymatic activity to function as adaptors for the assembly of signaling complexes (Tomlinson et al., 2000). One such adaptor is the linker for the activation of T cells (LAT) (Zhang et al., 1998a). LAT is a transmembrane adaptor protein containing multiple sites of tyrosine phosphorylation which allow for the recruitment of many essential signaling molecules including phospholipase Cγ-1 (PLCγ-1), Grb2, and Gads (Finco et al., 1998; Lin and Weiss, 2001a; Tomlinson et al., 2000; Zhang et al., 1999a; Zhang et al., 2000). Multiple groups have demonstrated that signals emanating from the TCR must be sustained for prolonged periods of time, on the order of hours, for T cells to proliferate (Iezzi et al., 1998; Lee et al., 2002). IL-2 production also requires a similar length of time, for instance, six hours of stimulation with Con A and PMA were required for commitment the cells to maximal IL-2 production in Jurkat T cells (Weiss et al., 1987).

In order for a productive T cell-APC conjugate to form, a number of cell surface molecules must bind their respective ligands. During this cell-cell interaction, many proteins localize to the area of cell-cell contact, whereas others are excluded. Studies originally performed in mouse T cell clones demonstrated that the contact point between the T cell and the APC is a highly ordered structure (Monks et al., 1998). This structure

has become known as of the immunologic synapse (Bromley et al., 2001). To mimic what was seen in mouse T cell clones, we used the Jurkat-superantigen (SAg) system, where Jurkat T cells are stimulated with staphylococcal enterotoxin SAg presented on B lymphoblastoid cells. SAgs function by simultaneously binding to the β chain of the TCR and MHC class II molecules on the APC. This interaction has a similar activation time course as that seen with peptide loaded onto MHC molecules (Lavoie et al., 1999). Molecules known to be excluded from the synapse in mouse clones, such as CD43 and CD45, are also excluded in the Jurkat-SAg system (Bunnell et al., 2002; Delon et al., 2001; Johnson et al., 2000; van der Merwe et al., 2000).

Many groups have studied the signals involved in activating T cells, but considerably less is known about how a T cell terminates its response to stimulation. A class of molecules thought to play a role in the downregulation process is tyrosine phosphatases. CD148, also known as Dep-1 and Byp, is a transmembrane tyrosine phosphatase that is upregulated on T cells following activation (Tangye et al., 1998b). The extracellular domain consists of eight fibronectin type III domains, with multiple glycosylation sites that add to the size of the molecule (Gaya et al., 1999). Because of its bulky extracellular domain, it is likely to be excluded from the immunologic synapse, similar to other proteins with bulky extracellular domains such as CD43 and CD45 (Delon et al., 2001; Johnson et al., 2000; van der Merwe et al., 2000). Previous work has shown that CD148 is a negative regulator of TCR signaling since the activity of the TCR-induced transcription factor NFAT (Nuclear factor of activated T cells) was reduced in CD148-expressing cells (Baker et al., 2001; Tangye et al., 1998b). CD148 can act as a negative regulator by causing the specific dephosphorylation of LAT and PLC-γ1 (Baker

et al., 2001). However, in these studies, cells were only stimulated with soluble anti-TCR antibodies that do not mimic the physiologic interaction that leads to the formation of an immunologic synapse.

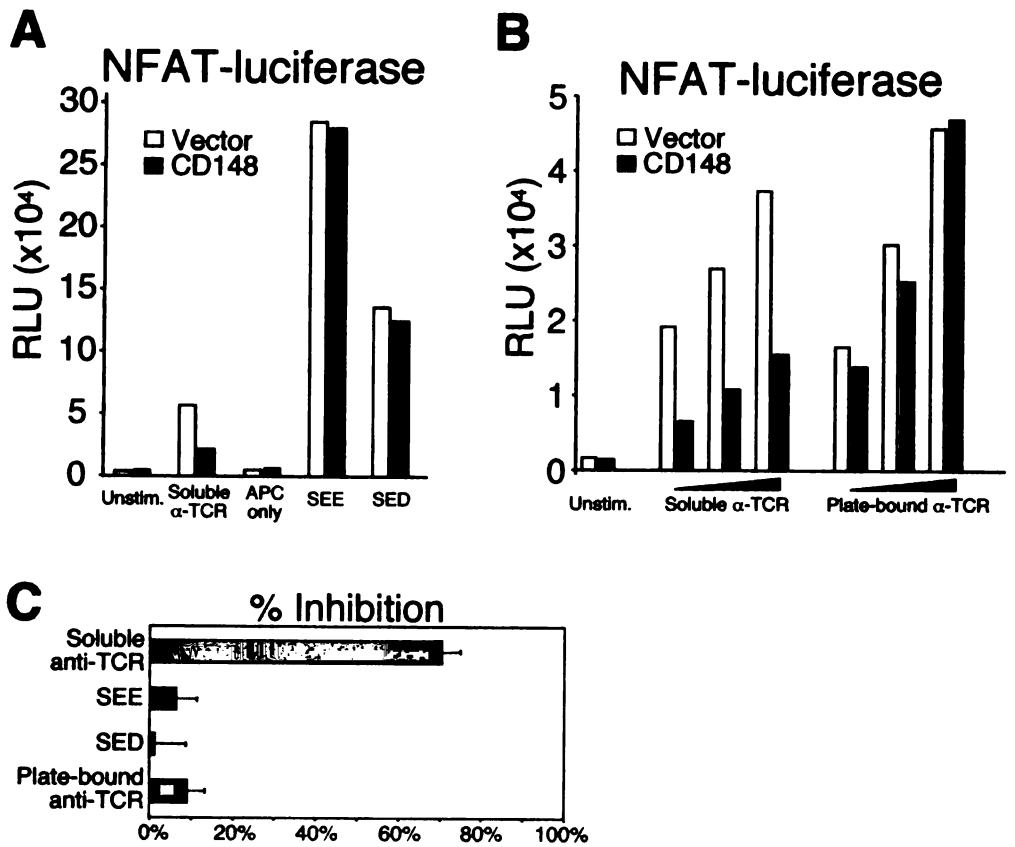
To further study the role of CD148 in T cell signaling, we investigated whether expression of CD148 inhibits T cell activation induced not only by soluble anti-TCR antibody (Ab) stimulation, but also with either SAg and APCs or immobilized anti-TCR Ab. We found that at eight hours following stimulation, CD148 only inhibits T cell activation stimulated with soluble anti-TCR. Immunofluorescence revealed that CD148 is excluded from the immunologic synapse and would therefore be unable to interact with substrates localized to the synapse such as LAT (Tanimura et al., 2003). This exclusion is mediated, at least in part, by the large extracellular domain of CD148. Forced targeting of the CD148 phosphatase domain to the synapse results in the potent inhibition of NFAT activation independent of the technique used to trigger the TCR. These data lead us to propose a model whereby CD148 activity during early T cell-APC interactions is regulated by exclusion from the immunologic synapse. However, following T cell-APC disengagement, CD148 is no longer excluded by the synapse and can then access and dephosphorylate substrates to downregulate prolongation of the response. Exclusion from the synapse could provide an important level of regulation, to prevent the premature termination of signaling pathways.

Results

Differential effects of CD148 depending on stimuli

To examine the negative regulatory role of CD148 on TCR signaling using a physiologic stimuli, we utilized APCs loaded with staphylococcal enterotoxin (SE) SAg. The SAg-coated APCs provide a localized stimulus to which a T cell will polarize to following appropriate TCR stimulation. These changes can be visualized as directed actin polymerization and microtubule organizing center (MTOC) re-orientation (Lowin-Kropf et al., 1998). Jurkat T cells were transfected with wild type murine CD148 together with an NFAT-luciferase reporter construct. Cells were stimulated the next day with either soluble anti-TCR (C305 MAb) or various types of SAg (SEA, SED, and SEE), that induce a broad range of signal strength, using Daudi B lymphoblastoid cells serving as APCs. As previously reported, expression of CD148 in Jurkat T cells inhibited soluble anti-TCR induced NFAT activation by about 60%-70% (Baker et al., 2001). Interestingly, stimulation with any three of the SAgs tested was not associated with substantial decreases in the NFAT response when CD148 was expressed (Figure 5.1A). A potential concern when comparing soluble anti-TCR with SAg and APCs is that APCs present a number of additional ligands to the T cell besides the SAg bound to MHC class II molecule during the interaction. To rule out the possibility that these other interactions accounted for the differences seen, we coated plates with the same anti-TCR used for the soluble MAb stimulation. Stimulation with immobilized anti-TCR mimics the localized stimulus provided by the SAg and APC. As seen with the SAg and APC stimulation, NFAT activation in response to various concentrations of plate-bound anti-TCR stimulation was not substantially inhibited by the expression of CD148 (Figure 5.1B).

Figure 5.1 Expression of CD148 only inhibits NFAT activation induced by soluble anti-TCR mAb. Jurkat T cells were transfected with 2 μg of pcdef3-mCD148 or vector control, along with 15 μg of a 3x-NFAT-luciferase reporter and rested overnight. (A) Cells were then stimulated with soluble anti-TCR mAb (1:2000 dilution of ascites; C305), or with the indicated SAg (250 ng/ml) and APCs. (B) Cells transfected as before were stimulated with increasing amounts of soluble anti-TCR mAb or plate-bound anti-TCR mAb. For (A) and (B), the mean of triplicate samples in one representative experiment is shown. (C) The mean percent inhibition for each stimuli is shown from multiple experiments. Error bars represent standard errors of the mean for at least 4 separate experiments, each done in triplicate. Relative light units (RLU) normalized to PMA/Ionomycin.


A
NFAT-luciferase
RLU (x10⁴)
30
25
20
15
10
5
0
Vector
CD148
Unstim.
Soluble α-TCR
APC only
SEE
SED
B
NFAT-luciferase
RLU (x10⁴)
5
4
3
2
1
0
Vector
CD148
Unstim.
Soluble α-TCR
Plate-bound α-TCR
C
% Inhibition
Soluble anti-TCR
SEE
SED
Plate-bound anti-TCR
0%
20%
40%
60%
80%
100%

The average percent inhibition induced by the various TCR stimuli is shown in Figure 5.1C. Thus, it seems from these data that CD148 preferentially inhibits a non-polarizing, soluble, anti-TCR stimulation. In contrast, CD148 expression has little effect on polarizing stimuli such as SAg with APCs or plate-bound anti-TCR.

To further examine the differences in T cell activation seen with the various methods of triggering through the TCR, we utilized a previously characterized CD148-inducible system in Jurkat T cells (Baker et al., 2001). The early activation marker CD69 is known to be upregulated in T cells stimulated through the TCR. As shown in Figure 5.2, expression of CD148 substantially inhibited the subsequent upregulation of CD69 induced by an overnight stimulation with soluble anti-TCR. However, in cells stimulated with anti-TCR-coated beads or SAg and APCs, expression of CD148 did not inhibit the upregulation of CD69 (Baker et al., 2001). These data demonstrate that endogenous responses reflecting T cell activation are also unaffected by CD148 expression if cells are stimulated by immobilized anti-TCR or SAg and APCs.

Previous studies have demonstrated that inducible expression of CD148 leads to a specific loss of LAT and PLCγ-1 phosphorylation induced by soluble anti-TCR mAb stimulation (Baker et al., 2001). To investigate whether immobilized anti-TCR mAb stimulation results in the same phosphorylation pattern, we stimulated Jurkat T cells that express CD148 with either soluble or bead coated anti-TCR mAb for various periods of time. Stimulation with soluble anti-TCR mAb in the presence of CD148 resulted in decreased tyrosine phosphorylation levels of pp140 (PLCγ-1) and pp36 (LAT), compared to cells not expressing CD148, as previously reported (Figure 5.3A) (Baker et al., 2001).

Figure 5.2 Only soluble anti-TCR mAb induced CD69 upregulation is inhibited by CD148 expression. Tet-inducible CD148 expressing Jurkats (clone L12) were induced with 1 μg/ml doxycycline for 48 prior to stimulation. Cells were then stimulated overnight with soluble anti-TCR mAb, bead-coated anti-TCR mAb, or SAg (1 μg/ml SEE) with Daudi B cells serving as APCs. Cells stimulated with 20 ng/ml PMA + 1 μM Ionomycin served as positive controls. Cells were then stained with anti-CD69-PE and analyzed by flow cytometry. For SAg and APC stimulation, T cells were gated with anti-CD5-FITC. The shaded histogram represents unstimulated samples or those stimulated with APCs only.

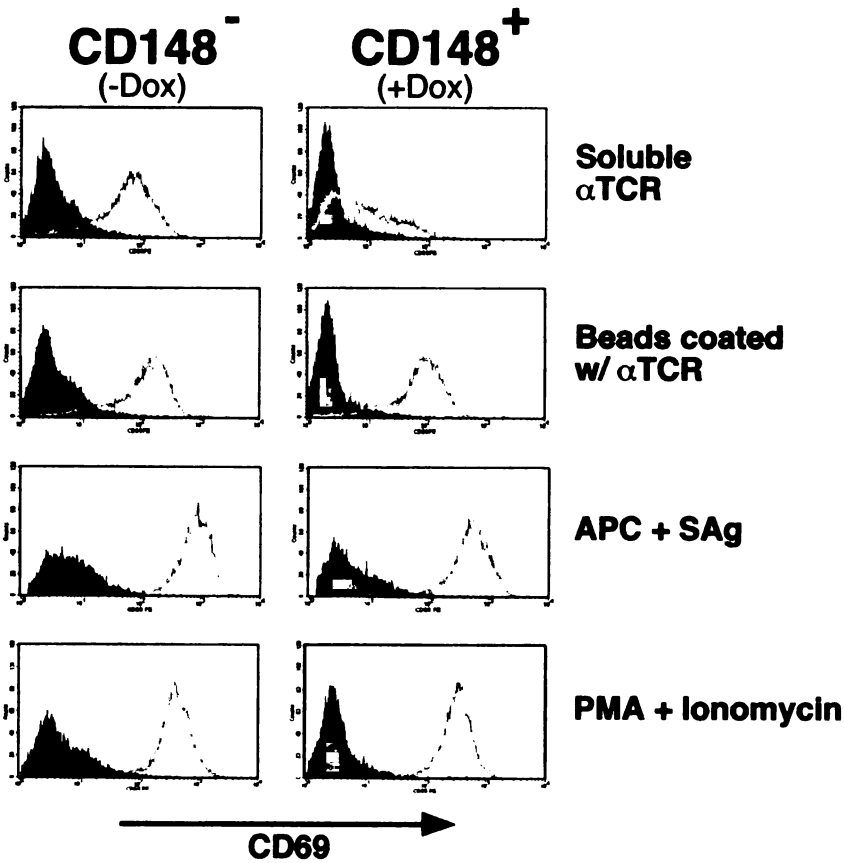
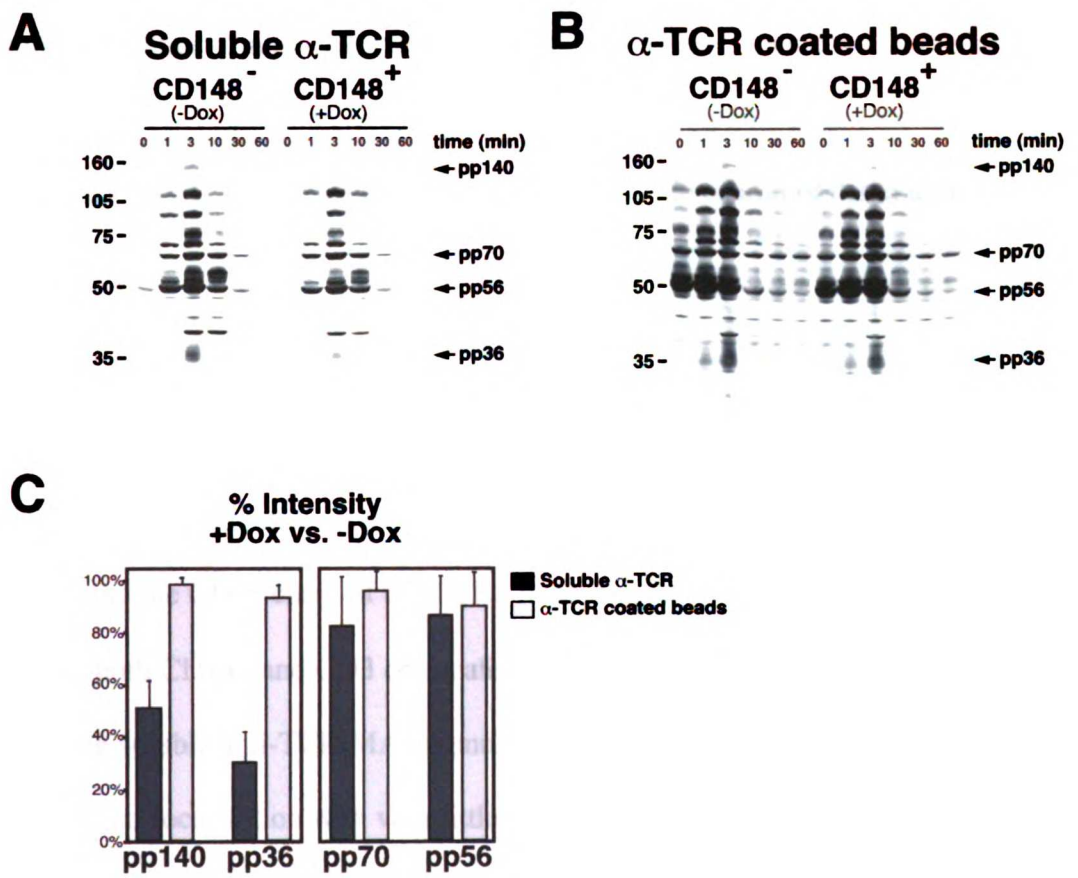

CD148-
(-Dox)
CD148+
(+Dox)
Soluble αTCR
Beads coated w/ αTCR
APC + SAg
PMA + Ionomycin
CD69

Figure 5.3 CD148 does not affect tyrosine phosphorylation induced with immobilized anti-TCR mAb. Tet-inducible CD148 expressing Jurkats were induced as before. Cells were washed once and rested in PBS for 30 min prior to stimulation with either (A) soluble anti-TCR mAb or (B) bead-coated anti-TCR mAb for the indicated time points. Cells were then lysed in buffer containing 1% NP-40 and proteins separated by SDS-PAGE. Tyrosine phosphorylated proteins were then visualized by western blotting with an anti-phosphotyrosine MAb followed by a goat anti-mouse-HRP secondary. (C) Average percent intensity of the indicated phosphotyrosine bands from 3 separate experiments is shown comparing soluble to bead-coated anti-TCR stimulation. Blots were developed on a Kodak Imaging Station and bands were quantitated using Kodak 1D imaging software.

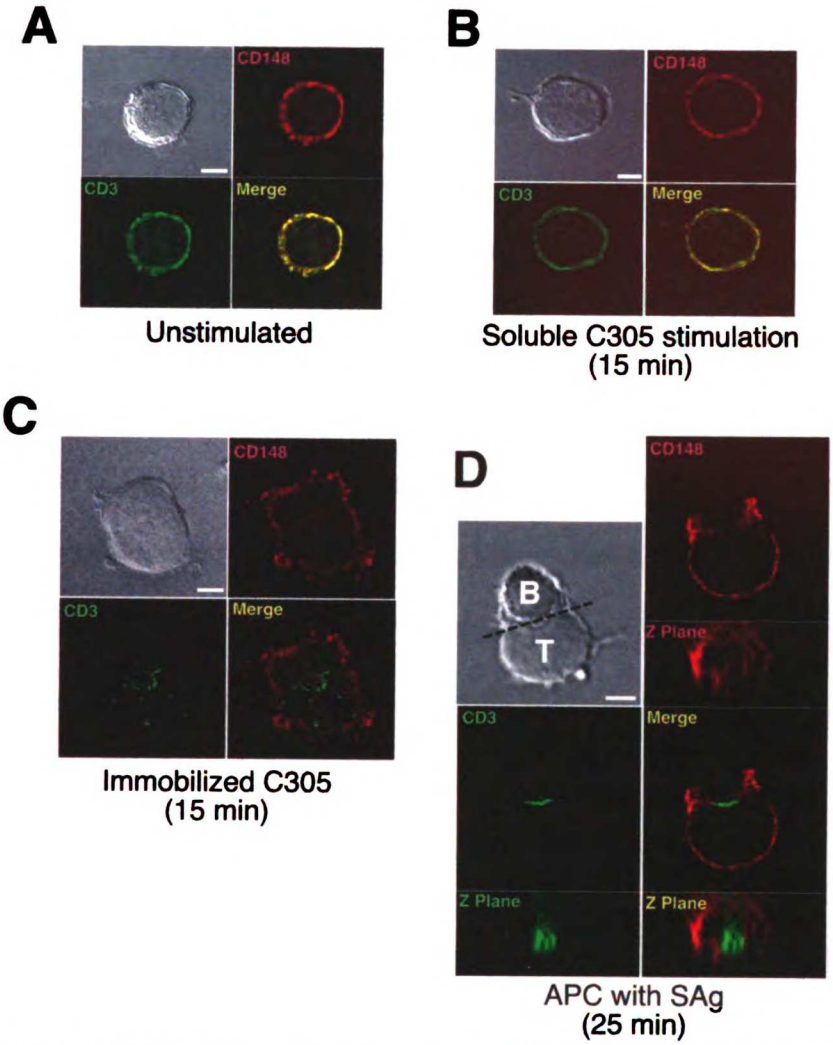

A
Soluble α-TCR
CD148-
(-Dox)
CD148+
(+Dox)
0 1 3 10 30 60 0 1 3 10 30 60
time (min)
160-
105-
75-
50-
35-
pp140
pp70
pp56
pp36
B
α-TCR coated beads
CD148-
(-Dox)
CD148+
(+Dox)
0 1 3 10 30 60 0 1 3 10 30 60
time (min)
160-
105-
75-
50-
35-
pp140
pp70
pp56
pp36
C
% Intensity
+Dox vs. -Dox
100%
80%
60%
40%
20%
0%
pp140
pp36
pp70
pp56
Soluble α-TCR
α-TCR coated beads

Quantitation of phosphotyrosine bands from three separate experiments showed that phosphorylation of pp140 and pp36 was decreased by approximately 50% and 70%, respectively (Figure 5.3C). Interestingly, cells expressing CD148 did not show decreased phosphorylation of pp140 and pp36 when stimulated with anti-TCR-coated beads (Figure 5.3B,C). The tyrosine phosphorylation state of other bands, such as pp70 and pp56, were similar for either stimulus independent of CD148. These data show that the initial events in proximal TCR signaling, as assessed by tyrosine phosphorylation, are affected differently by CD148, depending upon whether soluble or immobilized anti-TCR is used.

CD148 is excluded from the immunologic synapse

To study the cellular localization of CD148 during the response to the various TCR stimuli, immunofluorescence microscopy was performed with antibodies specific to CD148 and the CD3ε chain of the TCR. In resting Jurkat T cells inducibly expressing CD148, both CD148 and CD3 co-localize at the cell surface (Figure 5.4A). Similarly, following soluble anti-TCR MAb stimulation, CD148 and CD3 still show almost complete co-localization with very little compartmentalization of green and red staining (Figure 5.4B). In cells dropped onto coverslips coated with anti-TCR Ab, the cells initially make contact and then begin to spread, as previously reported by others (Bunnell et al., 2002). After 15 minutes, the cells were visualized at the cell-coverslip interface with the same antibodies as before. In striking contrast to a soluble anti-TCR mAb stimulation, cells stimulated by immobilized anti-TCR mAb showed small clusters of CD3 in the center of the cell-coverslip interface, while CD148 accumulated at the edges

Figure 5.4 CD148 does not co-localize with CD3 and is excluded from the immunologic synapse upon triggering with a polarizing stimulus. Cells were induced to express CD148 and stimulated with (A) PBS, (B) soluble anti-TCR mAb (C305), (C) anti-TCR mAb (C305) coated coverslips, or (D) APCs with SAg (1 μg/ml SEE). Immunofluorescence was performed using anti-hCD148 and anti-CD3ε and the appropriate secondary antibodies. CD148 is shown in red while CD3 is shown in green. For (D), the lower panel for each of stain represents the reconstructed Z stack contact view at the dotted line from the DIC image. (E) Jurkat cells were transfected with mCD148 and truncated hCD25, or (F) mCD148, mCD45RO, and truncated hCD25. Transfections were enriched for CD25+ prior to conjugate formation. Cells were stained for CD148 (red), CD3 (green), and either (E) hCD43 (blue) or (F) mCD45 (blue). Bar, 5 μm.

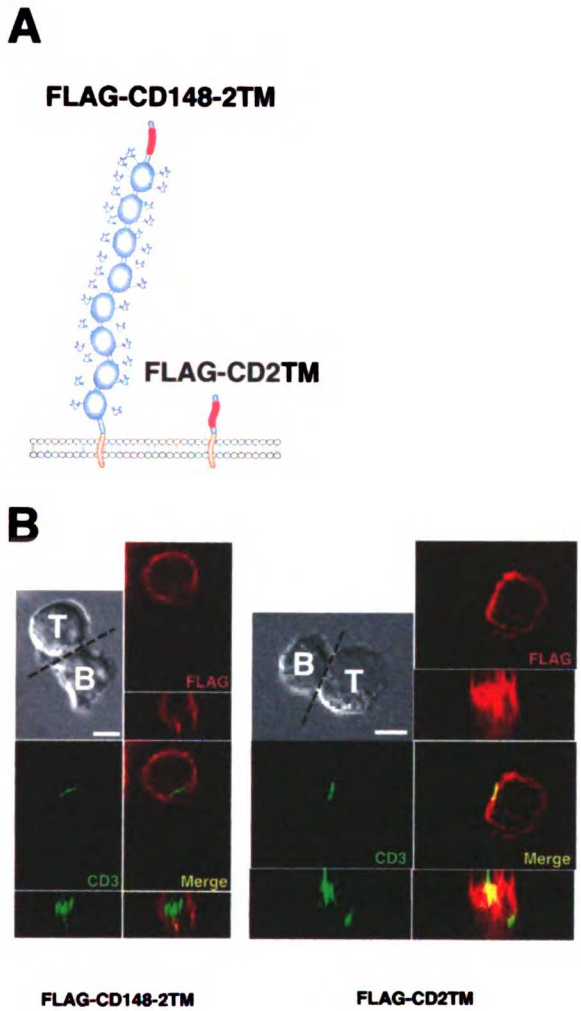

A
CD148
CD3
Merge
Unstimulated
B
CD148
CD3
Merge
Soluble C305 stimulation
(15 min)
C
CD148
CD3
Merge
Immobilized C305
(15 min)
D
B
T
CD148
Z Plane
CD3
Merge
Z Plane
Z Plane
APC with SAg
(25 min)
E
T
B
CD148
CD3
CD43
APC with SAg
(25 min)
F
CD148
CD3
CD43
APC with SAg
(25 min)

of the spread cell (Figure 5.4C). This pattern of CD148 staining is similar to those previously described for CD45 and CD43 using Ab-coated slides (Bunnell et al., 2002). When T cells stimulated with SAg and APCs were visualized, CD3 clustered tightly at the interface of the T cell and APC. Staining of CD148 showed that it was largely excluded from this region of CD3 staining (Figure 5.4D). To further confirm the exclusion of CD148 from the immunologic synapse, localization of CD148 was compared to that of CD43 and CD45, two molecules previously demonstrated to be excluded from the synapse (Delon et al., 2001; Johnson et al., 2000; van der Merwe et al., 2000). As shown in Figure 5.4E and 5.4F, both CD43 and CD45 co-localized with CD148, demonstrating that CD148 is indeed excluded from the immunologic synapse.

The extracellular domain mediates exclusion from the synapse

In order to examine the mechanism that mediates the exclusion of CD148 from the synapse, the entire extracellular domain of CD148, with a FLAG epitope inserted just c-terminal to the signal peptide, was fused to the transmembrane domain of CD2 (FLAG-CD148-2TM). This chimeric molecule was truncated after the sixth cytoplasmic amino acid to prevent its association with CD2AP, since previous reports had shown that CD2AP plays a role in receptor patterning and cell polarization (Dustin et al., 1998). The localization of FLAG-CD148-2TM protein was compared to a protein that only has the FLAG epitope on the extracellular portion of the molecule fused to the same CD2 transmembrane domain (FLAG-CD2TM). The endogenous CD148 signal sequence was used in both constructs for consistency (Figure 5.5A). Transfected Jurkat

Figure 5.5 The extracellular domain of CD148 plays a role in exclusion from the immunologic synapse. (A) A representation of the chimeric molecule containing the signal sequence of mCD148 followed by a FLAG epitope, and the full length mCD148 extracellular domain fused to a truncated hCD2 transmembrane domain (FLAG-CD148-TM2) was compared to an extracellular domain deletion chimera (FLAG-TM2). (B) Cells were transfected with one of the constructs along with truncated hCD25 and enriched for CD25+ cells prior to conjugate formation. Conjugates were fixed and stained with anti-FLAG and anti-CD3ε shown in red and green, respectively. The lower panel for each of stain represents the reconstructed Z stack contact view at the dotted line from the DIC image. Bar, 5 μm. (C) Quantitation of the exclusion of each chimeric molecule out of the immunologic synapse was performed. The graph depicts the mean of two or three experiments, where between 30 to 60 conjugates for each construct were analyzed for each experiment. Error bars represent the standard error of the mean.

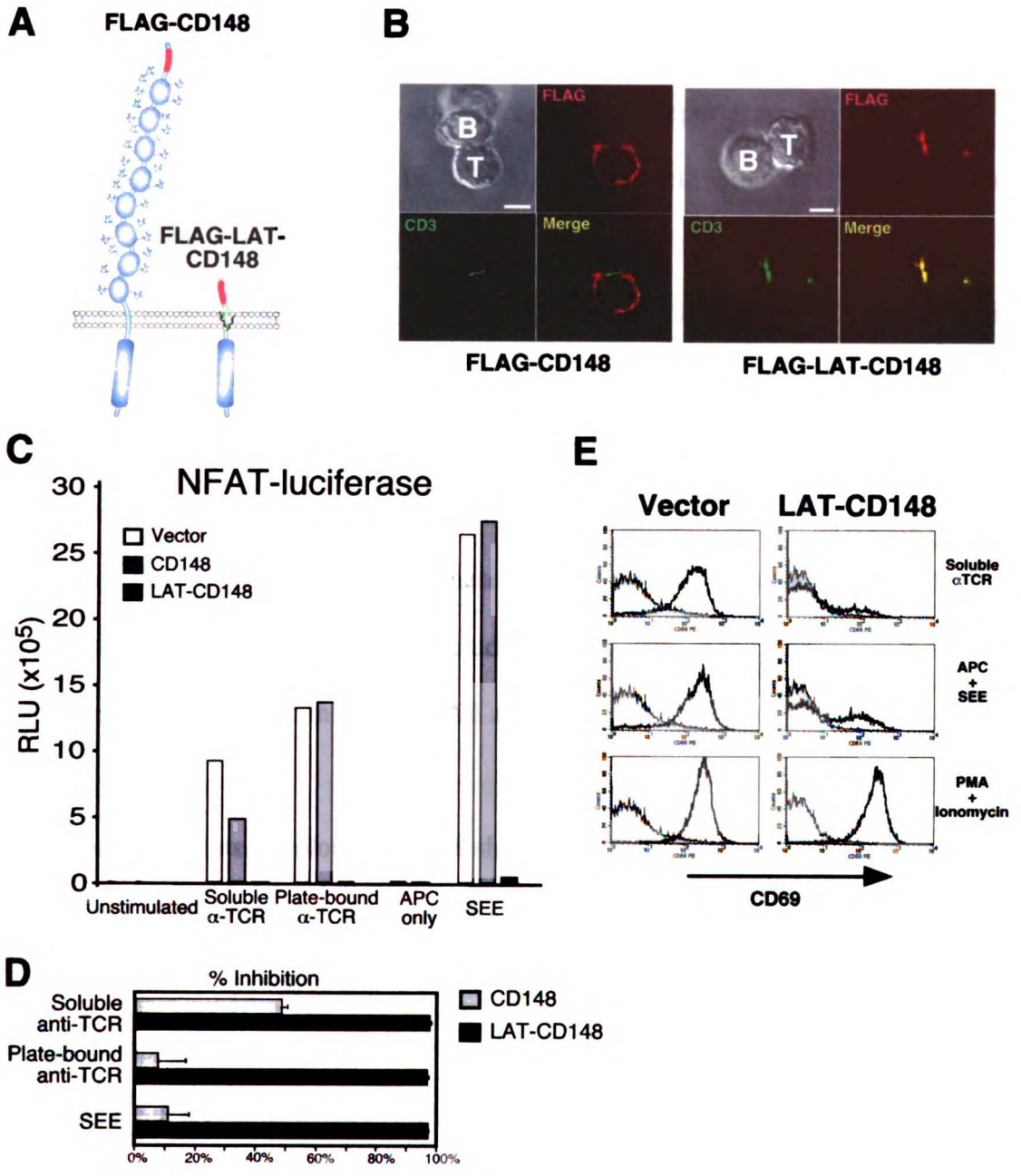

A
FLAG-CD148-2TM
FLAG-CD2TM
B
T
B
FLAG
CD3
Merge
B
T
FLAG
CD3
Merge
FLAG-CD148-2TM
FLAG-CD2TM
C
Exclusion from the synapse
% of Conjugates Excluding
100
80
60
40
20
0
FLAG-CD148
FLAG-CD148-2TM
FLAG-CD2TM

T cells were mixed with Daudi B cells pre-incubated with SAg as before. Conjugates were visualized by immunofluorescence of the FLAG epitope (in red) and CD3 (in green) (Figure 5.5B). The chimera containing the full-length extracellular domain of CD148 was generally excluded from the site of CD3 staining, whereas the molecule containing only the FLAG epitope in the extracellular domain showed considerable co-localization with CD3. Quantitation of multiple conjugates fixed at a 25 minute time point determined that the FLAG-CD148-2TM chimera was excluded a bit less frequently than wildtype CD148, however, much more frequently than just the FLAG-CD2TM (Figure 5.5C). Conjugates were scored as "excluded" if CD3 staining was tightly localized at the site of T cell-APC contact and if the majority of FLAG staining did not co-localize with CD3. These data demonstrate that the extracellular domain does indeed play a major role in the exclusion of CD148, but suggest that other factors may also be involved in exclusion of CD148 from the immunologic synapse.

Targeting of the CD148 phosphatase domain to the synapse inhibits signaling

To determine if exclusion from the immunologic synapse could limit the ability of CD148 to downregulate NFAT, we constructed a chimeric molecule that would target the CD148 phosphatase domain into the synapse (Figure 5.6A). A FLAG-tagged LAT extracellular and transmembrane region was used since previous studies had demonstrated that LAT co-localizes with CD3 (Bunnell et al., 2002; Harder and Kuhn, 2000; Tanimura et al., 2003). Immunofluorescence with antibodies to the FLAG epitope and the CD3ε chain of TCR was performed to demonstrate that the chimera was indeed targeted to the immunologic synapse (Figure 5.6B). It is interesting to note that the

Figure 5.6 Targeting of the CD148 phosphatase domain to the immunologic synapse inhibits NFAT activation. (A) shows a representation of the construct utilized for targeting the CD148 phosphatase domain to the synapse. FLAG-LAT-CD148 was made by inserting a FLAG epitope in the extracellular domain of LAT and fusing the phosphatase domain of CD148 after the LAT transmembrane domain. (B) Transfected Jurkat T cells were enriched for transfected cells as before. Conjugates were formed, fixed, and stained with anti-FLAG and anti-CD3ε shown in red and green respectively. Bar, 5 μm. (C,D) Jurkat T cells were transfected with vector, 2 μg of FLAG-CD148, or 2 μg of FLAG-LAT-CD148 along with 15 μg of a 3x-NFAT-luciferase reporter and rested overnight. Equal protein expression of the two constructs was assayed by flow cytometry for the FLAG epitope (data not shown). Cells were then stimulated with soluble anti-TCR mAb (C305), plate-bound anti-TCR mAb (C305), or with APCs and SAg as in figure 1. (C) The means of triplicate samples in one representative experiment are shown. (D) The mean percent inhibition compared to vector control of each construct for each stimuli is shown where error bars represent standard error from 3 separate experiments, each done in triplicate. Relative light units (RLU) normalized to PMA/Ionomycin. (E) Jurkat cells were transfected with vector or FLAG-LAT-CD148 and stimulated overnight with soluble anti-TCR, APCs and SEE, or PMA and Ionomycin. Cells were then stained for CD69 and analyzed by flow cytometry gating only on transfected cells. The shaded histogram represents CD69 levels on unstimulated cells.


A
FLAG-CD148
FLAG-LAT-CD148
B
FLAG
CD3
Merge
B
T
FLAG-CD148
FLAG-LAT-CD148
C
NFAT-luciferase
Vector
CD148
LAT-CD148
RLU (x10^5)
30
25
20
15
10
5
0
Unstimulated
Soluble α-TCR
Plate-bound α-TCR
APC only
SEE
D
% Inhibition
Soluble anti-TCR
Plate-bound anti-TCR
SEE
CD148
LAT-CD148
0%
20%
40%
60%
80%
100%
E
Vector
LAT-CD148
Soluble αTCR
APC + SEE
PMA + Ionomycin
CD69

efficiency of mature synapse formation was decreased in cells expressing FLAG-LAT-CD148 compared to wildtype FLAG-CD148 (data not shown).

To determine if targeting of the CD148 phosphatase domain to the synapse had a functional effect on T cell signaling, an NFAT-luciferase reporter was co-transfected. Similar to Figure 5.1, the wild type FLAG-CD148 construct inhibited NFAT activation induced by soluble anti-TCR stimulation, whereas plate-bound anti-TCR mAb or SAg and APC stimulation was only slightly affected. When the FLAG-LAT-CD148 chimera was expressed at the same level as the full length FLAG-CD148 molecule, as measured by flow cytometry using the FLAG epitope (data not shown), it completely inhibited NFAT activation for all of the stimuli used. The mean percent inhibition of NFAT activation is depicted in Figure 5.6D. These experiments demonstrate that when the phosphatase domain of CD148 is not excluded from the site of the immunologic synapse, it can potently inhibit TCR-induced NFAT activation.

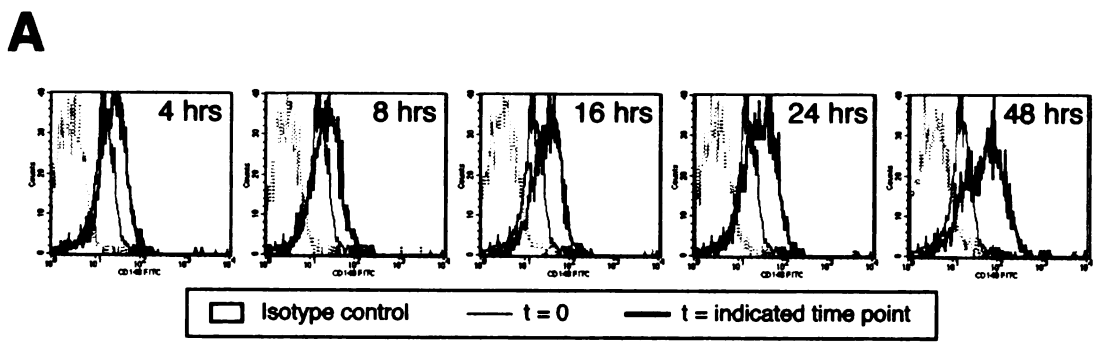
CD148 downregulates prolonged T cell signaling

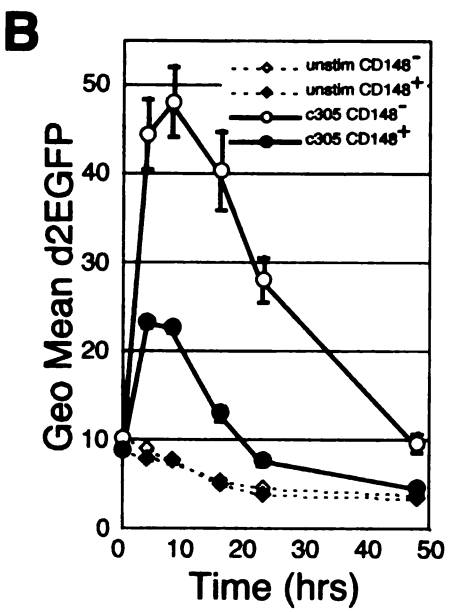
Since expression of CD148 did not have an effect on SAg and APC-induced NFAT activation, presumably due to exclusion from potential substrates associated with the TCR complex, we wanted to test whether CD148 plays a role in downregulating prolonged T cell signaling events. Previous studies had demonstrated that stimulation of primary human T cells with either PHA or anti-CD3 antibodies induces the upregulation of CD148 after 48 hours (Tangye et al., 1998b). To more carefully study the time-course of CD148 upregulation using more physiologic stimulus, we stimulated primary human CD4 T cells with a mixture of SAgs (SEA, SED, SEE) and assayed for CD148

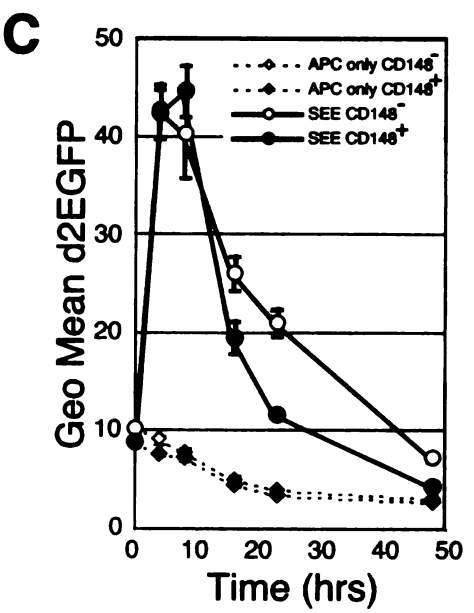
expression by flow cytometry at 0, 4, 8, 16, 24, and 48 hours following stimulation. As shown in Figure 5.7A, freshly isolated CD4+ cells express low levels of CD148. However, following stimulation, CD148 levels are increased, even at 4 hours, and continue to rise. CD148 expression was highest at 48 hours.

Since expression of CD148 continued to increase past 8 hours, we hypothesized that CD148 may play a role in downregulating the prolonged signaling events after the T cell has become activated. To test this hypothesis, an NFAT reporter construct was generated that expresses a destabilized form of the enhanced green fluorescent protein with a half-life of two hours (4xNFAT-d2EGFP). This destabilized form of GFP contains a PEST domain that has been shown to target the protein for more rapid degradation (Li et al., 1998). Response of the 4xNFAT-d2EGFP reporter could then be monitored at later time points by the loss of GFP fluorescence. When reporter-transfected Jurkat T cells inducibly expressing CD148 were stimulated with soluble anti-TCR mAb, NFAT activation was suppressed at all time points, similar to that seen with the NFAT-luciferase construct at eight hours (Figure 5.7B). When these same cells that were stimulated with SAg and APCs, early NFAT responses were similar regardless of CD148 expression. However, at time points after eight hours, cells expressing CD148 downregulated NFAT activation more rapidly than those not expressing CD148 (Figure 5.7C). These data indicate that CD148 does indeed play a role in downregulating the prolonged signaling events following SAg and APC stimulation.

Figure 5.7 Expression of CD148 inhibits prolonged NFAT signaling in T cell APC conjugates. (A) Human PBMCs were isolated and stimulated with a cocktail of SEA, SED, and SEE (300 ng/ml each). Cells were stained at the indicated time points with anti-CD148 and anti-CD4. Histograms represent CD148 expression gated on CD4+ cells. Shaded gray is the isotype-matched control at each time point, whereas the t=0 (thin line) and the indicated time point (thick line) are overlaid. (B,C) Tet-inducible CD148 Jurkats were induced for 48 hours and then transfected with the 4xNFAT-d2EGFP reporter construct and truncated CD25 as a transfection marker. Cells were rested for 6 hours, and then stimulated with the indicated stimuli. Cells were analyzed by flow cytometry at various time points by gating on CD25+, 7-AAD negative cells (live cell marker) and reading out the geometric mean fluorescence of the d2EGFP. (B) Cells + or – Dox were either mock stimulated or stimulated with soluble anti-TCR mAb. (C) Cells + or – Dox were stimulated with either Raji B cells alone or Raji B cells with 250 ng/ml SEE. Shown are the mean from 3 separate experiments where error bars indicate the standard error.


A
4 hrs
8 hrs
16 hrs
24 hrs
48 hrs
Isotype control
t = 0
t = indicated time point


B
unstim CD148-
unstim CD148+
c305 CD148-
c305 CD148+
Geo Mean d2EGFP
Time (hrs)
0
10
20
30
40
50

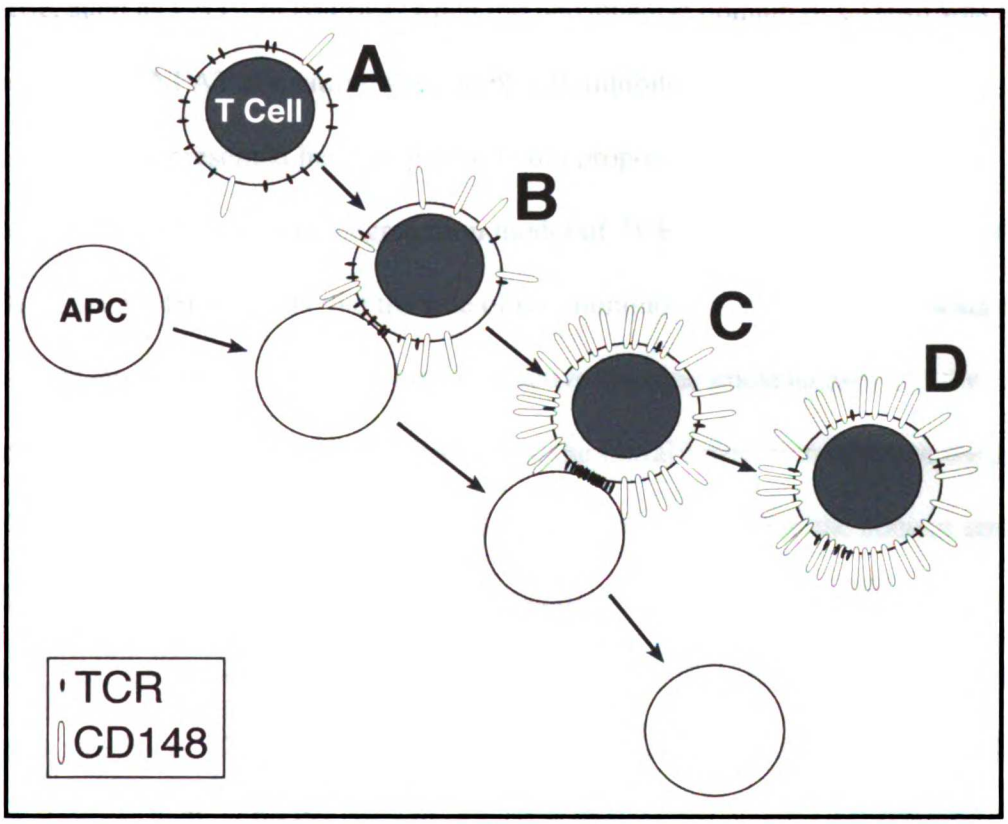

C
APC only CD148-
APC only CD148+
SEE CD148-
SEE CD148+
Geo Mean d2EGFP
Time (hrs)
0
10
20
30
40
50

Discussion

Based on this study, we propose the following model for the role of CD148 in T cell receptor signaling (Figure 5.8). Resting T cells express low levels of CD148 at the cell surface. Upon engagement of the TCR with the appropriate SAg or peptide-loaded MHC molecule, signals are generated to upregulate the expression CD148. The low amounts of CD148 that are present at the cell surface are excluded as the immunologic synapse forms. More importantly, the newly synthesized CD148 that is made in response to the initial T cell activation, are excluded as well. This is critical, since we know that a T cell must receive a continuous signal, on the order of hours, to proliferate and generate IL-2. If CD148 were not segregated away from potential substrates in the synapse, premature termination of T cell response might occur. Once the T cell has disengaged from the APC, CD148 can access its substrates leading to the dephosphorylation of LAT, PLC-γ1, and other substrates, thereby downregulating prolonged T cell activation

To date, the studies investigating the role of CD148 in TCR signaling have relied solely upon soluble stimuli such as PHA, PMA and ionomycin, or soluble anti-TCR Ab (Baker et al., 2001; Tangye et al., 1998b; Tangye et al., 1998c). These techniques, though effective in activating T cells, do not take into consideration T cell polarization following receptor engagement. To further investigate the function of CD148 in TCR signaling, we utilized more physiologic, polarizing stimuli. In experiments involving plate-bound anti-TCR MAb, bead-coated anti-TCR MAb, and APCs presenting SAg, expression of CD148 had only a minimal effect on TCR signaling events such as CD69 upregulation and NFAT activation at early time points. Previous studies have

Figure 5.8 Model of CD148 function in T cells. (A) A mature resting T cell in the periphery expresses low levels of CD148 at the cell surface. (B) When the T cell encounters an APC presenting the proper peptide or SAg, the T cell engages the APC and the TCR clusters at the site of APC contact. During this engagement, the T cell receives the proper signals for the upregulation of CD148. (C) A fully mature synapse stabilizes the T cell-APC contact and prevents the upregulated CD148 molecules from dephosphorylating its substrates within the immunologic synapse. (D) Once the T cell has finished receiving all of the signals required for proliferation and IL-2 secretion, it releases the APC allowing for CD148 to redistribute, access its substrates, and downregulate the sustained signaling events that are taking place at the synapse.


T Cell
A
B
APC
C
D
TCR
CD148

demonstrated that these polarizing stimuli induce the formation of an immunologic synapse-like structure enriched in the TCR and downstream signaling molecules (Bunnell et al., 2002; Lowin-Kropf et al., 1998; Roumier et al., 2001). During T cell engagement by an APC with SAg, CD148 was excluded from the immunologic synapse leading to the notion that this serves to sequester CD148 from substrates that are localized within the synapse, such as LAT. In contrast, when the phosphatase domain of CD148 was targeted to the synapse, NFAT activation was completely inhibited.

The data presented here support a model proposed by van der Merwe and colleagues termed the kinetic-segregation model of TCR triggering (van der Merwe et al., 2000). This model suggests that the role of the immunologic synapse is to create a "pro-signaling" environment by the exclusion of active tyrosine phosphatases and the enrichment of tyrosine kinases. In this model, the initial interaction between the APC and TCR is regulated by multiple small close-contact zones within the contact area that result in the "small scale" segregation of molecules (van der Merwe et al., 2000). Areas such as these could explain why the tyrosine phosphorylation induced with anti-TCR-coated beads is not affected by CD148 even at very early time points (1-3 min). These time points are well under the amount of time it takes to form a mature immunologic synapse (Bromley et al., 2001).

For the purposes of this study, the term immunologic synapse is used to describe the area of TCR enrichment. The immunologic synapse was originally described as being composed of two parts. The central supramolecular activation cluster (c-SMAC) and the peripheral-SMAC (p-SMAC) (Monks et al., 1998). The c–SMAC contains proteins such as the TCR complex, CD28, PKC-theta, and src family kinases. The p-

SMAC, consisting of proteins such as CD2, LFA-1 and talin, appears as a ring that surrounds the c-SMAC. Most studies of the immunologic synapse have been done using mouse T cell clones that respond to APC loaded with a specific peptide (Bromley et al., 2001; van der Merwe, 2002). Since a reactive peptide for Jurkat T cells is not known, the SAg system was used to mimic peptide dependent interactions between the TCR and MHC. In the case of Jurkats stimulated with SAg, we were never able to clearly discriminate between c-SMAC and p-SMAC structures as described in mouse clones and pre-activated T cells. In general, the site of APC contact in this system showed co-localization of molecules from both the c- and p-SMAC (data not shown). This may be a characteristic of Jurkat cells interacting with SAg and APCs or it may be a feature of T cells stimulated with SAg. It is important to note that proteins such as CD43 and CD45, excluded from the synapse in mouse clones, were also excluded in the Jurkat-SAg system as well.

With the elucidation of the ERM-mediated mechanism for CD43 exclusion from the immunologic synapse, the model of purely size-based exclusion has become less favored. However, many groups have demonstrated that altering the size of extracellular domains can alter the function of proteins such as CD45 and CD48 (Irles et al., 2002; Wild et al., 1999). The attachment of the CD148 extracellular domain onto the TM of CD2 alone was enough to cause the exclusion of the chimera from the immunologic synapse, albeit not to the same extent as native CD148. This suggests that size may play a predominant role, but other factors may also contribute to the exclusion of CD148 from the synapse.

Since CD148 has a very similar distribution on the cell surface to that of CD43, an obvious candidate for one of these other factors involved in the exclusion of CD148 was that of the ERM family. Previous studies have mapped the ERM proteins association with CD43, CD44, and ICAM-2 to a series of positively charged amino acids in the juxta-membrane cytoplasmic domain (Yonemura et al., 1998). CD148 does have a similar stretch of residues, but almost all transmembrane proteins do, presumably as a stop-transfer sequence for the transmembrane domain. Experiments attempting to co-immunoprecipitate Ezrin with CD148 did not reveal any association of the two proteins (data not shown). Moreover, the FLAG-LAT-CD148 chimera still contains this string of charged residues on the cytoplasmic face of the TM domain. This construct localized to the synapse, implying that the charged residues do not play a critical role in the exclusion of CD148. Another possible mechanism mediating the exclusion of CD148 from the synapse is the PDZ binding motif present at the very C terminus of CD148 which has been demonstrated to bind syntenin (Iuliano et al., 2001). When the GYIA motif was deleted, this truncated CD148 functioned similarly to wild type CD148 in its ability to downregulate NFAT in response to stimulation with soluble anti-TCR MAb. The PDZ mutant also did not inhibit the immobilized anti-TCR or SAg and APC stimulation, nor was it localized differentially by immunofluorescence microscopy compared to wildtype CD148 (data not shown).

The role of the immunologic synapse in T cell-APC interactions has remained a controversial topic. From studies examining tyrosine phosphorylation by biochemistry and synapse formation by microscopy, it is clear that signaling precedes the formation of the synapse (Lee et al., 2002). Observations such as these have led us to propose that the

synapse is a mechanism whereby the cell can prevent the premature termination of a productive signaling event, as inhibitory proteins such as CD148 become upregulated. Once the T cell has obtained the signal it requires for proliferation and other effector functions, it disengages from the APC, which then allows CD148 to redistribute and gain access to and dephosphorylate substrates. This segregation of phosphatases from their substrates is potentially yet another layer of regulation, beyond protein expression, enzymatic activation, or protein-protein interactions, for the control of T cell activation.

EXPERIMENTAL PROCEDURES

Antibodies and Cells

TCR stimulation was performed with the anti-Jurkat TCR Vβ8 monoclonal antibody (MAb) C305 (Weiss and Stobo, 1984). The anti-phosphotyrosine (4G10) and anti-FLAG (M2) MAb are from Upstate Biotechnology Inc. (Lake Placid, NY) and Sigma (St. Louis, MO) respectively. 7-AAD, anti-CD5 (L17F12), anti-CD25 (2A3), anti-CD69 (L78), anti-CD3ε (SK7) and anti-mCD45 (30F-11) are from BD Pharmingen (San Diego, CA). Anti-human CD148 (A3) has been previously described (Tangye et al., 1998b). Anti-human CD43 was a kind gift from Lewis Lanier. Streptavidin conjugated to Alexa-488, -555, and -647 are from Molecular Probes (Eugene, OR), and goat anti-mouse IgG-Cy5 and donkey anti-rat IgG-Cy3 are from Jackson ImmunoResearch (West Grove, PA). Jurkat T cells, Daudi B cells, and Raji B cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin. Tetracycline-inducible CD148 cells (clone L12) were previously described (Baker et al., 2001). For PBMC separation, buffy coats from whole blood were layered onto Ficoll-Hypaque (Sigma) and centrifuged at 350 x g for 30 min at room temp. Cells were washed and resuspended in RPMI 1640 with 10% fetal bovine serum, 2 mM glutamine, penicillin, and streptomycin.

Plasmids

The pcdef3-mCD148 was constructed by removing the mCD148 cDNA from pME18S-Byp (a kind gift from Dr. Hisashi Umemori) and inserting it into pcdef3 vector cut with Eco RV. This version of the mCD148 cDNA has a T175S compared to the

published sequence. The FLAG-mCD148 was generated by inserting a FLAG epitope between Ala38 and Ala39 (after the signal sequence) in pcdef3-mCD148. Residue numbers for FLAG-mCD148 do not include the amino acids from the FLAG epitope. The FLAG-CD148-2TM was created by fusing the FLAG-mCD148 extracellular domain at Asp870 to Ile210 of hCD2. The FLAG-CD2TM construct was made by fusing the signal sequence and FLAG epitope of FLAG-mCD148 at Ala48 to Gly207 of hCD2. Both CD2-containing constructs were truncated at Arg241. The FLAG-LAT-148 construct was made by first inserting a FLAG epitope after the start methionine of human LAT. The fusion joined LAT (Pro33) to mCD148 (Phe896) with a Ser linking the two fragments. Fusion constructs were made by multi-step PCR and all final products were sequenced. The 3xNFAT-luciferase plasmid was previously described (Shapiro et al., 1996). Murine CD45RO was expressed in a pcdef3 expression construct. The 4xNFAT-d2EGFP was made by replacing the EGFP from a 4xNFAT-EGFP with the d2EGFP (Clontech, Palo Alto, CA). The original 4xNFAT-EGFP was made by placing 4 copies of the NFAT/AP-1 binding site from IL-2 promoter in pEGFP-1 (Clontech).

APC and SAg stimulation

A 1:1 ratio of Daudi or Raji B cells to Jurkat T cells were used as APCs. Cells were loaded with the indicated staphylococcal enterotoxin (SE) SAg for 30 min prior to stimulation. SEE, SEA, and SED were purchased from Toxin Technology inc. (Sarasota, FL). For PBMC stimulation, a mixture of SEA, SED, and SEE were used at 300 ng/ml of each type. Cells were centrifuged to the bottom of a round bottom tube to initiate stimulation and left undisturbed until time of harvest.

Transfection and enrichment

Jurkat cells were transfected as previously described (Baker et al., 2001). For enrichment of transfected cells, cells were co-transfected with a truncated CD25 construct. 18 hours later, cells were labeled with anti-human CD25 MACS beads (Miltenyi Biotec, Auburn, CA) for 20 min on ice and then washed. Cells were positively selected with an LS column and MACS magnet (Miltenyi, Biotec). Enriched cells were resuspended in media and allowed to rest for 2 hours.

Lysates and western blots

Cells were lysed in 1% NP40 and visualized by western blotting as previously described (Lin and Weiss, 2001a). For quantitation of western blots, bands were quantitated on a Kodak Imaging Station using Kodak 1D image analysis software version 3.5 (Eastman Kodak Co., Rochester, NY).

Immunofluorescence

Cells were placed onto poly-L-lysine coated slides and allowed to settle for 5 min. Conjugates were made by pre-loading Daudi B cells with 1 μg/ml of SEE for 30 min prior to mixing 1:1 with T cells in complete media. Cells were then gently centrifuged for 30 sec and placed at 37 C for 25 min. Conjugates were gently resuspended and allowed to settle on slides as before. Paraformaldehyde was added to 3% final concentration for 30 min. Cells were then blocked in 1% bovine serum albumin and 10% rabbit serum in PBS. Cells were stained with the indicated antibodies, followed by the

appropriate secondary antibodies when necessary. Slides were visualized on a Marianas Turn-Key system from Intelligent Imaging (Denver, CO) and images were analyzed using SlideBook software (intelligent Imaging). Images were deconvolved by nearest neighbor and exported as TIFF files.

Reporter assays

Jurkat cells were transfected, as before, with 20 μg of a 3x NFAT-luciferase reporter construct and the indicated CD148 construct. Expression of the CD148 constructs was examined by flow cytometry. Stimulation for luciferase assays was carried out for 8 hrs. Cells were harvested, lysed, and assayed for luciferase activity as previously described (Shapiro et al., 1996). For NFAT activation time-course experiments, CD148 was induced on clone L12 for 48 hrs prior to transfection. 15 μg of a truncated CD25 construct was co-transfected with 20 μg of the 4xNFAT-d2EGFP reporter. The cells were allowed to recover for 6 hrs prior to stimulation. NFAT activation was assessed as the geometric mean fluorescence intensity of CD25 positive, 7-AAD negative cells.

Chapter 6

Implications and future directions

The data presented in this thesis have elucidated a few possible mechanism of how T cells regulate their activation through LAT and CD148. Multiple studies from various groups have convincingly shown that LAT plays a critical role for the development and activation of T cells (Finco et al., 1998; Zhang et al., 1999b). Also, the role of CD148 as a negative regulator is clear. Though much has been learned about the functions of both LAT and CD148 in T cells, many questions remain, a few of which will be discussed in this chapter.

Role of cysteine 26 and 29 for LAT localization in lipid rafts

Chapter 2 of this thesis demonstrates a functional requirement for the localization of LAT in lipid rafts, but why is this required? Does LAT need to be localized in lipid raft to be phosphorylated by ZAP-70? Since the C26/29S mutation of LAT can still be phosphorylated, this is unlikely. Dramatically, the C26/29S is phosphorylated even in the unstimulated state, but cannot reconstitute LAT function. This demonstrates that the phosphorylation state alone does not necessarily reflect LAT's ability to reconstitute signaling. Even though the C26/29S LAT mutant is phosphorylated in the resting state, the basal level of phospho-Erk or NFAT activation is not higher. This implies that recruitment to rafts may be required to interact with its binding partners. Another possibility is that lipid raft localization is required for the dephosphorylation of LAT or turnover of palmitoylation sites. It is important to keep in mind that phosphorylation of LAT based on phosphotyrosine immunoblotting does not provide information as to which

sites are phosphorylated. It may be that in the C26/29S LAT mutant, different tyrosine residues are phosphorylated compared to the wt LAT molecule.

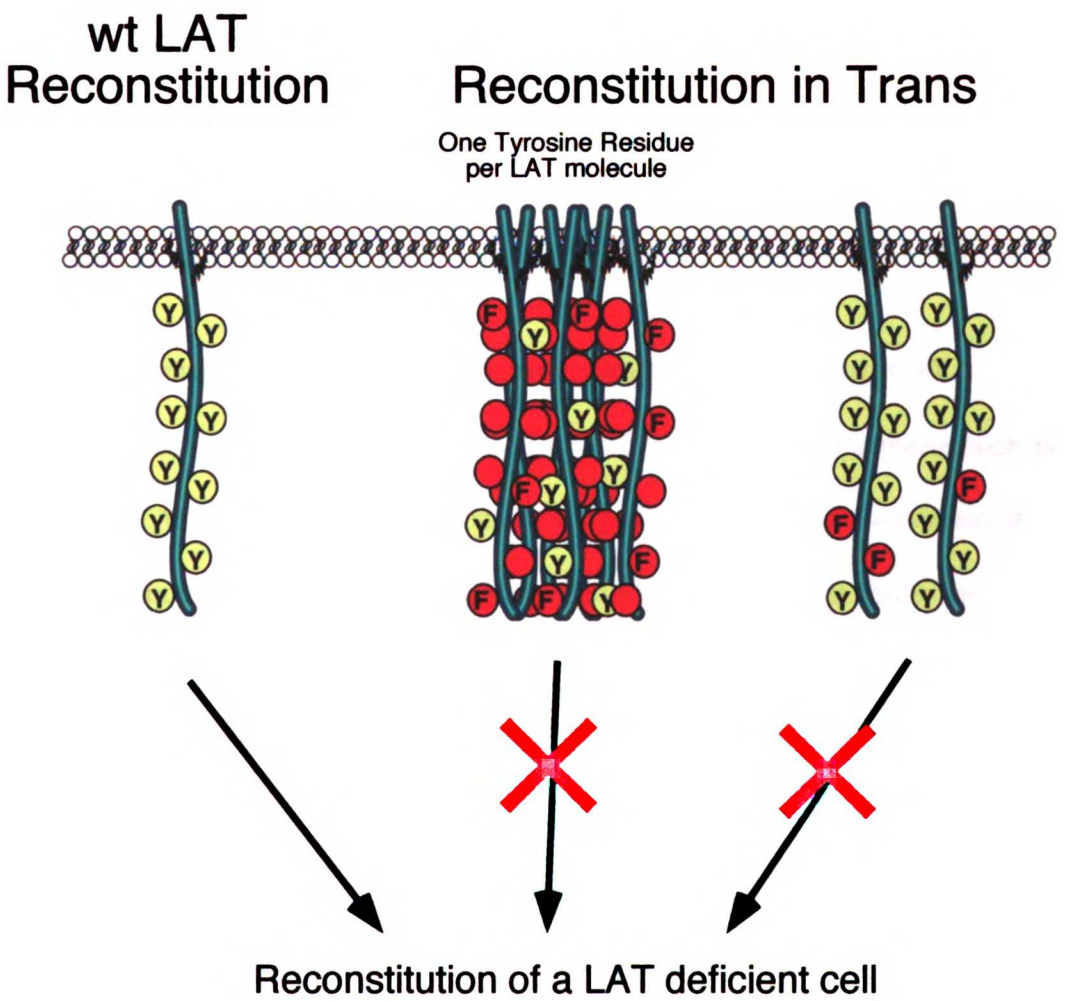
Other questions about the requirement of LAT in lipid rafts are derived from the observation that only about half of the LAT molecules exist in the insoluble fraction of a sucrose gradient. Is this due to depalmitoylation of LAT and could this be another regulatory mechanism? There is evidence that reversible palmitoylation can have functional effects on signaling proteins (Mumby, 1997). For example, in the case of $G_{s\alpha}$, the activating point mutant, R201C, causes the half-life of the attached palmitate group to go from approximately 2 min for wt $G_{s\alpha}$, to 90 min (Wedegaertner and Bourne, 1994). Also, there is evidence that differences in oxidative environment can alter the function of signaling molecules such as Lck and LAT. This could potentially be due to changes in the ability to attach palmityl groups to cysteines (Gringhuis et al., 2000; Gringhuis et al., 2002; Verweij and Gringhuis, 2002). These data support the idea that resident time of LAT in lipid rafts could be an important regulatory mechanism. Utilizing biochemical techniques of raft isolation may not be the best approach since we are assuming that the signaling events occur at the plasma membrane. One concern with sucrose gradient centrifugation is that intracellular membranes are also isolated during this procedure. Exploring other techniques such as immuno-gold electron microscopy (EM) to visualize the lipid rafts and their constituents could clarify contradictory data with various concentrations of different detergents. These types of studies have already been done on mast cells yielding important information on Fc receptor mediated signaling and LAT (Wilson et al., 2002; Wilson et al., 2001). These experiments demonstrated that LAT molecules exist in distinct pools separate from regions where the Fc receptor localizes

upon stimulation. Following stimulation, these pools of LAT aggregated into larger pools. Observations such as these could never have been made using conventional biochemical techniques.

Structure-function analysis of LAT and the proposed signalosome

Most groups who have studied LAT have focused on the role the multiple tyrosine residues within LAT play in mediating downstream signals (Lin and Weiss, 2001a; Zhang et al., 2000; Zhu et al., 2003). The initial models of LAT function suggested that it served as an anchor at the plasma membrane to recruit various proteins to the plasma membrane. However, if this were the case, transfection of LAT-deficient cells with a combination of LAT mutants should reconstitute the cells as long as all the tyrosine residues are present (Figure 6.1). Attempts to reconstitute the LAT-deficient cells in trans did not restore the signaling defect. One explanation comes from the idea that LAT does not simply recruit molecules to the plasma membrane. Instead, LAT may nucleate molecules into a larger complex, which has been termed a signalosome. Formation of the signalosome could require multiple inter-domain interactions, which are needed to stabilize the larger multimeric complex. Studies have previously demonstrated that the binding kinetics of single SH2-phosphotyrosine interactions are on the order of K_d=0.1 to 1.0 μM which are thought to be fairly transient (Kuriyan and Cowburn, 1997). SH3-Pro interactions were also shown to be in the low micromolar range (K_d=1.0-10 μM). However, increasing evidence suggests that simultaneous multiple domain interactions can significantly increase binding affinity and specificity. Shoelson and

Figure 6.1 LAT-deficient cell must be reconstituted with at least Tyr 132, 171, and 191 on the same molecule: Evidence for simultaneous multi domain interactions. Reconstitution of the LAT deficient J.CaM2 cell line with a mix of LAT constructs each containing only one tyrosine residue or a mix of two constructs (Y132F and Y171,191F) did not result in rescue of the T cell activation in these cells.

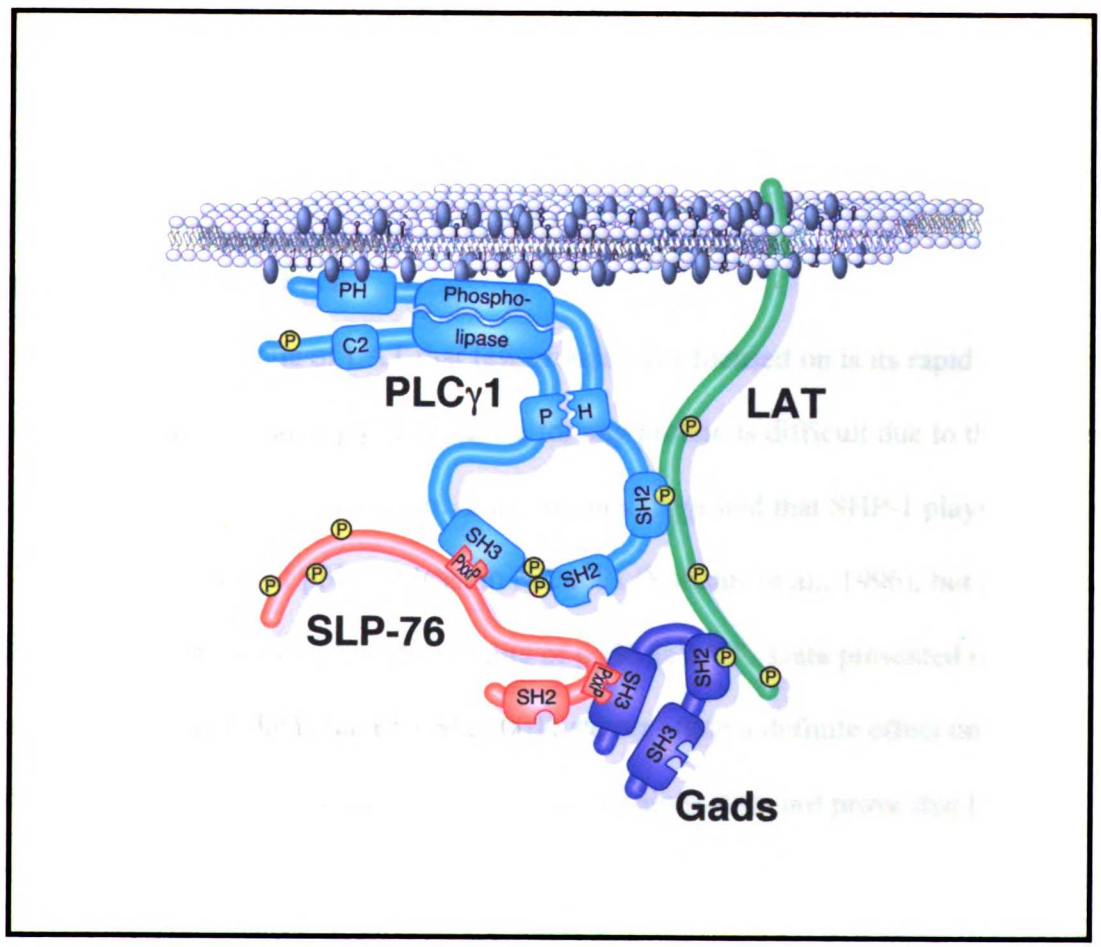

wt LAT
Reconstitution
Reconstitution in Trans
One Tyrosine Residue
per LAT molecule
Reconstitution of a LAT deficient cell

colleagues have shown that interactions increase the K_d from the low micromolar range for a single SH2-phosphotyrosine to the low nanomolar range for tandem SH2-phosphotyrosine interactions (Ottinger et al., 1998). Combining our own data and data from other groups, we hypothesized the existence of a tetrameric complex made up of LAT, PLC-γ1, SLP-76, and Gads (Figure 6.2) (Lin and Weiss, 2001a; Liu et al., 1999; Yablonski et al., 2001; Zhang et al., 2000). This stable complex could then recruit other proteins, such as Tec kinases, to the membrane to potentiate signaling.

Other LAT-like molecules

Obviously, many questions still exist surrounding the mechanism of LAT function in T cell activation. With the identification of new LAT-related proteins such as LAX and LAB/NTAL, more light has been shed on signaling pathways downstream of Ag receptors. Recently, other LAT-like molecules have been cloned that add to the growing list of transmembrane adaptor proteins (Brdicka et al., 2002; Janssen et al., 2003; Zhu et al., 2002) (Figure 1.5). The concept of a B cell equivalent to LAT has been considered for a long time, but no conclusive studies have demonstrated a complete LAT equivalent. It is not clear that a single molecule in B cells will have all the characteristics as LAT in T cells. Some of the hallmarks of LAT are its ability to recruit PLC-γ, Grb2, and the Gads/SLP-76 complex to lipid rafts. In the B cell, LAB/NTAL has been shown to recruit Grb2 and localize it to lipid rafts, but it does not associate with PLC-γ or BLNK, the SLP-76 equivalent in B cells (Brdicka et al., 2002; Janssen et al., 2003). One hypothesis is that multiple molecules in B cells contribute the same functions as a single

Figure 6.2 Model of the proposed four-part signalosome complex. LAT is known to recruit both PLC-γ1 and the SLP-76/Gads complex. There are also reports of PLC-γ1 binding to SLP-76. This model depicts a 4-molecule complex whereby the multiple simultaneous inter-domain interactions serve to stabilize a signalosome complex.


PH
Phospho-
lipase
C2
PLCγ1
P
H
LAT
SH2
SH3
PxxP
SH2
SLP-76
SH2
SH3
PxxP
SH2
SH3
Gads

molecule of LAT in T cells. It has been shown that the Igα subunit of the BCR is localized to lipid rafts following stimulation and that it associates with BLNK through a phosphotyrosine-SH2 interaction (Cheng et al., 2001; Engels et al., 2001; Kabak et al., 2002). PLC-γ recruitment to the plasma membrane has been demonstrated to involve BLNK and the Tec family kinase BTK (Chiu et al., 2002). These data along with the studies involving LAB/NTAL could explain why a single LAT equivalent in B cells has not been identified.

Dephosphorylation of LAT

One characteristic of LAT that few groups have focused on is its rapid dephosphorylation. Studying phosphatases for any protein is difficult due to the inherent promiscuity of phosphatases in vitro. Early studies suggested that SHP-1 plays a role in the dephosphorylation of pp36, at least in NK cells (Valiante et al., 1996), but conclusive evidence has been lacking since the cloning of pp36 as LAT. Data presented in chapter 5 along with published data (Baker et al., 2001), demonstrate a definite effect on LAT phosphorylation in the presence of CD148, but that alone does not prove that LAT is a direct substrate for CD148. Also, the fact that Jurkat T cells do not express endogenous CD148, yet LAT phosphorylation dramatically declines, on the order of minutes, following TCR stimulation. These data suggest that other phosphatases besides CD148 play a role in dephosphorylating LAT.

Attempts to search for the LAT phosphatase were carried out using an in-gel phosphatase assay. For this, recombinant LAT was produced in bacteria and purified. This LAT was then phosphorylated in vitro with recombinant Syk in the presence of γ-

^{32}P-ATP. The radio-labeled LAT was incorporated into an acrylamide gel, and lysates run into the gel. The gel was then washed to renature the proteins within the gel and incubated to allow for its dephosphorylation. The gel was dried and exposed on film. In places where dephosphorylation of LAT took place, a white band appeared against a black background. Though this technique did yield some potential candidates based on size, it was difficult to identify most bands. Now that nearly all mammalian protein tyrosine phosphatases have been identified, proteins with correct molecular weights could be examined for their specificity to substrates. By generating substrate-trapping mutants within the phosphatase domain, candidate phosphatases could be tested (Flint et al., 1997). These mutations prevent the removal of the phosphate group resulting in a change in V_{max} of the phosphatase without changing its K_d.

Regulation of CD148

Although much about CD148 has been learned, very simple questions still deserve investigation. Like most RPTPs, CD148 has a rather large extracellular domain. In the case of CD148, the extracellular domain is composed of 8 heavily glycosylated fibronectin type III domains. What role these domains play in function still remain a mystery. Only one published study has attempted to identify a ligand for CD148 (Sorby et al., 2001). By using extracellular matrix preparations, Sorby and colleagues demonstrated that an unidentified molecule within the Matrigel preparation used could affect the phosphatase activity of CD148. This study implies that there could be a CD148 ligand present, but this is far from conclusive evidence. Little effort has been expended in the search for a ligand. One could easily generate an Ig-fusion protein with the

extracellular domain of CD148 or set up a functional screen with the extracellular domain of CD148 fused to the zeta chain of the TCR. Importantly, the second idea is based on the assumption that a CD148 ligand would induce dimerization and would lead to downstream signaling events, such as activation of an NFAT reporter.

Some RPTPs have been shown to be regulated by dimerization (Desai et al., 1993; Jiang et al., 2000). Although currently no evidence exists for such regulation for CD148, the idea is intriguing. Antibodies to CD148 have been used in attempts to alter activity of CD148, however the exact mechanism of how such antibodies affect activity is not known (Tangye et al., 1998b). In other systems, such as with CD2 and CD45, different antibodies to the same molecule can elicit significantly different functional effects, therefore, one must be careful when interpreting such antibody data (Alsinet et al., 1990; Miller et al., 1993). To test the consequences of dimerization, one technique that has been utilized for other phosphatases is to fuse the extracellular domain of a known receptor that dimerizes, such as the epidermal growth factor receptor, to the normal phosphatase domain of the protein of interest (Desai et al., 1993). Such experiments have not been attempted with CD148. They could easily determine if forced dimerization of the CD148 phosphatase domain has an effect on the ability of CD148 to inhibit T cell activation.

A few questions about the mechanism of CD148 function in T cells also remain to be answered. One intriguing question is what other factors play a role in the exclusion of CD148 from the immunologic synapse that was described in chapter 5? Potential mechanisms center around protein-protein interaction regions within CD148. The cytoplasmic region contains only a single phosphatase domain, however, the amino acid

sequence GYIA at the C terminus has been shown to bind a PDZ domain containing protein called syntenin (Iuliano et al., 2001). No role of syntenin has been identified or reported in TCR signaling. However, the identification of this interaction opens the door for potential associations with other PDZ domain containing proteins. Interestingly, the cytoplasmic domain of CD148 consists of only a single phosphatase domain. Most transmembrane phosphatases have two, with only the membrane proximal one being functional (Li and Dixon, 2000). Initial theories were that the second phosphatase domain conferred substrate specificity or sterically regulated the proximal phosphatase domain, however this is apparently not the case with CD148.

Other clues regarding possible mechanisms of CD148 exclusion from the synapse comes from studies done with other molecules known to be excluded from the synapse, such as CD43. Recent papers have demonstrated that the ERM family of proteins plays a role in actively recruiting CD43 out of the immunologic synapse. The family of ERM proteins, which refers to ezrin, radixin, and moesin, bind to charged residues proximal to the transmembrane domain of CD43. Through interactions with actin, ERM proteins function to re-localize CD43 following APC engagement (Allenspach et al., 2001; Delon et al., 2001; Yonemura et al., 1998). Experiments to test whether these proteins play a role in CD148 exclusion from the immunologic synapse have not yet been performed.

The Role CD148 in the immune system

Much of the discussion sections from chapters 4 and 5 examine the signaling function of CD148 within a cell, however the role of CD148 in an immune response also deserves attention. These data suggest an important negative regulatory role for CD148

in response to antigen, however the most definitive experiments to study the role of CD148 in an immune response have yet to be done. Data from a tissue specific deletion in mice of CD148 in T cells, B cells, and other hematopoietic cells will hopefully answer many questions. One may predict that since CD148 plays a role in the downregulation of an immune response, loss of CD148 may lead to autoimmunity or even development of lymphoma. If fact, there is some evidence that CD148 acts as a tumor suppressor gene in colorectal cancer (Ruivenkamp et al., 2003; Ruivenkamp et al., 2002). Since CD148 is not highly expressed in the thymus, one would not foresee a defect in T cell selection. In contrast, B cells constitutively express CD148. Therefore, one might predict that loss of CD148 in the B cell lineage could cause defects in B cell tolerance or increased Ig production leading to loss of B cell regulation in response to antigen.

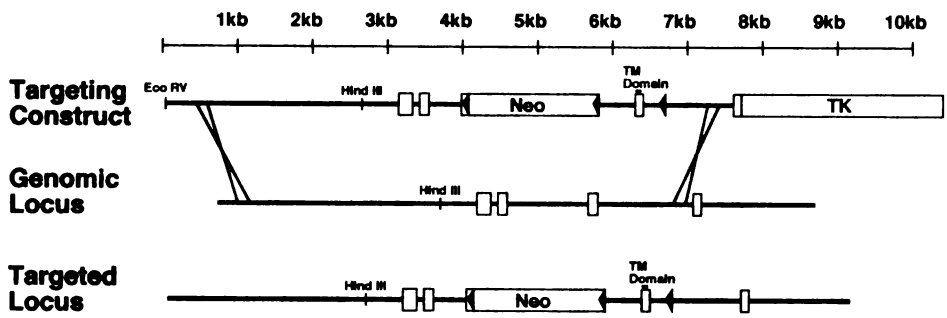
The complete inactivation of CD148 in the mouse leads to an early embryonic lethality due to angiogenic defects (Takahashi et al., 2003). We did try hematopoietic stem cell transfers from homozygous deficient fetal liver cells, but data were inconclusive due to strain differences. The CD148 deficiency on a pure inbred C57/BL6 background, leads to embryonic lethality before hematopoietic stem cells migrate to the liver from the yolk sac. When the mice were bred onto the out-bred Black Swiss strain of mice, the embryos survived long enough to harvest hematopoietic stem cells, however experiments using mice on the out-bred background did not provide conclusive data.

Therefore, we attempted to generate a tissue specific knockout. Progress has been made to create an allele of CD148 that encodes loxP recombination sequences flanking both sides of the exon encoding the transmembrane domain. Following expression of the

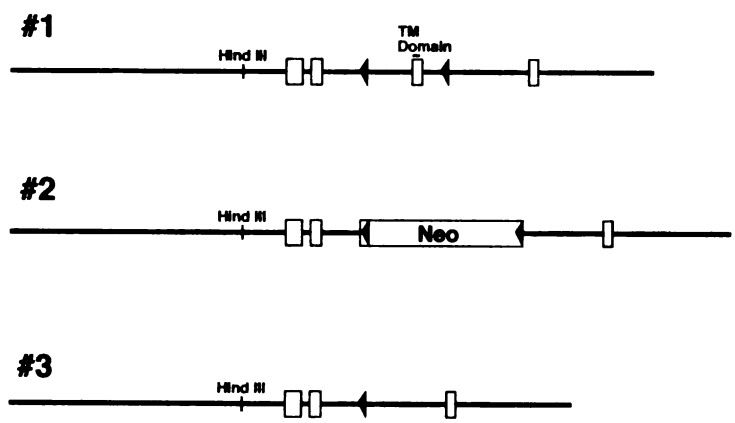
Cre recombinase, the exon flanked by the recombination sequences would be deleted resulting in the loss of functional CD148. A diagram of the targeting construct is shown in Figure 6.3. The construct was transfected into ES cells and homologous recombinants isolated. Positive clones were then transiently transfected with Cre to cause loss of the neomycin resistance cassette. Attempts to generate mice from these floxed ES cells by injection into blastocysts yielded no chimeras. A postdoctoral fellow in the lab will continue this project.

T cell signaling is a constantly changing field with significant questions still remaining unanswered. As more progress is made, many of the missing components of the various signaling pathways are falling into place. Eventually, through the continuing work of many labs, the many pathways involved in signaling through the TCR will be fully understood. Since T cells play various critical roles in orchestrating the immune responses, this knowledge should lead to an understanding of how breakdowns in immune regulation leads to autoimmune diseases, and how the immune system could be better manipulated to overcome afflictions such as cancer, infection, or autoimmunity.

Figure 6.3 Schematic of the CD148 conditional knockout. The CD148 targeting construct contains homologous arms that flank the exon containing the transmembrane (TM) domain of CD148. A floxed Neo cassette is placed 5' to the TM exon and a third lox site is placed 3'. After screening for homologous recombination in transfected embryonic stem (ES) cells, positive clones will be transfected with a Cre expression construct. Cre expression will induce recombination leading to three potential outcomes. Clones with recombination possibility #1 will be injected into blastocysts to generate chimeras. Chimeras will then be bred for germline transmission of the targeted allele.



Transfection of Cre leads to three possibilities



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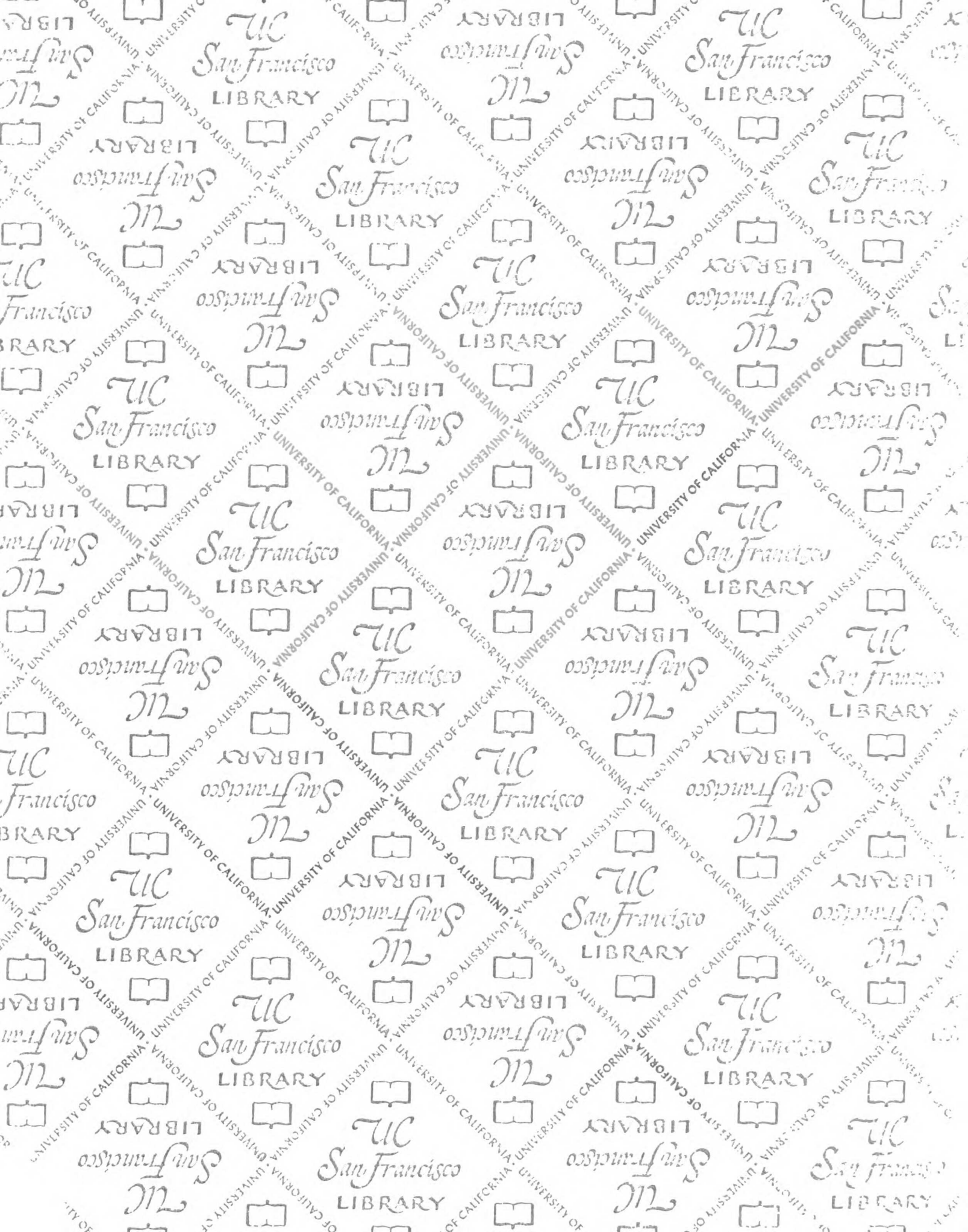
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