THE INTERACTIONS OF LCK IN T CELLS AND ITS REGULATION BY CD45

By

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ABSTRACT

Lck is a Src-family tyrosine kinase whose activity is essential for T cell activation and development. The phosphorylation of two key tyrosine residues in Lck, Tyr 505 and Tyr 394, regulate its activation by inducing profound changes in conformation. *In vitro*, CD45, a leukocyte-specific tyrosine phosphatase, preferentially dephosphorylated Tyr 394, as opposed to Tyr 505, and this specificity depended on the Lck non-catalytic domains and not the sequence specificity of the phosphatase catalytic pocket. The Lck non-catalytic domains enhanced the rate of CD45-mediated Tyr 394 dephosphorylation, and inhibited, but did not block catalysis at Tyr 505. A direct, specific, non-catalytic interaction was observed between the second phosphatase domain of CD45 (D2) and subdomain *X* (SD10) of Lck. This interaction was also shown to influence CD45 substrate specificity as the replacement of Erk1 SD10 with that of Lck resulted in the conversion of Erk1 into a more efficient CD45 substrate.

In T cells, the majority of CD45 is constitutively associated with a smaller protein aptly named CD45-associated protein (CD45AP). An interaction between Lck and CD45AP was determined to occur independently of CD45 in T cells. A direct CD45AP:Lck interaction was confirmed *in vitro*, and was shown to antagonize the ability of CD45 to interact with Lck. Moreover, the presence of CD45AP significantly inhibited CD45-mediated Lck dephosphorylation at Tyr 394, but not Tyr 505, suggesting a role for CD45AP in sustaining Lck activity in the cell.

CD44 is a widely expressed transmembrane adhesion molecule that requires the activity of Src-family kinases in T cells to induce cytoskeletal changes associated with cell adhesion. Here, Src-family kinases Lck and Fyn were both shown to associate with

CD44 in T cells. *In vitro*, the CD44 cytoplasmic domain bound to Lck directly in a zinc dependent manner, but did not bind to Fyn. The treatment of T cells with cation chelator 1,10-phenanthroline both disrupted the CD44:Lck association and blocked CD44-induced cytoskeletal changes. This suggests that the zinc-dependent Lck association with CD44 plays a key role in mediating CD44-induced cell signaling events required for cell adhesion.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
Arg	arginine
BSA	bovine serum albumin
cDNA	complementary DNA
CIP	calf intestinal phosphatase
CNBr	cyanogen bromide
Csk	C-terminal Src kinase
cyt	cytoplasmic domain
Cys	cysteine
D1	first, N-terminal protein tyrosine phosphatase domain
D2	second, C-terminal protein tyrosine phosphatase domain
DMEM	Dulbecco's modified Eagle medium
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
Erk1/2	extracellular signal related kinase 1/2
ERM	ezrin, radixin, moesin
FAK	focal adhesion kinase
Fyn	$p59^{fyn}$
GEM	glycolipid enriched membrane
GSH	glutathione coated sepharose beads
GST	glutathione-S-transferase
h	hour
HA	hyaluronan
His	histidine
HRP	horseradish peroxidase
IPTG	isopropyl β-D-thiogalactoside
ITAM	immunoreceptor tyrosine-based activation motif

kb	kilobase pair
kDa	kiloDalton
LAT	linker for activation of T cells
LFA-1	lymphocyte function associated antigen
Lck	$p56^{lck}$
Lys	lysine
mAb	monoclonal antibody
min	minute
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PKC	protein kinase C
PLCγ1	phospholipase C γ1
PMSF	phenylmethylsulfonyl fluoride
PTP	protein tyrosine phosphatase
pTyr	phosphorylated tyrosine
PVDF	polyvinylidene fluoride
SDS	sodium dodecyl sulfate
Ser	Serine
SH2	Src homology 2
SH3	Src homology 3
Src	$p60^{src}$
TCR	T cell receptor
Thr	threonine
Tyr	tyrosine
ZAP70	$p70^{ZAP}$

LIST OF PUBLICATIONS

Lefebvre, D. C., Felberg, J., Cross, J., and Johnson, P., The non-catalytic domains of Lck regulate its dephosphorylation by CD45; *Biochim. Biophys. Acta.*, In press (2003)

Felberg, J., Lefebvre, D. C., Wang, Y., Lam, M., Ng, D. H. W., Birkenhead, D., Cross, J., and Johnson, P., An interaction between the subdomain *X* of the kinase domain of Lck and the inactive second tyrosine phosphatase domain of CD45 augments dephosphorylation by CD45 and influences substrate specificity, *Mol. Cell. Biol.*, Submitted for review (2003)

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CHAPTER 1

Introduction

1.1 An introduction to T cell immunology

In 1796, Edward Jenner discovered that an injection of cowpox could provide protection from the fatal human disease, smallpox. This isolated finding led to the eradication of smallpox two centuries later, introduced vaccines to modern medicine, and initiated the study of immunology. The immune system is an intricate network of tissues, cells, and molecules that function in a coordinated manner to battle a multitude of foreign substances, infectious pathogens, and even the cancers of our own tissues. Jenner's smallpox vaccine highlighted the importance of our adaptive immune system, a sophisticated response that is highly specific, tremendously diverse, and able to provide lifelong immunity.

T lymphocytes play several important roles in the adaptive immune response. These include direct effector functions such as stimulating B cells to produce antibodies and the killing of virally infected cells, as well as indirect functions such as coordinating the immune response by secreting cytokines. The importance of these T cell functions is demonstrated in athymic *nu/nu* mice, and in AIDS patients. In both cases the subject is severely immuno-compromised due to a lack of T cells, and therefore often succumbs to the symptoms of microbial and viral infections.

Also critical is the proper control of T cell function since abnormal T cell activation can lead to cancers and autoimmune diseases. Upon recognition of antigen by the T cell receptor (TCR), intracellular protein kinases initiate and amplify a signaling cascade resulting in the phosphorylation and activation of many enzymes. Activated enzymes then signal for gene transcription resulting in T cell proliferation and differentiation. Conversely, protein phosphatases serve to dephosphorylate many

activated cellular proteins. Thus, T cell activation, the adaptive immune response, and the onset of disease all hinge on the delicate balance between kinases and phosphatases in the cell (reviewed in 1, 2). Lck, a tyrosine kinase, and CD45, a tyrosine phosphatase, are two enzymes that dictate this balance, and are critical for proper T cell activation and development.

1.2 Lck is a member of the Src family of tyrosine kinases

1.2.1 The discovery of Src-family kinases and Lck

Nearly a century ago, Peyton Rous observed a transmissible avian neoplasm of the common fowl, later found to be caused by a viral pathogen termed the Rous sarcoma virus. Sixty-five years after Rous' observation, the oncogenic potential of this virus was identified as being due to its expression of a gene encoding a subtle mutation in the normal cellular gene, c-Src (3). This viral version, v-Src, is the first oncogene discovered, and c-Src is now the prototype for the non-receptor Src-kinase family (reviewed in 4). Currently, there are nine members of the Src-kinase family in vertebrates including Src, Fyn, Yes, Fgr, Lyn, Hck, Lck, Blk and Yrk. All members share similarities in domain structure and in regulation via tyrosine phosphorylation (reviewed in 5, 6). However, they differ in tissue expression (reviewed in 7), subcellular localization (reviewed in 4), and in the amino acid sequence of their N-terminal "unique" domain (8). Of particular importance to the immune system are Src-family kinases expressed in hematopoietic cells: Fyn, Fgr, Lyn, Hck, Lck and Blk. Of these, only Lck and Fyn display significant expression in T lymphocytes.

Lck, like most members of the Src-family, was discovered due to its oncogenic potential and cell transforming abilities. Studies with the LSTRA murine T lymphoma cell line detected elevated levels of a 56-kDa phospho-protein (p56) that was potentially a tyrosine kinase distinct from other known Src-family kinases (9). The same group later isolated the cDNA for the entire p56 gene, determined it to be a novel protein tyrosine kinase (PTK) with extensive homology with p60 c-Src, and suggested the name p56tck, for T cell kinase (10). In parallel, a second study characterized a Src-related, lymphocyte-specific PTK gene that was rearranged and over-expressed in the T cell lymphoma LSTRA due to retroviral insertion adjacent to the gene (11). This gene, later named Lck (lymphocyte cell kinase), was localized to human chromosome 1 proximal to a site of frequent abnormalities in human lymphomas and neuroblastomas (12).

1.2.2 Lck genetics

The Lck gene is composed of 12 exons that show a common organization pattern throughout the Src-family kinases (reviewed in 7). In mice and humans, expression of the Lck gene is driven from two structurally independent promoters (reviewed in 13). Immediately adjacent to the first coding exon, the proximal promoter remains dormant in peripheral T cells, but is active in the thymus suggesting a role in T cell development (14, 15). In contrast, the distal promoter, located several kb 5' of the Lck coding region (35 kb in humans, 15), remains active in immature and mature T cells. Interestingly, the over-expression of Lck in LSTRA cells that led to its identification was due to the insertion of a Moloney murine leukemia virus long terminal repeat (LTR) upstream of the Lck proximal promoter. This insertion induced profound increases both in Lck gene transcription and mRNA translation resulting in a 50-fold enhancement of Lck protein

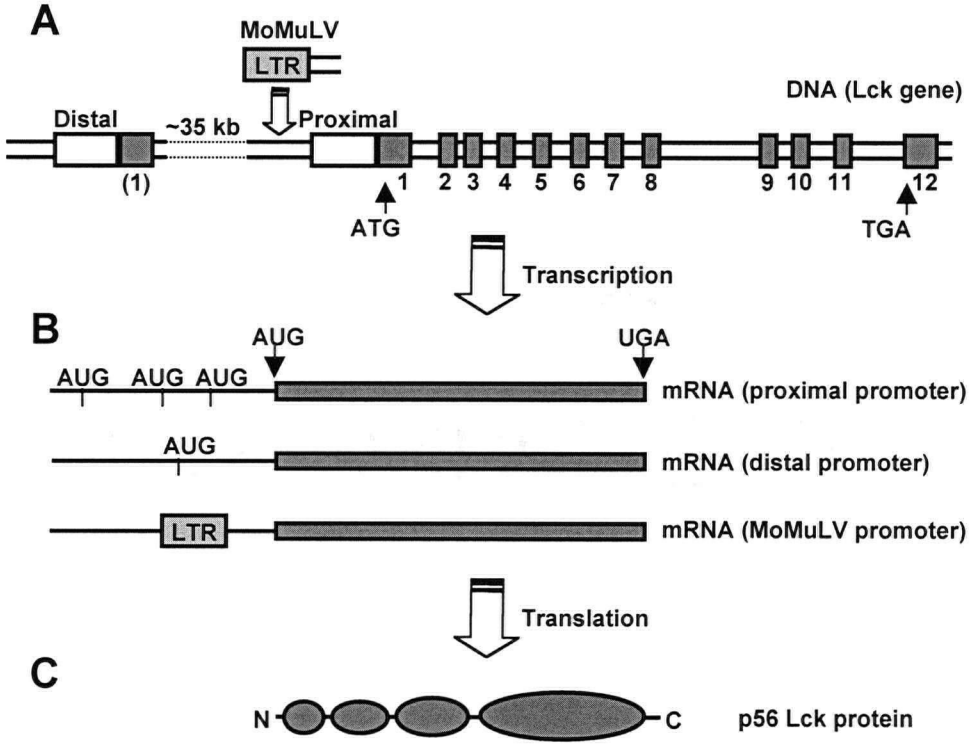


Figure 1.1. The Lck gene, transcripts and protein. (A) The structure of the Lck gene with exons numbered consecutively from 5' to 3' in green. Lck proximal and distal promoters are shown in dark gray with start (ATG) and stop (TGA) codons indicated. The position of insertion of the Moloney murine leukemia virus (MoMuLV) long terminal repeat (LTR) in LSTRA cells is shown in light gray. (B) mRNA transcripts created from the proximal promoter (top), distal promoter (middle) or viral LTR (bottom) with start (AUG) and stop (UGA) codons indicated. (C) Globular cartoon representing tertiary structure of Lck protein created from any of the above transcripts. The amino (N) and carboxy (C) termini are indicated.

expression in LSTRA cells (reviewed in 13). Despite having two promoters that create two transcripts (differing only in the 5' untranslated region), Lck is expressed as a single polypeptide. Moreover, sequence elements located upstream of the authentic Lck AUG initiation codon have been shown to reduce the efficiency of Lck protein translation *in vivo* (16), suggesting a method of translational regulation with two transcripts. An illustration of the Lck and its transcripts is provided in figure 1.1.

1.2.3 Lck expression

Currently, Lck is known to be expressed predominantly in T cells (11), including all immature and mature T cell forms (17). Moreover, its expression in human fetal thymocytes occurs in coordination with the appearance of lymphoid cells in the developing thymus (18). Lck expression has also been detected in natural killer (NK) cells (19), in some B cells (18, 20), and recently in rabbit cardiac myocytes (21).

1.2.4 Lck significance in T cell function

There is extensive genetic evidence that establishes Lck expression as being critical for both thymocyte development and T cell signaling via the TCR. Using homologous recombination in murine embryonic stem cells, an Lck null mutation was created to study mice lacking Lck expression (22). These Lck-knockout mice are viable, healthy, and fertile under pathogen free conditions but demonstrate substantial thymic atrophy due to a decrease in thymocyte numbers. More specifically, Lck-deficient mice exhibit a profound (10- to 60-fold) reduction in the double positive ($CD4^+CD8^+$) thymocyte population, indicating a sharp arrest in T cell development at the double negative ($CD4^-CD8^-$) stage. Moreover, the very few peripheral T cells that develop in Lck-knockout mice demonstrate diminished proliferation in response to CD3 and TCR

crosslinking, suggesting that Lck is essential for TCR signaling. Thus, Lck was established as a molecule that is crucial for proper T cell development, especially in maturation to the single positive stage, a role that is not compensated for by other Src-family kinases expressed in T cells, most notably Fyn.

The contribution of Lck in TCR signaling was further established using T cell lines. Most striking was a report that the Jurkat human leukemic T cell line (J.CaM1.6), lacking functional Lck due to a splicing defect, fails to induce intracellular tyrosine phosphorylation or mobilize calcium in response to TCR stimulation (23). Likewise, an Lck-deficient cytotoxic T cell line (CTLL-2) demonstrated a profound reduction in TCR-mediated cytolytic effector function that could be restored upon transfection with an expression vector encoding wild type Lck (24). Another study showed substantial increases both in IL-2 production and in cellular tyrosine phosphorylation upon TCR stimulation in cells over-expressing a constitutively active Lck mutant, Lck-F505 (25). Thus, in addition to being indispensable for T cell development, Lck is a key component in T cell function via TCR signaling. More recently, roles for Lck in T cell co-stimulation and adhesion have been established and are discussed later in this chapter.

1.2.5 Lck and Src-family kinase structure

All Src-family kinases share a common tertiary protein structure including a unique N-terminal domain, a Src homology 3 (SH3) domain, an SH2 domain, a catalytic tyrosine kinase domain, and a short carboxy-tail (reviewed in 4). Likewise, there are two key regulatory tyrosine residues conserved throughout the Src family that dictate the activation status of these kinases. An illustration of Src protein structure including all regions except the unique domain is provided in figure 1.2.

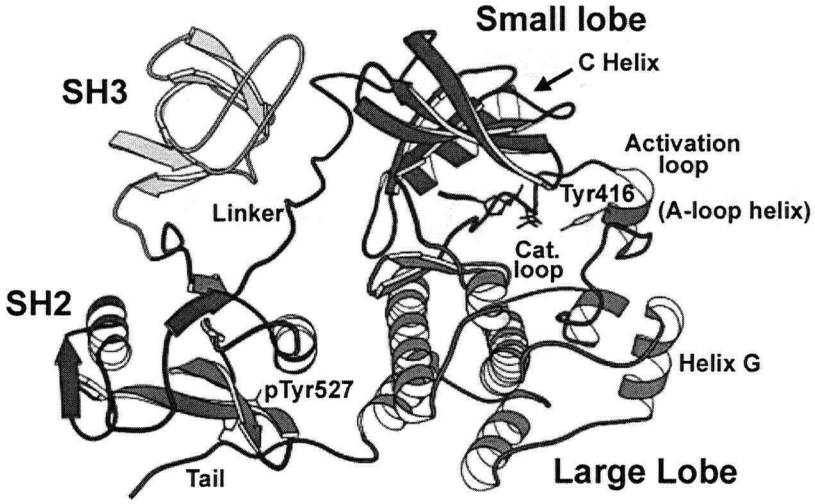


Figure 1.2. Ribbon diagram of C-terminally phosphorylated Src. The closed inactive conformation of Src phosphorylated at Tyr 527 (pTyr527) resulting in an intramolecular interaction with the SH2 domain (SH2). The linker region (Linker) between the SH2 and kinase domain small lobe forms a type-II proline helix that interacts with the SH3 domain (SH3). The catalytic loop encoding the catalytic Lys residue (position 409 in Src) is indicated as is the C Helix encoding the conserved Glu residue (position 310 in Src). Note the lack of phosphorylation at the Tyr 416 of the activation loop and the absence of the unique domain not included in the crystal structure. This diagram was modified from that produced elsewhere (26).

The N-terminal domain is referred to as the "unique" region owing to the substantial sequence variability between corresponding regions of the other Src-family kinases (reviewed in 7). A recent study showed that this domain in Lck lacks any defined structural elements suggesting the absence secondary protein structures in this region (27). Common to the first six residues of all Src-family kinase members is the consensus sequence Met-Gly-X-X-X-Ser/Thr required for the covalent linkage of myristate (14 carbon saturated fatty acid) to the glycine at position 2. Although the exact function of the unique domain remains elusive, mutation of the Lck Gly 2 residue blocks Lck myristylation, prevents stable membrane association (28), and thereby completely abolishes the ability of activated Lck to enhance cellular tyrosine phosphorylation in response to TCR stimulation (29). Post-translational palmitoylation of Lck can occur at both Cys 3 and Cys 5 (30-32), and there is evidence that palmitoylation is also required for Lck membrane association (33-35). Moreover, Lck palmitoylation is implicated in the specific targeting of Lck to glycolipid-enriched membranes (GEMs), although the relative contributions of Cys 3 and Cys 5 acylation to this localization are controversial. One study reported that an Lck-CD16 chimeric protein, with both Cys 3 and 5 mutated to Ala, was not present in the GEM fraction (35). In other studies, the mutation of Cys 3 to Ser specifically prevented Lck raft localization and TCR-induced signaling events such as NF-AT activation (34, 36). Conversely, a lack of palmitoylation at Cys 3 was shown to be important for Lck co-immunoprecipitation with GPI-anchored proteins that are localized in GEM domains (31). Despite this conflicting data, it is thought that a major role of the unique region is to localize Lck, via lipid modification, to specific plasma membrane domains where it can participate in T cell signaling events.

The Lck unique region also mediates important intermolecular interactions. Two cysteines, Cys 20 and Cys 23, have been shown to mediate the interactions of Lck with the cytoplasmic domains of both the CD4 and CD8α TCR co-receptors (37). This interaction is mediated by a tetrameric thiol-chelation of a single zinc cation that coordinates cytoplasmic cysteine residues on both Lck and CD4 or CD8α (38, 39). Interestingly, no other Src-family kinase unique region contains analogous tandem zinc-chelating cysteines, possibly explaining why Fyn cannot compensate for the loss of Lck in T cell development and activation (reviewed in 1). Moreover, the deletion of the Lck unique region, or its replacement with that of Fyn, was shown to increase Lck tyrosine phosphorylation at Tyr 505, suggesting a role for this region in Lck regulation via interactions with protein tyrosine phosphatases (40).

SH3 domains, containing a shallow hydrophobic groove, are well known for the ability to bind type-II proline helices created by P-X-X-P sequences (reviewed in 41). In contrast, SH2 domains contain tandem binding pockets separated by a β sheet, one pocket for phosphotyrosine binding, and one for determining binding specificity via residues downstream of the phosphotyrosine (reviewed in 42). Thus, the preferred SH2 binding motif of Src-family kinases is **pY**-E-E-I such that the pY+3 residue (I) inserts into the second hydrophobic SH2 pocket (43, 44). Studies using X-ray crystallography provided 3 dimensional structural comparisons of c-Src and Hck that further established the roles of SH2 and SH3 domains in Src-family kinase conformation and regulation (26, 45, 46). These crystals confirmed earlier work (47, 48) indicating that the SH2 domain bound to the phosphorylated conserved C-terminal tyrosine (Tyr 505 in Lck) resulting in an intramolecular interaction and a proposed "closed" inactive conformation.

Interestingly, the linker region between the SH2 and kinase domain was shown to adopt a type-II proline helix structure that bound the SH3 domain providing additional stability for the closed conformation. These intra-molecular interactions also have profound effects on Lck regulation, and are discussed later in this chapter.

The catalytic kinase domain is composed of a small upper lobe that binds ATP and contains mostly β strands, and a larger lower lobe that binds peptide substrates and is composed mostly of α helices. A critical feature of the catalytic domain is the auto-phosphorylation loop (or activation loop) that contains the conserved regulatory auto-phosphorylation tyrosine residue (Tyr 394 in Lck) and is located between the upper and lower lobes near the catalytic cleft. A comparison of the crystal structures of a phosphorylated loop of Lck and the non-phosphorylated loop of Src shows that the phosphorylation of the regulatory tyrosine disrupts the A-loop helix and flips the tyrosine out of the catalytic cleft (Fig. 1.3). Most protein kinase domains, including those of Src-family kinases, have a remarkably similar structure consisting of 8 major β strands and 9 major α helices that can be dissected into 12 subdomains. Intriguing is α helix G which comprises part of the conserved subdomain 10 of protein kinases. This structure is completely exposed at the base of the kinase domain (Fig 1.2), is present in most known protein kinases, but is highly variable in residue sequence and has no known function.

The short C-terminal tail is critical because it contains the regulatory tyrosine that binds the SH2 domain when it is phosphorylated by C-terminal Src kinase (Csk) (49, 50). This C-terminal tail phosphorylation has a profound impact on Src-family kinase conformation (described above) and activation (described later). Src-family kinases have

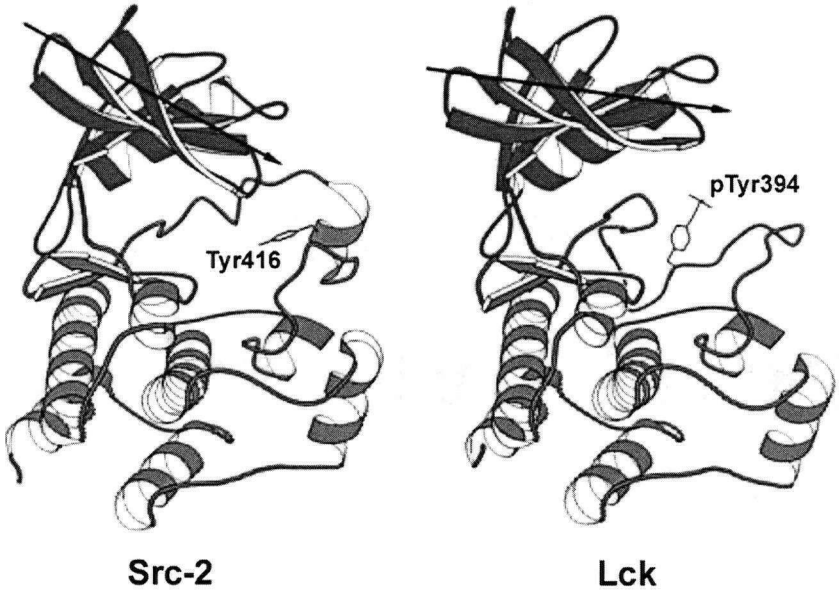


Figure 1.3. Ribbon diagrams comparing the activation loop structure of active and inactive Src-family kinases. Src-2 represents a Src kinase domain crystallized in the presence of the ATP analog and kinase inhibitor AMP-PNP (26). Lck represents the Lck kinase domain phosphorylated at the activation loop Tyr 394 (51). Activating tyrosine residues located on the activation loop of each kinase domain are indicated. Arrows are intended to underscore the much more open catalytic cleft of the active phosphorylated Lck kinase compared to Src-2.

a relatively conserved sequence around the C-terminal regulatory tyrosine of T-E-X-Q-**pY**-Q, with the E-X-Q-**pY** motif being important for Csk substrate recognition (52). Interestingly, although the preferred peptide sequence of the Src-family SH2 domains is **pY**-E-E-I (43), none of these kinases contain this sequence in their carboxy-tail. This suggests that the intramolecular interaction is weak and has the potential to be displaced or broken and replaced by a high affinity ligand.

1.3 Lck function in T cell biology

1.3.1 Lck substrates in T cells

Due to a close genetic relation to Src, and the discovery of elevated protein tyrosine kinase activity of p56 in the LSTRA murine T lymphoma cell line, Lck was identified as a protein tyrosine kinase (9, 11). However, despite the importance of Lck function in T cell development and TCR signaling, there remains a limited understanding of the molecules that Lck phosphorylates in T cells. Well-established substrates of Lck in TCR signaling include the immuno-receptor tyrosine based activation motifs (ITAMs) present in TCR ζ chains and CD3 γ, δ and ε subunits (53, 54), as well as the Syk-family kinase ZAP-70 (53, 55). In response to TCR stimulation, Lck has also been implicated in the phosphorylation of the adapter molecules p105(CasL) (56) and SHC (57), the Tec family kinase Itk (58), phosphatidylinositol 3-kinase (PI 3-kinase) (59), and the guanine nucleotide exchange factor Vav (60, 61). *In vitro*, Lck can phosphorylate the cytoplasmic tail of the T cell co-stimulatory molecule CD28 (62), and CTLA-4 (63, 64). In resting T cells, Lck has also been implicated in the constitutive phosphorylation of Cbp/PAG (65). More recently, Lck substrates were identified in integrin signaling components including FAK, ABP280 filamin (reviewed in 66), and ezrin (67). Src-family kinases have also

been implicated in the tyrosine phosphorylation of Pyk2, Cas, and to a lesser extent FAK in response to CD44 stimulation (68). Together, these Lck substrates support roles for Lck in signaling via the TCR, co-stimulation by CD28, and in adhesion signaling via CD44 and integrins.

Lck also has several binding partners in T cells. Aside from the zinc-dependent association with CD4 and CD8α, most Lck interactions are via the Lck SH2 and/or SH3 domain and do not necessarily involve Lck kinase activity. The Lck SH2 domain has been shown to bind several tyrosine phosphorylated proteins from TCR stimulated cell lysates (47). More specifically, the Lck SH2 domain binds tyrosine phosphorylated ZAP-70 (69), CD3 ζ chains (70), the adapter proteins Lad (71) and Dok (72), as well as FAK and Pyk2 (73), and paxillin (74) upon TCR stimulation. Interestingly, the SH2 domain also mediates an unconventional phosphotyrosine-independent interaction with CD45 (75), the tyrosine phosphatase known for its ability to up-regulate Lck via dephosphorylation of the Lck C-terminus. The Lck SH3 domain has been implicated in binding proline rich regions of CD28 (76), as well as the Cbl ubiquitin ligase (77), MAPK (70) and CD2 (78), although the significance of these interactions is not well understood.

The significance of some of these Lck substrates and binding partners in T cell signaling and adhesion are discussed later in this chapter.

1.3.2 Lck function in T cell development

Due to the destructive power of T cells seen in autoimmune diseases, and the susceptibility to infection of immuno-compromised individuals, it is important for the body to closely scrutinize T cell development. Thus, in the thymus, developing T cells

undergo a rigorous selection process such that the mature T cells released into the periphery are capable of recognizing self MHC (positive selection), but also distinguish self peptides from foreign (negative selection). The current affinity hypothesis for T cell selection (reviewed in 79) suggests that the intensity of signaling through the TCR determines the fate of developing thymocytes. Since Lck is intimately involved in proximal TCR signaling, its role in T cell development has been extensively studied. Early research with Lck-knockout mice showed a profound block in T cell development, establishing a critical role for Lck in this process (22). The importance of Lck kinase activity was highlighted in a study showing that thymocyte development was rescued in CD45-knockout mice expressing a constitutively active mutant form of Lck (80). Recent studies have demonstrated a role for Lck in T cell CD4/CD8 lineage commitment (reviewed in 81). Interestingly, Lck is more likely to associate with CD4 than CD8 in thymocytes since CD4 has a higher affinity for Lck, and spliced variants of CD8 molecules exist in the thymus that lack the full cytoplasmic tail (82). Moreover, hyperactive Lck can promote MHC class I restricted thymocytes to a CD4 lineage commitment, whereas inactive Lck expressed in MHC class II restricted thymocytes confers CD8 commitment (reviewed in 81). This suggests that not only is Lck indispensable for thymocyte development, but the degree of Lck activity also determines thymocyte lineage commitment.

1.3.3 Lck function in T cell receptor signaling: signal 1

Perhaps the best-understood role of Lck is that of its participation in signaling via the TCR. A common paradigm for signal transduction is the clustering of a cell surface receptor by an external ligand that leads to the activation of intracellular protein kinases.

Activated kinases then initiate a phosphorylation cascade throughout the cytoplasm, concluding in the activation of transcription factors that regulate gene expression.

Peripheral naïve T cells circulate through the bloodstream, periodically enter the lymphatic system and patrol lymph nodes for the presence of "non-self" peptides. Upon TCR recognition of a foreign peptide bound by major histocompatibility (MHC) molecules on the surface of antigen presenting cells (APCs), multiple TCRs become clustered on the surface of the T cell. The TCR however, has no intrinsic enzyme activity and thus relies on the kinase activity of Src-family kinases (Lck in particular) to initiate cell signaling. There are two current models for the association of Lck with the TCR during antigen recognition, although both mechanisms are likely to occur in concert: 1) CD4 and CD8 molecules, constitutively associated with Lck, bind MHC with the TCR and bring Lck close to cytoplasmic TCR components (83, 84), and 2) the coalescence of lipid-raft micro-domains where TCR clustering sequesters a number of GEM-associated signaling molecules including Lck (reviewed in 85).

Thus, like the TCR, Lck becomes clustered upon antigen recognition and becomes activated via auto-phosphorylation of Tyr 394 in the activation loop, an event required for full kinase activity (29, 86). Activated Lck is then able to phosphorylate multiple tyrosine residues present in the ITAMs of TCR ζ chains and CD3 subunits creating binding sites for the tandem SH2 domains of ZAP-70 molecules (reviewed in 1). Recruitment of ZAP-70 to the TCR places it in close proximity to activated Lck, resulting in Lck-mediated phosphorylation of the ZAP-70 activation loop Tyr 493 inducing ZAP-70 activation (55). During TCR stimulation Lck is also thought to activate the Tec kinase Itk via the phosphorylation of its activation loop, allowing Itk to induce phospholipase-Cγ

(PLCγ)-mediated intracellular calcium release (87, 88). Lck-activated ZAP-70 and Itk then phosphorylate two critical adapter proteins, LAT (linker of T cell activation) and SLP-76, respectively, to extend the signaling cascade (reviewed in 2) concluding in actin reorganization, transcriptional activation, and cell proliferation (Fig. 1.4).

The importance of Lck, ZAP-70 and the adapter molecules LAT and SLP-76 in TCR signaling should be noted. In response to TCR stimulation, cells lacking Lck exhibit meager cellular phosphorylation, defective ζ chain and ZAP-70 phosphorylation, and fail to activate the MAP kinase pathway, all of which are restored with Lck expression (23, 89, 90). Thus, Lck appears to be essential for proper TCR signaling, since other Src-family kinases provide poor compensation for this function. Likewise, patients lacking a functional ZAP-70 gene are severely immunocompromised due to limited and dysfunctional T cell populations (reviewed in 2). LAT and SLP-76 are absolutely essential for signaling via the TCR as indicated by the profound block in thymocyte development and the lack of peripheral T cells in LAT- and SLP-76-knockout mice (91, 92). These molecules become heavily phosphorylated during TCR signaling thereby providing multiple sites for the recruitment of SH2-expressing signaling proteins such as the Grb2/SOS complex, PLCγ, and Vav (reviewed in 93). An illustration of Lck function during TCR signaling is provided in figure 1.4.

1.3.4 Lck function in CD28 signaling: signal 2

It is generally accepted that naïve T cells receiving a strong signal 1 alone (TCR) are induced into a state of anergy. T cell activation requires a second signal, or co-stimulation, and the best-studied co-stimulatory molecule is CD28. CD28 ligation by B7

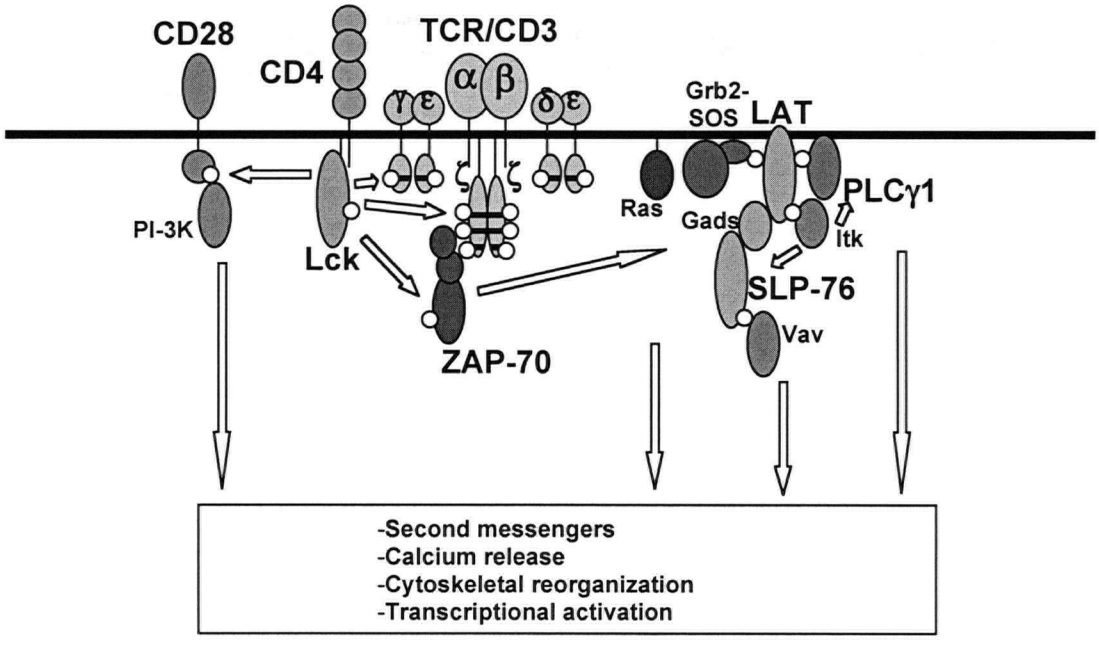


Figure 1.4. Proximal signaling events involved in T cell activation. Upon encountering processed antigen, CD4 (or CD8 in cytotoxic T cells) is positioned adjacent to the triggered TCR (composed of α and β subunits) associated with CD3 components (including γ, δ and two ε chains). Immunoreceptor tyrosine activation motifs (ITAMs) present on cytoplasmic TCR ζ chains and CD3 (black bars) are subsequently tyrosine phosphorylated (white dots) by activated Lck and Fyn. Phosphorylated ITAMs recruit ZAP-70 that, upon phosphorylation and activation by Lck, phosphorylates the linker for activation of T cells (LAT). LAT phosphorylation recruits Itk that is phosphorylated and activated by Lck resulting in Itk-mediated phosphorylation of PLCγ1, and SLP-76 which recruits Vav. Phosphorylated LAT further recruits the Grb2/SOS complex resulting in Ras activation and stimulation of the MAP kinase pathway. PLCγ1 is implicated in intracellular calcium release and Vav induces signaling for cytoskeletal rearrangement. Co-stimulatory molecule CD28 interacts with and is phosphorylated by Lck, and is subsequently associated with PI 3-kinase resulting in stimulation of the AKT pathway and transcriptional activation in concert with signals from the TCR.

on the surface of professional APCs optimizes TCR-induced cell proliferation by up-regulating IL-2 production (reviewed in 94). It also prevents T cell anergy induced by signal 1 alone (95). Thus, the "two signal" system of T cell activation was proposed where signal 1 is generated from the TCR and signal 2 from CD28 (96). However despite the age of this model, the intricacies of CD28 signaling remain unlcear. Currently there are models for CD28 in augmenting TCR signals, thereby lowering the threshold of T cell activation, as well as independent proximal CD28 signaling resulting in an integration of the TCR-CD28 pathways at the transcription factor level (97).

Interestingly, Lck has been implicated in the functions of both signal 1 and 2. Low levels of Lck were found to associate with CD28 in T cells, resulting in Lck activation upon CD28 crosslinking (98). Moreover, a proline rich segment in CD28 was shown to bind the Lck SH3 domain and induce kinase activation (76). Activated Lck can tyrosine phosphorylate the cytoplasmic tail of CD28 at Tyr 173 in the **Y**-M-N-M motif, providing an SH2 binding site for PI 3-kinase and the Grb2/SOS complex. Recruitment of these signaling molecules then promotes activation of the AKT and MAP kinase pathways, respectively. A recent study has also suggested that CD28 cooperates with CD4 to sustain Lck autophosphorylation in the interface between the T cell and APC (termed the "immunological synapse", (99)).

1.3.5 Lck function in down-regulating T cell activation

Like CD28, CTLA-4 binds to B7 molecules on the APC, but does so with up to 100-fold higher affinity (100). Lck is also associated with CTLA-4 in T cells and phosphorylates two cytoplasmic CTLA-4 tyrosine residues, inducing the recruitment of SH2 containing phosphatase SHP-2 (63, 64). This phosphatase recruitment results in

TCR ζ dephosphorylation (101) and decreased LAT phosphorylation (102). Therefore, Lck activation during T cell stimulation also serves as a negative feedback loop to control and extinguish T cell activation. More specifically, not only is Lck essential for the propagation of T cell activation via both the TCR and CD28, but it also serves as a key initiator of CTLA-4-mediated T cell down-regulation.

1.3.6 Lck localization in lipid-raft domains

The plasma membrane of T cells is complex and composed of a phospholipid bilayer containing independent microdomains enriched with specific populations of ordered lipids. These domains include molecules of cholesterol intercalated between glycosphingolipids and gangliosides creating a highly ordered "lipid-raft" platform capable of lateral movement in the plane of the cell membrane. These ordered lipid rafts have characteristically high melting temperatures, high buoyancy on sucrose gradients, and low solubility in cold non-ionic detergents in contrast to the bulk of cell membranes (reviewed in 103). Interestingly, certain transmembrane, or membrane-associated, proteins display an affinity for localizing to lipid-raft fractions, and Lck is one of these proteins (31).

Lck acylation, particularly the palmitoylation of Cys 3 and Cys 5 residues, have been implicated in Lck lipid-raft localization (31, 35, 36). However, Lck also exists in non-raft fractions of the cell membrane, and one study has reported that approximately equal amounts of Lck exist in raft and non-rafts domains (68), although this ratio is sensitive to cell lysis conditions. Interestingly, the lipid-raft localization of CD4, the TCR co-receptor, was shown to be mediated both by palmitoylation and by its association with Lck (104). Likewise, the palmitoylation of LAT (critical for TCR signaling)

localizes this transmembrane adapter protein to lipid-raft domains (105), where it recruits signaling molecules to rafts upon TCR signaling. Moreover, the adhesion molecule CD44 also localizes to lipid rafts in T cells where it then associates with both Lck and Fyn (68, 106). Although CD44 palmitoylation can occur upon CD44 ligation (107), it is not known if palmitoylation is required for CD44 raft localization. Thus, it is worthy to note that not all constitutively raft-associated proteins are palmitoylated, as is the case for the Tec kinase, Itk (108). Importantly, lipid-raft association specifically is crucial for the signaling functions of both Lck and LAT upon TCR stimulation (reviewed in 85).

Currently there is conflicting data on the localization of the TCR in lipid-raft domains of resting cells. Different raft detection techniques and different detergents used for cell lysis have been attributed to the cause of the discrepancies. However, relatively common are the findings that the TCR association with lipid rafts is sensitive to 1% Triton-X-100 detergent, and is enhanced upon cross-linking (109-112). Thus, it has been suggested that the putative constitutive association of TCR in lipid-raft domains is stabilized by raft aggregation in response to TCR clustering (85). More importantly, the aggregation of lipid rafts, either with the cross-linking of CD3 or of lipid rafts alone (via cholera toxin), induces Lck-dependent, lipid raft-localized tyrosine phosphorylation and ZAP-70 recruitment (112).

Lck phosphorylation and activation status in lipid rafts however remains controversial. Interesting is the general finding that CD45, a key regulator of Lck phosphorylation, is largely excluded from lipid-raft domains (68, 113). Moreover, Csk, a negative regulator of Lck activity, is associated with lipid rafts in resting T cells via its interaction with the raft-localized adapter protein Cbp/PAG. Thus, in accordance with

the absence of CD45, and presence of Csk in lipid-raft domains, Lck has been reported to be hyper-phosphorylated at its negative regulatory tyrosine (Tyr 505) in lipid rafts (113). However, these findings suggest that raft localized Lck is inactive, in contrast to findings described above where Lck-dependent tyrosine phosphorylation occurred in raft aggregations. Thus, it has been suggested that upon receptor ligation, raft-associated Lck encounters a high affinity SH2 ligand allowing the kinase to open and activate via Tyr 394 auto-phosphorylation (114). Moreover, since CD45 is largely excluded from aggregated rafts, it is possible that activated raft-associated Lck is protected from CD45-mediated dephosphorylation. Clearly, the activation state of Lck in lipid-raft domains remains a critical question in T cell signaling. Likewise, the intricacies of CD45-mediated, and Cbp/PAG-Csk-mediated regulation of Lck are discussed in detail later in this chapter.

A current model suggests that isolated lipid rafts, only 70 nm in diameter (115), contain only a few proteins that are easily accessible to phosphatases such as CD45. TCR engagement is then thought to aggregate and coalesce lipid rafts excluding CD45 and allowing sustained phosphorylation and the activation of downstream signaling. Consistent with this model is the finding that the cross-linking of lipid domains alone (via cholera toxin) produces similar signaling events to CD3 stimulation, including tyrosine phosphorylation and the exclusion of CD45 (85). An illustration of this model is shown in figure 1.5.

1.3.7 Lck localization in the immune synapse

Clearly, lipid rafts are becoming established as important platforms for T cell signaling. Recent studies using immunofluorescence to observe signaling machinery at

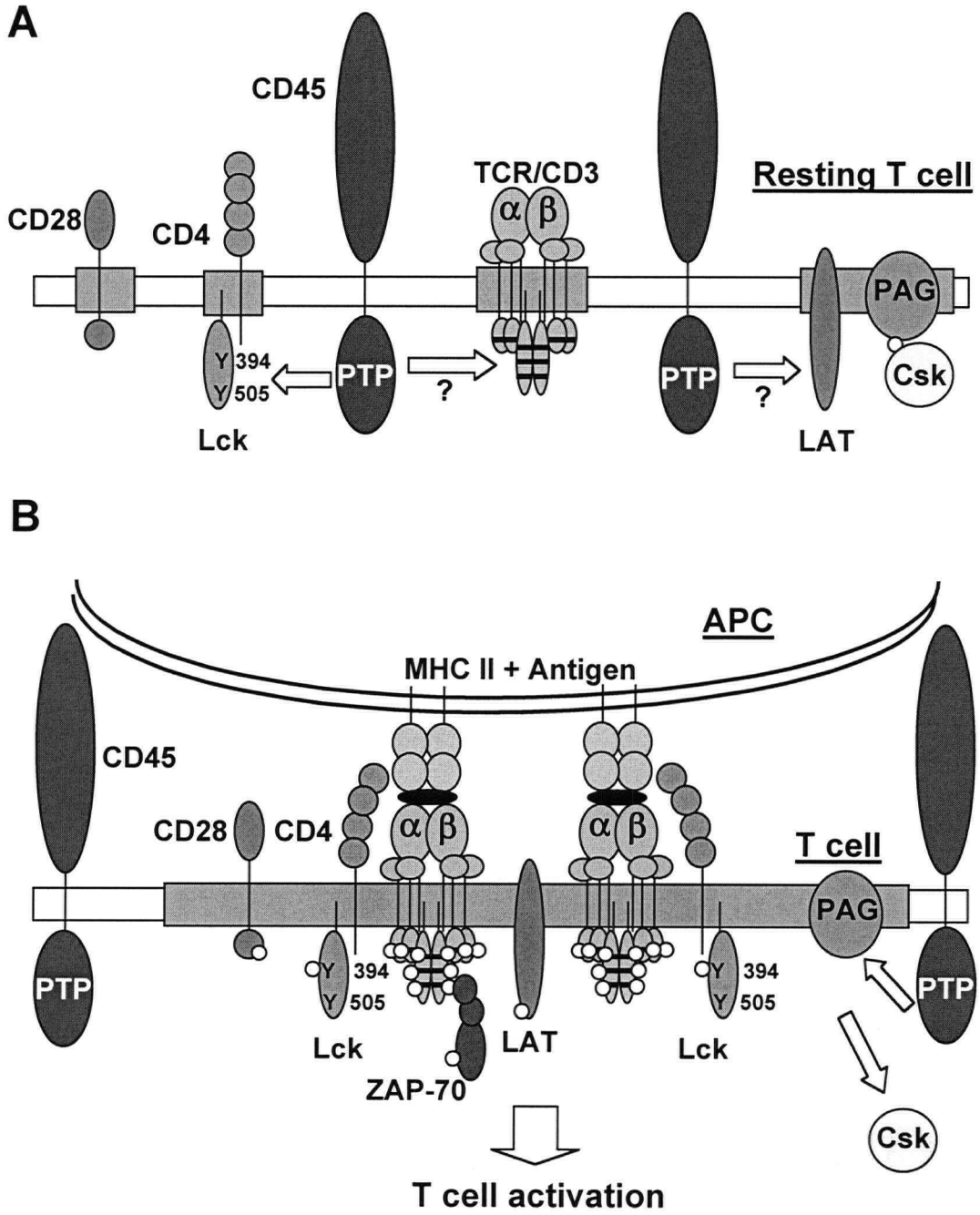

A
CD45
TCR/CD3
Resting T cell
CD28
CD4
α
β
PAG
Y 394
Y 505
PTP
?
PTP
?
Csk
Lck
LAT
B
APC
MHC II + Antigen
CD45
CD28
CD4
α
β
α
β
T cell
PAG
PTP
Y 394
Y 505
Y 394
Y 505
PTP
Lck
LAT
Lck
ZAP-70
Csk
T cell activation

Figure 1.5. Model for T cell activation via lipid-raft aggregation and immune synapse formation. All molecules are as defined in figure 1.4. (A) In resting T cells, lipid rafts (gray bars) are small and disperse such that raft-associated proteins are susceptible to phosphatase activity from non-raft phosphatases, particularly CD45. CD45 maintains Lck in a non-phosphorylated state due to continual dephosphorylation of Lck regulatory tyrosines 505 and 394. CD45 may also regulate TCR and LAT phosphorylation in resting cells, although currently this is not well defined. Resting T cells also localize Csk to the membrane via binding to constitutively phosphorylated, lipid raft-associated, PAG. (B) Ligation of TCRs by MHC/peptide presented by antigen presenting cells (APCs) results in lipid-raft aggregation at the APC – T cell interface (the immune synpase), the co-localization of raft-associated signaling molecules such as Lck, LAT and CD28, and the exclusion of non-raft molecules such as CD45. The exclusion of CD45 is further enforced by the relatively small space between T cell and APC in the immune synapse that is incompatible with the large extracellular domain of CD45. CD45 exclusion allows for sustained phosphorylation and activation of signaling kinases Lck and ZAP-70 resulting in the induction of downstream T cell signaling events and cell activation. Although Lck Tyr 394 is phosphorylated at the immune synapse, the phosphorylation status of Tyr 505 remains in question. However, TCR stimulation induces the rapid dephosphorylation of PAG by CD45 resulting in the release of Csk, suggesting a lack of Lck phosphorylation at Tyr 505 during synapse formation.

the T cell – APC interface (the "immunological synapse") revealed a 2- to 3-fold enhancement of lipid rafts (116) and the clustering of T cell receptors at the center of the interface. This central region is surrounded by a ring of integrin-dependent adhesion mediated by LFA-1 and ICAM-1 molecules (117). These 3 dimensional contact domains were named 'supramolecular activation clusters' (SMACs) with the TCR, Src kinases, and lipid rafts in the center "c-SMAC" and the peripheral integrin ring named the "p-SMAC" (117).

Both Lck and Fyn co-localize with the TCR at the center of the synapse, but do so in a time-dependent manner. One study revealed the enrichment of Lck and Fyn in the c-SMAC at 5 to 13 minutes after cell conjugation (117). Elsewhere, Lck was shown to be rapidly recruited to the center of the immune synapse along with CD3ζ upon T cell – APC interaction, but was later localized in the synapse periphery suggesting a limited timeframe in which Lck can phosphorylate CD3ζ (118). The same study revealed the translocation of endosomal Lck to the center of the synapse 10 minutes after cell – cell contact, suggesting a second source of Lck to sustain late signal transduction. Furthermore, CD4 was shown to deliver Lck to the immune synapse where it becomes phosphorylated at its activation site (Tyr 394), an event that is sustained by the presence of CD28 (99). Interestingly, CD28 and CD3 co-aggregation have been shown to cluster lipid domains (119), although CD28 is not required for raft localization in the c-SMAC (116). Together, these data indicate a spatial/temporal recruitment of Lck at the immune synapse where it becomes activated and initiates T cell signaling events.

Perhaps the most intriguing and controversial topic in immune synapse biology is the location of CD45. Topographical analysis suggests that the massive extracellular

domain of CD45 (extending up to 51 nm above the cell surface (120, 121)) is far too big to allow CD45 access into the immune synapse that spans only approximately 15 nm according to the sizes of the TCR and MHC (122). In accordance with this, studies have reported the exclusion of CD45 from lipid-raft fractions (described above), and the absence of CD45 from the TCR signaling domain (123). However, two separate groups have reported that a small amount of CD45 enters the c-SMAC adjacent to the TCR approximately 3 minutes after TCR ligation with MHC (124, 125). The inclusion of CD45 in the c-SMAC has been suggested as being required to "reset" (dephosphorylate) basal levels of tyrosine phosphorylation found on TCR signaling components to allow for TCR-mediated phosphorylation on appropriate sites (125), or to maintain Lck activation via constant dephosphorylation of the inhibitory Tyr 505 residue (124). Thus, it is clear that signaling molecules such as Lck and the TCR localize both to lipid rafts and the c-SMAC during T cell – APC interactions. However the regulation of Lck phosphorylation, and the role and localization of CD45 in this process is uncertain.

1.3.8 Lck function in T cell adhesion

T cell functions such as extravasation from the blood stream into tissues, migration through the thymus, lymphocyte homing, and even sustained TCR signaling at the immune synapse, all require the involvement of adhesion molecules as well as cytoskeletal changes. Perhaps the most important, and most intriguing, function in cell adhesion is the ability of circulating lymphocytes to rapidly convert between adherent and non-adherent phenotypes. Controlled adhesion allows lymphocytes to migrate into lymph nodes as well as target their extravasation from the blood specifically at sites of tissue inflammation and infection. Alternatively, a lack of control in lymphocyte

adhesion leads to then onset of pathologies including atherosclerosis and other inflammatory diseases (reviewed in 126). Thus, understanding the molecular mechanisms of cell adhesion will provide insight into the functions of lymphocytes in the immune system as well as strategies for disease prevention.

Cell adhesion molecules can be categorized into the cadherins, selectins, integrins, and CD44. Cadherins serve important functions in embryogenesis and tissue structure (reviewed in 127). L-selectins are expressed on leukocytes specifically and mediate transient binding of leukocytes to high walled endothelial venules (HEVs). Integrins are widely expressed transmembrane hetero-dimers composed of various α and β subunits. Much of what we know about T cell adhesion comes from the analysis of T cells binding to immobilized ligands, interactions that are mediated by integrins, and LFA-1 in particular. CD44 is a widely expressed type-1 transmembrane glycoprotein that is an additional member of the adhesion molecule family.

Leukocyte adhesion to the endothelium can be roughly broken down into stages of selectin-mediated rolling (transient adhesion) followed by integrin-mediated adhesion, arrest and subsequent migration from blood vessel into the tissue (diapedesis, (reviewed in 128)). The role of CD44 in this process remains under investigation, however anti-CD44 antibodies have been shown to delay leukocyte infiltration at the cutaneous site at delayed-type hypersensitivity (129) and block CD44-mediated rolling of T lymphocytes on an endothelial cell line (130). Most convincing, is the finding that CD44 binding to hyaluronic acid (HA, a component of the extracellular matrix and a well established ligand of CD44) is inducible with superantigen stimulation, resulting in CD44:HA-dependent rolling and CD44-dependent extravasation at the inflammatory site (131, 132).

Thus, CD44 is thought to function in concert with selectins and integrins to mediate inducible rolling, adhesion and extravasation of T cells at inflammatory sites.

Adhesion molecules also play important roles in initiating and sustaining the interaction between the T cell and the APC during antigen recognition. A major hurdle in prolonged TCR binding to MHC/peptide is the relatively low affinity of this interaction with a dissociation constant of 10^{-4} to 10^{-7} M, which are several orders of magnitude below that of the immunoglobins ($<10^{-9}$ M, (reviewed in 122)). Thus, adhesion molecules such as CD2 and LFA-1 have been implicated in bringing T cell and APC membranes close together and sustaining the interaction (reviewed in 122; 133, 134). Consistent with this is the identification of an integrin adhesion ring localized in the p-SMAC of the immune synapse around the c-SMAC cluster of TCRs and signaling machinery (135). Likewise, CD44 has also been implicated in T cell activation. Two independent studies have reported enhanced anti-CD3-induced IL-2 production when CD44 is crosslinked (136, 137).

Thus, LFA-1 and CD44 appear to share similar functions in facilitating lymphocyte adherence to endothelium as well as sustaining T cell – APC interactions. Another key link between these two molecules is a common signaling component in Src-family kinases. Integrin signaling has been studied extensively in several cell types (reviewed in 138, 139, 140), and is known to induce the activation of several non-receptor protein tyrosine kinases, including both Src and Fyn in platelets and fibroblasts (reviewed in 138). Likewise, in T cells, CD44-mediated cytoskeletal changes are known to require Src-family kinase activity (68), and CD44 crosslinking induces Lck activation (141). Thus, it is of interest to compare CD44 and integrin function in adhesion and T

cell signaling. Moreover, given the potential role of Lck in CD44-mediated signaling events, it is tempting to further investigate the interaction between CD44 and Lck.

CD44 and Lck are known to associate exclusively in lipid-raft domains (68, 142). Likewise, CD44-mediated cytoskeletal reorganization involves the recruitment of Lck and Fyn into lipid-rafts domains (106). *In vitro*, the CD44 cytoplasmic domain mediates a direct protein-protein interaction with Src (143), however the details of the interactions between CD44 and Src-family kinases in T cells are lacking. Moreover, despite the involvement of Lck in CD44-mediated signaling, the significance of ZAP-70 in this process remains in question. ZAP-70 phosphorylation was detected upon CD44 crosslinking in one study (141), although this has not been seen elsewhere (68).

A hallmark of integrin signaling is the recuitment and activation of focal adhesion kinase (FAK). In fibroblasts, integrin mediated adhesion induces FAK auto-phosphorylation at Tyr 397 creating a high affinity SH2 site for Src (145). Recruitment of Src then allows for the phosphorylation of several adhesion molecules including paxillin, tensin, Cas, and FAK (reviewed in 146). Likewise, in T cells, CD44-induced cytoskeletal changes are associated with the phosphorylation of Pyk2, Cas and to a lesser extent, FAK (68); however details of the association of CD44 with these molecules are lacking. Pyk2 is a tyrosine kinase that is expressed in the central nervous system and hematopoietic cells, and shares ~45% sequence identity with FAK. Like FAK, Pyk2 has multiple sites of tyrosine phosphorylation for the recruitment of SH2 containing proteins, including Src-family kinases. Thus, it is tempting to compare the signaling events of CD44, Lck and Pyk2 in T cells with those of integrin, Src and FAK in fibroblasts. An illustration of the rudimentary CD44 signaling cascade is provided in figure 1.6.

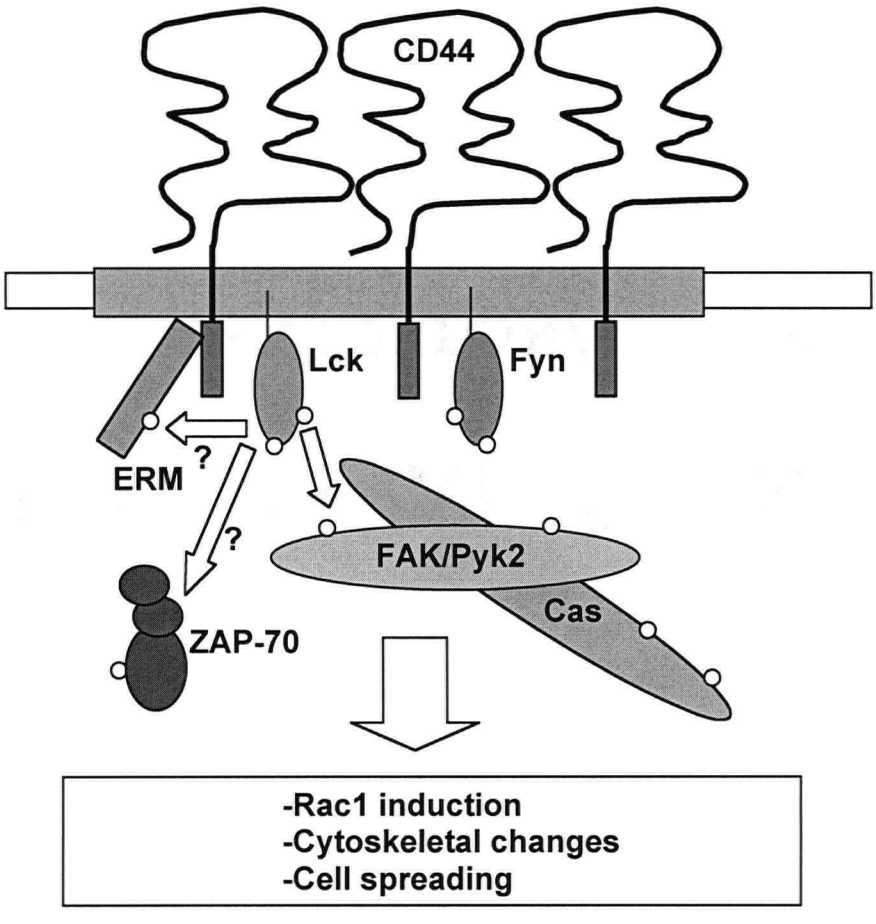


Figure 1.6. Proximal signaling events induced by CD44 clustering. The crosslinking of CD44 leads to intracellular tyrosine phosphorylation (white dots) and Rac1-mediated cytoskeletal changes involved in cell adhesion, a process that is dependent on Src-family kinase activity (Lck and Fyn) in T cells. Lck and Fyn associate with CD44 exclusively in lipid rafts (gray bar) resulting in the phosphorylation of Pyk2, Cas, FAK, and possibly ZAP-70 and ERM proteins. Although Lck and Fyn phosphorylation status during CD44 signaling is not known, these kinases are possibly phosphorylated at their carboxy-tails as well as the activation loops since cell adhesion is enhanced in $CD45^-$ cells.

Another trait common to both CD44- and integrin-mediated cell adhesion is the negative effect of CD45 expression. In contrast to the positive effect that CD45 has on TCR signaling, $CD45^{-/-}$ bone marrow derived macrophages exhibit increased Src-family kinase activity and more rapid β2 integrin-mediated cell spreading (cytoskeletal changes involved in cell adhesion) compared to $CD45^{+/+}$ cells (147). Likewise, cell spreading on anti-CD44 antibody was shown to occur in $CD45^{-}$ T lymphoma cells, but not in $CD45^{+}$ cells (68). Thus CD45 appears to down-regulate cell adhesion, a role that has been suggested to occur via the dephosphorylation of Src-family kinase activation loops (114).

1.4 Lck regulation

1.4.1 Tyrosine phosphorylation, conformation and activation

Several years before the crystal structures were resolved, it was understood that the activity of Src-family kinases was regulated by their phosphorylation status. Indeed, the oncogenic and transforming v-Src gene was known to encode a deletion in the C-terminal tail that removes Tyr 527, a substrate of C-terminal Src kinase (Csk), suggesting that a phosphorylated tail represses Src activity. Likewise, the expression of an Lck protein mutated at the C-terminal Tyr 505 resulted in an increase in kinase activity, as detected by increased Lck phosphorylation at Tyr 394, increased tyrosine phosphorylation of cellular proteins, and the oncogenic transformation of transfected cells (28, 148, 149). Further studies revealed that the Lck SH2 domain, and to a lesser extent the SH3 domain, was implicated in down-regulating Lck kinase activity (150), and that the SH2 domain was capable of binding peptides corresponding to the phosphorylated C-terminus of Lck (47, 151). Thus, it became understood that an

intramolecular interaction between the Lck SH2 domain and the phosphorylated Tyr 505 served to down-regulate kinase activity, possibly by blocking or distorting the activation site (reviewed in 13).

In 1997, two Src-family kinases, Src and Hck, were crystallized in their auto-inhibited, and C-terminally phosphorylated forms (45, 46). These crystal structures supported the suggested intramolecular interaction between the SH2 domain and the carboxy tail, but surprisingly this interaction was shown to occur on the opposite side of the kinase domain from the catalytic site, and did not distort kinase domain structure. A ribbon model of the closed inactive Src structure is provided in figure 1.2. The mechanism for the inhibition of kinase activity is that in the closed inactive form, helix C of the kinase domain (containing the conserved glutamate, Glu 310 in Src) is rotated, disrupting the Glu 310 – Lys 295 salt bridge required to position the lysine side chain for interactions with the α and β phosphates of ATP during catalysis. In agreement with this structural data are studies showing that the disruption of the interaction between the SH2 domain and carboxy tail results in kinase activation, thought to coincide with an open Src-kinase conformation (reviewed in 152). Indeed, the release of the intramolecular interaction either with a high affinity SH2 or SH3 domain ligand can stimulate Src (153) and Hck activation (154), akin to Lck activation via mutations to the SH2 domain or carboxy-tail (reviewed in 13). Thus, the inhibitory conformation by C-terminal Src kinase phosphorylation can be reversed by high affinity ligands or mutations that disrupt the intramolecular interaction, and "open" the kinase.

Src-family kinase C-terminal phosphorylation however, is not the only event affecting activation. Most tyrosine kinases and serine/threonine kinases maintain a

similar structured catalytic domain containing a flexible activation loop. Src-family kinases share a conserved tyrosine residue (Tyr 394 in Lck) in this loop whose phosphorylation correlates with kinase activation, such as in fibroblasts transfected with the Lck Y505F mutant (148, 149). The importance of Tyr 394 phosphorylation was highlighted by studies showing that the activation of Lck and subsequent oncogenic transformation of fibroblasts via the Y505F mutation could be prevented by a simultaneous mutation of Tyr 394 (28). Likewise in T cells, the mutation of Tyr 394 to Phe (Y394F) was shown to diminish the enhanced TCR-induced tyrosine phosphorylation mediated by the Lck Y505F mutant (29). Moreover, upon isolation from transfected YAC T lymphoma cells, the Lck Y505F mutant displays kinase activity substantially above that of wild type Lck (86). However the Lck Y394F mutant and the Lck Y394F Y505F double mutant display meager kinase activity that is several-fold lower than wild type Lck. Thus, although the absence of phosphorylation at the C-terminus potentiates Lck kinase activity, the critical parameter for kinase activation is the subsequent phosphorylation at Tyr 394.

The crystal structure of the Tyr 394 phosphorylated Lck kinase domain revealed that, in constrast to the non-phosphorylated Src and Hck, the phosphorylated activation loop loses its short A-loop helix structure resulting in a flipping of the regulatory tyrosine from inside the catalytic cleft to the exposed periphery. This dynamic relocation of Tyr 394 serves an activating function for two reasons: 1) the unphosphorylated A-loop helix physically occupies a substrate binding area, and 2) helix formation stabilizes an inactive catalytic conformation to prevent any inappropriate kinase activity (26). A comparison of phosphorylated and non-phosphorylated activation loops is provided in figure 1.3.

Thus, Tyr 505 and Tyr 394 are two key regulatory residues that control Lck activation through phosphorylation-induced conformational changes.

It is worthy to note that phosphorylation of Lck Tyr 192 (located in the SH2 domain) has been detected *in vitro*, and was shown to increase Lck activity (155). However, there is little data elsewhere to support this, and other studies have shown that a Y192F mutation has no effect on Lck kinase activity (86).

1.4.2 Lck serine phosphorylation

The N-terminal unique region of Lck can become serine phosphorylated in response to T cell stimulation via the TCR (156-159), the IL-2 receptor (160), or phorbol ester treatment (161, 162). Several Lck phospho-serine residues have been identified in these studies including Ser 42, 59, 158, and 194, and a consistent observation is the shift in Lck SDS-PAGE gel mobility from 56 to 60 kDa upon serine phosphorylation. However, there is considerable controversy regarding the kinases responsible for the serine phosphorylation of Lck and the significance of this phosphorylation in Lck function. Some groups report that Erk mediates Lck Ser 59 phosphorylation (163, 164), while others observe this event despite the presence of Erk pathway inhibitors (165). Protein kinase C has also been identified as being capable of inducing Lck serine phosphorylation (163). There is also conflicting data from various studies reporting that serine phosphorylation inhibits Lck activity (158, 159), has no effect on Lck activity (162), or even stimulates Lck activity (160).

Interestingly, there is evidence for a role of Lck Ser 59 phosphorylation in a TCR-mediated negative feedback pathway (166). It was reported that TCR engagement with a low-affinity peptide-MHC complex (antagonist) stimulates Lck-induced SHP-1

phosphorylation, followed by the binding of Lck to phosphorylated SHP-1 and the subsequent inactivation Lck by SHP-1. In contrast, high-affinity TCR ligands (agonists) rapidly induce Erk activation and the phosphorylation of Lck Ser 59 which prevents the SHP-1:Lck interaction, resulting in elevated TCR ζ chain phosphorylation. Thus, this data supports a role for Lck serine phosphorylation in SHP-1-mediated Lck regulation during different TCR responses to high- and low-affinity ligands.

1.4.3 CD45, a tyrosine phosphatase with a role in Lck up-regulation

CD45 is a transmembrane protein tyrosine phosphatase expressed on the surface of all nucleated hematopoietic cells. Alternative splicing of three exons (A, B and C) yields various isoforms that differ in the length of the glycosylated extracellular domain. Thus, exon splicing creates a range in CD45 size from 28 to 51 nm above the cell surface, and a range in molecular mass from 180 to 220 kDa (reviewed in 167). As in most receptor-like phosphatases, CD45 expresses two tandem cytoplasmic phosphatase domains, with the catalytic activity belonging to the first domain (D1, Fig. 1.7). The function of the second domain (D2) is unclear, although there is evidence that it stabilizes and augments D1 phosphatase activity (168-170). Moreover, there is evidence that D2 may have roles in determining CD45 substrate specificity and in regulating CD45 activity. Reports have suggested that Csk-mediated phosphorylation of D2 enhances CD45 activity *in vitro* and facilitates CD45 binding to Lck (171). Likewise, the serine and tyrosine phosphorylation of D2 have been shown to enhance CD45 activity against certain artificial substrates (172). In agreement, the dephosphorylation of D2 serine residues in response to the addition of calcium ionophore in thymocytes was shown to reduce CD45 phosphatase activity (173).

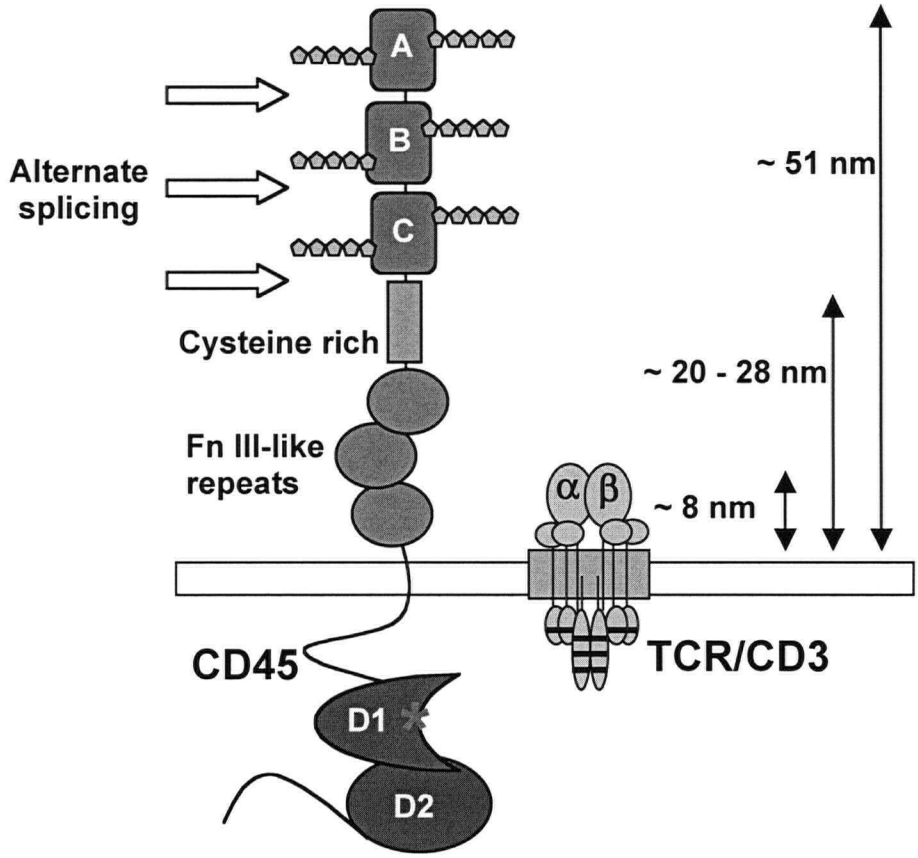


Figure 1.7. Schematic representation of CD45 structure. The cytoplasmic region of CD45 includes a membrane-proximal region, two tandem phosphatase domains (D1 and D2) of which only D1 has any catalytic activity (star), and a carboxy tail. The extracellular domain has multiple sites for N-linked glycosylation and is predicted to have three fibronectin (Fn) III-like domains (178), as well as a cysteine rich region. The CD45R0 isoform expresses the Fn III-like domains and the cysteine rich region, but lacks any of the alternately spliced exons, and thus is predicted to extend 20 to 28 nm above the cell surface. The three alternatively spliced exons (A, B and C) are heavily O-glycosylated and are predicted to extend the length of the extracellular domain to 51 nm. The height of the TCR is provided as a comparison and is predicted to be at least 6-fold shorter than the largest CD45 isoform (122).

CD45 is known to regulate various Src-family kinases in hematopoeitic cells (114). In T cells specifically, Lck (174, 175) and Fyn (176, 177) have been identified as major CD45 substrates due to their hyper-phosphorylation in $CD45^-$ cells. More recent studies have further established Lck, and to a lesser extent Fyn, as CD45 substrates in T cells and *in vitro* (reviewed in 152). However, there is evidence that CD3 ζ (179), ZAP-70 (180), Janus kinase (181), and possibly LAT (182) are CD45 substrates as well. It is also worthy to note the significance of CD45 expression in T cells. CD45-knockout mice display a severe block in T cell development at the immature double positive ($CD4^+CD8^+$) stage (183). Moreover, CD45 deficient T cells are unable to proliferate, produce cytokines, or induce target cell lysis in response to TCR engagement (reviewed in 167). CD45 is highly expressed in T cells (representing up to 10% of cell surface proteins), and demonstrates a 7- to 10-fold greater phosphatase activity than RPTPα against artificial substrates (184). Thus, CD45 clearly plays a prominent role in T cell biology, and bestows an incredible phosphatase presence due its sheer abundance and potent intrinsic activity.

Consistently, CD45 deficient T cells demonstrate Lck hyper-phosphorylation at the inhibitory Tyr 505 residue (48, 174, 185-188). Thus, CD45 is considered to be a positive regulator of Lck activity in resting T cells. This role is further supported by the demonstration of direct CD45-mediated Lck Tyr 505 dephosphorylation *in vitro* (185), and the restoration of thymocyte development in CD45 deficient mice by the expression of Lck Y505F (80). Csk, a cytosolic tyrosine kinase, is the only known kinase capable of phosphorylating Src-family kinases at their C-terminus, and its function thereby directly

opposes that of CD45. Thus, the balance of Lck activation, and T cell activation is thought to hinge on an equilibrium between CD45 and Csk function (Fig. 1.8).

1.4.4 A role for CD45 in Lck down-regulation

Interestingly, in some CD45 deficient cell lines, Lck activity is significantly elevated (188, 191, 192) despite hyper-phosphorylation at the negative regulatory Tyr 505. This dilemma was attributed to the fact that, in certain cases, Lck isolated from $CD45^-$ cells is also hyper-phosphorylated at the activation loop Tyr 394, albeit to a much lesser extent than Tyr 505 (reviewed in 193). Moreover, CD45 was shown to directly dephosphorylate purified auto-phosphorylated Lck *in vitro* (86), further supporting a role for CD45 in Lck down-regulation via Tyr 394 dephosphorylation. Perhaps the most physiologically relevant study of CD45-mediated Lck down-regulation was that in which mice expressing sub-oncogenic levels of constitutively active Lck Y505F were shown to develop aggressive thymic lymphomas on a $CD45^{-/-}$ background (194). In this study CD45 was shown to suppress the onset of tumors presumably via the dephosphorylation of Lck Tyr 394. Thus, the role of CD45 in Lck regulation is more complex than initially believed. Clearly, a dichotomy exists in the ability of CD45 to both up- and down-regulate Lck activity (Fig. 1.8), however the variables determining the balance between these opposing events remain unknown.

1.4.5 SHP-1, PEP and Csk, roles in Lck down-regulation

SHP-1 is a cytosolic protein tyrosine phosphatase that is expressed predominantly in hematopoietic cells. Unlike most phosphatases, SHP-1 expresses two tandem N-terminal SH2 domains (reviewed in 195) that allow for the recruitment of SHP-1 to immunoreceptor tyrosine based inhibitory motifs (ITIMs) in response to lymphocyte

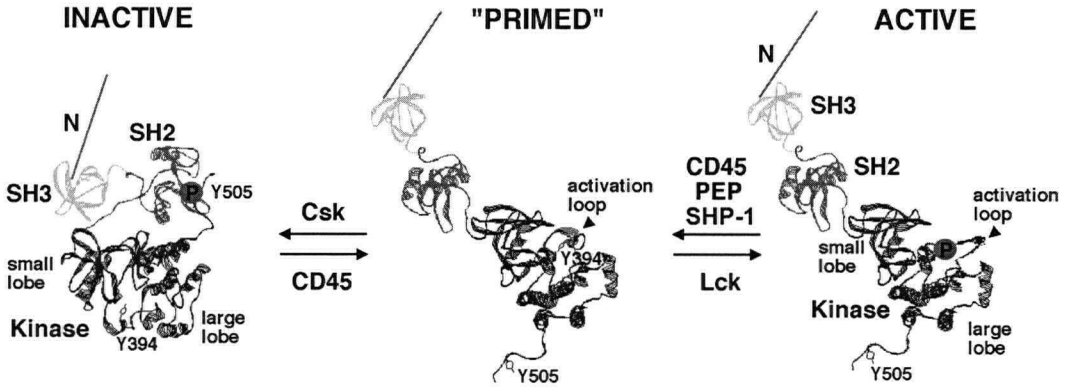


Figure 1.8. The equilibrium of Lck activation. When phosphorylated at Tyr 505, Lck exists in a closed inactive conformation that is stabilized by the SH2:pTyr 505 and SH3:linker intramolecular interactions. The representation of the closed form was generated by SWISS-MODEL (189) and was based on crystal structures of Src, Hck and the Lck kinase domain (26, 45, 46, 51, 190). Due to a lack of defined structural elements, the unique (N) region of Lck is indicated as a line. Dephosphorylation of Lck Tyr 505 by CD45 is predicted to induce an open Lck conformation. This however creates an intermediate or "primed" activation state where Lck is still inactive due to a lack of phosphorylation at Tyr 394 and the blocking of the catalytic site by the activation loop. Tyr 394 auto-phosphorylation displaces the activation loop generating an active form of Lck. Phosphatases CD45, PEP and SHP-1 function in direct opposition to Csk and Lck creating an equilibrium of Lck phosphorylation, conformation and activation. This figure is modified from a similar version shown elsewhere (152).

activation (reviewed in 196). This serves as negative feedback loop in T cells, since SHP-1 recruitment results in the dephosphorylation of SLP-76 (197) and ZAP-70 (198). The analysis of *motheaten* mice, with a genetic mutation resulting in a SHP-1 deficiency, revealed lymphocyte hyper-responsiveness and increased Lck activation in response to TCR stimulation (199). Recently, SHP-1 was shown to specifically dephosphorylate Lck at its activating Tyr 394 *in vitro* and in an embryonic kidney cell line (200). This data suggests that the absence SHP-1-mediated Lck down-regulation may contribute to the lymphoid abnormalities of *motheaten* mice, and establishes SHP-1 as a possible regulator of activated Lck in stimulated T cells.

Another cytosolic protein tyrosine phosphatase, PEP, is expressed exclusively in lymphoid cells (201). Like SHP-1, PEP inhibits TCR stimulation events including the transcriptional activation of c-fos, and the IL-2 gene (202). In the same study, the analysis of proximal signaling events revealed that PEP reduced the TCR-induced tyrosine phosphorylation of the Lck Y505F mutant (phosphorylated at Tyr 394). Thus, CD45, SHP-1 and PEP are three phosphatases implicated in Lck down-regulation via Tyr 394 dephosphorylation.

Interestingly, a proline rich segment in PTP PEP links this phosphatase to the SH3 domain of Csk in T cells (201). Csk is widely expressed and known to down-regulate the activity of Src-family kinases via its unique ability to phosphorylate the inhibitory tyrosine at the carboxyl tail, including those of Lck and Fyn in T cells (50, and reviewed in 203). Thus, Csk over-expression potently inhibits TCR-induced protein tyrosine phosphorylation (204), and Csk-knockout murine embryos exhibit the enhanced activity of Src, Fyn and Lyn (205). This data further supports a role for Csk in the negative

regulation of Src-family kinases *in vivo*. Csk resembles Src-family kinases in structure (containing an SH3, SH2 and a catalytic kinase domain), but lacks sites of N-terminal acylation, or either regulatory tyrosine (49). Thus, Csk is cytosolic and its regulation is not entirely understood. However, the understanding of Csk function in T cell has recently been enhanced by studies showing its association with the PEP phosphatase and the membrane adapter molecule PAG/Cbp (PAG hereafter).

The association of Csk with PEP supports an interesting model for Lck regulation. Since ~60% of PEP is localized to cell membranes (206), it has been suggested that PEP recruits Csk to the membrane where these two enzymes combine to simultaneously dephosphorylate Lck Tyr 394, and phosphorylate Lck Tyr 505 respectively, acting as a dual switch of Lck down-regulation (202).

PAG (phosphoprotein-associated with glycosphingolipid enriched domains) was originally identified as a tyrosine phosphorylated, transmembrane adapter protein that binds the SH2 domain of Csk and is localized in lipid-raft domains of the cell membrane (65). In unstimulated T cells, Src kinases maintain PAG tyrosine phosphorylation and the accompanied association of PAG with Csk in lipid rafts (65, 207). This is in agreement with raft-localized Lck exhibiting Tyr 505 phosphorylation and reduced activity (113). However, this also presents a paradox, such that Lck activity is thought to provide constitutive PAG phosphorylation in rafts. The same studies revealed that upon T cell stimulation PAG is dephosphorylated, likely by CD45 (208), resulting in the dissociation of Csk. Moreover, the over-expression of PAG in murine T cells results in the inhibition of TCR-mediated IL-2 production, cell proliferation and intracellular tyrosine phosphorylation (208). Thus, it is currently believed that TCR-induced Lck

activation can be sustained due to the release of Csk from coalesced lipid rafts at the immune synapse. Subsequent re-association of Csk with PAG several minutes after TCR stimulation would then provide a means of Lck down-regulation via Tyr 505 phosphorylation (207).

1.4.6 CD45AP, an enigma in T cell signaling

On the surface of T cells, the majority of CD45 is constitutively associated with a 30-kDa lymphocyte-specific protein (209, 210) that was named CD45-associated protein (CD45AP) upon being cloned from murine T cells (211) or lymphocyte phosphatase-associated phosphoprotein (LPAP) in human T cells (212). The molecular cloning of the CD45AP gene revealed the presence of a short extracellular region (9 amino acids), a single transmembrane domain, and a cytoplasmic domain encoding approximately 145 residues (211, 212). Interestingly, the expression of CD45AP is limited to T and B cells (213), despite the expression of CD45 throughout the nucleated hematopoietic lineage. Moreover, in some $CD45^-$ T cell lines, there is little or no CD45AP protein expression despite normal levels of CD45AP-encoding mRNA (212, 213), suggesting that CD45AP protein is rapidly degraded in the absence of CD45. The CD45:CD45AP association occurs at a 1:1 stoichiometry in T cells (209, 212) and this interaction is localized to the transmembrane domain of each protein (214-216).

However, the role of CD45AP in lymphocyte function remains unclear, and there are conflicting results from three separate strains of CD45AP-knockout mice. The first CD45AP-knockout strain exhibited a marked reduction in lymphocyte proliferation in response to antigen receptor stimulation and a reduction in the CD45:Lck association (217). However, two subsequent strains of CD45AP-knockout mice later revealed no

significant difference in the association of CD45 and Lck, or in cell proliferation upon stimulation (218, 219). In support of the findings from first CD45AP-knockout strain (217), recent work in our laboratory has shown that upon CD3 stimulation, T cells over-expressing CD45AP exhibit enhanced and sustained intracellular tyrosine phosphorylation compared to wild type cells (D. Wong, unpublished data). Thus, it is tempting to suggest a role for CD45AP in augmenting lymphocyte antigen receptor signaling events. In support of this model, recent studies have revealed direct interactions of CD45AP with Lck and ZAP-70 *in vitro*, as well as in T cells in response to TCR stimulation (220). Likewise, CD45AP associates with the TCR, CD4 and CD8 co-receptors, and activated forms of Lck in T cells (221). Thus there is an emerging role for CD45AP in T cell signaling, although further experiments are required to reveal its mechanism.

1.5 Lck in health and disease

In its identification in the mid 1980s, Lck was isolated as the proto-oncogene responsible for the transformation of murine T lymphoma LSTRA cells (9, 11). Later studies revealed the neoplastic transforming ability of a mutated Lck gene in murine fibroblast cells (148-150), similar to the v-Src oncogene known for decades to be potently oncogenic. Likewise, the analysis of transgenic mice expressing constitutively active forms of Lck revealed the induction of aggressive tumors (194, 222). Lck is also implicated in a variety of human cancers (reviewed in 223), and thus has been identified as a bona fide oncogene, capable of cell transformation if mutated or over-expressed.

In contrast to the cancers produced by excessive Lck activity, the absence of Lck activity is detrimental owing to deficient signaling in T cells. Severe combined immune deficiency (SCID) is a disease characterized by the lack of antibody production or T cell function in an immune response. Thus, SCID patients are severely immuno-compromised and are highly susceptible to infection due to an ineffective adaptive immune system. Recently, T cells isolated from an infant who had been diagnosed with SCID responded poorly to stimulation and displayed a marked reduction in Lck expression (224). Genetic analysis revealed the presence of an alternatively spliced Lck transcript lacking exon 7. The patient suffered from sepsis, dehydration, weight loss, and cytomegalovirus infection and underwent a bone marrow transplant in an attempt to restore T cell function with wild type Lck expression. This case study highlights the importance of Lck function in the immune system, and suggests that a lack of Lck activity can induce SCID in humans.

Lck has also been implicated in the infection of T cells by the human immuno-deficiency virus (HIV). The HIV Nef protein, essential for HIV propagation and AIDS progression, has been shown to interact with the SH3 domain of several Src-family kinases leading to kinase activation (225). Interestingly, both T cell activation and Nef-mediated phosphorylation of the viral capsid correspond with increased viral replication. More specifically, the conserved core region of Nef was shown to be responsible for both its interaction with Lck and the enhanced replication of HIV particles in T cells (226). Together, these data suggest that the binding of Nef to Lck is central during an HIV infection of T lymphocytes.

A good example of T cell-mediated autoimmune disease is that of type-1 diabetes in which T cells destroy the insulin producing pancreatic islet β cells. Patients with this disease must endure a lifetime of insulin injections and profound daily changes in blood sugar levels. Recently, a cohort of such patients was analyzed for the signaling capabilities of their peripheral T lymphocytes. Consistently, these patients exhibited T cell hypo-responsiveness correlated with a reduction in Lck expression (227). Likewise, the alteration of Lck expression was also detected in patients suffering from systemic lupus erythematosus (228), although the mechanism of this pathology is not understood.

Clearly, Lck is a key molecule in T cell biology with critical roles in T cell development, response to antigen recognition, and adhesion. Thus, due to its significance in T cell function, Lck is often associated with T cell related pathology and remains a key target in drug design. In furthering the understanding of Lck function and regulation, it is the hope of scientists and clinicians that the intricacies of this molecule can provide insights into novel treatments for disease.

1.6 Thesis Objectives

Controlled Lck activity is paramount to proper T cell function, such that too little or too much activity produces profound and adverse effects. Clearly, the regulation of this kinase in T cells is critical to ensure that signaling events during development, antigen recognition, and adhesion occur in a controlled manner. Thus, the overall goal of this thesis is to better understand Lck regulation. Crystal structure studies have revealed how the intramolecular mechanisms of Lck phosphorylation mediate its conformation, and thereby its activation. To date, the CD45 is the tyrosine phosphatase most

established in the regulation of Lck phosphorylation. Although there is extensive evidence for the role of CD45 in Lck up-regulation (via Tyr 505 dephosphorylation), recent studies continue to show support for an additional role in Lck down-regulation (via Tyr 394 dephosphorylation). However, the balance between Lck up- and down-regulation achieved by CD45 is not well understood. Thus, the first objective of this thesis is to establish and evaluate the variables affecting the ability of CD45 to dephosphorylate Lck at Tyr 394 or Tyr 505.

An enigma in CD45 function and T cell signaling is the role of CD45AP. Despite conflicting knockout-mouse data, there is evidence that CD45AP associates with TCR machinery and may serve to augment TCR signaling via its ability to associate with both CD45 and Lck. However, the details of the CD45AP:Lck association are not well defined, nor are the effects of this association on the critical enzyme-substrate interaction of CD45 and Lck. Thus, the second objective of this thesis is to characterize the CD45AP:Lck interaction, and determine the effect of this interaction on CD45-mediated Lck regulation.

CD44 is a widely expressed cell adhesion molecule that upon clustering at cell surface induces an intracellular signaling cascade resulting in cytoskeletal reorganization despite lacking any enzymatic activity of its own. In T cells, Src-family kinases have been implicated in providing the necessary kinase activity for proximal CD44 signaling events. However, the relative contributions of Lck and Fyn to this process remain unclear, as do the details of their ability to associate with CD44. Thus, the third objective of this thesis is to investigate and characterize the association of Lck and Fyn with CD44 in T cells.

CHAPTER 2

Materials and methods

2.1 Materials

2.1.1 Cell lines

Murine BW5147 T lymphoma cells ($CD45^+$ and $CD45^-$; ATCC) transfected with CD3 ζ and δ to express surface T cell receptor (TCR)/CD3 were used (229), and are referred to hereafter as BW+ and BW- respectively. BW+ cells were subsequently transfected with c-myc-tagged CD45AP, and two clones (clone 10 "BW+cAP.1", and clone 13 "BW+cAP.2") were used (D. Wong, M.Sc. thesis). Clone 10 was sorted by flow cytometry to select for high c-myc-CD45AP expression. Murine AKR1 T lymphoma cells (AKR1) transfected with the full-length hematopoietic form of CD44.1 were from B. Hyman (230). AKR1 cells transfected with truncated CD44.1, residues 1 to 274 (numbering as in 231), expressing only the first two amino acids of the cytoplasmic domain (from B. Hyman), or full-length CD44.1 with a cysteine 268 to alanine point mutation (from A. Maiti) were also used. All AKR1 and BW cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle Medium (DMEM, Invitrogen Life Technologies, Burlington, ON) supplemented with 10% horse serum (HS, Hyclone, Mississauga, ON), 1 mM sodium pyruvate (Invitrogen Life Technologies), 2 mM L-glutamine (Sigma-Aldrich, Oakville, ON), 100 U/mL penicillin (Invitrogen Life Technologies), and 100 μg/ml streptomycin (Invitrogen Life Technologies). BW+ and BW- were grown in the presence of 3 mM L-Histidinol (Sigma-Aldrich) to maintain CD3 expression. AKR1 cells were grown in 1.5 mg/ml G418 (~0.75 mg/ml of active G418, Invitrogen Life Technologies) to maintain CD44 expression. BW+ cells transfected with c-myc-CD45AP were grown in both 3 mM Histidinol and 1.5 mg/ml G418.

2.1.2 Antibodies

2.1.2.1 Primary antibodies

Rabbit antisera specific for the amino-terminal region of Lck (R54, 75), the cytoplasmic domain of CD45 (RO2.2, 184), cytoplasmic CD45AP residues 42 to 157 (WW1B2, D.Wong, M.Sc. thesis) or cytoplasmic CD44 residues 314 to 343 (J1WBB, 68) were used. The 4G10 anti-phosphotyrosine monoclonal antibody was from Upstate Biotechnology Inc. (Lake Placid, NY), the anti-6His antibody was from Amersham Pharmacia Biotech (Baie d'Urfe, Quebec), and the rabbit anti-Erk1 polyclonal IgG (C-16) and rabbit anti-Lck polyclonal IgG (2102) were from Santa Cruz Biotechnology (Santa Cruz, CA). Rat anti-mouse CD44 monoclonal antibodies (mAbs) KM201 (232), and IM7.8.1 (IM7, 233), and rat anti-mouse mAbs CD4 GK1.5 (ATCC) and CD45 I 3/2 (234) were used. Rabbit antisera specific for Fyn (235) was kindly provided by Dr. A. Veillette (McGill University, Montreal). Anti-GST antisera was generated by immunizing rabbits with GST (D. Wong, unpublished). Rabbit antisera specific to the autophosphorylation site of Lck (Tyr 394) was kindly provided by A. Shaw (99). The rabbit polyclonal antibody specific to the autophosphorylation site of Src (Tyr 416) was from Cell Signaling Technology (Mississauga, ON)

2.1.2.2 Secondary Antibodies

Protein A horseradish peroxidase (HRP) was purchased from Biorad Laboratories (Mississauga, ON), goat anti-mouse IgG-HRP (H+L) was from Southern Biotechnology Inc. (Birmingham, AL), and goat anti-rabbit IgG-HRP (H+L) was from Jackson Immunoresearch Laboratories Inc. (West Grove, PA).

2.1.3 Bacterial strains and expression vectors for cloning

Four *Escherichia coli* (*E. coli*) strains were used for expression of recombinant proteins. XL-1 Blue (Stratagene, Cedar Creek, TX) and DH5α (New England Biolabs Ltd., Mississauga, ON) were used for expression of GST fusion proteins except where noted, or where GST fusion proteins were co-expressed with other plasmids. K12 UT5600 *E. coli* (New England Biolabs Ltd.), deficient in the OmpT outer membrane protease, were used for GST and MBP fusion proteins where indicated. BL21 (DE3), from F. Studier (236) was used for the expression of 6His-tagged proteins, or for co-expression of GST fusion proteins with the pT-Trx plasmid (237). pT-Trx was used to produce thioredoxin protein that has been shown to increase Lck solubility in *E. coli* lysates (237).

GST fusion proteins were created by ligation of various cDNA constructs into a variety of pGEX vectors (Amersham Pharmacia Biotech) as indicated, or were supplied in pGEX vectors as described. The pRSET A (Invitrogen Life Technologies), pET-28a (Novagen, Madison, WI), and pET-3D-6His-IEGR (238) vectors were used to create 6His-tagged constructs. The latter vector was modified previously to encode a kanamycin resistance cassette in place of the ampicillin resistance gene of the pET-3D-6His-IEGR vector (J. Felberg, Ph.D. thesis). MBP-fusion proteins were expressed from the pMAL-cri vector (New England Biolabs Ltd.) and the pBCMGSneo vector (239) was used to clone constructs for eukaryotic expression.

2.1.4 Plasmids and recombinant proteins

2.1.4.1 Plasmids encoding recombinant proteins used as negative controls

Plasmids encoding irrelevant recombinant proteins used as negative controls for *in vitro* binding assays are as follows: GST and MBP alone were expressed and purified from pGEX-3X (Amersham Pharmacia Biotech) and pMAL-cri vectors respectively, the plasmid encoding GST-SHC SH2 (human) was from T. Pawson (240), the GST-Grb2 plasmid (murine (241)) was from M. Gold, and the GST-Erk1 plasmid (human) was from S. Pelech (242).

2.1.4.2 Lck related plasmids

Murine Lck was provided by B. Sefton (10) and the following Lck mutants were provided by A. Veillette (28, 243) and subcloned by D. Ng (Lck residues -8 to 509) into pGEX 4T-2 (Amersham Pharmacia Biotech): Lck F505, where tyrosine 505 has been mutated to a phenylalanine; Lck F394, where tyrosine 394 has been mutated to a phenylalanine, and Lck R273, where the active site lysine has been mutated to an arginine to generate a kinase inactive form of Lck. The plasmid encoding Lck R154K, with arginine 154 has been mutated to a lysine, was provided by D. Littman (244). This was incorporated into GST-Lck F394 to create GST-Lck F394/R154K by subcloning a *Bam*HI – *Nco* I fragment (containing Lck residues -8 to 233). GST-Lck N32, containing the non-catalytic regions of Lck (the unique amino-terminal region, SH3 and SH2 domains (residues -8 to 233) was created by ligation of a *Stu* I - *Nco* I fragment of Lck into pGEX-2T by D. Ng. Constructs expressing GST-Lck kinase domain proteins (wild-type, F394, F505 and R273 mutants) were created by subcloning a *Nco* I - *Stu* I fragment containing residues 232 to 509 of Lck into pGEX 4T-3. All full-length Lck and Lck kinase domain constructs were co-expressed in BL21 (DE3) *E. coli* with the pT-Trx plasmid. The Lck SH2 (residues 122 to 234) and SHC SH2 (residues 366 to 473)

domains were subcloned into the pRSET A vector to create 6His-Lck SH2 and 6His-SHC SH2 fusion constructs. The cDNA for rat Csk (49) was provided by F. Jirik and subcloned into pGEX-2T by R. Tang, and into pET-3D-6His-IEGR (with a kanamycin resistance gene) by J. Cross, and these were used to express GST-Csk and 6His-Csk respectively. Murine Fyn cDNA was obtained from R. Perlmutter and was then subcloned into the pGEX-4T-2 vector by P. Mendoza to generate the GST-Fyn construct.

The GST-Erk-Lck SD10 chimera (GST-Erk(L-SD10)) was constructed previously (M. Lam, J. Cross) by creating two silent mutations (*Stu* I and *Xba* I) flanking the Erk1 SD10 region in pGEX-2T Erk1 by PCR. The murine Lck SD10 region, residues 444-466 (MTNPEVIQNLERGYRMVRPDNCP), was generated by PCR and ligated into pGEX-2T Erk1 to create pGEX-2T-Erk1-Lck SD10 such that Lck SD10 was inserted between Erk1 residues Gly 247 and Ser 301 (242), and was then expressed in XL-1 Blue *E. coli.* Immobilized GST-Erk(L-SD10) was auto-phosphorylated by incubation of the purified fusion protein (0.2 μM) with 0.2 mM ATP prior to use in dephosphorylation assays.

2.1.4.3 CD45 related plasmids

The construct of murine 6His-tagged CD45 cytoplasmic domain (CD45), encoding residues 565 to 1268 (as numbered in 245), and the 6His-tagged construct encoding catalytically inactive protein with a mutation of Cys 817 to Ser (CD45C817S), were created previously (238). The GST-CD45C817S plasmid was created previously (J. Cross) by subcloning the cytoplasmic domain of CD45C817S into the pGEX-3X vector and expressed in XL1 blue *E.coli.* The 6His-D2 construct (residues 903-1268) containing part of the spacer region, the entire second phosphatase domain and the carboxy tail is as described (169).

2.1.4.4 CD45AP related plasmids

Murine cDNA of CD45AP was provided by A. Takeda (211) and was subcloned previously by D. Wong into pGEX-4T-1 to create a GST-CD45AP construct encoding residues 48 to 197 (CD45APcyt, including the entire cytoplasmic domain) that was transfected into UT5600 *E coli* (numbering as in, 212). A fragment encoding a GST-CD45APcyt construct lacking the C-terminal 40 residues (encoding residues 48 to 157) was ligated previously (D. Wong) into pGEX-4T-1 and pET-28a vectors to create the GST- and 6His-tagged CD45APcyt Δ157-197 constructs, respectively. For eukaryotic expression, a cDNA fragment encoding a c-myc CD45AP fusion protein (residues 34 to 197) was subcloned previously into the pBCMGSneo vector and transfected into BW+ T lymphoma cells by D. Wong.

2.1.4.5 CD44 related plasmids

The cDNA sequence of the murine CD44.1 cytoplamic domain (residues 272 to 343, (numbering as in, 231)) was created by PCR (R. Tang) and was previously ligated (R. Li) into pGEX-2T to create the GST-CD44 construct containing the entire cytoplasmic domain (GST-CD44cyt). Truncated CD44, lacking 13 membrane-proximal residues (encoding residues 285 to 343), was created previously by R. Li and was ligated into pGEX-2T to create GST-CD44cyt Δ272-284. To create the plasmid encoding full-length CD44 cytoplasmic domain fused to MBP, CD44cyt in pGEX-2T was used as a template to generate cDNA encoding the cytoplasmic domain of CD44 by PCR using forward primer 5'-GACCAGATCTCGGATCCTCAATAGTAGGAGAAGG-3' and reverse primer 5'-CACTGGATCCGAATTCAATGACGT TTC-3'. This product was then cut with *Bam* HI and *Eco* RI (overhangs filled-in with Klenow), ligated into an *Eco*

RI site (also filled-in with Klenow) of the pMAL-cri vector, and transfected into UT5600 *E. coli*.

The CD44 cysteine 277 to alanine mutant was generated previously by J. Cross and was then subcloned into a *Bam* HI – *Pst* I cut pMAL-cri vector to create the MBP-CD44cyt C277A fusion. The CD44 cysteine 268 to alanine mutant was created previously by A. Maiti, subcloned into the pBCMGSneo vector and then transfected into AKR1 cells for eukaryotic expression.

2.2 Methods

2.2.1 Expression of recombinant proteins

2.2.1.1 Expression of GST fusion proteins

GST-Lck fusion proteins were co-expressed with thioredoxin from the pT-Trx plasmid (encoding chloramphenicol resistence) to increase the solubility of Lck protein (237) in BL21 (DE3) *E. coli*. With the exception of Lck-R273 and Kin-R273, all GST-Lck fusion proteins were also co-expressed with 6His-Csk from the pET-3D-6His-IEGR plasmid encoding kanamycin resistence. GST fusion proteins were produced in *E. coli* and purified according to manufacturer's recommendations (Amersham Pharmacia Biotech). Briefly, *E. coli* cultures were grown at 37°C, 180 rpm to an $O.D._{600nm}$ of 0.6 to 0.8 and were then treated with 0.1 mM isopropyl β-D-thiogalactoside and incubated at 26°C, 180 rpm for a minimum of 3 h. Cultures ranged in volume from 5 ml to 1 L depending on the yield of individual fusion proteins. Cells were then pelleted and lysed in 10 ml GST lysis buffer (0.1 mg/ml lysozyme, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X-100, 2 mM EDTA) including 0.2 mM phenylmethylsulfonyl fluoride, and 1

μg/ml each of aprotinin, leupeptin and pepstatin (protease inhibitors). The soluble lysate was then incubated with glutathione sepharose (GSH) beads (500 μl of a 50% slurry, Amersham Pharmacia Biotech) for 2 h at 4°C to allow for the coupling of GST fusion proteins. Protein-coupled GSH beads were then washed vigorously three times in wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton-X-100, plus protease inhibitors) and were quantitated as described below (section 2.2.3).

For some experiments, soluble forms of GST fusion proteins were required, therefore immobilized GST fusion proteins were eluted or enzymatically cleaved from GSH beads. In this case, GST-Lck fusion proteins were purified from a 1L bacterial culture and washed three times with 1X TBS (20 mM Tris-HCl pH 7.5, 150 mM NaCl). To elute immobilized GST fusion proteins, the beads then underwent two consecutive incubations, end-over-end for 30 min with 1 ml of 15 mM glutathione pH 8.0 (Sigma-Aldrich). The 2 ml of eluted protein was pooled and passed through a PD10 column (Amersham Pharmacia Biotech) to remove the glutathione. Fractions from the PD10 containing protein were pooled and the eluted protein was quantitated (see section 2.2.3). To cleave protein from immobilized GST, the beads were washed with 1X TBS and then resuspended in 1X TBS to create a 50% bead slurry that was then incubated end-over-end for 4 h at 4°C with 0.5 NIH units of thrombin. Cleaved Lck present in the supernatant was quantitated (section 2.2.3) and used for binding assays and dephosphorylation assays.

2.2.1.2 Expression of 6His-tagged proteins

Recombinant 6His-tagged CD45 cytoplasmic domain proteins (6His-CD45, 6His-CD45C817S and 6His-D2) were purified as described elsewhere (169). Other 6His-tagged proteins were treated essentially the same as the GST fusion proteins except that

the bacteria were lysed in 6His lysis buffer: 0.1 mg/ml lysozyme, 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% Triton-X-100, 1 mM EDTA, 20 mM imidazole, 0.025% β-mercaptoethanol and protease inhibitors. 6His-proteins from a 1 L culture were bound to 1 ml Ni^{2+}-NTA-Agarose (50% slurry, Qiagen Inc., Mississauga, ON) and washed with lysis buffer, followed by high salt buffer 1 (20 mM Tris-HCl pH 7.2, 0.5 M NaCl, 0.5% Triton-X-100), lysis buffer, high salt buffer 2 (20 mM Tris-HCl pH 7.2, 1.0 M NaCl, 0.5% Triton-X-100), and lysis buffer again before being quantitated (see section 2.2.3) or eluted (as described for GST fusion proteins) with 1 M imidazole pH 7.2, 150 mM NaCl.

2.2.1.3 Expression of MBP-fusion proteins

MBP-fusion proteins were expressed and purified essentially as described for GST fusion proteins except that soluble lysates were incubated with 500 µl of amylose resin (50% slurry, New England Biolabs) to produce purified immobilized MBP-fusion proteins.

Purified GST, 6His and MBP recombinant fusion proteins were all snap frozen in liquid nitrogen and stored at –80°C.

2.2.2 Immunoblotting

Following separation by SDS polyacrylamide gel electrophoresis (PAGE), proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon -P, Millipore Canada Ltd., Mississauga, ON) according to the manufacturer's instructions (Biorad). Membranes were then left to dry overnight, or were submerged briefly in methanol and left to dry for 20 minutes. Due to the hydrophobicity of the PVDF membrane, all immunoblotting was done without an initial blocking step. Dry membranes were incubated for 1 h with primary antibody diluted in 5% skim milk

powder or 1% bovine serum albumin (BSA, Sigma-Aldrich) in TBST (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20). Membranes were then washed 3 to 5 times (1 to 5 minutes per wash) in TBST before adding the secondary antibody, also diluted in 5% skim milk or 1% BSA in TBST, and incubated for 40 minutes. Membranes were then washed as described above, submerged in enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham Pharmacia Biotech), and exposed to Kodak Biomax film for various amounts of time.

Dilutions for primary antibodies were as follows: 4G10 anti-phosphotyrosine antibody was diluted 1/5000 in 1% BSA in TBST. All other primary antibodies were diluted in 5% skim milk in TBST. R54 anti-Lck, RO2.2 anti-CD45, anti-CD45AP and anti-GST antisera were all diluted 1/5000. JIWBB anti-CD44 antiserum, anti-Fyn antiserum, and anti-6His, 2102 anti-Lck and anti-Erk1 antibodies were all diluted 1/1000. KM201 anti-CD44 from hybridoma tissue culture supernatant was diluted 1/10.

Protein A-HRP diluted 1/5000 in 5% skim milk in TBST was used as the secondary antibody to detect R54, RO2.2, WW1B2, anti-Fyn, anti-GST, JIWBB and anti-Erk1. Goat anti-rat HRP diluted 1/5000 in 5% skim milk in TBST was used to detect KM201, and goat anti-mouse HRP diluted 1/5000 in 1% BSA or 5% skim milk in TBST was used to detect 4G10 and anti-6His antibodies, respectively.

2.2.3 Quantitation of purified recombinant proteins

Various dilutions of known amounts of BSA were generated to create a standard curve for quantitation. Proteins were then boiled in reducing SDS sample buffer, separated by SDS-PAGE and the gel was subsequently stained with Coomassie blue. Spot densitometry was then used to determine the concentration of purified proteins

relative to the BSA standard curve using AlphaImager^{TM} 1200 software (Alpha Innotech, San Leandro, CA).

2.2.4 *In vitro* phosphorylation of 6His-CD45 with recombinant Csk

6His-CD45 cytoplasmic domain protein was purified, washed and left immobilized on Nickel-NTA-agarose beads as described above. Immobilized 6His-CD45 (500 ng, 150 nM final concentration) was washed twice in kinase buffer (50 mM Tris-HCl pH 7.2, 3 mM $MnCl_2$, 1 mM sodium orthovanadate) and resuspended in 40 µl of kinase buffer containing 1 mM ATP (Amersham Pharmacia Biotech) and with or without 500 ng (245 nM final concentration) of thrombin-cleaved recombinant Csk. After a 60-minute incubation at 30°C, the immobilized CD45 was washed twice with wash buffer and then used in binding or dephosphorylation assays with Lck. Initial experiments were performed using 500 ng (245 nM final concentration) of recombinant Csk to phosphorylate 4 µg (1200 nM final concentration) of 6His-CD45 for 30 min in 40 µl of kinase buffer plus sodium orthovanadate (vanadate). However, this method was later optimized to use the conditions described above.

2.2.5 Dephosphorylation of immobilized GST-Lck with calf intestinal phosphatase

Recombinant GST-Lck and Lck kinase domain proteins were dephosphorylated using calf intestinal phosphatase (CIP, New England Biolabs). Immobilized GST-Lck fusion protein (2 µg) bound to GSH beads was washed twice with 1X CIP buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 1 mM dithiothreitol), resuspended in 100 µl of 1X CIP buffer to which 1 µl of CIP (10,000 units/ml) was added and incubated at 37°C for 1 h. Proteins were then washed twice in binding buffer and used in an *in vitro* binding assay (described below). The sample was divided, subjected to SDS-PAGE and

transferred to separate PVDF membranes such that 10% of the sample was immunoblotted with an anti-phosphotyrosine antibody (4G10) and the remaining 90% was immunoblotted with anti-CD45 anti-serum (RO2.2).

2.2.6 Dephosphorylation of Lck proteins with recombinant CD45

Dephosphorylation assays were preformed essentially as previously described for GST-Lck (184). Briefly, 5 µl of 120 nM 6His-CD45 was added to 5 µl of 110 nM thrombin-cleaved Lck at 30°C. Reactions were stopped at various time points by immersion into a dry-ice ethanol bath. Proteins were then subjected to SDS-PAGE and the phosphorylation status and Lck protein amounts were determined by Western blot analysis with 4G10 and R54 or 2102, respectively. The amount of phosphotyrosine per unit Lck was calculated after densitometric scanning using AlphaImager™ 1200 software by dividing the phosphotyrosine value by the Lck value. Lck phosphorylation at time 0 was taken as 100%.

Alternatively, immobilized 6His-CD45 that had been incubated in kinase buffer with or without recombinant Csk to create phosphorylated and non-phosphorylated forms of CD45 respectively (see above) was washed twice with ice-cold wash buffer, and resuspended in phosphatase buffer (50 mM Tris-HCl pH 7.2, 1 mM EDTA, 0.1% β-mercaptoethanol) to a concentration of 120 nM. Immobilized CD45 (5 µl of 120 nM) was then added to 5 µl of 120 nM Lck and treated as described above.

For assays in which recombinant protein competitors were included, soluble Lck protein or 6His-CD45 were diluted to 120 nM with 600 nM of 6His-CD45AP protein or 6His-SHC SH2. CD45 (5 µl) was then added to 5 µl of Lck at 30°C to create final

concentrations of 60 nM (CD45 and Lck) and 300 nM (CD45AP or SHC SH2). All other conditions are as described above.

2.2.7 Dephosphorylation of immobilized GST fusion proteins with CD45

These assays were performed at 30°C in 10 µl of phosphatase buffer essentially as described above. Purified recombinant 6His-CD45 (60 nM) was incubated with various auto-phosphorylated recombinant GST fusion proteins, either 25 nM GST-Lck, or 380 nM GST-Erk1 or GST-Erk(L-SD10) for increasing amounts of time. These concentrations were shown by densitometric analysis using Alphaimager™ software to contain approximately equal levels of phosphotyrosine. Assays were performed as described in 2.2.6 and the tyrosine phosphorylated bands were analyzed by densitometric analysis using Alphaimager™ software and subsequently graphed. The amount of tyrosine phosphorylation at time 0 was taken as 100% for all of the substrates.

2.2.8 *In vitro* binding assays

All binding assays were performed with purified recombinant proteins such that an excess molar, or µg, amount of a bead-immobilized protein was assayed with a limiting soluble protein in a 40 µl volume. Soluble proteins not purified by FPLC (such as thrombin-cleaved or eluted GST fusion proteins, 6His-CD45AP and 6His-Lck SH2) often exhibited low levels of background binding, thus binding buffers were supplemented with 0.05% Triton-X-100 in these assays to reduce background effects. The concentration of proteins used in different binding assays was variable owing to the high degree of variability in the yield of different proteins obtained upon purification. To aid the visualization of bead-bound protein after centrifugation, each binding assay was

supplemented with Sepharose CL-4B (Sigma-Aldrich) such that the packed bead volume was 10 µl.

2.2.8.1 Binding assays with immobilized GST-Lck and soluble 6His-CD45 or 6His-CD45AP proteins

In 40 µl of binding buffer 1 (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol plus protease inhibitors), 2 µg of immobilized recombinant GST-Lck, GST-Kin, GST-N32, GST-SHC SH2, or GST alone (610, 880, 960, 1250 and 1900 nM final concentrations, respectively) were incubated with 1 µg of soluble recombinant 6His-CD45 protein (300 nM final concentration) in the presence or absence of 0.5 mM vanadate for 2 h, with shaking, at 4°C. The beads were then washed vigorously three times with RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS and protease inhibitors). The beads were then boiled in reducing SDS sample buffer, and the proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and the relative amount of CD45 protein that had bound to the immobilized Lck proteins was determined by blotting with RO2.2 antiserum. Coomassie blue protein stain was applied to the PVDF membrane to confirm the presence of equal amounts of GST fusion protein used in each assay. In some cases, 10% of the sample was run on a separate gel, transferred to PVDF and probed with the 4G10 antibody to determine the tyrosine phosphorylation status of the Lck proteins. The amount of CD45 bound per unit of GST fusion protein was determined by densitometric scan analysis of the RO2.2 Western blot and the Coomassie blue stain with AlphaImager™ 1200 software.

Alternatively, in 40 µl of binding buffer 2 (20 mM Tris-HCl pH 7.2, 150 mM NaCl, 0.05% Triton-X-100 and protease inhibitors), 2 µg of recombinant immobilized GST-Lck fusion proteins (final concentrations as above), or GST-Erk1 and GST-Erk(L-SD10) (725 and 760 nM final concentrations, respectively) were incubated with 100 or 540 ng of soluble 6His-CD45AP Δ157-197 (195 nM or 1050 nM final concentrations, respectively). The samples were then treated as described above except that the CD45AP protein that bound to the immobilized Lck was detected by immunoblotting with an anti-6His antibody.

2.2.8.2 Binding assays with immobilized GST fusion proteins and soluble 6His-D2

In 40 µl of binding buffer 1 (plus 0.05% Triton-X-100), 2 µg of recombinant immobilized GST fusion proteins (final concentrations as above) were incubated with 540 ng (300 nM final concentration) of soluble 6His-D2 for 2 h, with shaking, at 4°C. The samples were then treated as described above, and the amount of 6His-D2 that had bound to the GST fusion proteins was detected by blotting with RO2.2 antiserum.

2.2.8.3 Binding assays with immobilized 6His-CD45, or GST-CD45AP, and soluble Lck proteins

Immobilized 6His-CD45 (500 ng) was initially incubated in kinase buffer with or without recombinant Csk to create phosphorylated and non-phosphorylated forms of CD45 respectively (see above). The beads were then washed twice with cold binding buffer 2 (plus 1 mM vanadate). Ten percent of the immobilized CD45 was then subjected to SDS-PAGE for determination of CD45 phosphorylation status with a 4G10 immunoblot. The remaining 90% (450 ng, 135 nM final concentration) of the CD45 was incubated with 100 ng (30 nM final concentration) of eluted GST-Lck F505 or GST-Lck

F394 for 2 h, with shaking, at 4°C. The samples were then treated as described above, except that Lck bound to the immobilized CD45 was detected by blotting with R54 antiserum, while Coomassie blue staining was used to detect equal amounts of CD45 used in each assay. Ni^{2+}-NTA agarose beads alone (10 µl) were used as a negative control for Lck binding.

Alternatively, in 40 µl of binding buffer 2, 2 µg of recombinant immobilized GST-CD45APcyt, GST-CD45APcyt Δ157-197, GST alone or 6His-CD45C817S protein (1150, 1290, 1900, and 600 nM final concentrations, respectively) was incubated with 100 ng of thrombin-cleaved Lck (45 nM final concentration). The samples were treated as described above, and bound Lck was detected by immunoblotting with R54 antiserum.

2.2.8.4 Binding assays with immobilized GST- and MBP-CD44 fusion proteins to soluble Src-family kinases

Immobilized GST or MBP alone (3.1 and 5.1 µg respectively, 3.0 µM final concentration), or GST-CD44cyt, GST-CD44cyt Δ272-284, MBP-CD44cyt or MBP-CD44cyt C277A (2.0, 1.9, 3.0 and 3.0 µg respectively, 1.5 µM final concentration) fusion proteins were incubated with 0.1 µM (final concentration) of soluble thrombin-cleaved Lck, N32, or Kin protein (220, 100, and 120 ng, respectively) or eluted GST-Lck (0.33 µg) or GST-Fyn (0.34 µg), in 40 µl of binding buffer 3 (10 mM Tris-HCl pH 7.2, 140 mM KCl, 0.05% Triton-X-100 and protease inhibitors) in the presence or absence of various divalent cations at final concentrations of 50 µM. Proteins were incubated for 2 h, with shaking, at 4°C. Immobilized MBP fusion proteins were washed twice with binding buffer 3, or three times with RIPA depending on the degree of background binding. Immobilized GST fusion proteins were washed with RIPA, high salt buffer1,

RIPA, high salt buffer 2, and RIPA again, all containing 1 mM EDTA (except experiments with N32 where wash buffers had 5 mM EDTA). EDTA was included in wash buffers to chelate ions inducing non-specific background binding. Samples were then boiled in reducing SDS sample buffer and subjected to SDS-PAGE for Western blot analysis. Src-family kinase proteins that bound to the immobilized CD44 fusion proteins were detected with anti-GST antiserum, R54 anti-Lck antiserum or 2102 anti-Lck antibody. The amounts of immobilized GST and MBP fusion proteins were detected using Coomassie blue protein stain.

2.2.9 Conformation assay

In 40 µl of binding buffer 2, 2 µg of immobilized GST-Lck and GST-Kin fusion proteins (610 and 880 nM final concentrations, respectively) were incubated with 1 µg (1450 nM final concentration) of soluble 6His-tagged Lck SH2 domain for 2 h, with shaking, at 4°C. The beads were then washed three times in RIPA buffer and treated as described in 2.2.8.1. Phosphotyrosine coupled to beads (65% coupling of 10 µmol (approximately 6.5 µmol) of O-phospho-L-tyrosine (Sigma-Aldrich) to 1 ml of packed CNBr activated Sepharose CL-4B beads) was used as a positive control for SH2 binding.

2.2.10 *In vitro* competition binding assays

2.2.10.1 Binding of 6His-CD45 C817S and Lck SH2 to GST-Lck fusion proteins

Immobilized GST-Lck F394, GST-Kin F394, and GST alone were resuspended in 40 µl of binding buffer 2 to final concentrations of 610 nM, 610 nM, and 1900 nM respectively. Immobilized proteins were incubated with either 61 nM or 610 nM final concentrations of 6His-Lck SH2 or 6His-CD45C817S for 2 h, with shaking, at 4°C. Immobilized proteins were then washed once with RIPA buffer, followed by high salt

buffer 1, RIPA, high salt buffer 2, and RIPA again. The samples were then boiled in reducing SDS sample buffer, separated by SDS-PAGE, transferred to a PVDF membrane and immunoblotted with an anti-6His antibody. The amounts of immobilized proteins used were detected with Coomassie blue protein stain.

2.2.10.2 Binding of 6His-CD45C817S or 6His-D2 and 6His-CD45APcyt Δ157-197 to immobilized GST-Lck kinase domain

Immobilized GST-Lck proteins, GST-Grb2 or GST alone were resuspended in 40 µl of binding buffer 2 to a final concentration of 610 nM and were incubated without, or with equal molar amounts (610 nM), or 5-fold excess molar amounts (3050 nM) of 6-His CD45C817S or 6His-D2 and/or 6His-CD45APcyt Δ157-197 for 2 h, with shaking, at 4°C. Assays containing D2 and CD45AP proteins underwent three washes with RIPA buffer. Alternatively, assays containing CD45C817S and CD45AP protein were washed as described in 2.2.10.1. After washing, samples were treated as described in 2.2.10.1. The amount of soluble 6His protein bound per unit of immobilized GST fusion was determined by densitometric scan analysis of the 6His immunoblot and the Coomassie blue stain with AlphaImager^{TM} 1200 software. The binding of soluble 6His protein in the absence of competition was standardized to 100%.

2.2.11 Immunoprecipitation

2.2.11.1 Lck, CD45, and CD44 immunoprecipitations

R54 anti-Lck antiserum (1 µl) was coupled to 10 µl of packed protein A sepharose beads (Repligen, Waltham, MA) in 500 µl of lysis buffer (10 mM Tris-HCl pH 7.2, 140 mM KCl, 1% Brij-58 and protease inhibitors) for 1 h, end-over-end, at 4°C. BW5147 or AKR1 T cells (10^7) were lysed in 500 µl of cold lysis buffer on ice for 10

min, then centrifuged for 10 min at 16,000 x *g*. The supernatant was collected and transferred to the pre-coupled R54 anti-Lck protein A sepharose beads, or to 50 µl of I 3/2 anti-CD45 mAb (50% slurry), or to 50 - 62.5 µl of IM7 anti-CD44 mAb (20% slurry) previously conjugated to CNBr-Sepharose beads (4 mg/ml, Sigma-Aldrich), and then incubated for 2 h, end-over-end, at 4°C. The beads were then washed 1 to 2 times in lysis buffer, boiled in reducing SDS sample buffer and subjected to SDS-PAGE for Western blot analysis (10 µl from the soluble lysate was also loaded directly onto the gel as a lysate control representing $2x10^5$ cell equivalents). PVDF membranes were immunoblotted with R54, RO2.2, or KM201 to detect Lck, CD45 or CD44 respectively, or with indicated antibodies to detect proteins that were co-immunoprecipitated. In some cases, known amounts of recombinant proteins were loaded onto the gel as standards to allow for the quantitation of proteins that were immunoprecipitated and co-immunoprecipitated using densitometric scan analysis with AlphaImager™ 1200 software.

To deplete cell lysates of CD45, four consecutive anti-CD45 immunoprecipitations were performed as described above, except that incubations were for 1 h. Depleted lysates were then transferred to pre-coupled R54 anti-Lck protein A sepharose beads for an anti-Lck immunoprecipitation (described above).

2.2.11.2 CD44 and CD4 immuno-precipitations in the presence of phenanthroline

AKR1 T lymphoma cells (10^7) were resuspended in 2 mls of warm DMEM (plus 10% horse serum) containing 5 mM 1,7- or 1,10-phenanthroline dissolved in ethanol, or ethanol alone, and were incubated at 37°C for 20 min. Cells were then pelleted, the media was removed and cells were lysed in 1 ml of lysis buffer with or without 5 mM

1,7- or 1,10-phenanthroline (Sigma-Aldrich) as described above. Detergent soluble lysate was then added either to 50 - 62.5 µl of IM7 anti-CD44 mAb previously conjugated to CNBr-Sepharose beads (4 mg/ml), or to 10 µl of packed protein G sepharose beads (Amersham Pharmacia) pre-coupled with 250 µl of GK1.5 anti-CD4 hybridoma tissue culture supernatant. The lysates were then incubated with immobilized antibody for 2 h, end-over-end, at 4°C, and the beads were subsequently treated as described in 2.2.11.1.

2.2.12 Cell spreading assays

Fifty microlitres of purified KM201 anti-CD44 mAb (40 µg/ml), purified GK1.5 anti-CD4 (40 µg/ml), or 2% BSA in phosphate buffered saline (PBS) were immobilized to the wells of a 96-well tissue culture plate (Nalge Nunc International, Rochester, NY) overnight at 4°C. The wells were then washed once with 200 µl of PBS, blocked with 50 µl of 2% BSA for 60 min at 37°C, and then washed twice with 200 µl of PBS.

BW5147 or AKR1 T cells ($5x10^4$) were resuspended in 50 µl of binding media alone (DMEM with 0.1% fetal calf serum), or in binding media containing 2.5 mM 1,7- or 1,10-phenanthroline, and were incubated at 37°C for 20 min. The supernatant was then removed, and the cells were resuspended in binding media alone, transferred to antibody- or BSA-coated wells, and incubated for 2 h at 37°C. The cells were then fixed with 16% para-formaldehyde and were photographed. To determine the percentage of cell spreading, the number of cells that were flattened and elongated was compared to the total number of cells incubated.

CHAPTER 3

The role of the Lck non-catalytic domains in CD45-mediated Lck regulation

3.1 Introduction and rationale

Like all members of the Src-kinase family, Lck activity is regulated by its conformation, which is regulated by its phosphorylation status. All nine Src-family kinase members share a common multi-domain structure composed of a unique region, an SH3 domain, an SH2 domain, a catalytic tyrosine kinase domain and a short carboxy-tail. Upon its phosphorylation, the carboxy-tail of these kinases mediates an intramolecular interaction with the SH2 domain creating a closed and inactive conformation (reviewed in 4). The disruption of this interaction by mutation of the carboxy-tail (149), deletion of the SH2 domain (150), or the presence of a high affinity SH2 or SH3 ligand (246) results in kinase activation, implicating an important role for the non-catalytic domains in Src-family kinase regulation.

CD45, a protein tyrosine phosphatase (PTP), is known to up-regulate Lck activity via dephosphorylation of the Lck carboxy-tail (Tyr 505) (reviewed in 13, 124). However, there is mounting evidence that CD45 is also capable of dephosphorylating Lck at its activation loop (Tyr 394), suggesting an additional role for CD45 in Lck down-regulation (reviewed in 193; 247). Thus, CD45 acts as a "molecular switch", capable of inducing or inhibiting Lck activity by controlling its phosphorylation, conformation and activation status.

A consistent finding in resting $CD45^+$ T cells, is the presence of Lck in a largely dephosphorylated form, suggesting continual CD45 activity. Moreover, CD45 and Lck can be co-immunoprecipitated from T cells under mild lysis conditions suggesting that an association is maintained other than that expected for a transient enzyme–substrate interaction (248). Previous work from our laboratory has demonstrated that a direct

interaction occurs between CD45 and Lck *in vitro* involving the Lck unique region and the SH2 domain, but not the SH3 domain (75). This suggests that Lck non-catalytic domains play an important role in Lck regulation via their ability to mediate both an intramolecular interaction and an intermolecular interaction with CD45.

Despite these findings, details of the interaction between CD45 and Lck are lacking, particularly since Lck can exist in closed and open conformations and CD45 can dephosphorylate both positive and negative regulatory tyrosines. Likewise, little is known about the role of the second phosphatase domain of CD45 and how it may influence Lck regulation. Thus, the goal of this study was to characterize the direct CD45:Lck interaction *in vitro* using purified recombinant proteins. In particular, the roles of the Lck non-catalytic domains in CD45-mediated Lck regulation were addressed.

3.2 Experimental approach

Much of what we currently know about the ability of CD45 to regulate Lck comes from studies using T cells lacking CD45, either from knockout mice or deficient T cell lines. A consistent phenotype of CD45-deficient T cells is the hyper-phosphorylation of Lck and Fyn at the carboxy-tail tyrosine (Tyr 505 in Lck), suggesting a role for CD45 in the up-regulation of these kinases. Moreover, recent studies with $CD45^{-/-}$ and Lck transgenic mice have indicated that CD45 is also capable of down-regulating Lck via dephosphorylation Tyr 394 (194, 247).

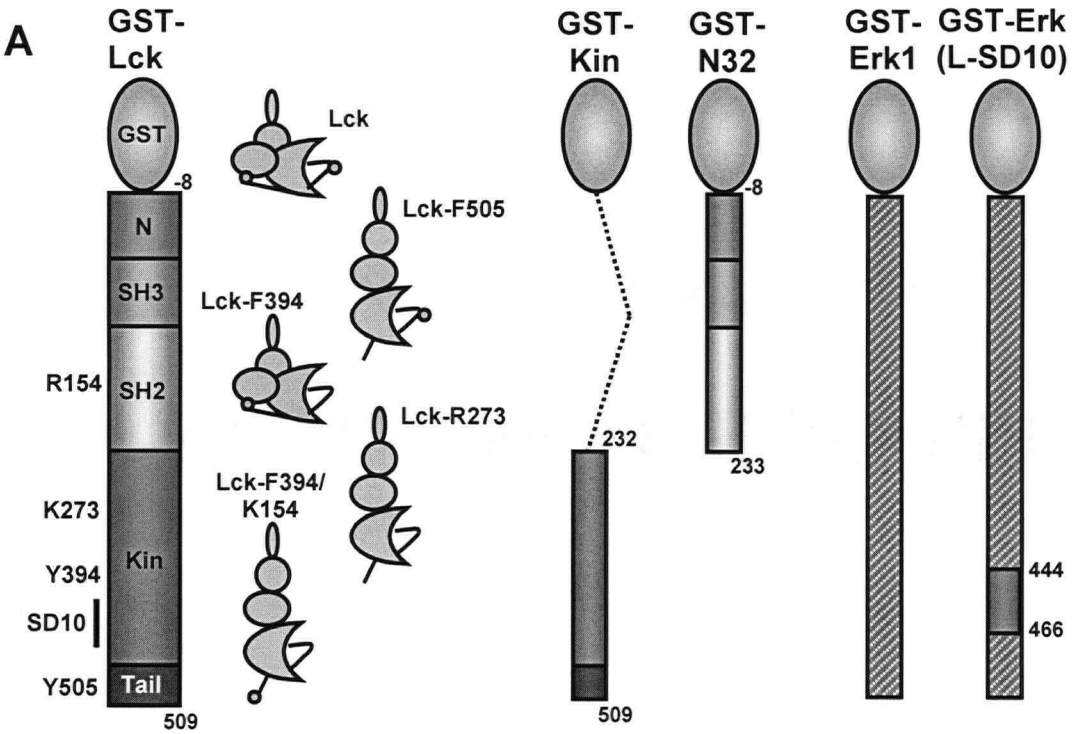
However, despite the evidence for these contradictory roles of CD45 in Lck regulation, little is known about the variables governing the CD45:Lck interaction or if a preference exists for the CD45-mediated dephosphorylation of Lck at Tyr 394 or Tyr

505. Therefore, various forms of CD45 and Lck were produced in bacteria and purified as recombinant proteins to establish and characterize the direct CD45:Lck interaction *in vitro*. The use of recombinant Lck protein is supported by the crystal structure studies of Src-family kinases (26, 46, 51), all of which produced folded proteins from insect cells expressing recombinant baculovirus vectors. Likewise, enzyme analyses investigating the K_m and V_{max} values of both Lck and CD45 have been performed using purified recombinant proteins and revealed values comparable to proteins isolated from natural sources (169, 249). Thus, recombinant proteins were employed in the present study to investigate the role of the Lck non-catalytic domains in the regulation of Lck by CD45. Mutant forms of Lck and CD45 proteins were also produced to localize the specific regions of the interaction and to determine the effects of Lck phosphorylation and conformation status on its interaction with CD45 (Fig. 3.1).

3.3 Results

3.3.1 Generation of purified recombinant proteins

To better determine how the tyrosine phosphatase, CD45, binds and dephosphorylates its physiological substrate, Lck, GST fusion proteins consisting of full-length murine Lck (Lck), the Lck non-catalytic domains (including N, SH3 and SH2 (N32)), the catalytic kinase domain and carboxy-tail (Kin), various Lck mutants, human Erk1 (Erk1) and an Erk1-Lck chimeric protein (Erk(L-SD10) were expressed and purified from *E. coli*. (Fig. 3.1A) These proteins were then immobilized on glutathione sepharose (GSH) beads and incubated with various forms of purified, recombinant soluble 6His-tagged murine CD45 cytoplasmic domain proteins (Fig. 3.1C).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GST
-8
N
SH3
SH2
R154
K273
Kin
Y394
SD10
Y505
Tail
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Lck-F394
Lck-R273
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-8
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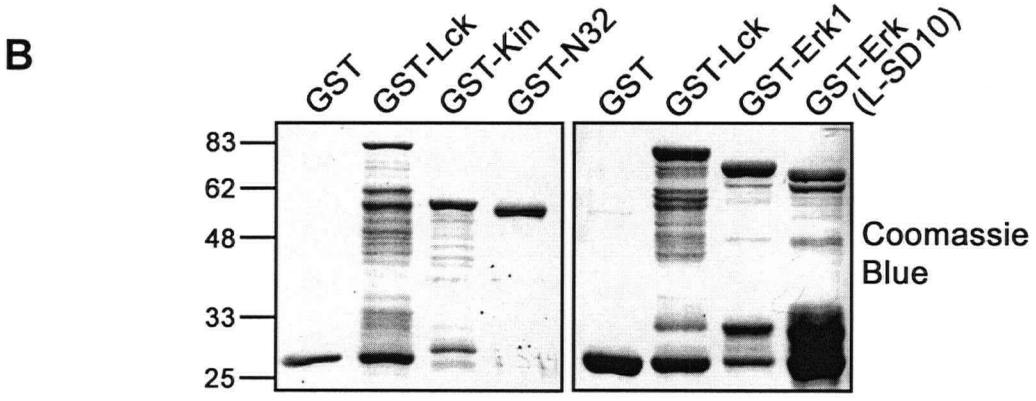
GST-Erk (L-SD10)
444
466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GST-Lck
GST-Kin
GST-N32
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GST-Erk1
GST-Erk (L-SD10)
83
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48
33
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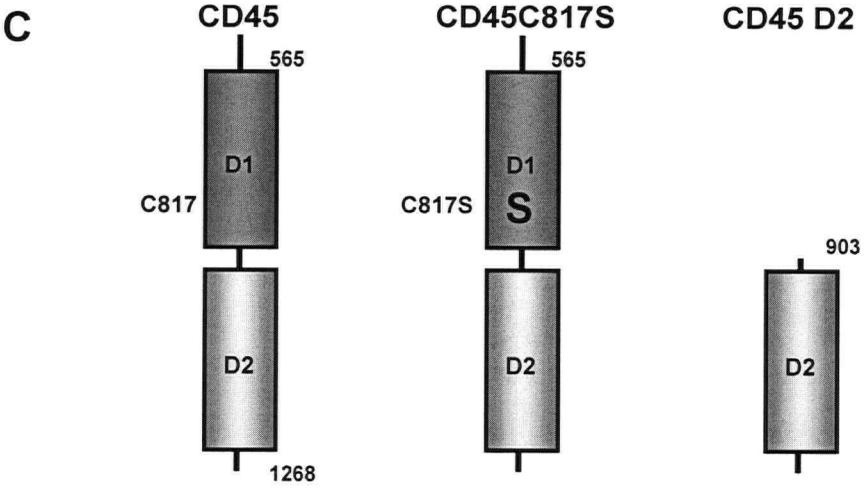
C
CD45
565
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903
D2

Figure 3.1. Recombinant GST and 6His fusion proteins. (A) Primary (blocks) and tertiary (globular) structure cartoons of GST fusion proteins purified from bacteria. All numbers represent residue positions from murine Lck. "Lck" represents wild type full-length Lck, "Lck-F505" has a Y505F mutation, "Lck-F394" has a Y394F mutation, "Lck-R273" has a K273R mutation, and Lck-F394/K154 has both Y394F and R154K mutations. "SD10" represents Lck subdomain *X*. "N32" represents a construct expressing Lck N, SH3 and SH2 domains, "Kin" represents the Lck kinase domain and the carboxy-tail ("tail"). "Erk1" represents wild type Erk1, and "Erk(L-SD10)" represents an Erk1 mutant expressing the Lck SD10 in place of its own. Globular cartoons indicate the predicted conformations of open and closed Lck mutants, and orange circles indicate tyrosine phosphorylation. (B) Analysis of purified recombinant GST fusion proteins. Proteins were boiled in reducing SDS sample buffer, separated with SDS-PAGE and detected with Coomassie blue protein stain. (C) Primary structure representation of 6His-CD45 proteins purified from bacteria. All numbers represent murine CD45 residue positions. "CD45" represents cytoplasmic CD45 containing both domain 1 (D1) and domain 2 (D2), "CD45C817S" has a C817S mutation creating an inactive PTP, and "D2" represents half of the spacer region, the entire second phosphatase domain, and the carboxy tail of CD45. A Coomassie blue protein stain of recombinant 6His-CD45 proteins is presented elsewhere (169).

Recombinant Lck proteins were co-produced in *E. coli* with thioredoxin protein resulting in a dramatic increase in Lck solubility and yield as reported previously (237). Upon purification, active forms of Lck were tyrosine auto-phosphorylated suggesting proper protein folding in bacteria upon production. Moreover, unlike the recombinant Lck kinase domain, N32, and CD45 proteins that were purified to near homogeny, full-length Lck, Erk1 and Erk(L-SD10), were consistently partially degraded despite the presence of protease inhibitors during bacterial lysis (Fig. 3.1B). However, purified full-length Lck displayed substantial kinase activity *in vitro* as determined by auto-phosphorylation in the presence of (γ-^{32}P)ATP (data not shown). Likewise, purified Erk1 and Erk(L-SD10) were both capable of auto-phosphorylation *in vitro* in the presence of ATP as detected by the 4G10 anti-phosphotyrosine antibody (data not shown). This suggests that the proper structure and folding of these proteins was maintained upon purification despite the presence of some degradation.

3.3.2 Both the catalytic and non-catalytic regions of Lck participate in the interaction with CD45

To determine the importance of Lck tyrosine phosphorylation in the CD45:Lck interaction, immobilized recombinant GST-Lck proteins were incubated with purified soluble 6His-tagged CD45 (CD45C817S). This is an inactive CD45 phosphatase, where the active site cysteine (Cys 817) present in the first PTP domain has been mutated to serine. After washing the beads (see Materials and methods for details), the amount of CD45C817S that had bound to the immobilized GST proteins was detected after SDS-PAGE and Western blot analysis with RO2.2 anti-CD45 antibody (Fig. 3.2A). The interaction of the inactive CD45C817S protein with Lck was largely attributed to its

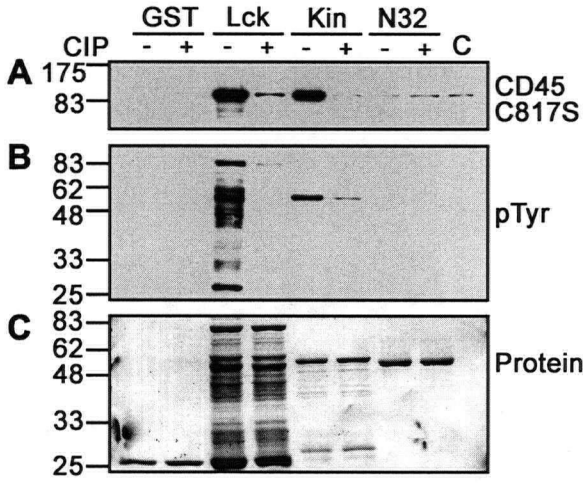

Figure 3.2. The effect of phosphorylation of GST-Lck fusion proteins on the binding of 6His-CD45C817S cytoplasmic domain. (A) Western blot of CD45C817S with RO2.2 indicating amount that bound to immobilized GST fusion proteins (GST, Lck, Kin and N32) that were treated (+) or untreated (-) with calf intestinal phosphatase (CIP) prior to performing the binding assay (see Materials and methods for details). "C" is 10 ng of CD45C817S loaded directly onto the gel. (B) Western blot of GST fusion proteins probed with an anti-phosphotyrosine antibody (4G10). (C) Coomassie blue protein stain of the PVDF membrane indicating actual amounts of each GST fusion protein used in the assay.

interaction with the Lck kinase domain, although a persistent, low-level interaction was also observed with the non-catalytic domains of Lck (N32). Pre-treatment of the Lck proteins with calf alkaline phosphatase (CIP) to dephosphorylate Lck (Fig. 3.2B) resulted in a major decrease in the binding of CD45C817S to Lck and the kinase domain, indicating that binding was largely phosphotyrosine-dependent, and therefore likely occurring via the catalytic pocket. Since both Lck and Kin proteins are auto-phosphorylated when produced in *E. coli*, the phosphotyrosine-dependent interaction is likely to be mediated by the catalytic pocket of CD45 and the auto-phosphorylated Tyr 394 of Lck. In this case, Lck and Kin proteins were not co-expressed with Csk and thus were not phosphorylated at Tyr 505. Some residual binding of CD45C817S to dephosphorylated Lck and Kin was detected and CIP treatment had no effect on the binding of N32 to CD45C817S. This indicates that for the inactive CD45 phosphatase, the binding to Lck is determined largely by the phosphorylation state of Lck. However, both the kinase domain and the non-catalytic domains of Lck can also interact with CD45 in a phosphotyrosine-independent manner, albeit to a much lesser extent.

To gain insight into the relative contributions of catalytic and non-catalytic regions in CD45 binding to Lck, this interaction was examined in binding buffer containing 0.5 mM phosphatase inhibitor sodium orthovanadate (vanadate). The ability of vanadate to block phosphatase activity stems from its ability to bind to the catalytic pocket of the phosphatase enzyme (250), thus allowing for the observation of binding contributions from other regions of CD45. Figure 3.3A shows that in the absence of vanadate, Lck binds substantially more CD45C817S than wild type CD45 (densitometric analysis revealed this difference to be approximately 10-fold, as shown in figure 3.3C).

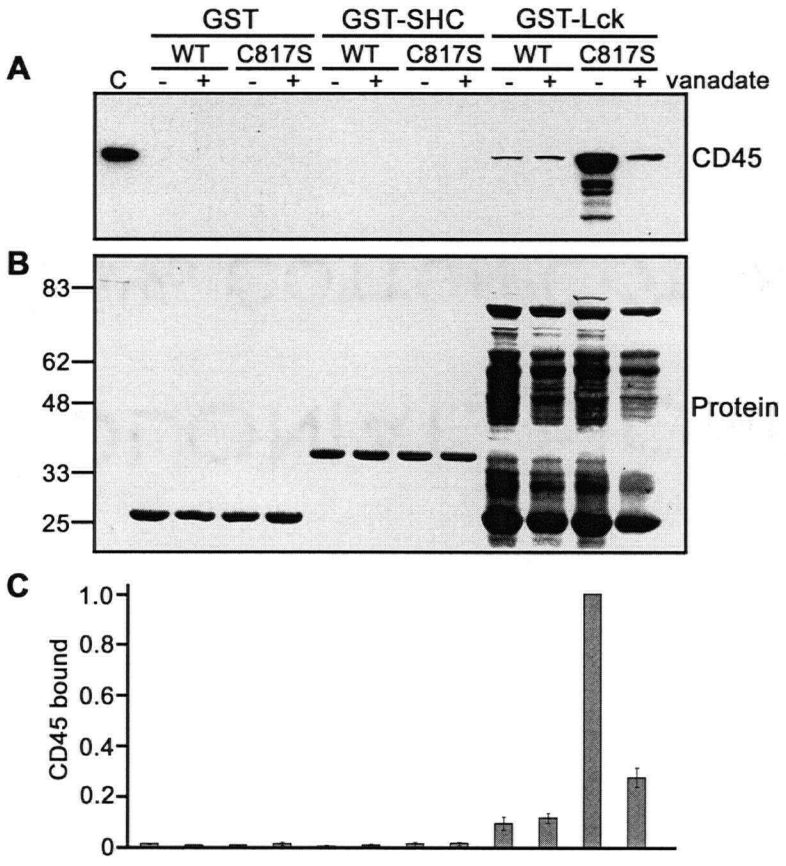

Figure 3.3. Binding of 6His-CD45 and 6His-CD45C817S to GST-Lck. (A) Western blot of soluble CD45 protein with RO2.2 to detect the amount of wild type CD45 (WT) and CD45C817S (C817S) that bound to immobilized GST fusion proteins: GST alone (GST), SHC SH2 domain (GST-SHC), and Lck (GST-Lck). "C" represents 50 ng of 6His-CD45 loaded directly onto the gel as a control. The presence and absence of 0.5 mM vanadate is indicated by "+" and "-" respectively. (B) Protein stain of the PVDF membrane with Coomassie blue to indicate the amounts of GST fusion proteins used in each assay. (C) Graphical representation of the amount of CD45 protein bound per unit of GST fusion protein. CD45C817S binding to Lck in the absence of vanadate was standardized to 1. Values represent the average from 2 experiments (error bars show standard error).

This is likely due to the transient nature of the enzyme-substrate interaction with the active CD45 form. However, in the presence of vanadate, the interaction between CD45C817S and Lck was reduced to approximately 25%, and approached the level seen with wild type CD45 and Lck (plus vanadate). Control proteins GST alone, and GST-SHC SH2 did not significantly bind CD45 or CD45C817S under any conditions. Thus, in agreement with figure 3.2, the binding of CD45 to Lck is comprised of a major catalytic interaction, and a minor but persistent interaction mediated by CD45 regions other than the catalytic pocket. Together, this data suggests the relative contributions of catalytic and non-catalytic regions of CD45 in binding to Lck are approximately 75 and 25% respectively.

3.3.3 CD45C817S preferentially binds to Lck that is auto-phosphorylated at Tyr 394

To determine if the phosphorylation site or conformation of Lck affected its interaction with CD45, a series of phosphorylated GST-Lck mutants were generated. Active GST-Lck proteins were produced in *E. coli* and isolated from the soluble fraction. Isolated Lck and Lck F505 were auto-phosphorylated, but Lck F394 and the inactive Lck R273 were not. In addition, Lck, Lck F394 and Lck F505 were produced in *E. coli* that had been co-transfected with a plasmid expressing Csk, a tyrosine kinase that phosphorylates Lck at Tyr 505. This yielded GST-Lck proteins that were differentially phosphorylated. Lck and Lck F505 were auto-phosphorylated at Tyr 394 as determined by the detection of these proteins with antibodies specific for Lck pTyr 394 and Src pTyr 416 (which cross reacts with Lck, data not shown). The co-expression of Csk with Lck proteins in bacteria resulted in a significant increase in Lck and Lck F394

phosphorylation, but did not affect Lck F505 phosphorylation. This suggested that Lck F394 was phosphorylated at Tyr 505 alone, and that Lck was phosphorylated at both Tyr 394 and Tyr 505. The kinase dead Lck, Lck R273, was produced in *E. coli* lacking Csk, and was not detectably tyrosine phosphorylated (Fig. 3.4B). The relative amount of phosphorylation per unit of Lck protein was determined for each construct. The phosphorylated proteins were all found to be phosphorylated to similar extents (Fig. 3.4E).

Binding assays with these proteins indicated that CD45C817S bound approximately equally to Lck and Lck F505, bound less to Lck F394 and even less to Lck R273 (Fig. 3.4A). The same pattern of binding was observed with differentially tyrosine phosphorylated Lck kinase domain proteins (Fig. 3.4A, right panel). Although not apparent on this exposure, a longer exposure revealed a low level of binding of CD45C817S to Kin R273 that was above background levels (data not shown). These data indicate that CD45C817S binds preferentially to tyrosine phosphorylated Lck, as would be predicted by binding via the inactive catalytic pocket. However, it was also noted that CD45C817S had a preference for binding to Lck phosphorylated at Tyr 394 over Tyr 505 that could not readily be accounted for by different levels of phosphotyrosine (Fig. 3.4D and E). It has a similar preference for the kinase domain alone phosphorylated at Tyr 394, indicating that the preference is not affected by the non-catalytic domains of Lck. In the previous figures we had shown that the binding of CD45C817S is largely determined by the interaction of the catalytic pocket with phosphorylated Lck and so it is possible that this preference is created by an interaction between the catalytic pocket of CD45 and the sequence surrounding Lck pTyr 394.

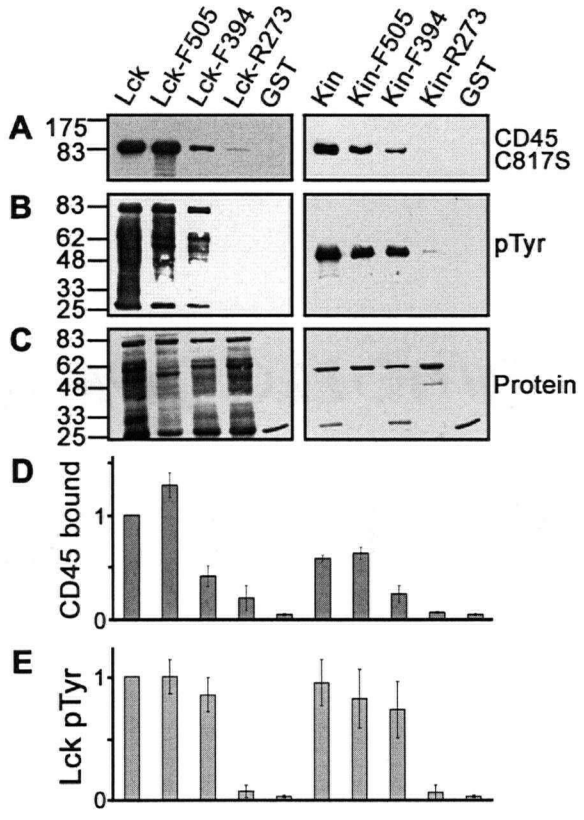

Figure 3.4. Binding of 6His-CD45C817S to differentially phosphorylated GST-Lck fusion proteins. (A) Western blot of soluble CD45C817S protein with RO2.2 to detect the amount of CD45 remaining bound to immobilized GST fusion proteins. (B) Anti-phosphotyrosine Western blot with 4G10 to measure the tyrosine phosphorylation state of the GST fusion proteins. (C) Protein stain of the PVDF membrane with Coomassie blue to indicate that approximately equal amounts of GST fusion proteins were used in each case. (D) Graphical representation of the amount of CD45C817S bound per unit GST protein. CD45 binding to Lck was standardized to 1. Values represent the average from 4 experiments (error bars show standard deviation). (E) Graphical representation of the amount of tyrosine phosphorylation divided by the amount of Lck protein, determined by Western blotting with 4G10 and Coomassie blue staining, respectively. The ratio of GST-Lck phosphorylation: protein was standardized to 1 and other values compared to this. Values represent the average from 7 experiments (error bars show standard deviation).

3.3.4 Non-catalytic regions of CD45 augment binding to the predicted open form of Lck

Since the CD45C817S protein largely represents binding via the catalytic pocket of CD45, we next examined the contribution of the non-catalytic regions of CD45 to the interaction with the two forms of Lck: the predicted closed, Tyr 505 phosphorylated form and the predicted open, Tyr 394 phosphorylated form. To do this, we used the active CD45 cytoplasmic domain protein diluted in binding buffer containing 0.5 mM vanadate. This is predicted to block interactions with the catalytic pocket thereby allowing interactions mediated by other regions of the CD45 cytoplasmic domain to be observed.

As with CD45C817S, more CD45 bound to Lck F505 than to Lck F394 (Fig. 3.5). However, unlike CD45C817S, CD45 also bound well to the non-phosphorylated Lck R273. The average binding from 4 experiments is shown graphically (Fig. 3.5D) and indicates that CD45 prefers to bind to Lck R273 and Lck F505, both of which are predicted to exist in the open conformation, over Lck F394 and Lck, which are both predicted to exist in the closed conformation. In this case, binding does not correlate with phosphorylated forms of Lck, consistent with the involvement of regions other than the catalytic site. This suggests that non-catalytic regions of CD45 preferentially bind to the open form of Lck, independent of Tyr 394 phosphorylation. When CD45 binding to the differentially phosphorylated Lck kinase domains alone was examined (Fig. 3.5A), CD45 bound all to the same extent and bound to a lesser extent than with the predicted open forms of full-length Lck. Together, this indicates that the regions of the cytoplasmic domain of CD45 outside the catalytic pocket interact with both the non-catalytic domains of Lck and the kinase domain of Lck.

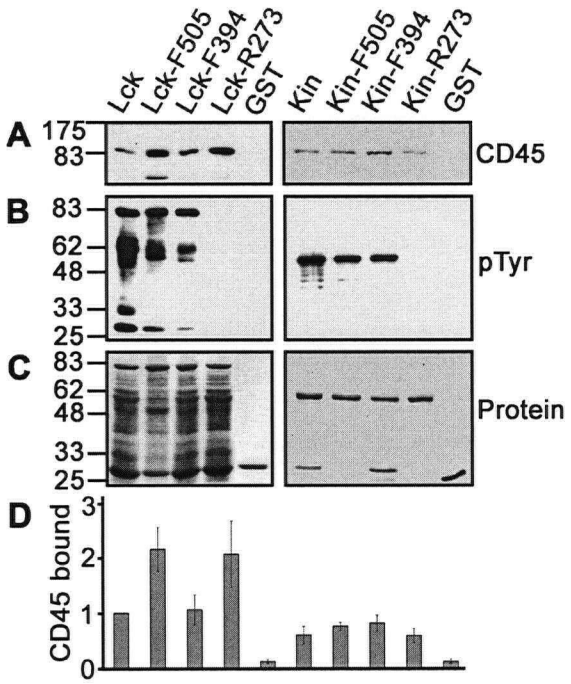

Figure 3.5. Binding of 6His-CD45 plus vanadate to differentially phosphorylated GST-Lck fusion proteins. Details as described in figure. 3.4 except that 6His-CD45 plus vanadate was used instead of CD45C817S. (D) Graph showing the amount of CD45 bound per unit GST protein and is the average from 4 experiments.

3.3.5 The non-catalytic domains of Lck differentially affect the dephosphorylation rates of Tyr 394 and Tyr 505

To evaluate the role of the non-catalytic interactions between CD45 and Lck on the dephosphorylation of Lck and to determine if the preferential binding of CD45C817S to autophosphorylated Lck F505 translated into the preferred dephosphorylation of Tyr 394, recombinant, active CD45 cytoplasmic domain protein was incubated with soluble Lck proteins (cleaved from GST by thrombin) and their dephosphorylation was monitored over time by Western blot analysis with an anti-phosphotyrosine antibody (4G10). Western blotting with 4G10 indicated that the different Lck and Kin constructs had approximately similar levels of tyrosine phosphorylation (Figs. 3.4B – 3.7B). To determine if there was a preference for dephosphorylation of Tyr 394 over Tyr 505 due to the sequence surrounding the phosphotyrosine, as suggested by the preferential binding of CD45C817S to Lck Kin F505 over Lck Kin F394, CD45-mediated dephosphorylation of the Lck kinase domain mutants was analyzed. Figure 3.6 demonstrates that both Tyr 394 and Tyr 505 phosphorylated kinase domains were dephosphorylated at similar rates. This indicates that there is no preference for Tyr 394 or Tyr 505 when non-catalytic Lck domains are absent. This suggests that the Lck kinase domain and the sequences surrounding Tyr 394 or Tyr 505 do not influence the substrate specificity of CD45 for either site.

It was next determined if the non-catalytic domains of Lck influenced the rate of dephosphorylation by comparing the dephosphorylation of full length Lck with the kinase domain alone. Figure 3.7 shows that Lck F505 was dephosphorylated at a faster rate than Kin F505 whereas Lck F394 was dephosphorylated at a slower rate than Kin F394. This

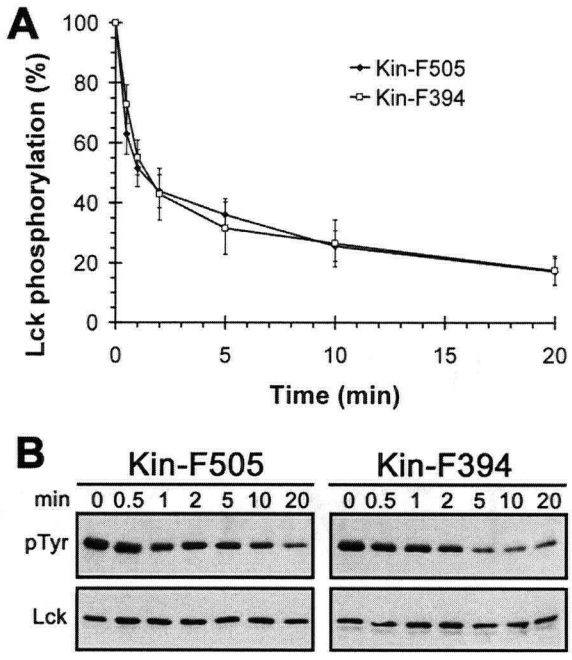

Figure 3.6. *In vitro* dephosphorylation of thrombin-cleaved Lck kinase domain proteins by 6His-CD45. (A) Graph representing the rate of dephosphorylation of the Lck kinase domain by CD45. Values were obtained by dividing the amount of phosphotyrosine by the amount of Kin protein present at each time point (see Materials and methods for details). The value at time 0 was taken as 100% and values represent the average of 7 experiments with error bars representing standard deviation. (B) Representative phosphotyrosine (pTyr) and Lck kinase domain Western blot (Lck) with 4G10 and 2102, respectively. After the indicated incubation times, the reaction was stopped, the samples divided in two and analyzed separately by Western blotting as described above.

indicates that the non-catalytic domains of Lck facilitate the dephosphorylation of Tyr 394 but reduce the dephosphorylation of Tyr 505. The dephosphorylation of Tyr 505 Lck was approximately 2-fold slower than Tyr 394 Lck. To make sure this was not due to slight differences in phosphorylation between the two constructs, dephosphorylation assays were performed using both equal molar amounts and equal phosphotyrosine amounts. Similar results were obtained in both cases. It is worthy to note that CD45 was able to effectively dephosphorylate Tyr 505, which is predicted to be protected by an intramolecular interaction with the Lck SH2 domain. This suggests that in the absence of a high affinity SH2 or SH3 domain ligand, CD45 is able to displace the SH2 domain of Lck and dephosphorylate Tyr 505.

To determine if the reduced dephosphorylation rate of Tyr 505 was due to its intramolecular association with the Lck SH2 domain, initial attempts were made to establish whether the phosphorylated Tyr 505 Lck was indeed present in a closed conformation. To do this, the binding of exogenous Lck SH2 domain to exposed phosphorylated Tyr 505 was examined. Figure 3.8 shows that Lck SH2 binds much better to Kin F394 than to Lck F394, consistent with the notion that phosphorylated Tyr 505 is exposed and accessible in the kinase domain alone, but is protected by the endogenous SH2 domain in Lck F394. Furthermore, when a critical residue in the SH2 domain (Arg 154) is mutated to a Lys so that the SH2 domain can no longer bind phosphotyrosine (251), binding to the exogenous SH2 domain occurs (Fig. 3.8). This evidence thus supports the prediction that phosphorylated Tyr 505 Lck exists in a closed conformation and that Lck F394/K154, exists in an open conformation with an accessible

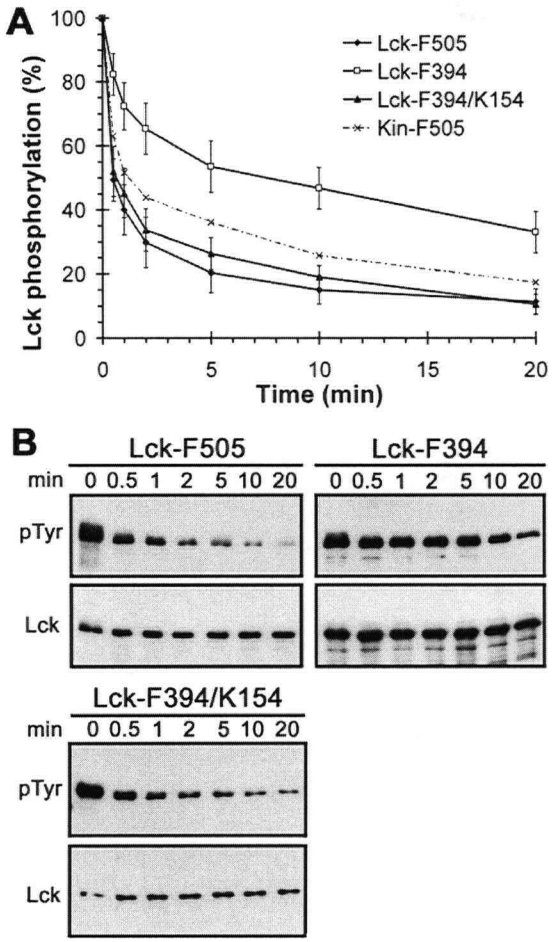

Figure 3.7. *In vitro* dephosphorylation of thrombin-cleaved Lck proteins by 6His-CD45. (A) Graphical representation of Lck dephosphorylation by 6His-CD45, details as described in legend to figure 3.6. Values represent the average from 6 experiments and the dotted line depicts the dephosphorylation rate of Kin F505 taken from figure 3.6. (B) Western blot analysis is as described in figure 3.6 except that Lck antisera (R54) was used to detect Lck.

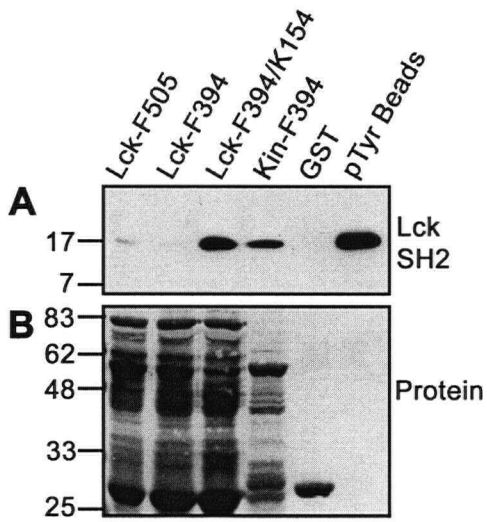

Figure 3.8. Analysis of Lck conformation. (A) Western blot of 6His-Lck SH2 remaining bound to various GST proteins (indicated above the blot) detected with an anti-6His antibody. Phosphotyrosine coupled to beads served as a positive control for SH2 binding (pTyr Beads, see Materials and methods for details). (B) Protein stain with Coomassie blue demonstrated that approximately equal amounts of GST fusion proteins were used in each case.

phosphorylated Tyr 505. Interestingly, when the dephosphorylation rate of Csk phosphorylated Lck F394/K154 was determined, it was found to be virtually identical to the dephosphorylation of Lck F505 (Fig. 3.7). These results could not be explained by differences in the stoichiometry of phosphorylation as the phosphorylation level of Lck F394, Lck F394/K154 and Lck F505 were all similar. A comparison of the phosphorylation/protein ratio for each construct, normalizing the value for Lck F394 to 1, yielded values of 1.1 ± 0.2 for Lck F394/K154 and 1.2 ± 0.3 for Lck F505 (n=4).

Therefore, these results indicate that the slower dephosphorylation rate of Tyr 505 in Lck F394 was due to its interaction with the SH2 domain, because in the absence of the intramolecular interaction in the Lck F394/K154 protein, dephosphorylation of Tyr 505 proceeded at an equal rate to that of Tyr 394.

3.3.6 CD45C817S binds more effectively than exogenous Lck SH2 to Lck F394

To determine whether CD45 gains access to phosphorylated Tyr 505 because it has a greater affinity for phosphorylated Tyr 505, a competition assay was performed between 6His-CD45C817S and exogenous 6His-Lck SH2 for binding to the exposed, phosphorylated Tyr 505, present in Kin F394. At a 1:1 molar ratio, about 60% of the molecules bound to Csk phosphorylated Kin F394 were CD45C817S and 40% were Lck SH2 (data not shown). In addition, the binding of each individually to Kin F394 was approximately equal (Fig. 3.9). These data suggest that both CD45C817S and the exogenous Lck SH2 domain have approximately similar affinities for an exposed pTyr 505. In a second experimental approach, the ability of 6His-CD45C817S and exogenous 6His-Lck SH2 to bind to the protected, phosphorylated Tyr 505 in full-length Lck was examined. When CD45C817S or Lck SH2 has to compete with endogenous SH2 domain

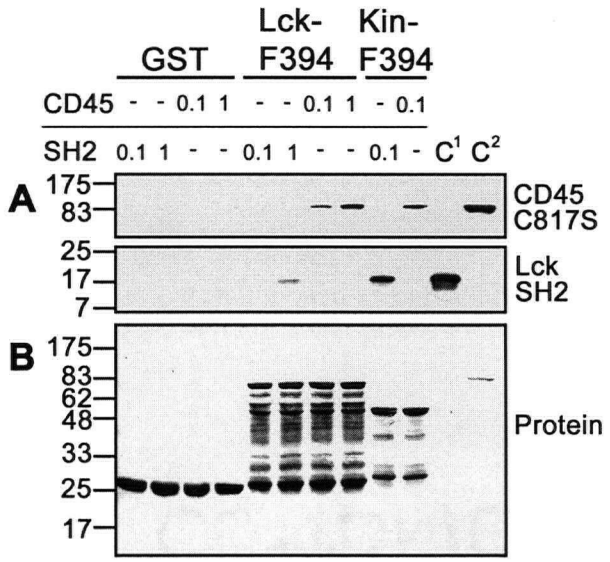

Figure 3.9. Binding of soluble 6His-CD45C817S and 6His-Lck SH2 to immobilized GST fusion proteins, Lck F394 and Kin F394. (A) Western blot of 6His-CD45C817S (upper panel) or 6His-Lck SH2 (lower panel) that bound to GST proteins. CD45 and SH2 were added to the assay either in equal molar (1) or at one-tenth molar (0.1) amounts relative to the immobilized GST proteins (as indicated above the gel) and were both detected with an anti-6His antibody. C^1 and C^2 represent approximately 2.4 pmoles of SH2 or CD45 respectively, that were loaded directly on the gel. (B) Protein stain with Coomassie blue to indicate the amount of GST fusion protein used in this assay.

for binding to Csk phosphorylated F394 Lck, CD45C817S bound more effectively than the exogenous SH2 domain (Fig. 3.9). This indicates that CD45 is more effective than Lck SH2 in competing for binding to phosphorylated Tyr 505 when protected by endogenous SH2 domain. This suggests that the interaction of CD45 with Lck facilitates the release of the intramolecular Lck SH2 domain interaction resulting in the dephosphorylation of Tyr 505. The former experiment suggests that this is not due to a higher affinity of CD45 for phosphorylated Tyr 505, but is likely due to the interaction of CD45 with the non-catalytic domains of Lck.

3.3.7 Csk-mediated tyrosine phosphorylation of CD45 has no effect on its ability to regulate Lck

To investigate if CD45 phosphorylation has any effect on its ability to regulate Lck, purified recombinant Csk was employed to phosphorylate CD45 prior to use in binding and dephosphorylation experiments. Figure 3.10 (lower panel) represents a 4G10 Western blot, and shows that Csk is indeed capable of producing tyrosine phosphorylated CD45. This phosphorylation however, was only seen when phosphatase inhibitor sodium orthovanadate (vanadate, 1 mM) was also present in the buffer suggesting rapid CD45 auto-dephosphorylation *in vitro*. Phosphorylated and non-phosphorylated forms of CD45 (both present in 1 mM vanadate) were then assayed for their ability to interact non-catalytically with open and closed forms of Lck. Figure 3.10 shows no difference between phosphorylated and non-phosphorylated forms of CD45 in their abilities to bind to either form of Lck.

Although the binding of CD45 to Lck was unaffected by Csk-mediated CD45 phosphorylation, we further investigated whether Csk-mediated phosphorylation of CD45

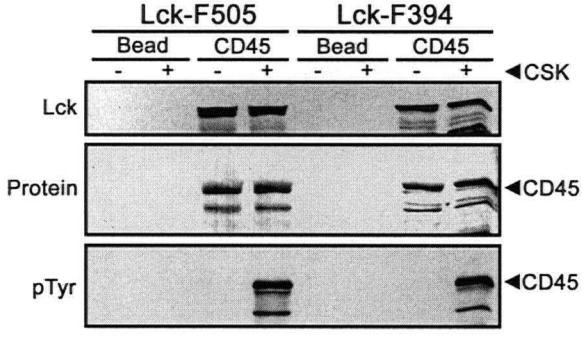

Figure 3.10. The effect of Csk-mediated CD45 phosphorylation on the *in vitro* binding of Lck F505 and Lck F394 to 6His-CD45. Immobilized 6His-CD45 (500 ng) was incubated with 100 ng of eluted GST-Lck proteins for 2 hours at 4°C in binding buffer (see Materials and methods). Immobilized CD45 or beads alone were then washed and subjected to Western blot analysis. 80% of the assay was immunoblotted with an anti-Lck antibody, R54, and the remaining 20% for phosphotyrosine. Upper panel: Western blot of soluble thrombin-cleaved Lck F505 or Lck F394 binding to immobilized 6His-CD45 that had been pre-incubated in the absence (-) or presence (+) of recombinant Csk (see Materials and methods). Middle Panel: Protein Stain of the PVDF membrane with Coomassie blue to indicate that approximately equal amounts of 6His-CD45 were used in each assay. Lower panel: 4G10 anti-phosphotyrosine Western blot depicting 6His-CD45 phosphorylation status. All blots are representatives of three separate experiments.

affected its ability to dephosphorylate predicted open and closed Lck substrates. CD45 was phosphorylated by Csk and then subsequently washed twice in phosphatase buffer to remove vanadate prior to incubation with Lck. As shown in figure 3.11B, despite substantial tyrosine phosphorylation of CD45 immediately after incubation with Csk (lane 7, "CD45"), the removal of vanadate resulted in rapid CD45 autodephosphorylation resulting in a non-phosphorylated CD45 enzyme by the 30 second time point of the Lck dephosphorylation assay. Further experiments optimized the Csk-mediated phosphorylation of CD45 (see Materials and methods for details), and in these assays some CD45 phosphorylation could still be detected after 20 minutes (data not shown). However, in these experiments, the dephosphorylation rates of open (F505) and closed (F394) forms of Lck were similar for Csk phosphorylated, and non-phosphorylated forms of CD45. Figure 3.11A shows a difference in Lck-F505 phosphorylation at the 0.5 minute time point, however this difference was found to be insignificant over the course of several experiments.

Taken together, these results indicate that Csk is capable of transiently phosphorylating CD45, but as far as can be determined, this phosphorylation does not significantly affect CD45 in its ability to regulate Lck in these *in vitro* assays.

3.3.8 6His-D2 binds to a specific region of the Lck kinase domain and plays a role in substrate specificity

Demonstrated above is an interaction between the non-catalytic domains of CD45 and Lck. It has also been demonstrated that the second, catalytically inactive domain of CD45 (D2, residues 903 to 1268) can bind to the kinase domain of Lck in a phosphotyrosine-independent manner (J. Felberg, unpublished data). This D2:Lck kinase

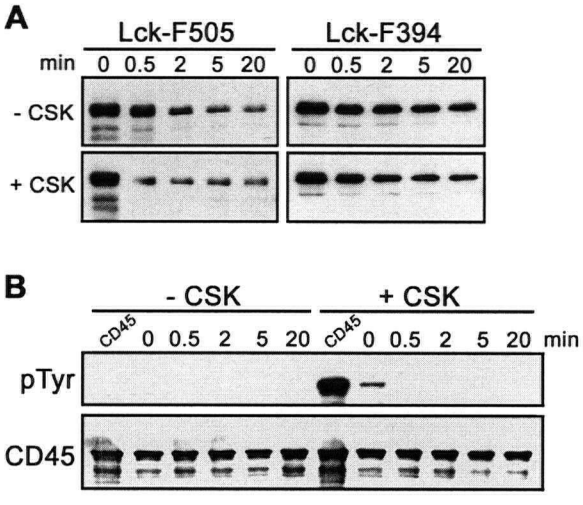

Figure 3.11. The effect of Csk-mediated CD45 phosphorylation on the *in vitro* dephosphorylation of Lck F394 and Lck 505. (A) Phosphotyrosine Western blot with 4G10 representing the rate of Lck-F505 and Lck-F394 dephosphorylation by CD45. After the indicated time points, the reactions were stopped, and samples were analyzed by Western blotting. Upper and lower panels show Lck dephosphorylation rates using CD45 that was pre-incubated in the absence or presence of recombinant Csk respectively (see Materials and methods for details). (B) Representative phosphotyrosine (pTyr) and 6His-CD45 (CD45) Western blots with 4G10 and RO2.2, respectively, depicting CD45 phosphorylation and protein amounts during the Lck dephosphorylation assay shown in (A). Lanes 1 and 7 indicate the phosphorylation state of CD45 immediately after incubation with or without Csk (upper panel). (A) and (B) are representative Western blots from four separate experiments.

domain interaction correlated with CD45 dephosphorylation ability, such that D2 bound similarly to Src-family kinases, but bound Csk to a lesser extent and did not bind significantly to a less related kinase, Erk1. Likewise CD45 dephosphorylated Lck and Src at similar rates, but dephosphorylated Csk and Erk1 at lower rates (J. Felberg, unpublished data). Therefore, to further localize the site of interaction and determine its role in influencing substrate specificity, the interaction between CD45 and Lck was modeled using SWISS-MODEL (189) based on the *in vitro* data and the 3D structures of the two-domain PTP, LAR (252) and the Src-family kinases (45, 46, 51) (J.Felberg, unpublished). One region of interest revealed by this analysis was the subdomain *X* (SD10) region of the Lck kinase domain.

Since the catalytic domains of tyrosine and serine/threonine kinases are highly conserved in 3D-structure and in the existence of 12 subdomains (253, 254), the overall kinase domain structures of Lck, Csk and Erk1 are quite similar. However, the SD10 sequence between Lck, Csk and Erk1 is poorly conserved and this divergence correlated well with the binding ability of CD45 D2. Thus, it is possible that a specific region within the kinase domain may have evolved as a substrate recognition domain, to mediate the regulatory interaction with other kinases and phosphatases. Such a region would be predicted to be divergent between distantly related kinases. Thus, our attention was focused on SD10, the most poorly conserved kinase subdomain. Currently, the function of this region is unknown despite being exposed at the base of the large kinase lobe (253, 254). A comparison of this region in Lck with Src, Fyn, Csk and Erk1 revealed 60% identity with Src and Fyn, 40% with Csk and essentially no identity with Erk1 (J. Felberg, unpublished). Likewise, a 3D comparison of Lck and Erk2 SD10 regions

revealed substantial structural differences, in agreement with the lack of sequence identity (Fig. 3.12D).

To determine if Lck SD10 has a role in binding 6His-D2, a chimeric protein was made in which the Erk1 SD10 region was replaced by that of Lck (M. Lam, unpublished). This chimeric protein was expressed in *E. coli* as a soluble GST fusion protein (GST-Erk(L-SD10)). Like GST-Erk1 (242, 255), GST-Erk(L-SD10) was capable of *in vitro* tyrosine autophosphorylation, indicating that the chimeric kinase was active (Fig. 3.13B). However, unlike GST-Erk1, 6His-D2 bound significantly to the chimeric GST-Erk(L-SD10) protein, to a level almost approaching that observed with GST-Lck (Fig. 3.12A). Figure 3.12B shows that equal amounts of GST-Erk1 and GST-Erk(L-SD10) were present. This demonstrates that Lck SD10 can mediate binding to 6His-D2 and that transfer of this region to a distantly related kinase, which does not normally bind to 6His-D2, is sufficient to induce binding.

Given the binding of 6His-D2 to the Erk(L-SD10) chimera but not to Erk1, it was next investigated whether the presence of Lck SD10 in the Erk(L-SD10) chimera also affected its dephosphorylation by CD45. GST-Erk1 and GST-Erk(L-SD10) were phosphorylated to approximately equal levels and the rate of dephosphorylation of each protein by 6His-CD45 was compared to that of GST-Lck. Figure 3.13 indicates that when the Erk SD10 region is replaced with the Lck SD10 in the GST-Erk(L-SD10) chimera, Erk1 now becomes an efficient substrate for CD45, being dephosphorylated at a similar rate to Lck. This provides evidence that the interaction of CD45-D2 with the SD10 region of Lck plays a significant role in determining the substrate specificity of CD45 for Lck *in vitro*.

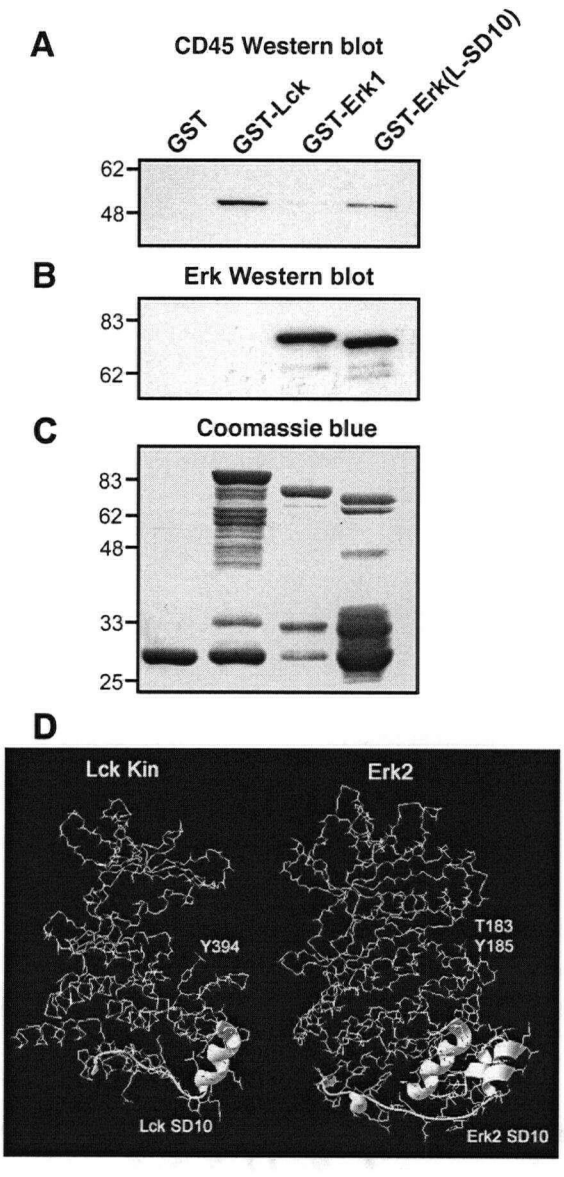

Figure 3.12. Binding of 6His-D2 to GST-Lck, GST-Erk1 and GST-Erk(L-SD10). (A) Soluble 6His-D2 (0.54 μg) was added to 2 μg of immobilized GST fusion protein and incubated for 2 h at 4°C in an *in vitro* binding assay, and then subjected to SDS-PAGE for Western blot analysis. (A) Anti-CD45 Western blot (RO2.2) showing the amount of 6His-D2 remaining bound to the GST fusion proteins. GST fusion proteins used in the binding assay are shown in an Erk Western blot (B), and in a Coomassie Blue protein stain (C). Prestained molecular mass standards are indicated on the left in kDa. (D) 3D analysis of murine Lck kinase domain and Erk2 structure using SWISS-MODEL. Key activating residues on respective activation loops are indicated to provide orientation. Lck and Erk2 SD10 regions (residues 444 to 466 and 229 to 281, SWISS pdb files 3LCK and 2ERK, respectively) are highlighted.

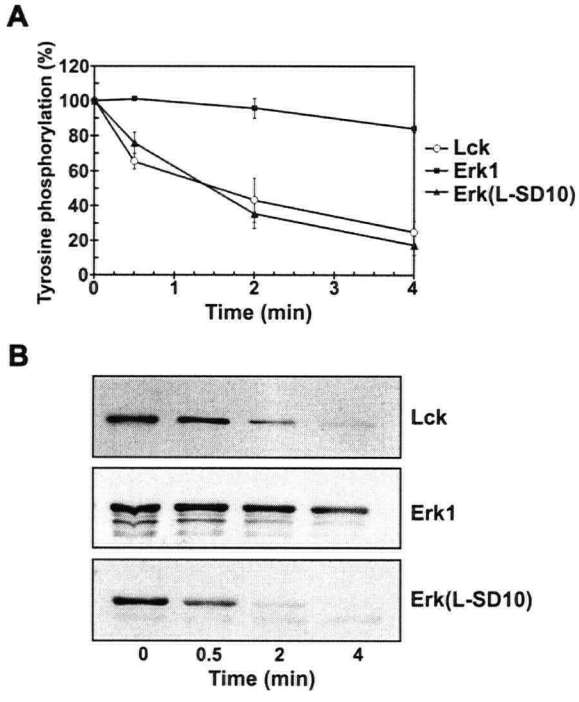

Figure 3.13. Dephosphorylation of tyrosine-phosphorylated GST fusion proteins. 60 nM of 6His-CD45 was incubated with tyrosine phosphorylated 25 nM GST-Lck, 380 nM GST-Erk1 or 380 nM Erk(L-SD10) in 10 μl of PTP buffer for the time points indicated. (A) Graphical analysis of data averaged from 3 separate dephosphorylation assays. The amount of phosphorylation at time 0 for each substrate was taken as 100%. (B) Anti-phosphotyrosine Western blot analysis of GST fusion protein substrates used in dephosphorylation assays.

3.4 Discussion

3.4.1 The use of recombinant proteins *in vitro*

Ideally, the analysis of protein-protein interactions should occur in the cell under physiological conditions. Thus, methods such as co-immunoprecipitation from cells, the yeast-2-hybrid system, or fluorescent resonance energy transfer (FRET) analysis of labeled proteins are all advantageous due to the cellular context of the protein interaction. However, these methods can be complicated by sub-cellular protein localization, interference from other binding proteins, and the lack of definitive evidence for a direct interaction. In the case of CD45 and Lck, the association of these molecules in T cells and the significance of this association in T cell biology was already well established (reviewed in 13, 152). What remained unclear were the variables governing this interaction, such as Lck conformation and the contributions of non-catalytic interactions, and how these variables affect the opposing roles of CD45 in both the up- and down-regulation of Lck activity. Moreover, the dual roles of CD45 in Lck regulation, the differential localization of CD45 and Lck in the plasma membrane, and the adverse effects of mutant Lck expression in cells complicate the analysis of this enzyme-substrate interaction in T cells. Thus, although out of context with regards to cell physiology, these *in vitro* experiments were intended to complement previous data from T cells and further the understanding of the interaction between CD45 and Lck on a molecular level.

Clearly, the advantage of analyzing protein-protein interactions *in vitro* is that this system allows for the determination of a direct interaction in the absence of confounding variables present in the cell. However, the *in vitro* assays described in chapters 3, 4 and 5 of this thesis must be analyzed critically with respect to the caveats of these assays. Most

notably are kinetic considerations with regards to *in vitro* binding, competition and dephosphorylation assays described. The concentrations of purified proteins used in these assays were largely dictated by the amount of protein that could be expressed and purified from a litre of bacterial culture. Thus these assays were often performed under limiting conditions such that binding and catalysis did not occur under saturating substrate concentrations. Ideally, initial experiments should be conducted with increasing concentrations of substrate to determine the value required to reach saturating equilibrium binding, or to approach the V_{max} of the enzyme. However, despite being performed under limiting conditions, *in vitro* binding assays of this thesis did provide reproducible data allowing for the determination and comparison of soluble protein binding to various immobilized substrates. Kinetic parameters must also be considered in competition assays, in which soluble proteins are analyzed for the ability to bind to an immobilized substrate in the presence of a binding competitor. Data shown here provides substantial evidence for the ability of two soluble proteins to bind to substrate in either a synergistic or antagonistic manner. However, it is important to consider that the degree of binding and competition is likely to vary between assays performed under limiting and saturating conditions.

3.4.2 The complexity of the interaction between CD45 and Lck

Previous work in our laboratory established a direct, and non-catalytic interaction between cytoplasmic CD45 and the N and SH2, but not SH3, domains of Lck (75). Here, it has been shown that the Lck kinase domain alone is also capable of interacting with CD45. Lck and Kin interactions with inactive CD45 were both largely mediated by a "catalytic" interaction between Lck phosphotyrosine and the catalytic pocket of CD45

since prior dephosphorylation of Lck, or the presence of vanadate, repressed but did not eliminate the interaction. Inactive CD45C817S was used to characterize the CD45:Lck interaction due to the transient nature of the catalytic interaction with wild type CD45 (Fig. 3.3). This Cys to Ser mutant maintained its ability to bind to phosphotyrosine in agreement with studies elsewhere in which a similar CD45 mutant was used to isolate phosphorylated substrates from the lysates of stimulated T cells (179). Thus these inactive phosphatases are often referred to as "substrate-trapping mutants" (256). A comparison of CD45C817S binding to phosphorylated Lck in the presence and absence of vanadate allowed for the determination that approximately 25% of the CD45:Lck interaction is mediated by non-catalytic components.

It is well established that the enzyme-substrate interaction between CD45 and Lck is critical for Lck regulation and T cell function. Thus, the presence of a substantial non-catalytic interaction between CD45 and Lck suggests that regions other than the pTyr residue and the phosphatase catalytic pocket play a role in regulating Lck activity. Indeed, it has been shown previously that a 46-residue deletion in the Lck unique region interferes with CD45-mediated Lck Tyr 505 dephosphorylation in transfected fibroblasts (257) and in T cells (40). The latter study, employing a Fyn – Lck chimeric protein, further revealed that the expression of the Fyn unique domain in place of the Lck unique domain was unable to restore Lck Tyr 505 dephosphorylation in T cells. Moreover, deletion of the Lck SH2 domain in the Lck F505 mutant was shown to enhance the ability of Lck F505 to induce cellular tyrosine phosphorylation in unstimulated T cells, suggesting a role for the SH2 domain in Lck downregulation independent of pTyr 505 binding (258). Given that non-catalytic components mediate approximately 25% of the

CD45:Lck interaction (Fig. 3.3), together these data suggest that Lck non-catalytic domains participate in Lck regulation by facilitating the interaction with CD45 in the cell.

These findings indicate a complex, multi-domain, enzyme-substrate interaction between Lck and CD45. This involves not only the necessary interaction between the catalytic pocket of CD45 and the phosphotyrosine of Lck, but also substantial non-catalytic components of both Lck and CD45.

3.4.3 The role of Lck non-catalytic domains in CD45-mediated Lck regulation

Binding assays between CD45 and Lck in the presence of vanadate allowed for the observation of interactions mediated by regions other than the catalytic site of CD45. These non-catalytic interactions were enhanced 2-fold for Lck constructs predicted to exist in an open conformation, suggesting roles for Lck non-catalytic domains in its interaction with CD45. Consistent with binding data, the non-catalytic domains of Lck are shown to enhance the rate of dephosphorylation of the auto-phosphorylation site (Tyr 394) in the predicted open form of Lck. Contrarily, non-catalytic domains are shown to retard dephosphorylation of the negative regulatory site (Tyr 505), when Lck is in its predicted closed form due to the pTyr 505:SH2 intra-molecular bond. This difference in dephosphorylation rates was not due to CD45 sequence specificity surrounding Tyr 394 and Tyr 505, since the dephosphorylation of these sites in Lck kinase domain mutants (lacking non-catalytic domains) occurred at equal rates. Moreover, the ability of the closed form of Lck to retard Tyr 505 dephosphorylation was localized to the pTyr 505:SH2 bond, since a point mutation in the SH2 domain (R154K, known to abolish its phosphotyrosine binding ability), improved the rate of Tyr 505 dephosphorylation to that of Tyr 394 in the predicted open form of Lck.

It has been suggested in the literature that CD45-mediated dephosphorylation of Lck Tyr 505 may require the initial binding of a high-affinity SH2/SH3 ligand in order to release the pTyr 505:SH2 interaction and expose pTyr 505 (114, 243). Previous data has suggested total protection of pTyr 505 from CD45 catalysis due to the Lck SH2 domain (243). In that study, recombinant GST-CD45 could dephosphorylate Tyr 505 from pervanadate-induced phosphorylated Lck-R273 lacking the SH2 domain, but not from an intact Lck. In contrast to these findings, it has been shown here that CD45 is indeed capable of dephosphorylating SH2 bound pTyr 505 in the absence of any high affinity ligands, albeit at a slower rate compared to open forms. Moreover, despite the relatively equal affinities of CD45C817S and exogenous Lck SH2 for the exposed pTyr 505 of an Lck kinase domain alone, CD45C817S is superior to exogenous SH2 in gaining access to the pTyr 505 of a closed form of full-length Lck. Together, these data suggest that non-catalytic interactions between CD45 and Lck may facilitate the disruption of the pTyr 505:SH2 bond allowing CD45 access to pTyr 505 in the absence of a high affinity ligand. Furthermore, this *in vitro* data supports in vivo data that CD45 can dephosphorylate Lck at both Tyr 505 and 394 (reviewed in 193). Work shown here further extends in vivo data by showing that CD45 is more efficient at dephosphorylating open forms of Lck. This suggests that in the cell, there is a preference for CD45 in Lck down-regulation and that Lck activation via Tyr 394 phosphorylation is only transient.

3.4.4 A role for CD45 D2 in determining CD45 substrate specificity, and the significance of non-catalytic phosphatase-kinase interactions

Despite the presence of a catalytically inactive second phosphatase domain in several transmembrane phosphatases (259), the role of this structure remains elusive.

Recent work in our laboratory identified a CD45 D2 interaction with the kinase domain of Lck that occurred independently of Lck phosphorylation or conformation status, and was specific to the Src-family kinases (J. Felberg, unpublished data).

Here we demonstrate that the SD10 region of the kinase domain of Lck was capable of inducing an interaction with D2, and that this region alone could convert a poor substrate, Erk1, into an efficient CD45 substrate. Attempts were made create an Lck protein expressing the Erk1 SD10, however this protein was not readily expressed in bacteria suggesting improper folding. Together, this data demonstrates that the interaction between Lck and the second phosphatase domain CD45 can influence substrate specificity. It further suggests that the interaction of two poorly understood protein regions, the SD10 of protein kinases and the second phosphatase domain of transmembrane phosphatases, could be key in determining phosphatase substrate specificity in the cell.

Already, there is evidence to support this as the co-crystal of the CDK2 kinase and KAP phosphatase reveals a substantial non-catalytic interaction mediated by the SD10 expressing C-terminal region of the kinase and the carboxy-terminal helix of the phosphatase (260). The same study further revealed that the major interface between the dual specificity KAP phosphatase and the CDK2 kinase is actually non-catalytic, occurring distant from the pThr 160 of CDK2 and the catalytic pocket of KAP. Moreover, this non-catalytic interaction allowed for the correct orientation of pThr 160 with the phosphatase catalytic site underscoring the importance of this contribution to substrate determination (260). In another example, the MKP3 phosphatase was shown to have its activity induced upon an amino-terminal non-catalytic interaction with its

substrate Erk2 (261). Likewise, amino-terminal non-catalytic residues in the catalytic domain of PTP1B were shown to be dispensable for activity, but important for binding to its insulin receptor substrate (262).

Therefore, it is becoming clear that the interactions of phosphatases with their substrates are potentially complex, requiring the guidance of non-catalytic components for successful binding, activation and orientation for efficient dephosphorylation. Thus, in addition to mechanisms already suggested for phosphatase specificity such as specificity of the catalytic pocket and sub-cellular localization (reviewed in 263), the specificity of second phosphatase domains for particular SD10 regions of kinases may also prove to be key in many phosphatase–kinase interactions.

3.4.5 Differences between RPTPα regulation of Src and CD45 regulation of Lck

Analogous to CD45, the two-domain receptor protein tyrosine phosphatase α (RPTPα) has been implicated in Src activation (264) via dephosphorylation of the C-terminal negative regulatory tyrosine (Tyr 527) (265). Unlike CD45 however, RPTPα has been shown to be constitutively phosphorylated in cells at a C-terminal tyrosine (Tyr 789) (266). Recently, RPTPα pTyr 789 and the Src SH2 domain were shown to be important for RPTPα-mediated dephosphorylation of Src Tyr 527 (267). It was suggested that pTyr 789 of RPTPα provided an SH2 binding ligand to displace the Src pTyr 527:SH2 interaction and allow for dephosphorylation to occur. CD45 tyrosine phosphorylation can be detected in T cells upon treatment with pervanadate (171), although there is no evidence for CD45 phosphorylation in resting or TCR stimuated T cells. Csk-mediated tyrosine phosphorylation of CD45 Tyr 1193 by ATPγS *in vitro* was shown to increase its phosphatase activity and its ability to interact with Lck (171).

In the present study, all assays were performed with recombinant CD45 that was not detectably tyrosine phosphorylated. It should be noted that, despite this lack of phosphorylation, CD45 maintained the ability to bind and dephosphorylate an SH2-bound Lck pTyr 505 residue. However, efforts were made to determine if Csk-mediated phosphorylation of CD45 could affect the CD45:Lck interaction. Significant CD45 phosphorylation was achieved with Csk, however this required the presence of a phosphatase inhibitor indicating that CD45 was capable of rapid auto-dephosphorylation, perhaps explaining why CD45 tyrosine phosphorylation in T cells is not detectable. Moreover, CD45 phosphorylation did not result in any increase in its ability to bind to open or closed forms of Lck. Also, as far as could be determined, given the limitations of rapid CD45 auto-dephosphorylation, we could find no evidence that CD45 phosphorylation by Csk affected CD45 ability to dephosphorylate Lck. Thus, in contrast to previous work, this data suggests that CD45 phosphorylation is not a factor in its regulation of Lck. This also suggests that the interaction between CD45 and Lck occurs in a different manner to that of RPTPα and Src.

3.4.6 Summary

Overall, this work demonstrates that CD45 dephosphorylates Tyr 394 at a faster rate than Tyr 505 in Lck. This is not due to sequence specificity surrounding the phosphotyrosine but due to the intramolecular interaction of the SH2 domain with Tyr 505, which reduces, but does not inhibit, dephosphorylation of Tyr 505 by CD45. It is also shown that the non-catalytic regions of Lck and CD45 contribute to the enzyme-substrate interaction and facilitate the dephosphorylation of both Tyr 394 and Tyr 505 when Lck is in the open conformation. However, these interactions were not affected by

Csk-mediated tyrosine phosphorylation of CD45, as is the case for other phosphatase-kinase interactions. Finally, the non-catalytic CD45 D2 interaction with the Lck SD10 is shown to be a key determinant of CD45 substrate specificity *in vitro*.

CHAPTER 4

The role of CD45AP in CD45-mediated regulation of Lck

4.1 Introduction and rationale

Various studies with CD45-knockout mice or CD45-deficient cell lines have provided extensive evidence that this enzyme is essential for T cell development and activation (reviewed in 1). In contrast, little is known about the significance of CD45-associated protein (CD45AP), a 30-kDa protein that is constitutively associated with CD45 on the surface of T cells. The CD45:CD45AP association occurs at a 1:1 stoichiometry (209) and is exclusive to their respective transmembrane regions (214-216), indicating the absence of a cytosolic protein-protein interaction. However, despite a wealth of information on CD45 function, the role of CD45AP in T cell biology remains poorly understood.

Since Lck is a well-established CD45 substrate in T cells, studies have begun to investigate a potential role for CD45AP in facilitating CD45-mediated Lck regulation. Although controversial, data from one strain of CD45AP-knockout mice revealed impaired TCR-induced cell proliferation, decreased T cell cytotoxic function, and a reduction in the CD45-Lck interaction compared to their wild type littermates (217). Likewise, recent work in our laboratory has shown that CD45AP over-expression in T cells both enhances and sustains intracellular tyrosine phosphorylation upon CD3 stimulation (D. Wong, unpublished data). Thus, it is tempting to speculate a role for CD45AP in augmenting TCR-induced T cell proliferation by facilitating the association between CD45 and Lck in T cells. In support of this, CD45AP has been shown to associate with TCR signaling components, including Lck and ZAP-70 in T cells (220, 221), and there is evidence that CD45AP has a preference for associating with activated forms of Lck (221). Thus, suggested roles for CD45AP have included the recruitment of

activated Lck to CD45 for Tyr 394 dephosphorylation, or to the TCR for enhanced stimulation (221). Moreover, the ability of CD45AP to mediate both a transmembrane association with CD45, and a cytoplasmic association with Lck, raises the possibility of a CD45:CD45AP:Lck tri-molecular complex in resting T cells.

Although there is evidence of a direct CD45AP:Lck interaction *in vitro* (220), the details of this interaction are not known. Thus, given the increasing evidence for a CD45AP role in T cell signaling, the goal of this study was to characterize the CD45AP:Lck interaction and investigate the effects of CD45AP on CD45-mediated Lck regulation.

4.2 Experimental approach

To further explore the interactions of CD45AP with Lck and CD45 in T cells, a cDNA fragment encoding a c-myc-tagged CD45AP fusion protein (Fig. 4.1) was transfected into the T lymphoma cell line, BW5147. This created a system to study the effects of CD45AP over-expression in T cells.

To investigate and characterize the direct interaction between the cytoplasmic domain of CD45AP and Lck, various truncated and mutated forms of each protein were produced in bacteria and purified as GST or 6His fusion proteins (Figs. 3.1 and 4.1). This allowed for the demonstration of a direct CD45AP:Lck interaction *in vitro* and the determination of the specific regions involved. Moreover, the using mutant Lck proteins, the effects of Lck phosphorylation and conformation status on the CD45AP:Lck interaction were examined. Furthermore, purified recombinant CD45 was introduced to study the effects of the CD45AP:Lck interaction on CD45-mediated Lck regulation.

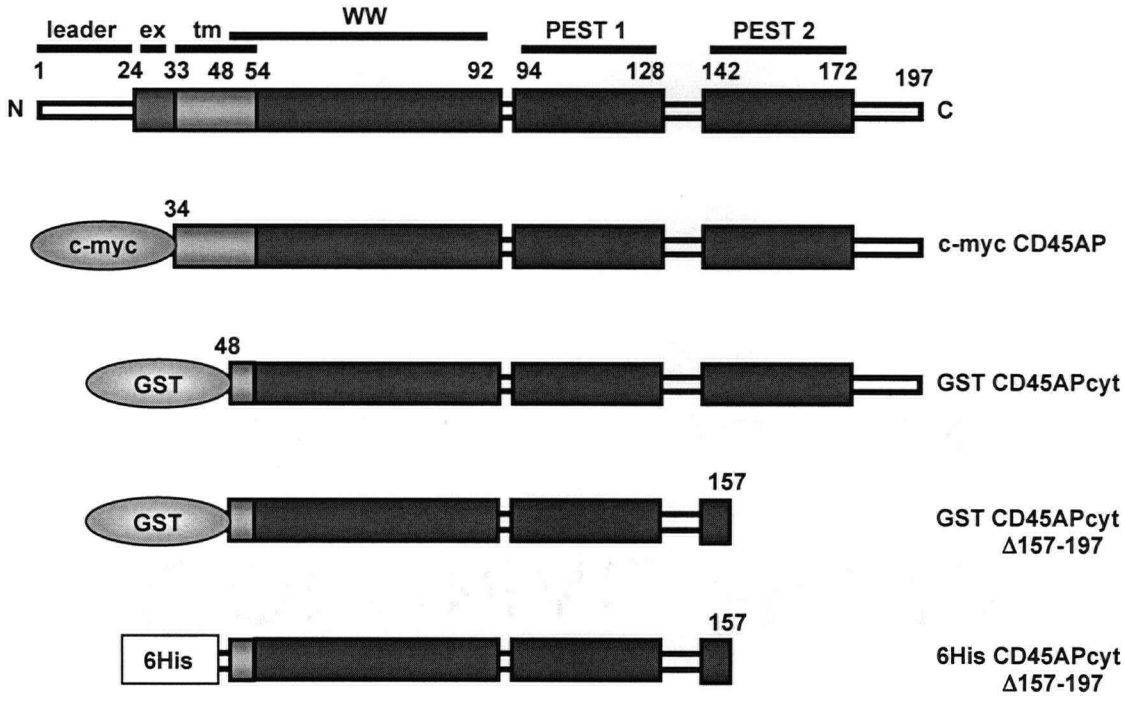

Figure 4.1. CD45AP recombinant proteins used. Endogenous T cell CD45AP structure is indicated (upper construct), and consists of a 23 amino acid N-terminal leader sequence (leader), a 9 amino acid extracellular domain (ex), a 22 amino acid transmembrane domain (tm) whose c-terminal 7 residues overlap with a 44 amino acid WW domain (WW), and two putative PEST sequences (PEST 1 and 2). "C-myc", "GST" and "6His" represent protein tags fused to the N-terminus of CD45AP. The residue numbers of various regions and truncations are marked (numbering as in, 212).

4.3 Results

4.3.1 Lck associates with CD45 and CD45AP to the same extent in T cells

To investigate the potential role of CD45AP in Lck regulation, the ability of CD45AP to associate with Lck in T cells was examined, and compared to that of CD45 and CD44. To determine this, 10^7 BW5147 T lymphoma cells (referred to hereafter as BW+ cells) were lysed in 500 µl of 1% Brij-58 lysis buffer and subjected to Lck immunoprecipitation (see Materials and methods). Lck immunoprecipitations (I.P.) were performed in triplicate, washed twice in lysis buffer and subjected to SDS-PAGE for Western blot analysis. Known amounts of purified recombinant CD45AP, CD45 or CD44 were loaded directly on gel as standards to provide a means of quantitating these molecules co-immunoprecipitated with Lck from T cells.

Figure 4.2A shows that in accordance with previous work (214, 221, 248), CD45AP is associated with Lck in unstimulated T cells. Interestingly, the amount of CD45AP (18 $\pm$ 6 fmoles per Lck I.P.) associated with Lck is comparable to that of CD45 (21 $\pm$ 6 fmoles per Lck I.P.). Figure 4.2B represents the average of four separate experiments and shows no significant difference in the amounts of CD45AP and CD45 that co-immunoprecipitated with Lck ($p=0.478$, two tailed t-test). In contrast, the amount of CD44 that co-immunoprecipitated with Lck (9 $\pm$ 6 fmoles per Lck I.P.) suggests that Lck is associated with approximately 2-fold more CD45AP than CD44 in T cells. This data provides further evidence for a significant CD45AP:Lck association in T cells and demonstrates upon lysis in 1% Brij-58, Lck is associated with similar amounts of CD45AP and CD45. Moreover, this data is consistent with the existence of a tri-

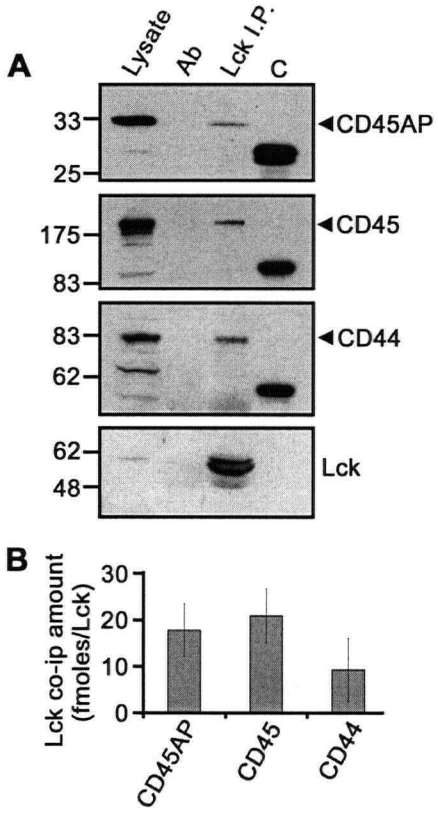

Figure 4.2. The association of CD45AP, CD45 and CD44 with Lck immuno-precipitated from BW+ T cells. BW+ T lymphoma cells (10^7) were lysed in 1% Brij-58 lysis buffer. Each lysate was subjected to immuno-precipitation with immobilized R54 anti-Lck antiserum (1 µl) for 2 hours at 4°C followed by washing and SDS-PAGE. (A) Western blot analysis of different T cell proteins that co-immunoprecipitated with Lck. The top, second and third panels represent individual immunoblots probed with anti-CD45AP (WW1B2), anti-CD45 (RO2.2) and anti-CD44 (JIWBB) antiserum respectively. The bottom panel depicts a representative anti-Lck blot probed with R54 anti-Lck antiserum. "Lysate" represents soluble cell lysate from $2x10^5$ cell equivalents. "Ab" represents a control lane loaded with anti-Lck antiserum alone. "C" indicates a control lane in which 0.12 pmoles of purified recombinant 6His-CD45APcyt Δ157-197, 6His-CD45 cytoplasmic domain, or MBP CD44 cytoplasmic domain was included. Relative molecular mass of the pre-stained markers is indicated to the left in kDa. (B) Graphical representation of the amount of CD45AP, CD45 or CD44 that co-immunoprecipitated with Lck. Values represent the average from 4 separate experiments with error bars representing standard deviation.

molecular complex between CD45, CD45AP and Lck in T cells.

4.3.2 Over-expression of CD45AP in T cells leads to an increase in its association with both CD45 and Lck

Upon establishing the relative amounts of CD45 and CD45AP that co-immunoprecipitated with Lck from T cells, we next sought to investigate the stoichiometry of the CD45AP:Lck association, and determine if the over-expression of CD45AP affected its association with Lck and CD45. Thus, BW+ cells were transfected with a c-myc-tagged CD45AP construct, creating a cell line that over-expresses CD45AP due to the combination of endogenous and c-myc-tagged forms. Two separate clones expressing c-myc CD45AP were identified and cultured (referred to hereafter as BW+cAP.1 and BW+cAP.2). Western blot analysis of the lysates from these cells shows the endogenous CD45AP at approximately 30 kDa, and the transfected c-myc CD45AP at approximately 35 kDa expressed by both clones (Figs 4.3A and 4.3C, upper panels).

Lck and CD45 immunoprecipitations were performed from separate lysates of BW+, BW+cAP.1 and BW+cAP.2 cells and were analyzed for the co-immunoprecipitation of CD45AP. Known amounts of purified recombinant CD45AP, Lck, and CD45 were included in the Western blot analysis to provide a means of quantitating these molecules isolated from T cells. The over-expression of CD45AP led to increases in its associations with both Lck and CD45 (Figs. 4.3A-D). Interestingly, the association of endogenous CD45AP with Lck or CD45 occurred to a similar extent in transfected and un-transfected cells, suggesting minimal competition between endogenous and c-myc CD45AP for these interactions. Figure 4.3B demonstrates a CD45AP:Lck association stoichiometry of approximately 1:10 in un-transfected cells that

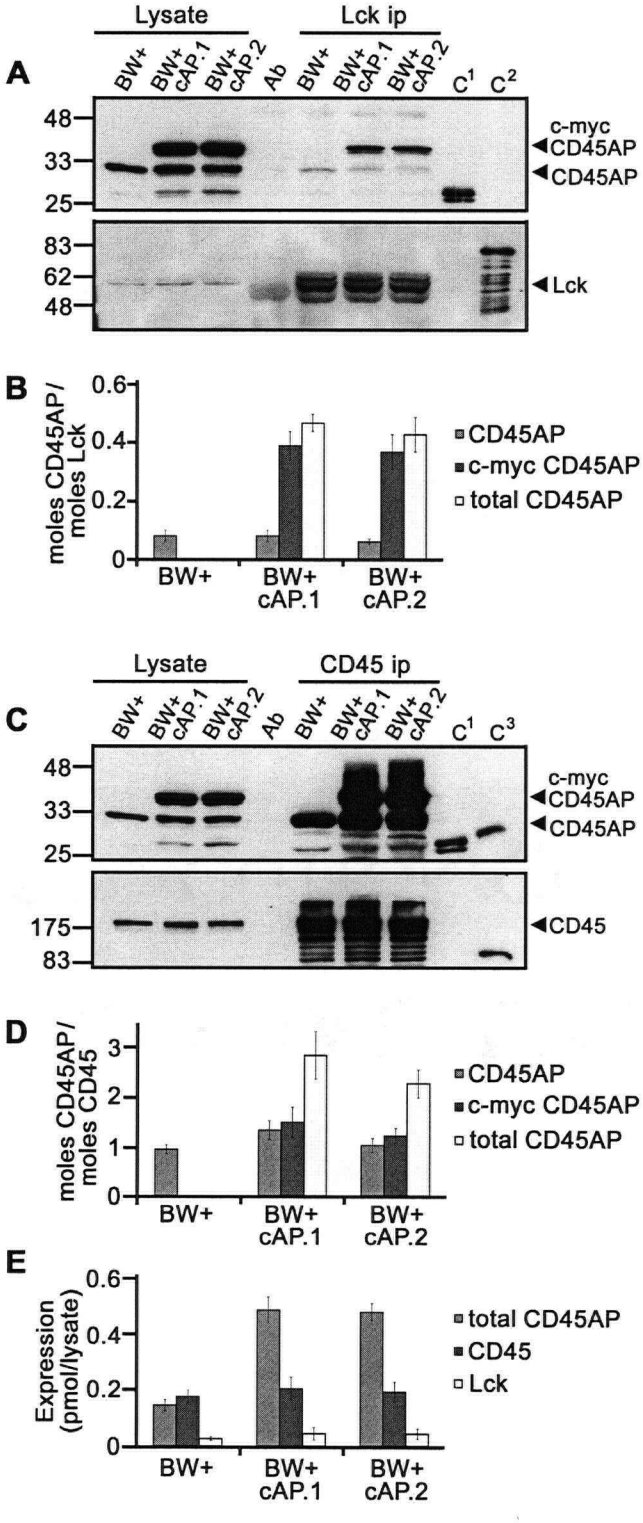
A
Lysate
Lck ip
BW+
BW+ cAP.1
BW+ cAP.2
Ab
C1
C2
48
33
25
83
62
48
c-myc CD45AP
CD45AP
Lck
B
moles CD45AP/ moles Lck
0.6
0.4
0.2
0
CD45AP
c-myc CD45AP
total CD45AP
C
Lysate
CD45 ip
C3
175
83
CD45
D
moles CD45AP/ moles CD45
3
2
1
0
E
Expression (pmol/lysate)
total CD45AP
CD45
Lck

Figure 4.3. The association of CD45AP with Lck and CD45 in CD45AP over-expressing T cells. BW+, BW+cAP.1 or BW+cAP.2 T cells (10^7) were lysed in 1% Brij-58 lysis buffer. Lysates were then either incubated with immobilized I3/2 anti-CD45 antibody (25 µl packed beads) or with immobilized R54 anti-Lck antiserum (1 µl) to immunoprecipitate CD45 or Lck ("CD45 ip" or "Lck ip"). (A, C) Western blot analyses of CD45AP (upper panels) and Lck or CD45 (lower panels) probed with anti-CD45AP (WW1B2), anti-Lck (R54), or anti-CD45 antiserum (RO2.2), respectively. "Lysate" represents soluble lysate from $2x10^5$ cell equivalents. "Ab" represents a control lane loaded with anti-Lck antiserum (A) or anti-CD45 antibody alone (C). "C^1", "C^2" and "C^3" represent 0.12 pmoles of purified recombinant 6His-CD45APcyt Δ157-197, GST-Lck and 6His-CD45 cytoplasmic domain, respectively. The band seen in the "C^3" lane of the CD45AP immunoblot represents a cross-reactive protein co-purified with recombinant CD45. The relative molecular mass of the pre-stained markers is indicated to the left in kDa. (B, D) Graphical representation of the number of moles of CD45AP, c-myc CD45AP or total CD45AP protein that were co-immunoprecipitated per mole of Lck or CD45. Values represent the average from 2 separate experiments with error bars representing standard error. (E) Graphical representation of the number of moles of total CD45AP protein, CD45 and Lck that are present in the soluble lysate of $2x10^5$ cells. Values represent the average from 4 separate experiments with error bars representing standard deviation.

is increased approximately 5-fold (to 1:2) when CD45AP is over-expressed. Likewise, the approximate 1:1 CD45AP:CD45 association stoichiometry in wild type cells is increased approximately 2-fold (to 2:1) when CD45AP is over-expressed (Fig 4.3). This indicates a correlation between CD45AP expression and the degree to which it associates with CD45 and Lck in the cell.

Figure 4.3E shows that BW+ cells express comparable amounts of CD45 and CD45AP that are approximately 5-fold above that of Lck expression. These values correlate well with the association stoichiometries of these molecules as the endogenous CD45AP:CD45 and CD45AP:Lck ratios were approximately 1:1 and 1:10, respectively. This suggested that every CD45 molecule, and 10% of the Lck molecules in a cell could be associated with CD45AP. The increase in the CD45AP:Lck stoichiometry from 1:10 to 1:2 upon CD45AP over-expression suggests that half of the Lck molecules in the transfected cells were coupled to CD45AP. Interestingly, the increase in the CD45AP:CD45 stoichiometry from 1:1 to 2:1 upon CD45AP over-expression suggests that a single CD45 molecule is capable of interacting with more than one CD45AP molecule (Fig 4.3). This 2:1 stoichiometry is a possibility given the range of hydrophobic residues involved in the transmembrane association.

Moreover, the 10-fold difference in the endogenous CD45AP association stoichiometries between CD45 and Lck is not consistent with the model of these proteins existing in a tri-molecular complex. This suggested that CD45AP might associate with CD45 and Lck individually via distinct bi-molecular interactions.

4.3.3 CD45AP and Lck can exist as a bi-molecular complex in T cells

Given the above data (Fig. 4.3), it was unlikely that the majority of CD45,

CD45AP, and Lck existed in a tri-molecular complex, however these results did not preclude the existence of such an interaction. Thus, we next sought to establish whether the association between CD45AP and Lck in T cells was direct, or indirect and possibly occurring via CD45. To investigate this, the lysates of BW+, BW+cAP.1 and BW+cAP.2 were depleted of CD45 prior to Lck immunoprecipitation. Lysates from 10^7 cells underwent four consecutive one-hour incubations in the presence or absence of 25 µl of packed I3/2 anti-CD45 antibody conjugated beads. Although CD45 was not totally eliminated from the cell lysates, the amount of CD45 was reduced considerably to 14 ± 5% (n=2) compared to untreated lysates and is shown in figure 4.4 (lower panel).

As expected, the depletion of CD45 also resulted in a reduction in the amount of CD45AP remaining in the lysate since significant amounts of CD45AP are associated with CD45 in the cell (Fig. 4.4). This reduction was more obvious for endogenous CD45AP (approximate 4-fold reduction) than for the over-expressed c-myc-tagged CD45AP (approximate 2-fold reduction). However, despite an approximate 7-fold reduction in CD45 levels upon its depletion, figure 4.4 shows only a slight difference in the amount of CD45AP that co-immunoprecipitates with Lck from CD45-depleted and untreated lysates. Spot densitometry analysis from 4 separate experiments revealed that upon CD45 depletion, the amount of endogenous CD45AP co-immunoprecipitated with Lck from BW+ cells was reduced modestly from 35 ± 17 to 18 ± 7 fmoles. Likewise, the total amount of CD45AP (including endogenous and c-myc CD45AP) co-immunoprecipitated with Lck from BW+cAP.2 cells was reduced slightly from 215 ± 51 to 145 ± 57 fmoles upon CD45 depletion. Given the profound reduction in CD45 levels upon its depletion, the relatively modest reduction observed in the CD45AP:Lck

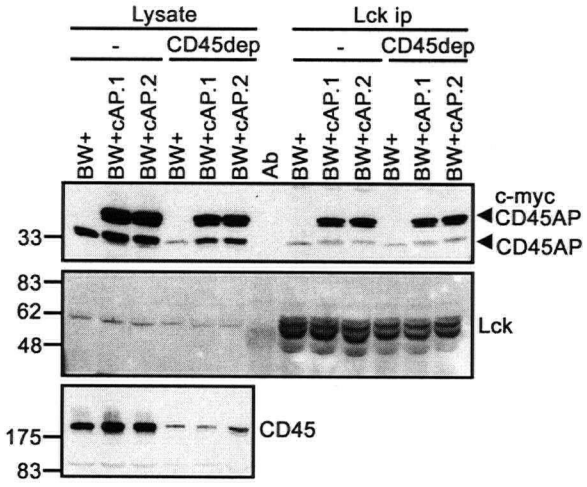

Figure 4.4. The effect of CD45 depletion on the CD45AP:Lck association in T cells. BW+, BW+cAP.1 or BW+cAP.2 T cells (10^7) were lysed in 1% Brij-58 lysis buffer. Lysates then underwent 4 consecutive 1-hour incubations in the presence or absence of immobilized I3/2 anti-CD45 antibody. Untreated (-) and CD45-depleted (CD45dep) lysates were then subjected to anti-Lck immunoprecipitation as described in figure 4.2. "Lysate" represents the soluble lysate from $2x10^5$ cells, "Ab" represents a control lane loaded with anti-Lck antiserum alone, and "Lck ip" represents lanes loaded with Lck immunoprecipitations. The upper, middle and lower panels represent Western blot analyses probed with anti-CD45AP (WW1B2), anti-Lck (R54) or anti-CD45 (RO2.2) antiserum, respectively. Relative molecular mass of the pre-stained markers is indicated to the left in kDa.

association suggests that this interaction in T cells is largely bi-molecular and independent of CD45.

4.3.4 CD45AP and Lck interact directly *in vitro*

The above data suggested a bimolecular interaction between CD45AP and Lck in T cells, thus we proceeded to investigate a direct interaction *in vitro* using purified recombinant proteins. Lck, cytoplasmic CD45AP (CD45APcyt), and cytoplasmic CD45AP lacking the C-terminal residues 157 to 197 (CD45APcyt Δ157-197) were all purified from bacteria as immobilized GST fusion proteins. Inactive cytoplasmic CD45 C817S was also purified from bacteria as an immobilized 6His-tagged protein to serve as a positive control for Lck binding (CD45C817S). Upon purification from bacteria, CD45APcyt was heavily degraded, however the deletion of the C-terminal 40 residues (CD45APcyt Δ157-197) resulted in a more stable and pure fusion protein. Immobilized GST-Lck was incubated with thrombin (see Materials and Methods) to cleave Lck from GST allowing for the collection of soluble, untagged Lck from the supernatant.

To investigate the interaction between Lck and CD45AP, 100 ng of soluble Lck was incubated with 2 μg of each bead-immobilized protein (listed above) in 40 μl for 2 hours at 4°C. The beads were then washed, boiled in reducing SDS sample buffer and subject to SDS-PAGE for Western blot analysis. Lck bound equally well to both CD45APcyt and the deletion mutant indicating that: 1) a direct protein–protein interaction occurs between CD45AP and Lck, and 2) the C-terminal 40 amino acids of CD45AP are dispensable for this interaction (Fig. 4.5, upper panel). This data provides evidence for a direct interaction between Lck and the cytoplasmic domain of CD45AP *in vitro*, and suggests that these molecules interact directly in T cells given the above data

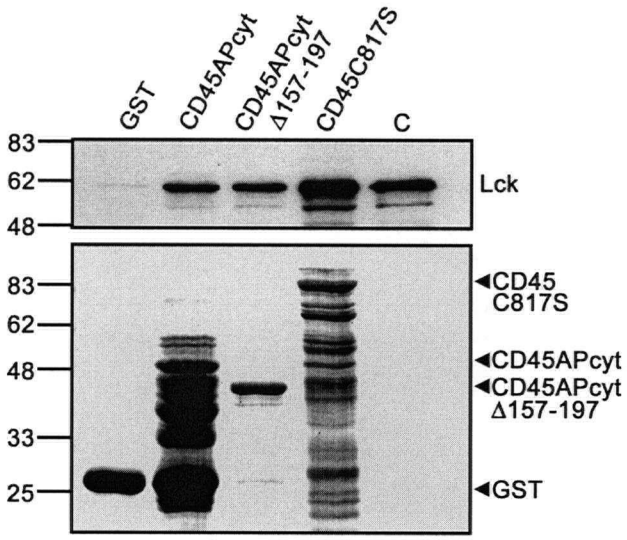

Figure 4.5. Lck binding to immobilized CD45AP and CD45 fusion proteins. Recombinant, soluble, thrombin-cleaved Lck (Lck, 100 ng) was incubated with 2 μg of immobilized GST alone (GST), GST cytoplasmic CD45AP (CD45APcyt), GST cytoplasmic CD45AP lacking the C-terminal 40 amino acids (CD45APcyt Δ157-197), or 6His-CD45 C817S (CD45C817S), in 40 μl for 2 h at 4°C before Western blot analysis. Soluble Lck that had bound to immobilized proteins was detected by probing the PVDF membrane with R54 anti-Lck antiserum (upper panel). "C" represents 10 ng of Lck loaded directly onto the gel as a control. Immobilized proteins used in the assay are shown as a Coomassie protein stain (lower panel). The upper two bands in the Coomassie stain of CD45APcyt were determined to be contaminants (Western blot analysis, data not shown). The relative molecular mass of the pre-stained markers is indicated to the left in kDa.

supporting a bi-molecular association in cells.

4.3.5 CD45AP interacts with Lck via the Lck kinase domain alone

The discovery of a direct interaction between Lck and CD45AP *in vitro* raised further questions as to the region of Lck responsible for the association and the effect that Lck phosphorylation and conformation status may have on the interaction. To address these questions, mutant forms of immobilized GST-Lck were purified from bacteria and employed in *in vitro* binding assays with soluble 6His-CD45APcyt Δ157-197. The CD45AP deletion construct was used since the C-terminal 40 amino acids were shown to be dispensable for the Lck interaction, and because it can be purified as a stable and uniform product (unlike the full-length cytoplasmic CD45AP which is impure and degraded, see figure 4.5).

All full-length GST-Lck fusion proteins except Lck R273 (Lck, Lck F505 and Lck F394) were purified from bacteria co-expressing Csk (see Materials and methods for details). Thus, wild type Lck (Lck), which can auto-phosphorylate at Tyr 394 in bacteria, is potentially phosphorylated both at Tyr 394 and Tyr 505. The phosphorylation of Tyr 505 is predicted to induce an intramolecular interaction between pTyr 505 and SH2 resulting in the adoption of the "closed" conformation of Lck. Thus, Lck and Lck F394 are predicted to exist in the closed conformation due to the presence of Csk-mediated Tyr 505 phosphorylation. Lck F505, phosphorylated at Tyr 394, and Lck R273 (an inactive, non-phosphorylated kinase) are thought to represent differentially phosphorylated "open" forms of Lck due to a lack of phosphate at Tyr 505. Data shown in chapter 3 (Fig. 3.8) supports the existence of these open and closed Lck forms.

To localize the Lck regions important for mediating a direct interaction with CD45AP, GST fusion proteins containing the N-terminal "unique" region of Lck alone (N), the Lck non-catalytic domains (N, SH3 and SH2 (N32)), and the Lck kinase domain alone (Kin) were produced. GST fusion proteins including SHC SH2, Grb2 and the cytoplasmic domain of CD45 served as negative controls for the binding of CD45APcyt Δ157-197. All GST proteins used, and their phosphorylation status, are represented in figure 4.6A with a Coomassie blue protein stain (middle panel) and a 4G10 immunoblot (bottom panel), respectively.

To characterize the direct interaction between CD45AP and Lck, 100 ng of soluble 6His-CD45APcyt Δ157-197 was incubated with 2 μg of various immobilized Lck proteins in 40 μl for 2 hours at 4°C. The amount of CD45AP protein bound to each Lck protein after was detected by Western blot analysis with an anti-6His antibody (Fig. 4.6A, upper panel). CD45APcyt Δ157-197 bound equally to all four full-length Lck constructs despite their differences in phosphorylation and conformation status. In figure 4.6A, CD45APcyt Δ157-197 is shown to bind slightly more to Lck F394 and Lck R273 than to other full-length Lck constructs, however the average binding over 5 separate experiments shows no significant difference in binding to any full-length Lck construct (Fig. 4.6B). CD45APcyt Δ157-197 also bound significantly to the Lck kinase domain alone, and did so to the same degree as binding to full-length Lck. Despite a faint band in the N32 lane, CD45APcyt Δ157-197 did not bind to Lck non-catalytic regions significantly above background over the course of 5 experiments (Fig 4.6B).

Together, this data localizes CD45AP binding to the Lck kinase domain alone. Moreover, it suggests that the CD45AP:Lck interaction may be further localized to a

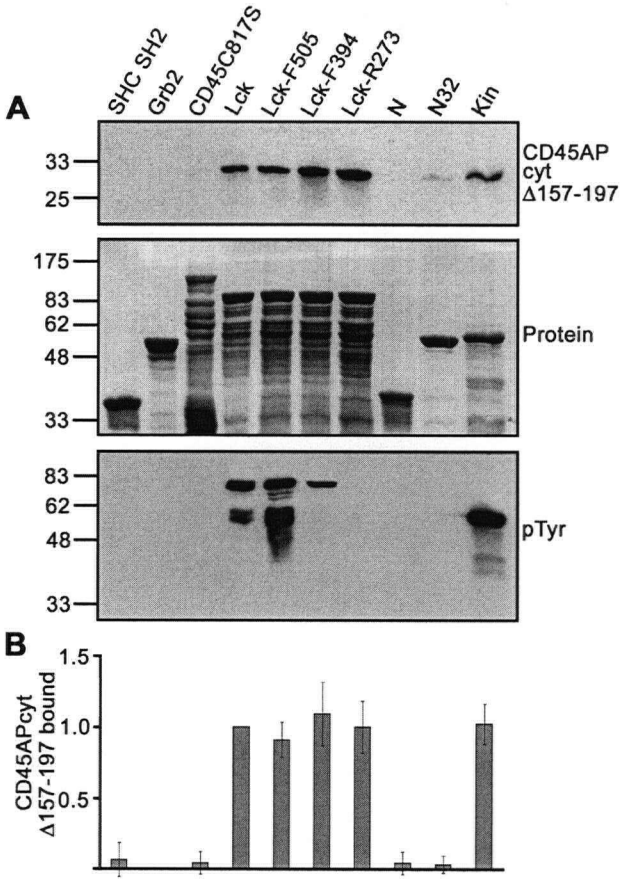

Figure 4.6. Binding of CD45APcyt Δ157-197 to immobilized Lck mutant proteins. Recombinant, soluble, 6His-CD45APcyt Δ157-197 (100 ng) was incubated with 2 μg of immobilized GST fusion proteins in an *in vitro* binding assay as described in figure 4.5. (A) Western blot analysis of CD45AP protein remaining bound to immobilized GST fusion proteins detected with anti-CD45AP antiserum (WW1B2, upper panel). Immobilized GST fusion proteins included the SHC SH2 domain (SHC SH2), Grb2 (Grb2), cytoplasmic CD45 C817S (CD45C817S), wild type Lck (Lck) Lck with Tyr 505 mutated to Phe (Lck-F505), Lck with Tyr 394 mutated to Phe (Lck-F394), Lck with Lys 273 mutated to Arg (Lck-R273), the unique "N" region of Lck (N), Lck N, SH2 and SH3 domains (N32), and the Lck kinase domain alone (Kin), and are shown in a Coomassie blue protein stain (middle panel). The tyrosine phosphorylation status of GST fusion proteins was detected with the 4G10 anti-phosphotyrosine antibody (lower panel, pTyr). The relative molecular mass of the pre-stained markers is indicated to the left in kDa. (B) Graphical representation of the relative amount of CD45APcyt Δ157-197 bound to each GST fusion protein from 4 separate experiments (binding to Lck was standardized to 1.00) with error bars representing standard deviation.

region of the kinase domain distant from the activation loop or carboxy-tail since differences in Lck phosphorylation and conformation status did not affect the interaction.

4.3.6 CD45AP does not bind to Lck subdomain *X* (SD10)

The localization of the CD45AP:Lck interaction to the Lck kinase domain was intriguing since the second phosphatase domain of CD45 (D2) has also been shown to bind to the Lck kinase domain (J. Felberg, unpublished data). Akin to CD45AP, the interaction between D2 and the Lck kinase domain was also shown to occur independently of Lck phosphorylation conformation status. Moreover, in chapter 3 of this thesis, D2 binding to Lck was further localized to a region that is poorly conserved among protein kinases, SD10. Despite functional differences, kinase domains typically share a similar 3-D structure that can be dissected into 12 separate subdomains. SD10 is located at the base of the large lobe of kinase domains, is fully exposed and consists of a single helix (helix G) and a random coil leading to helix H (see chapter 3 for further information on SD10). Given the apparent similarities of CD45AP and D2 interactions with Lck, we next investigated the ability of CD45AP to bind to Lck SD10.

CD45AP is not known to interact with any members of the MAP kinase family in T cells, thus an Erk1 GST fusion protein was employed as a negative control for CD45AP binding. Moreover, SD10 regions of Erk1 and Lck have essentially no sequence identity, and thus a mutant Erk1 protein was engineered to express the Lck SD10 in place of its own, Erk(L-SD10) (see chapter 3). To determine if Lck SD10 influenced interactions with CD45AP, 0.54 μg of soluble 6His-D2 or 6His-CD45APcyt Δ157-197 were incubated with 2 μg of immobilized GST-Lck, GST-Erk1 or GST-Erk(L-SD10) protein in 40 μl for 2 hours at 4°C. The amount of CD45AP protein that bound to

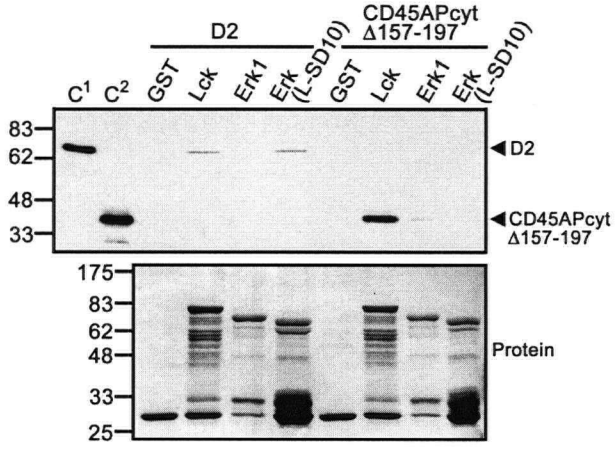

Figure 4.7. Binding of CD45 D2 and CD45APcyt Δ157-197 to Lck SD10.
Recombinant soluble 6His-CD45 D2 (D2) or 6His-CD45APcyt Δ157-197 (CD45APcyt Δ157-197) (0.54 μg) were incubated with 2 μg of immobilized GST fusion proteins in an *in vitro* binding assay as described in figure 4.5. The amount of D2 and CD45AP protein that bound to immobilized GST fusion proteins was detected by Western blot analysis with an anti-6His antibody (upper panel). "C^1" and "C^2" represent 50 ng of 6His-D2 and 6His-CD45APcyt Δ157-197 respectively loaded directly onto the gel as controls. Immobilized GST fusion proteins included GST alone (GST), Lck (Lck), Erk1 (Erk1), and an Lck-Erk1 chimera in which the native Erk1 SD10 was replaced with the SD10 of Lck (Erk(L-SD10)), and are shown in a Coomassie blue protein stain (lower panel). The relative molecular mass of the pre-stained markers is indicated to the left in kDa.

each immobilized GST fusion protein after washing the beads was detected by Western blot analysis with an anti-6His antibody (Fig. 4.7, upper panel). In accordance with chapter 3, figure 4.7 shows that 6His-D2 bound both to Lck and the Erk(L-SD10) chimera, but not to Erk1. Interestingly, CD45APcyt Δ157-197 bound only to Lck, and not to Erk1 or Erk(L-SD10) suggesting that unlike D2, CD45AP binds to a region of the Lck kinase domain other than SD10.

4.3.7 CD45AP and D2 compete weakly for binding to the Lck kinase domain

To determine the relative of affinities of CD45AP and D2 for the Lck kinase domain, an *in vitro* competition assay was performed. The concentration of immobilized GST-Lck kinase domain (Lck-Kin) was 610 nM in each assay and various molar ratios of 6His-CD45APcyt Δ157-197 and/or 6His-D2 were added. After a 2-hour incubation at 4°C, immobilized GST-Lck beads were washed and the amount of bound CD45AP and D2 was determined by Western blot analysis with an anti-6His antibody.

A comparison of CD45APcyt Δ157-197 and D2 binding in the absence of competition at a 1:1 molar ratio with Lck-Kin shows that approximately 6-fold more CD45AP protein than D2 bound to the Lck kinase doman (Fig. 4.8, lanes 5 and 8). Interestingly, despite the introduction of CD45APcyt Δ157-197, at a 1:1:1 molar ratio (Lck-Kin:D2:CD45AP), the binding of D2 was reduced only by approximately 2-fold (Fig. 4.8, lanes 6 and 9). Even at 1:1:5 molar ratios (Lck-Kin:D2:CD45AP) the relatively weak binding of D2 was not eliminated (Fig 4.8, lane 7). Moreover, CD45APcyt Δ157-197 binding to Lck-Kin was not significantly affected by an equal molar amount of D2 protein, and at 5:1 (D2:AP) molar ratio, only a modest reduction was observed (Fig. 4.8, lanes 9 and 10). It was an initial concern that D2 and CD45AP proteins could be

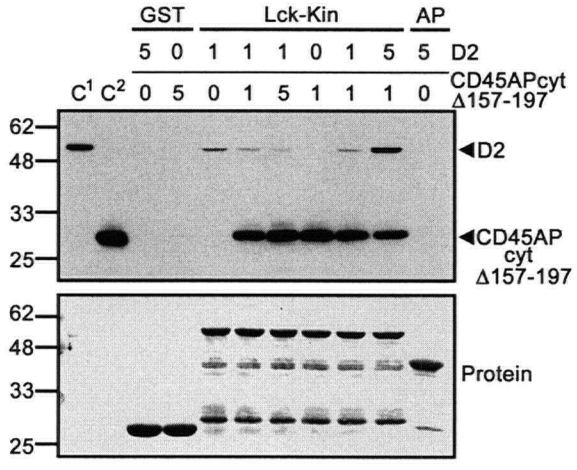

Figure 4.8. Competition assay between CD45 D2 and CD45APcyt Δ157-197 for binding to the Lck kinase domain. Immobilized GST alone (GST), GST-Lck kinase domain (Lck-Kin), and GST-CD45APcyt Δ157-197 (AP) were incubated at 610 nM final concentration without (0), or with equal molar (1) or 5-fold excess molar (5) concentrations of soluble 6His-CD45 D2 (D2) and/or 6His-CD45APcyt Δ157-197 (CD45APcyt Δ157-197) in 40 µl for 2 hours at 4°C before being washed and subjected to SDS-PAGE. The amount of 6His-D2 or 6His-CD45AP protein remaining bound to immobilized GST fusion proteins was detected by Western blot analysis with an anti-6His antibody (upper panel). Immobilized GST proteins used in the assay are shown in a Coomassie blue protein stain (lower panel). "C^1" and "C^2" represent 4.8 and 9.6 pmoles of 6His-D2 and 6His-CD45APcyt Δ157-197 respectively, loaded directly onto the gel. The relative molecular mass of the pre-stained markers is indicated to the left in kDa.

interacting with each other in the assay, however soluble D2 showed no binding to an immobilized GST-CD45APcyt Δ157-197 control (Fig 4.8, far right lane).

This data indicates that the interaction of CD45AP with Lck does not prevent the ability of CD45 D2 to bind to the Lck kinase domain at SD10. Moreover, this finding is in agreement with a role for CD45AP in facilitating the interaction of CD45 and Lck.

4.3.8 CD45AP inhibits the Lck:CD45C817S interaction

Since CD45AP and D2 bind to different regions of the Lck kinase domain and compete relatively weakly for interactions with Lck, we next investigated CD45AP has an effect on the interaction between cytoplasmic CD45 and Lck. To determine this, the binding of CD45 C817S to Lck was assayed in the presence and absence of CD45AP. Immobilized GST-Lck, GST-Lck R273 and GST-Grb2 were incubated at 610 nM final concentrations with or without an equal molar amount of soluble 6His-CD45C817S. Soluble CD45APcyt Δ157-197 was also added to certain assays at a 1:1 or 1:5 molar ratio (CD45C817S:CD45AP). The amount of 6His-CD45C817S and 6His-CD45AP Δ157-197 that bound to immobilized proteins were detected by Western blot analysis with an anti-6His antibody.

A comparison of CD45AP and CD45 binding alone at a 1:1 molar ratio with Lck protein shows that these proteins bind in comparable levels to Lck (Fig. 4.9A). Surprisingly, at a 1:1:1 molar ratio (Lck:CD45C817S:CD45AP), CD45APcyt Δ157-197 binds significantly better to Lck than CD45C817S and reduced the interaction of CD45C817S with Lck proteins to approximately 35% (Fig 4.9C). A 1:5 molar ratio of CD45C817S:CD45AP further reduced CD45C817S interactions with Lck and Lck R273 to approximately 20% (Fig 4.9C). This CD45AP-mediated inhibition of the

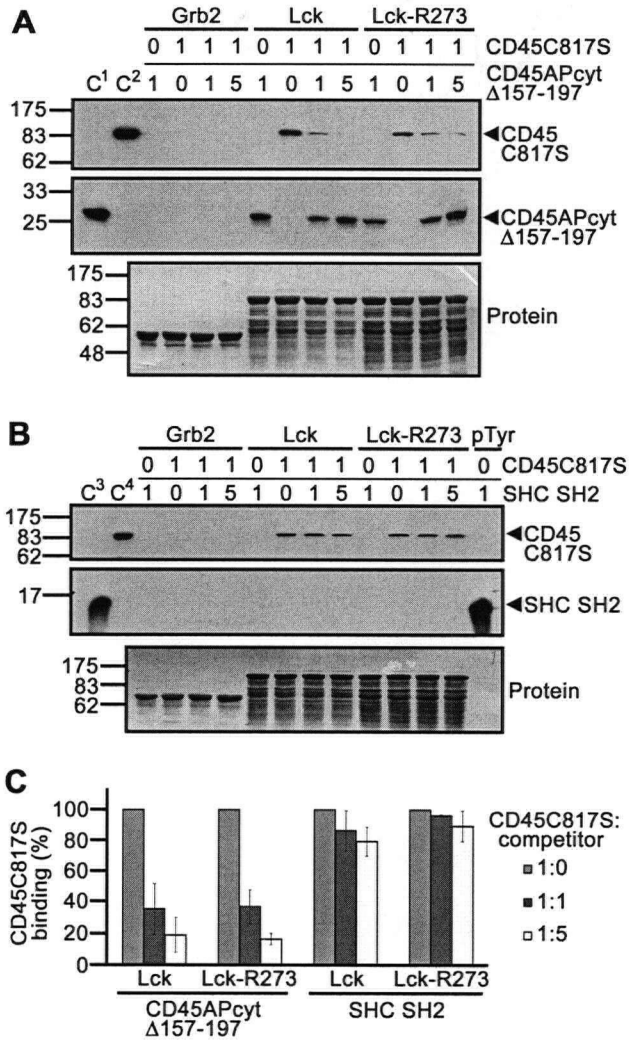

Figure 4.9. CD45C817S and CD45APcyt Δ157-197 competition for Lck. GST-Lck (Lck), GST-Lck R273 (Lck-R273) or GST-Grb2 (Grb2) were incubated at 610 nM without (0) or with equal molar (1) or 5-fold excess molar (5) concentrations of soluble 6His-CD45C817S (CD45C817S, A and B) and/or 6His-CD45APcyt Δ157-197 (CD45APcyt Δ157-197, A) and/or 6His SHC SH2 domain (SHC SH2, B) as described in figure 4.8. 6His-tagged CD45C817S, CD45APcyt Δ157-197, or SHC SH2 proteins that bound to immobilized GST fusion proteins were detected by Western blot analysis with an anti-6His antibody (A and B, upper and middle panels). Immobilized GST proteins used in each assay are shown as a Coomassie blue protein stain (A and B, lower panels). "C^2" and "C^4", "C^1", and "C^3" represent 2.4 pmoles of 6His-CD45C817S, 6His-CD45APcyt Δ157-197, and 6His-SHC SH2 respectively, loaded directly onto the gel. The relative molecular mass of the pre-stained markers is indicated to the left in kDa. (C) Graphical analysis of CD45C817S binding to Lck proteins. Binding in the absence of competitor was standardized to 100%, and values represent the average of 4 (CD45AP) or 2 (SHC SH2) experiments. Molar ratios of CD45C817S:CD45APcyt Δ157-197 or SHC SH2 (competitor) are provided in the legend.

CD45C817S:Lck interaction was not due to non-specific steric blocking since 6His-SHC SH2 had no effect on this interaction even at a 1:5 molar ratio (CD45C817S:SHC SH2, figure 4.9B). Proper folding and function the 6His-SHC SH2 protein is shown by its ability to bind to phosphotyrosine (pTyr) coupled beads. Together, these findings indicate that CD45AP provides specific and substantial inhibition of the enzyme–substrate interaction between CD45 and Lck.

This is an interesting and surprising finding suggesting that in contrast to the proposed role for CD45AP in facilitating the CD45:Lck interaction, CD45AP has the opposite effect: the ability to *block* the CD45:Lck interaction. Moreover, this suggests that CD45AP may block CD45 access to Lck Tyr 394 or Tyr 505 thereby altering the ability of CD45 to regulate Lck activity.

4.3.9. CD45AP specifically inhibits CD45-mediated dephosphorylation of Lck Tyr 394

Given the ability of CD45AP to inhibit CD45 binding to Lck, it was next determined whether this inhibition could also interfere with CD45-mediated Lck dephosphorylation. Thus, *in vitro* dephosphorylation assays were performed in the presence and absence of CD45AP. Since CD45AP was shown to bind to the Lck kinase domain alone, assays were initially performed with thrombin-cleaved soluble Lck kinase domain mutants Kin F505 and Kin F394 (phosphorylated at Tyr 394 and Tyr 505 respectively). Dephosphorylation assays consisted of 60 nM CD45 and 60 nM Lck protein incubated for various time points at 30°C in the presence or absence of 300 nM CD45APcyt Δ157-197. Figure 4.10A shows that the presence of CD45APcyt Δ157-197 reduced the rate of CD45-mediated Lck kinase domain phosphorylation with a significant

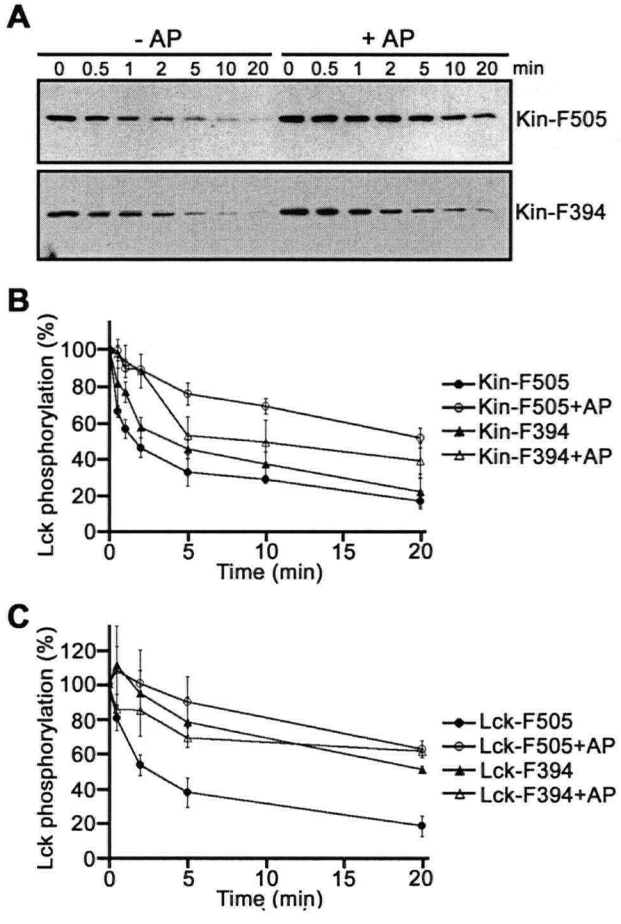

Figure 4.10. The effect of CD45AP on CD45-mediated Lck dephosphorylation. Sixty nM of soluble thrombin-cleaved full-length Lck mutants (Lck F505 and Lck F394) or Lck kinase domain alone mutants (Kin F505 and Kin F394) were incubated with 60 nM soluble 6His-CD45 in the presence or absence of 300 nM CD45APcyt Δ157-197 at 30°C for various time points. (A) Western blot analysis of Lck kinase domain phosphorylation using the 4G10 anti-phosphotyrosine antibody. The presence or absence of 300 nM CD45APcyt Δ157-197 is shown as "AP+" and "AP-" respectively. (B and C) Graphical analysis of Lck kinase domain and full-length Lck dephosphorylation, respectively. Phosphorylation at time 0 was taken as 100%, and values represent the average of 3 experiments with error bars error bars representing standard deviation.

preference for inhibition at Tyr 394 (Kin-F505) compared to Tyr 505 (Kin-F394). The average of 3 experiments is represented in figure 4.10B. Assays were then repeated with the predicted open and closed forms of the full-length Lck mutants (Lck F505 and Lck F394). In this case, the presence of CD45AP clearly inhibited the rate of Lck dephosphorylation at the Tyr 394 residue, whereas no significant effect was observed at Tyr 505 (Fig. 4.10C). This data suggests that in the cell, a function of CD45AP could be to sustain Lck activation by inhibiting CD45-mediated Tyr 394 dephosphorylation upon TCR signaling.

4.4 Discussion

4.4.1 CD45AP associations with Lck and CD45 in T cells

Previous work has provided support for a CD45AP:Lck association in T cells. One study reported an association of CD45AP with Lck only under mild lysis conditions with 1% digitonin (248), while another could only detect the presence of an association due to CD45AP-associated Lck kinase activity (214). Here, figure 4.2 provides supporting evidence that Lck can associate with CD45AP in T cells and reveals that this association occurs to a similar degree to that of Lck and CD45. Moreover, in the cell, approximately 10-fold more CD45AP associates with CD45 than with Lck (Fig. 4.3), however the expression of CD45 protein (175 ± 23 fmol/$2x10^5$ cell equivalents) is also substantially higher than that of Lck protein (25 ± 7 fmol/$2x10^5$ cell equivalents). Thus, the preference of CD45AP for the association with CD45, as opposed to Lck, could be due to differences protein expression levels rather than a binding affinity property.

A major caveat to the quantitation of T cell proteins in this chapter is the lack of a defined standard curve of known recombinant proteins included in the analysis (Figs. 4.2, 4.3, 4.4). In these experiments, spot densitometry analysis from only a single sample point allowed for a rough quantitation of proteins isolated from T cells. A second caveat of these experiments is the absence of a negative control for co-immunoprecipation. Future experiments should include controls to show that these associations defined by co-immunoprecipitation are specific and not due to background non-specific binding alone.

The association of CD45AP with CD45 is well established, and one study has shown that most of the CD45 in T lymphocytes is associated with CD45AP in T cells with an estimated 1:1 stoichiometry (209). In contrast, the stoichiometry of the CD45AP:Lck association in T cells remained unclear. Here, in agreement with previous work, the CD45AP:CD45 stoichiometry was determined to be 1:1, while the CD45AP:Lck stoichiometry was approximately 1:10 (Fig. 4.3). Moreover, upon CD45AP over-expression, these stoichiometries were increased to 2:1 and 1:2 respectively, suggesting a correlation between CD45AP expression and its ability to associate with CD45 and Lck. Studies investigating the transmembrane CD45:CD45AP interaction were unable to definitively localize this association to any particular region of the CD45 transmembrane domain, and reported that no single amino acid in this domain was essential for the interaction with CD45AP (268). This, together with the 2:1 stoichiometry determined for the CD45AP:CD45 association upon CD45AP over-expression, suggests that the transmembrane domain of CD45 may contain multiple sites capable of mediating interactions with more than one CD45AP molecule.

Figure 4.4 shows that in accordance with a recent study (220), significant amounts of CD45AP are maintained in T cell lysates that have been depleted of CD45, and that a population of CD45AP, but not CD45, sediments at a much slower rate during sucrose gradient centrifugation (data not shown). Together, these data suggest that a pool of non-CD45-associated CD45AP exists in the cell and that this pool may represent a lipid raft-localized population of CD45AP. Moreover, despite an approximate 7-fold reduction in CD45 levels upon its depletion from T cell lysates, the pool of CD45AP associated with Lck was only modestly affected. This suggests that the CD45AP:Lck association can occur as a bimolecular complex in T cells expressing CD45, and raises the possibility that it occurs in lipid-raft fractions of the cell membrane where a population of Lck, but not CD45 to any significant extent, has been shown to exist (68).

This data does not eliminate the possibility of the putative tri-molecular complex between CD45, CD45AP and Lck in cells since approximately 14% of CD45 remained in cell lysates upon CD45 "depletion". Moreover, the slight reduction in the CD45AP:Lck association upon CD45 depletion suggests that low amounts of a tri-molecular complex may indeed exist in resting T cells.

4.4.2 Characterizing the CD45AP:Lck interaction *in vitro*

Figure 4.5 shows that in accordance with another study (220), purified recombinant forms of cytoplasmic CD45AP and Lck are capable of a direct protein–protein interaction *in vitro*. Furthermore, the C-terminal 40 amino acids of CD45AP are shown to be dispensable for its interaction with Lck since a truncated CD45AP mutant (CD45cyt Δ157-197) bound similar amounts of Lck protein as the full-length CD45APcyt construct. This is in agreement with previous work where the

CD45AP:Lck association in COS-1 cells was localized to a 60 amino region in the cytoplasmic domain of CD45AP encoding the N-terminal putative PEST region (PEST 1, see figure 4.1) or "acidic domain" (221). Here, the interaction between CD45AP and Lck was shown to occur independently of Lck phosphorylation or conformation status, and was localized to the kinase domain of Lck in agreement with non-catalytic Lck domains being dispensable for the CD45AP:Lck association in COS-1 cells (221). Together, these findings suggest a direct cytoplasmic interaction between CD45AP and Lck in the cell, localized to the Lck kinase domain and the PEST 1 region of CD45AP.

Recent work in our laboratory identified an Lck association with the second phosphatase domain of CD45 (D2) that was also localized to the kinase domain of Lck and was phosphorylation-independent (J. Felberg, unpublished data). The interaction of D2 and Lck was further localized to Lck SD10 at the base of the large lobe of the kinase domain (see chapter 3). Figure 4.7 shows that CD45AP does not bind to Lck SD10 suggesting that D2 and CD45AP bind separate epitopes of the Lck kinase domain. Consistent with this, despite binding approximately 6-fold stronger to Lck, cytoplasmic CD45AP was unable to eliminate D2 binding to the Lck domain even when present in 5-fold molar excess (Fig. 4.8), suggesting a relatively weak ability to prevent binding. The occurrence of some CD45AP-induced reduction in D2 binding could be due to steric hindrance despite binding to different sites.

Thus a candidate region in the Lck kinase domain for CD45AP binding is the exposed face of the small upper lobe (see Fig. 1.3). It is worthy to note that this region remains exposed even in the closed conformation of Src-family kinases (Fig. 1.2). This is also in agreement with the independence of the CD45AP:Lck interaction with respect

to Lck phosphorylation and conformation (Fig. 4.6). Moreover, it is plausible that an interaction between the PEST1 region of CD45AP and the upper lobe of the kinase domain could place the C-terminus of the PEST1 region near the Lck activation loop. Such an interaction could then block CD45 access to pTyr 394 (Fig 4.10), while creating little competition for D2 access to Lck SD10 (Fig. 4.8).

4.4.3 The role of CD45AP in CD45-mediated regulation of Lck

Currently there is conflicting evidence about the role of CD45AP in CD45-mediated Lck regulation. Studies in LPAP deficient Jurkat T cells (269), and two separate strains of CD45AP-knockout mice (218, 219), revealed that the loss of CD45AP has no effect on the CD45:Lck interaction. Conversely, a CD45AP preference for the activated forms of Lck led to speculation that CD45AP may recruit these molecules to CD45 for down-regulation, or to the TCR to amplify signaling (221). Interestingly, as shown in figure 4.9, we find that cytoplasmic CD45AP has a comparable affinity to CD45C817S for binding Lck, and inhibits the CD45C817S:Lck interaction. Given that CD45C817S binds Lck largely via its catalytic pocket (chapter 3, Figs. 3.1 to 3.3), and that CD45AP and D2 compete weakly for binding to the Lck kinase domain, this data suggests that CD45AP blocks the catalytic interaction between CD45 domain 1 and Lck.

These findings must be considered in light of the fact that cytoplasmic CD45AP does not interact with cytoplasmic CD45, since this interaction occurs exclusively via their transmembrane domains. Thus, this *in vitro* data does not preclude the possibility that CD45AP recruits Lck to CD45 in the cell via its ability to bind to Lck in the cytoplasm, and to CD45 in the membrane. However, data shown here suggests that the association of CD45AP with Lck is largely bi-molecular in resting T cells. Thus further

investigation is required to determine the ability of CD45AP to affect Lck localization and regulation in the cell.

Nevertheless, the CD45AP-mediated inhibition of the CD45:Lck interaction was also shown to specifically inhibit CD45-mediated dephosphorylation of the Lck activation loop (Tyr 394). This finding is in accordance with recent data from our laboratory showing that CD45AP over-expression in T cells produces enhanced and prolonged phosphorylation in response to CD3 stimulation (D. Wong and P. Johnson, unpublished), and with data from one strain of CD45AP-knockout mice showing reduced T cell proliferation compared to wild type (270). This suggests a role for CD45AP in protecting Lck from CD45-mediated down-regulation of during T cell signaling events allowing for sustained Lck activation.

Although this study has provided novel evidence supporting a role for CD45AP in CD45-mediated Lck regulation, the role of CD45AP in a bi-molecular association with CD45 remains unclear. Given the findings that a population of CD45AP is present in the lipid-raft fractions of sucrose gradients (data not shown, (220)), and the indication that a small amount of CD45 transiently enters the immune synapse (125), it is possible that CD45AP serves a role in facilitating CD45 entry into coalesced lipid rafts during T cell signaling.

In summary, this work further characterizes the interactions of CD45AP with CD45 and Lck both in T cells and *in vitro*. Moreover, these results suggest a role for CD45AP in potentiating TCR signaling events as an antagonist to CD45-mediated Lck down-regulation by retarding the dephosphorylation of the activating Tyr 394 residue in the Lck kinase domain.

CHAPTER 5

A characterization of the interaction between CD44 and Lck

5.1 Introduction and rationale

CD44 is a widely expressed transmembrane adhesion molecule that binds to hyaluronan (HA), a component of the extracellular matrix. An isolated definitive function of CD44 has been difficult to determine since CD44-knockout mice appear normal (271). However, CD44 has been implicated in several biological processes including leukocyte extravasation, wound healing and tumour metastasis (reviewed in 128). Although devoid of any intrinsic enzyme activity, upon clustering CD44 is known to induce an intracellular signaling cascade resulting in cytoskeletal reorganization (reviewed in 272).

In T cells, Lck co-immunoprecipitates with CD44 and has been implicated in proximal signaling events induced by CD44 ligation (141). The CD44:Lck association was also shown to occur in low-density sucrose fractions exclusively, suggesting that this interaction occurs in the lipid-raft domains of T cell membranes (68, 142). Moreover, upon CD44 crosslinking, both Lck and Fyn are recruited to lipid-raft domains where they associate with CD44 (106). The treatment of T cells with Src-family kinase inhibitor PP2 has also been shown to block CD44-mediated cytoskeletal changes (68). Likewise, the clustering of CD44 is known to activate Lck (141) and induce the tyrosine phosphorylation of Pyk2, Cas and FAK (68). Thus, a role for Lck in CD44 signaling is becoming increasingly apparent.

There is also evidence of a CD44:CD4 association on the surface of T cells (273). The same study revealed that CD4 stimulation with gp120 potentiates CD44-mediated cell adhesion, indicating a functional interplay between CD44 and CD4 in this process. The apparent synergy between CD44 and CD4 stimulation in T cell adhesion is of

particular interest since both molecules are known to associate with Lck. It is well established that Lck interacts with CD4 and CD8α in a zinc-dependent manner (38, 39) and that dual cysteine residues in the membrane-proximal regions of both Lck and CD4/CD8α are critical for this interaction (274). Moreover, the function of CD4 in TCR signaling is dependent on its association with Lck (275, 276). Together, these findings prompted studies to investigate the possibility of a zinc-mediated CD44:Lck interaction.

Despite the mounting evidence for a role of Lck in CD44 function, the molecular basis of the CD44:Lck interaction remains poorly understood. Interestingly, the cytoplasmic domain of CD44 has been shown to mediate a direct interaction with c-Src *in vitro* (143), supporting the ability of CD44 to interact with Src-family kinases directly. However, evidence for a direct protein–protein interaction between CD44 and Lck is lacking. Thus, the goal of this study was to investigate a direct CD44:Lck interaction *in vitro*, characterize the CD44:Lck association in T cells, and determine if this association was required for CD44 function.

5.2 Experimental approach

Current knowledge of the association between CD44 and Lck is limited to a mutual localization of these molecules to lipid-raft domains in the T cell. In light of the fact that Src-family kinase activity is essential for CD44-induced cytoskeletal changes in T cells, the interaction of CD44 and Lck was investigated and characterized in the present study. To investigate the potential involvement of a zinc cation in the CD44:Lck interaction, both CD44-mediated cell spreading and the ability of CD44 to co-immunoprecipitate Lck were assayed their sensitivity to the cation chelator, 1,10-

phenanthroline. Although cell spreading is an artificial process induced *in vitro*, the cytoskeletal changes that result in the spread phenotype (cell flattening and elongation) are thought to mimic potential CD44-mediated cytoskeletal reorganization during lymphocyte extravasation *in vivo*.

To investigate a possible direct protein-protein interaction between the cytoplasmic domain of CD44 and Lck, recombinant forms of these proteins were produced in bacteria and purified. Various truncations and mutations of these recombinant proteins were also produced to characterize the CD44:Lck interaction and identify the specific regions involved.

5.3 Results

5.3.1 CD44-mediated T cell spreading is blocked by 1,10-phenanthroline

To determine if CD44-mediated T cell spreading requires the presence of a divalent cation, $CD45^-$ $TCR/CD3^+$ BW5147 T lymphoma cells (BW- cells) underwent a 20 minute, 37°C incubation in the presence or absence of the membrane-permeable, divalent cation chelator 1,10-phenanthroline prior to plating on immobilized anti-CD44 monoclonal antibody, KM201 (which recognizes the HA binding site of CD44). Figure 5.1 shows that in the absence of the cation chelator, BW- cells underwent the distinct morphological change of flattening and elongating (referred to hereafter as "spreading") when incubated on immobilized KM201. However, prior cell treatment with 2.5 mM 1,10-phenanthroline blocked the ability of BW- cells to undergo CD44-mediated cell spreading, and induced a cell phenotype comparable to those incubated on the bovine

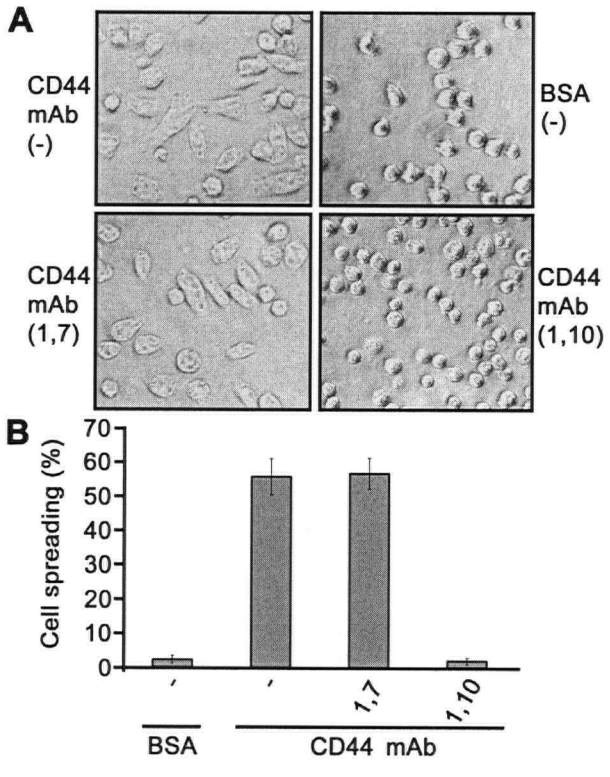

Figure 5.1. The effect of 1,10-phenanthroline on CD44-mediated spreading of CD45$^-$ BW5147 T cells. (A) BW- cells ($5x10^4$) were resuspended in 25 µl of binding media alone (see Materials and methods) or in binding media with 2.5 mM 1,7- or 1,10-phenanthroline and then incubated at 37°C, for 20 minutes. Cells were then pelleted, and resuspended in binding media alone before being immobilized for 2 h at 37°C on either BSA or anti-CD44 mAb (KM201) coated tissue culture plates. (B) Graphical analysis of the percentage of spread cells after 2 h. Values represent the average of 3 separate experiments, and error bars represent standard deviation.

serum albumin (BSA) control (Fig. 5.1). This inhibition of spreading was not due to the hydrophobicity of phenanthroline since cell spreading was maintained when treated with a non-chelating analog, 1,7-phenanthroline, nor was it due to the presence of the ethanol solvent alone (data not shown).

Additional experiments revealed that prior cell treatment with 1,10-phenanthroline concentrations as low as 0.5 mM could reduce the percentage of spread cells from 58 $\pm$ 5% (in the absence of phenanthroline) to 37 $\pm$ 12% (data not shown). However, a titration of 1,10-phenanthroline indicated that a concentration 2.5 mM was required to abolish cell spreading via CD44. It is worthy to note also, that at concentrations of 5 mM, prior cell treatment with 1,7-phenanthroline induced a modest reduction in the percentage of spread cells from 58 $\pm$ 5% (in the absence of phenanthroline) to 48 $\pm$ 3% (data not shown). Together, these data indicate that prior cation chelation by 1,10-phenanthroline results in the disruption of cell spreading. This suggests that the presence of a divalent cation is required for this process, possibly in CD44 signaling events that result in cytoskeletal changes.

5.3.2 CD4 and CD44 both induce 1,10-phenanthroline-sensitive T cell spreading

The dependency on divalent cations for CD44-induced T cell spreading was reminiscent of the divalent cation dependence of the interaction between Lck and CD4 or CD8α. To investigate if there were similarities between CD44 and CD4 signaling, $CD4^+$ TCR/$CD3^+$ $CD45^+$ AKR1 T lymphoma cells transfected with the hematopoietic form of CD44 were assayed for their ability to flatten and elongate on immobilized anti-CD44 (KM201) or anti-CD4 (GK1.5) antibodies. Figure 5.2 shows that some AKR1 cells underwent CD44- and CD4-mediated cell spreading in the absence of a cation chelator,

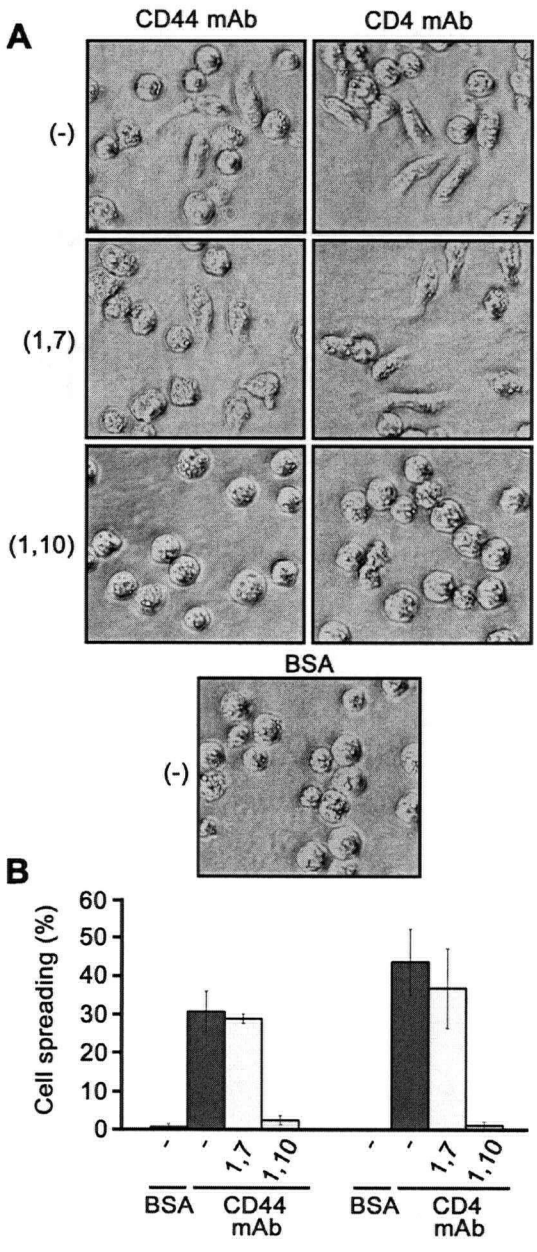

Figure 5.2. The effect of 1,10-phenanthroline on CD44- and CD4-mediated spreading of AKR1 T cells. (A) CD44.1-transfected AKR1 cells ($5x10^4$) were treated as described in figure 5.1 except in addition to BSA and KM201, cells were also immobilized on anti-CD4 mAb (GK1.5) coated tissue culture plates. (B) Graphical analysis of the percentage of cell spreading after 2 h. Values represent the average of 3 separate experiments, and error bars represent standard deviation.

albeit in lower percentages compared to BW- cells (Fig. 5.1). One reason for this could be the presence of CD45 (in AKR cells), a molecule that has been shown previously to negatively regulate CD44-mediated T cell spreading (68). Furthermore, the cell spreading mediated by both CD44 and CD4 molecules was abrogated by the pre-treatment of cells with 2.5 mM 1,10-phenanthroline, but was unaffected by the 1,7 analog (Fig. 5.2).

These findings indicate that the ligation of CD44 and CD4 can induce similar changes in cell morphology, suggesting similar signaling functions between these two molecules. Moreover, the functional sensitivity to 1,10-phenanthroline by both molecules suggests a common cation component in their signaling events.

5.3.3 The interaction between CD44 and Lck is reduced by 1,10-phenanthroline

To determine if the role of divalent cations in CD44-mediated cell spreading is to link CD44 to proximal signaling kinases, the associations of CD44 with Lck and Fyn were assayed for their sensitivity to 1,10-phenanthroline. Figure 5.3A shows that a 20 minute, 37°C incubation of AKR1 cells with 5 mM 1,10-phenanthroline (referred to as "cell treatment") prior to lysis reduced the CD4:Lck interaction by approximately 2-fold, and nearly abrogated the interaction when the chelator was also present in the lysis buffer. Likewise, figure 5.3B shows a similar effect of 1,10-phenanthroline on the CD44:Lck interaction. Spot densitomety analysis revealed that AKR1 cell treatment alone with 5 mM 1,10-phenanthroline reduced the interaction to approximately 60%, and to approximately 15% if also present in the lysis buffer. This effect was also observed with a 2.5 mM concentration of 1,10-phenanthroline, however at 1 mM a more modest effect was observed (data not shown). The reduction in the CD44:Lck and CD4:Lck

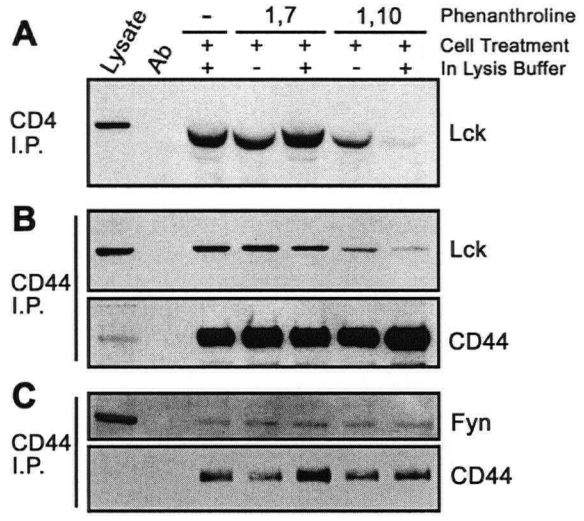

Figure 5.3. The effect of 1,10-phenanthroline on the association of CD44 with Lck and Fyn in AKR1 T cells. CD44.1-transfected AKR1 cells (10^7) were resuspended in 2 ml of media alone (DMEM plus 10% horse serum, see Materials and methods), or in media containing 5 mM 1,7- or 1,10-phenanthroline and incubated at 37°C for 20 minutes (Cell Treatment). Cells were then lysed in 1% Brij-58 lysis buffer alone, or buffer containing 1,7- or 1,10-phenanthroline (In Lysis Buffer). Lysates underwent anti-CD44 or anti-CD4 immuno-precipitations with bead-immobilized IM7 or GK1.5 antibody respectively, before SDS-PAGE and Western blot analysis. The presence and absence of phenanthroline in the media or buffer is indicated with (+) and (-) respectively. (A) Western blot analysis of Lck (R54 anti-Lck antiserum) from a CD4 immuno-precipitation. (B) Western blot analysis of a CD44 immuno-precipitation; upper panel blotted for Lck (R54), lower panel blotted for CD44 (JIWBB anti-CD44 antiserum). (C) As described in (B) except upper panel blotted for Fyn (anti-Fyn antiserum). "Lysate" represents the soluble lysate from $2x10^5$ cell equivalents. "Ab" represents a control lane loaded with immunoprecipitating antibody alone.

associations with cell treatment alone suggests that 1,10-phenanthroline is capable of crossing the cell membrane and chelating cations in the cytoplasm. Interestingly, the association of CD44 with Fyn was unaffected by the presence of 5 mM 1,10-phenanthroline under any conditions tested. The presence of 5 mM 1,7-phenanthroline did not affect the ability of CD44 or CD4 to interact with their respective Src-family kinases indicating that the effects of 1,10-phenanthroline were specific to its cation chelating ability, and not to other properties such as its hydrophobicity.

These findings indicate that like CD4, CD44 interactions with Lck are dependent on a divalent cation. Furthermore, CD44 associations with the Src-family kinases Lck and Fyn are shown to be distinct in AKR1 T cells.

5.3.4 Zinc mediates a direct interaction between CD44 and Lck *in vitro*

The common cation dependency between CD44 and CD4 in their induction of cell spreading and association with Lck (Figs. 5.2 and 5.3) suggested that, like CD4, CD44 might also interact with Lck via a single zinc cation. Since the CD44:Fyn association in T cells was not affected by 1,10-phenanthroline (Fig. 5.3), it was hypothesized that this interaction could be cation-independent and possibly indirect in the cell.

To investigate these interactions directly, GST-Lck and GST-Fyn were purified from *E. coli*, eluted from glutathione sepharose beads, and utilized as soluble fusion proteins. Cytoplasmic CD44 (residues 272 to 343) was purified from bacteria as an MBP fusion protein and left immobilized to amylose beads. A summary of recombinant CD44 proteins used is provided in figure 5.4A. GST-Lck and GST-Fyn were incubated at a final concentration of 0.1 μM with 1.5 μM MBP-CD44 or 3.0 μM MBP alone in the presence or absence of 50 μM $ZnCl_2$. Figure 5.5 shows that in the absence of zinc,

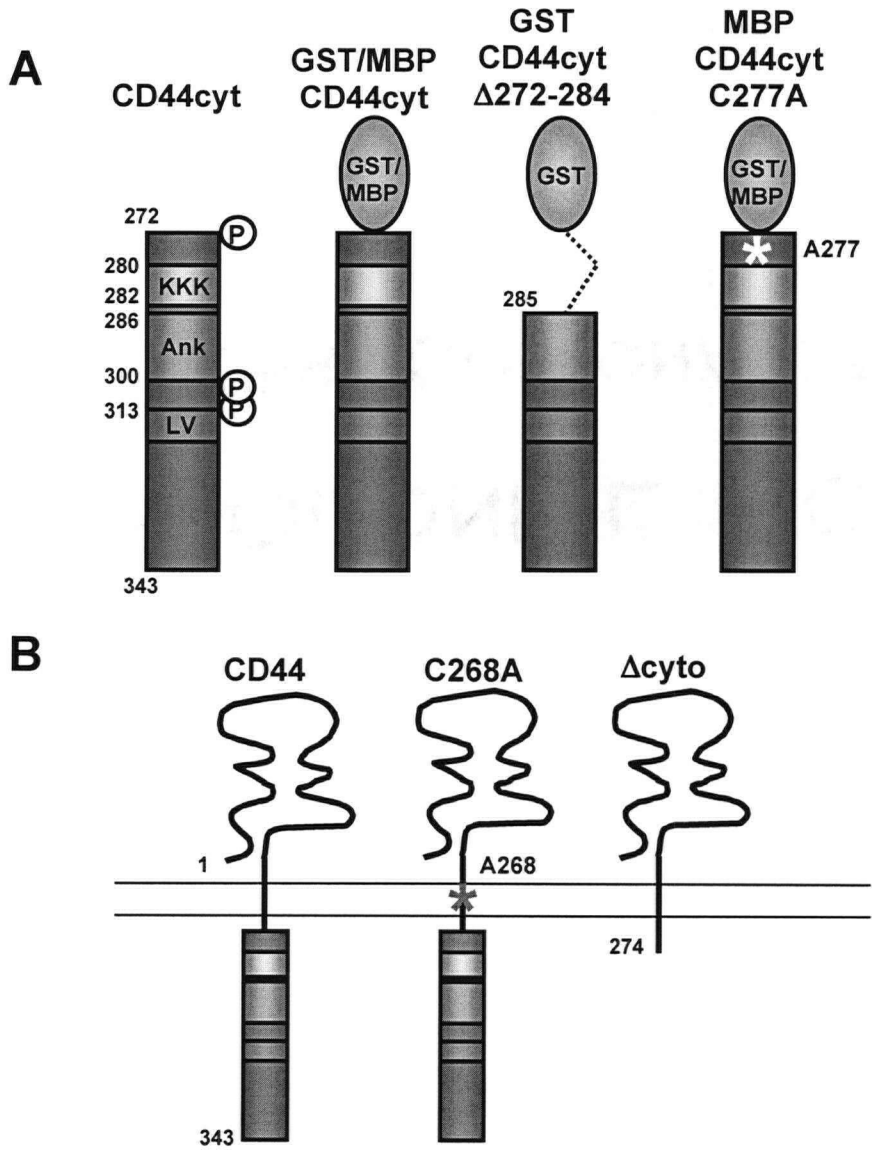

Figure 5.4. Schematic diagram of various CD44 constructs. (A) Primary structure illustration of recombinant cytoplasmic CD44 constructs purified from bacteria as GST or MBP fusion proteins. The far left structure represents the cytoplasmic domain of wild type CD44 from T cells. "TM" represents the transmembrane domain, "KKK" represents a basic region implicated in binding the ezrin, radixin and moesin (ERM) proteins, "Ank" represents a region implicated binding ankyrin, "LV" represents a motif implicated in CD44 basolateral sorting, and "P" represents potential sites of serine phosphorylation. (B) Illustration of various CD44 constructs transfected into AKR1 cells. "CD44" represents wild type hematopoietic CD44.1, "C268A" has a Cys 268 to Ala point mutation in the transmembrane domain, and "Δcyto" represents a construct expressing only the first two amino acids of the cytoplasmic domain. All numbers represent murine CD44 residue positions as reported (231).

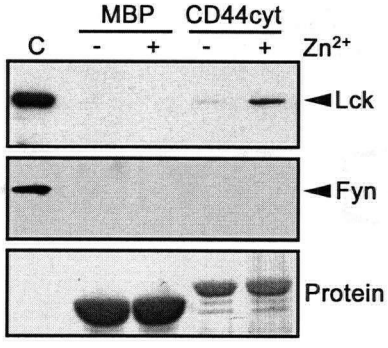

Figure 5.5. The effect of zinc on the binding of recombinant Lck and Fyn to cytoplasmic CD44. Upper and middle panels represent Western blot analyses of soluble GST-Lck and GST-Fyn (probed with anti-GST antiserum) that bound to immobilized cytoplasmic CD44 fused to MBP (CD44cyt) or to MBP alone (MBP) in the presence (+) or absence (-) of 50 μM $ZnCl_2$ (see Materials and methods assay details). Lower panel is a Coomassie blue protein stain showing the amounts of immobilized MBP protein used in each assay. "C" represents a control lane in which 50 ng of GST-Lck or GST-Fyn was loaded directly onto the gel.

cytoplasmic CD44 maintained a direct, cation-independent interaction with soluble GST-Lck *in vitro*. However, this interaction was induced approximately 10-fold in the presence of 50 μM $ZnCl_2$. Additional experiments revealed that the induction of GST-Lck binding to MBP-CD44 could occur at $ZnCl_2$ concentrations of 50 and 100 μM, but not 5 μM (data not shown). GST-Fyn did not bind detectably to MBP-CD44 *in vitro* in the presence or absence of zinc (Fig. 5.5) suggesting that the CD44:Fyn interaction may be indirect in the cell.

Despite demonstrating that Zn^{2+} potently induces the CD44:Lck interaction specifically, it remained unclear if other cations were also capable of this induction. Thus, the specificity of this interaction with regards to the zinc cation was investigated. Recombinant cytoplasmic CD44 and Lck were produced in *E. coli* as GST fusion proteins. Upon purification from bacteria, GST-CD44 cytoplasmic domain and GST-Lck were immobilized to glutathione sepharose beads and washed repeatedly. Immobilized GST-Lck was subsequently incubated with the thrombin protease to cleave Lck from GST allowing for the recovery of soluble Lck from the supernatant. Soluble thrombin-cleaved Lck (at 0.1 μM) was then used in an *in vitro* binding assay to characterize the direct interaction with immobilized GST-CD44 (at 1.5 μM). GST-CD44 was assayed at 1.5 μM since in a 40 μl volume, this concentration is equivalent to 2 μg of immobilized protein, an amount consistently used in *in vitro* binding assays of this thesis (see Materials and methods). The relatively poor yield of soluble Lck protein upon purification from bacteria resulted in the use of this protein at 0.1 μM in binding assays with immobilized CD44.

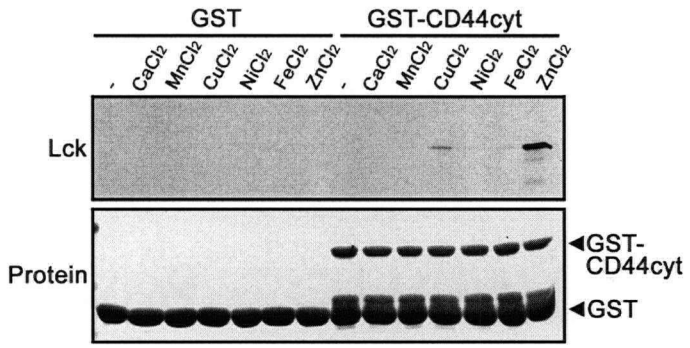

Figure 5.6. The effect of various cations on the binding of recombinant Lck to cytoplasmic CD44. Upper panel: Western blot analysis of soluble thrombin-cleaved Lck (probed with R54 anti-Lck antiserum) that bound to immobilized cytoplasmic CD44 fused to GST (CD44cyt) or to GST alone (GST) in the presence or absence of various cations at 50 μM (see Materials and methods assay details). Lower panel: Coomassie blue protein stain showing amounts of immobilized GST protein used in each assay.

Various divalent cations at a final concentration of 50 μM were tested for their ability to induce the CD44:Lck interaction. Figure 5.6 shows that this interaction was specific to the zinc cation, although copper and iron cations were also capable of a moderate augmentation. Although not apparent in this figure, longer exposures indicated a persistent CD44:Lck interaction in the absence of any cation that occured above background binding. Longer exposures also revealed that 50 μM zinc could induce Lck binding to GST alone by approximately 2-fold (data not shown). However this background induction is inferior when compared to the approximately 10-fold induction observed for the GST-CD44:Lck interaction.

Together, this data demonstrates a direct interaction between the cytoplasmic domain of CD44 and Lck that is composed of a major zinc-dependent interaction and a minor zinc-independent interaction *in vitro*.

5.3.5 The CD44:Lck association involves zinc-dependent and zinc-independent components in the cell

Given the above data indicating a direct cytoplasmic interaction between CD44 and Lck *in vitro*, we next investigated the contribution of the CD44 cytoplasmic domain to the CD44:Lck association in the cell. To determine this, AKR1 T cells were transfected with plasmids encoding full-length CD44.1, CD44 C268A (Cys 268 to Ala mutation in the transmembrane domain), and CD44Δcyto (lacking all but 2 cytoplasmic residues, see Fig.5.4B), which were all expressed to comparable levels on the cell surface (FACS analysis, data not shown). Figure 5.7 shows that the cytoplasmic domain of CD44 was critical for its association with Lck. The deletion of this region reduced the CD44:Lck interaction by approximately 6-fold, comparable to the reduction observed in

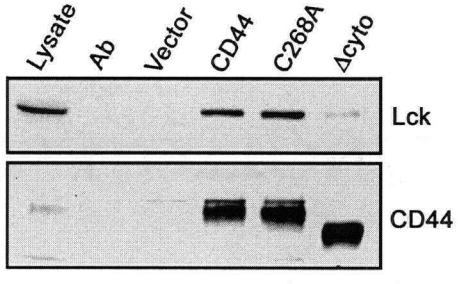

Figure 5.7. Determining the regions of CD44 involved in its association with Lck in AKR1 T cells. AKR1 cells (10^7), transfected with various forms of CD44, were lysed in 1% Brij-58 lysis buffer and incubated for 2 h with immobilized anti-CD44 antibody (IM7) to immunoprecipitate CD44. Upper and lower panels represent Western blot analyses of Lck and CD44 probed with R54 anti-Lck anitserum and KM201 anti-CD44 antibody respectively, from various CD44 immunoprecipitations. "CD44" represents wild type hematopoietic CD44.1, "C268A" represents full-length CD44 with a Cys 268 to Ala mutation, and "Δcyto" represents CD44 expressing only the first two amino acids of the cytoplasmic domain. "Lysate" represents soluble cell lysate from $2x10^5$ cells, and "Ab" represents a control lane loaded with IM7 anti-CD44 antibody alone.

the presence of 1,10-phenanthroline (Fig. 5.3). A point mutation of Cys 268 to Ala located in the transmembrane region of CD44 had no effect on the association of CD44 with Lck in AKR1 T cells. However, the necessity of the cytoplasmic region of CD44 suggested that a cytoplasmic cysteine might mediate the zinc-dependent interaction with Lck.

Together, data showing a weak association of CD44Δcyto with Lck and a persistent CD44:Lck association in the presence of 1,10-phenanthroline (Fig. 5.3) suggested that the CD44:Lck association in cells involves a minor component that is independent of the zinc-induced cytoplasmic interaction. To investigate this, the CD44Δcyto mutant was tested for its ability to associate with Lck in the presence of 1,10-phenanthroline. Figure 5.8 shows that at 2.5 mM concentrations of phenanthroline, the full-length CD44:Lck interaction maintained its sensitivity to the cation chelator (reduced to approximately 15%), but the CD44Δcyto:Lck interaction was not reduced significantly (Fig. 5.8B). CD44 immunoprecipitations initially conducted under 5 mM concentrations of phenanthroline revealed that 1,10-phenanthroline could modestly reduce the CD44Δcyto:Lck to approximately 75% (data not shown).

Together this data suggests that the CD44:Lck association in T cells is mediated both by a direct zinc-inducible cytoplasmic interaction, and by a weaker non-cytoplasmic association that is likely via a common association with lipid-raft domains.

5.3.6 Membrane-proximal regions of CD44 and Lck mediate the zinc-dependent interaction

To determine if the membrane-proximal regions of CD44 and Lck mediated the direct zinc-dependent interaction, purified recombinant proteins were assayed *in vitro*.

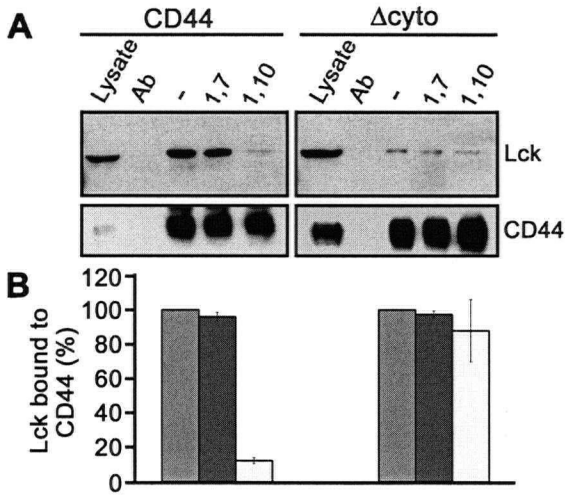

Figure 5.8. Determining the region of CD44 affected by the 1,10-phenanthroline-mediated disruption of the CD44:Lck association in AKR1 T cells. Conditions are as described in figure 5.3. Upper and lower panels represent Western blot analyses of Lck and CD44 probed with R54 anti-Lck and KM201 anti-CD44 antibody respectively from anti-CD44 immuno-precipitations. "CD44" represents wild type hematopoietic CD44.1, and "Δcyto" represents CD44 expressing only the first two amino acids of the cytoplasmic domain. "Lysate" represents soluble cell lysate from $2x10^5$ cells. "Ab" represents a control lane loaded with anti-CD44 antibody alone. (B) Graphical representation of the amount of Lck that co-precipitates with CD44 in the presence (1,7 or 1,10) or absence (-) of 2.5 mM phenanthroline. Lck amount in the absence of phenanthroline was standardized to 100%. Values represent the average of 4 experiments with error bars representing standard deviation.

Full-length cytoplasmic CD44 and a deletion mutant lacking cytoplasmic membrane-proximal residues 272 to 284 (Δ272-284) were purified from *E. coli* as immobilized GST fusion proteins. Bead-immobilized GST-Lck kinase domain (Kin) and GST-Lck non-catalytic domains (N32; composed of Lck N, SH3 and SH2 domains) were also produced and were subsequently treated with the thrombin protease to create soluble Kin and N32 proteins that were collected from the supernatant. Immobilized CD44 constructs were then tested for the ability to bind to soluble Kin and N32 in the presence or absence of 50μM $ZnCl_2$.

Figure 5.9 shows that Lck N32 (containing the zinc-binding Cys 20 and Cys 23 residues) was induced by zinc to bind to GST-CD44 while the Lck kinase domain bound to GST-CD44 in a zinc-independent manner. Interestingly, the kinase domain exhibited a reduced but persistent ability to bind to the CD44 Δ272-284 mutant independently of zinc. This suggests that membrane distal regions of CD44 might be involved in a zinc-independent interaction with the Lck kinase domain. In contrast, Lck N32 did not bind significantly to the CD44 Δ272-284 mutant in the presence or absence of zinc suggesting that this region is critical for the zinc-dependent interaction. These results demonstrate a complex cytoplasmic CD44:Lck interaction composed of both a zinc-dependent interaction between Lck N32 and a 13 amino acid membrane-proximal region of CD44, and a zinc-independent interaction largely mediated by the Lck kinase domain.

5.3.7 Cys 277 in the cytoplasmic domain of CD44 is required for the zinc-dependent interaction with Lck

Data shown in figure 5.9 localized the zinc-dependent interaction between CD44 and Lck to a 13 amino acid region in the membrane-proximal region of CD44. To

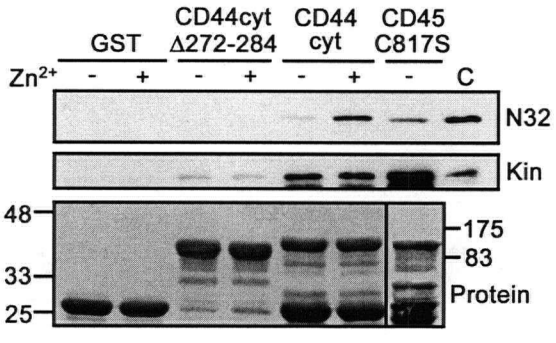

Figure 5.9. ***In vitro*** **binding assay defining the zinc-dependent binding of CD44 to Lck.** The upper and middle panels represent Western blot analyses of soluble thrombin-cleaved Lck N32 and Lck Kin (probed with R54 anti-Lck anti-serum, and 2102 anti-Lck antibody respectively) that bound to immobilized cytoplasmic GST-CD44 (CD44cyt), a 13 amino acid cytoplasmic CD44 deletion mutant (CD44cyt Δ272-284), or GST alone (GST) in the presence (+) or absence (-) of 50 μM $ZnCl_2$ (see Materials and methods assay details). "C" represents a control lane in which 50 ng of N32 or Kin were loaded directly onto the gel. The lower panel represents a Coomassie blue protein stain showing amounts of immobilized GST protein used in each assay. Molecular mass standards are indicated in kDa.

determine if a cysteine residue in this region mediates the zinc-dependent CD44:Lck interaction, a CD44 Cys 277 to Ala mutant was created and expressed as an immobilized MBP fusion protein. This construct was then tested for its ability to bind to soluble eluted GST-Lck in the presence and absence of $ZnCl_2$. Figure 5.10 shows that unlike wild type CD44, the C277A mutant cannot be induced to bind Lck in the presence of zinc. However, this mutant does maintain the relatively weak zinc-independent Lck interaction shown in figures 5.5 and 5.9. This indicates that the CD44 membrane-proximal Cys 277 residue is important in the zinc induction of the CD44:Lck interaction.

5.4 Discussion

5.4.1 The effects of CD45 expression and divalent cation chelation on CD44 function

Previous work in our laboratory established that a $CD45^-$ T cell line underwent distinctive cell spreading when incubated on immobilized anti-CD44 antibody (68). The negative effect of CD45 expression on CD44-mediated T cell spreading suggested a correlation between CD44 function and the activity of Src-family kinases Lck and Fyn, both known to be regulated by the tyrosine phosphatase, CD45 (reviewed in 1).

Consistent with previous findings (68), $CD45^-$ BW5147 T cells displayed a significantly higher percentage of cell spreading compared to the $CD45^+$ AKR1 T cell line. This supports a role for CD45 in the down-regulation of CD44-mediated T cell adhesion in stark contrast to its role in the up-regulation of TCR signaling via its ability to dephosphorylate Src-family kinases at their inhibitory C-terminal tyrosine, a process required for T cell development and activation (80, 184, and reviewed in 277).

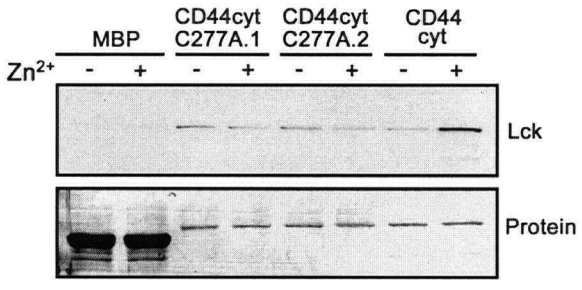

Figure 5.10. The effect of the C277A mutation on binding of CD44 to Lck *in vitro*. The upper panel represents the Western blot analysis of soluble GST-Lck (probed with R54 anti-Lck antiserum) that bound to immobilized cytoplasmic MBP-CD44 (CD44cyt), two clones of MBP-cytoplasmic CD44 with a Cys 277 to Ala mutation (CD44cyt C277A.1 and C277A.2), or MBP alone (MBP) in the presence (+) or absence (-) of 50 μM $ZnCl_2$ (see Materials and methods assay details). The lower panel represents a Coomassie blue protein stain showing amounts of immobilized GST protein used in each assay.

However, the enhancement CD44-mediated cell adhesion observed in $CD45^-$ cells presents a paradox due to the requirement of Src-family kinase activity (68) and the hyper-phosphorylation of these kinases at their inhibitory C-terminal tyrosine in absence of CD45 (reviewed in 1). Thus, the inhibition of T cell adhesion in $CD45^+$ cells is thought to occur by CD45-mediated down-regulation of Src-family kinases via dephosphorylation of the activating tyrosine residue (114). Also, Src-family kinases phosphorylated at the C-terminus may encounter high affinity SH2/SH3 ligands during adhesion signaling allowing for the release of the intramolecular bond and full kinase activation. Although the mechanisms determining the contradictory roles of CD45 in T cell signaling remain poorly understood, data shown here suggests that CD45 may specifically down-regulate a pool of CD44-associated kinases to prevent cell adhesion.

However, the use of a $CD45^-$ line in this study presents an artificial absence of CD45 that is difficult to justify in a physiological context. It is possible that the interaction between CD44 its physiological substrate, HA, during adhesion *in vivo* creates an intimate interaction between the T cell and the endothelium excluding CD45 due to its massive extracellular domain. This, combined with the fact that CD44 and Lck associate in lipid rafts alone (a domain that excludes CD45), suggests that during CD44-mediated T cell adhesion, the T cell – endothelium interface may create a membrane domain that is devoid of CD45 akin to the $CD45^-$ line studied here.

A recent study showing an interaction between CD44 and CD4 on the cell surface suggested a potential functional interplay between these two molecules, perhaps via a common signaling component in Lck (273). Interestingly, both CD44 (68, 142) and CD4 (278, 279) have been localized to lipid-raft domains where they associate with Lck, and

both have short cytoplasmic domains devoid of any known enzyme activity. Although CD4 is known to interact directly with Lck via a single zinc cation (38, 39), the details of the CD44:Lck association were unclear. Thus, given the proposed synergy between CD44 and CD4, and their common association with Lck, CD44 was investigated for the involvement of a zinc cation in a direct interaction with Lck.

Here, the treatment of T cells with 2.5 mM 1,10-phenanthroline blocked CD44-induced cell spreading. This effect was not due to the chelation of ions in the media, since after treatment with phenanthroline, the supernatant was removed, and the cells were resuspended in binding media alone (see Materials and methods for details). This suggested that 1,10-phenanthroline, known to be membrane permeable, was blocking CD44-mediated cell spreading by chelating ions within the cell. This is supported by the ability of 1,10-phenanthroline to disrupt the cytosolic, zinc-mediated CD4:Lck interaction (39), and highlights a role for divalent cations in CD44-mediated T cell adhesion.

Also important in this analysis was the observation of a modest spreading inhibition mediated by 5 mM concentrations of 1,7-phenanthroline, despite its inability to chelate cations (data not shown). This effect was likely due to phenanthroline toxicity at 5 mM, since cell membranes often appeared ruffled at this concentration (data not shown). However, cells treated with 2.5 mM 1,7-phenanthroline did not display membrane ruffling, and showed no inhibition in the ability to undergo CD44-mediated cell spreading, in contrast to treatment with 2.5 mM 1,10-phenanthroline. Together, this indicates that the cation chelating property of 1,10-phenanthroline was responsible for the inhibition of CD44-mediated T cell spreading.

Interestingly, AKR1 T cells also exhibited substantial spreading when incubated on an anti-CD4 antibody. Known for its role as a co-receptor during TCR antigen recognition and the delivery of Lck to TCR/CD3, the present study suggests a role for CD4 in T cell adhesion as well. Furthermore, like CD44, CD4-mediated T cell spreading was sensitive to the chelation of cations by 1,10-phenanthroline. Given the ability of 1,10-phenanthroline to disrupt the CD4:Lck interaction (39), this data suggested that the association of Lck with CD4 is essential for CD4-mediated cell spreading, and implied that the CD44:Lck association may be cation dependent as well.

Figure 5.3 confirmed that both the CD4:Lck and CD44:Lck interactions were sensitive to the presence of 1,10-phenanthroline. This suggested that the 1,10-phenanthroline-mediated inhibition of cell spreading was due to a common disruption in the association of Lck with CD4 and CD44. This finding is in agreement with the requirement of Src-family kinase activity for CD44-mediated cell spreading (68), and further suggested that CD44 may interact with Lck via a divalent cation. Interestingly, the association of CD44 with Fyn was not affected by cation chelation establishing Lck as the dominant Src-family kinase involved in CD44 function in T cells. Moreover, this suggests minor role for Fyn in CD44-mediated T cell spreading, possibly in the amplification of signaling initiated by Lck.

Since the association between CD44 and Lck is known to occur exclusively in lipid-raft domains, it was an initial concern that 1,10-phenanthroline may disrupt this interaction by altering the composition of plasma membrane lipids. However, sucrose gradient centrifugation studies revealed no effect of 1,10-phenanthroline on the migration of Lck to the high- and low-density fractions (data not shown).

5.4.2 Evidence for a direct, specific and zinc-induced interaction of CD44 and Lck

Figure 5.5 demonstrates that Lck maintained a weak direct interaction with the cytoplasmic domain of CD44 in the absence of a divalent cation. This interaction however, was enhanced substantially in the presence of the zinc cation. Typically, the presence of 50 μM zinc-induced the CD44:Lck interaction *in vitro* by approximately 10-fold. However, the extent of this induction varied between experiments and between various preparations of recombinant proteins, such that in certain assays, the induction of the CD44:Lck interaction was only 3- to 4-fold (Fig. 5.10). This *in vitro* data is in agreement with cell data in which cation chelation reduced, but did not abolish the CD44:Lck association. This indicates a complex interaction between Lck and the cytoplasmic domain of CD44 composed of a major zinc-dependent interaction and a minor zinc-independent interaction. This is in contrast with another study demonstrating a single high affinity site of interaction between Src and cytoplasmic CD44 *in vitro* (143). Fyn, however, did not bind to cytoplasmic CD44 in the presence or absence of zinc in agreement with this association in T cells being resistant to phenanthroline treatment. This suggests that the interaction between CD44 and Fyn is indirect in T cells and may occur due to a common localization in lipid-raft domains.

Since 1,10-phenanthroline is capable of chelating a variety of divalent cations, it was not clear if the induction of CD44 binding to Lck was specific to the zinc cation. Figure 5.6 shows that, like the CD4:Lck interaction (39), the binding of CD44 to Lck was specific to Zn^{2+} since other divalent cations including Ca^{2+}, Mn^{2+}, Cu^{2+}, Ni^{2+}, Fe^{2+} had little or no effect on the CD44:Lck interaction.

To further establish the direct, zinc inducible CD44:Lck interaction in the cell, AKR1 T cells transfected with CD44 mutants were employed. Despite evidence elsewhere that CD44 transmembrane Cys 268 mediates the interaction with Lck (280), this residue is shown here to be dispensable for the co-immunoprecipitation of Lck with CD44 from T cells. In contrast, a CD44 mutant lacking the cytoplasmic domain (CD44Δcyto) exhibited a profound reduction in its ability to associate with Lck in T cells. This data suggests that the direct cytoplasmic protein-protein interaction mediates the majority of the CD44:Lck association in T cells, but also reveals the presence of a non-cytoplasmic interaction likely mediated by a common lipid-raft association in cells.

Furthermore, unlike full-length CD44, the association of Lck with CD44Δcyto was not significantly affected by the presence of 2.5 mM phenanthroline, consistent with a membrane-mediated indirect interaction (Fig. 5.8). However, cell lysis in the presence of 5 mM 1,10-phenanthroline reduced this interaction to approximately 75% (data not shown) suggesting that this weak membrane-localized association has a slight sensitivity to the chelation of divalent cations in the lysate.

5.4.3 Cys 277 in the cytoplasmic domain CD44 mediates the zinc-dependent binding to Lck

Here, the zinc-dependent CD44:Lck interaction was localized to the membrane-proximal non-catalytic domains of Lck (N32) that contain a pair of cysteine residues (Cys 20 and 23) capable of binding zinc in an interaction with CD4 (38). In contrast, the Lck kinase domain (Kin) bound to the CD44 cytoplasmic domain in the presence or absence of zinc. Interestingly, Lck Kin, but not N32, demonstrated a low level of binding to cytoplasmic CD44 lacking 13 residues in the membrane-proximal region. Furthermore,

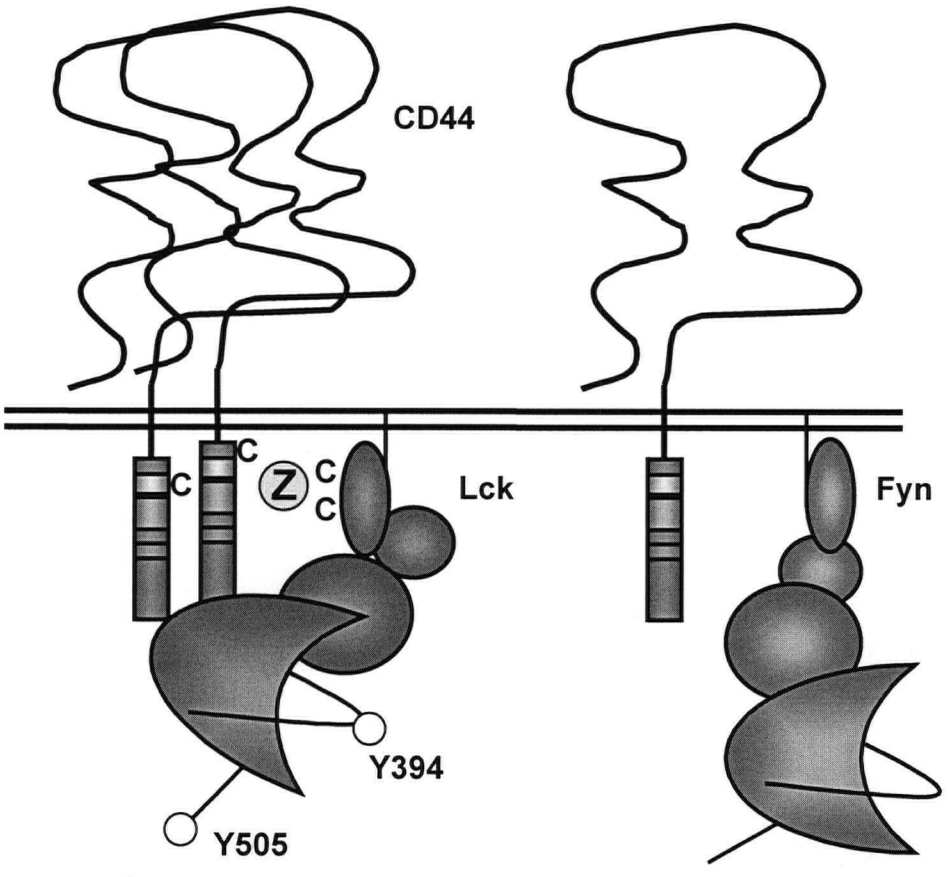

Figure 5.11. Schematic model of the CD44 association with Lck and Fyn in T cells. Globular cartoons represent individual Lck and Fyn domains, white circles represent tyrosine phosphorylation, "C" represents cysteine residues and "Z" represents a single zinc cation. Horizontal bars represent a lipid-raft domain in the T cell plasma membrane. Highlighted bands in the CD44 cytoplasmic domain are as described in figure 5.4.

this 13-residue deletion in CD44 abrogated the zinc induction of the CD44:N32 interaction localizing the zinc effect to this region. Together these results indicate two direct interactions between Lck and the CD44 cytoplasmic domain: a zinc-inducible interaction between N32 and a membrane-proximal region of CD44, and a zinc-independent interaction mediated by the Lck kinase domain and various regions of the CD44 cytoplasmic domain (see Fig. 5.11).

A mutation in CD44 Cys 277 (a residue within the 13 amino acid CD44 deletion mutant) was shown to abrogate the zinc-inducible interaction with Lck. This is consistent with findings elsewhere that show a single Cys mutation in CD4 can abolish zinc-mediated interactions with Lck (38). However, unlike CD4 and CD8α, the membrane-proximal region of cytoplasmic CD44 contains only a single cysteine, not the C-X-C motif required to chelate zinc. Currently the location of a second cysteine from CD44 is debatable. There is evidence indicating that CD44 can self-associate in T cell membranes (281), an association that promotes hyaluronan binding. Thus, perhaps the second cysteine comes from the Cys 277 of a second CD44 molecule. It is conceivable that CD44 clustering and self-association serves both to promote hyaluronan binding and to induce the zinc-mediated interaction with Lck. A model of this interaction is provided in figure 5.11. The cytoplasmic domain of murine CD44 does contain a second and distal cysteine residue, although this cysteine is not conserved in human CD44 making its significance somewhat questionable.

Zinc-finger protein structures are well known for the ability chelate zinc in a tetrahedral manner through the side chains of cysteine or histidine residue motifs (reviewed in 282). Although classical zinc fingers are referred to as "CCCC" (for four

cysteines) and "CCHH" (two cysteines and two histidines), recent evidence demonstrates protein-protein interactions mediated by the "CCHC" zinc finger from the erythroid transcription factor FOG (283), indicating that the CCHC motif is capable of zinc chelation. Interestingly, the cytoplasmic domain of CD44 contains a conserved histidine residue downstream of the membrane-proximal Cys 277, suggesting that this histidine could provide the fourth zinc-chelating residue in the CD44:Lck interaction.

It is also interesting to speculate that the zinc-dependent CD44:Lck interaction may have some effect on Lck activity. Work shown here and in other studies (68) indicates the necessity of Lck activity in CD44-mediated adhesion. However, as described above, there is an apparent contradiction since $CD45^-$ cells are known to have enhanced adhesion abilities via CD44 (68) and integrins (284), despite the hyper-phosphorylation of Src-family kinases at the inhibitory carboxy-tail tyrosine. Thus, perhaps the association of Lck with CD44 via zinc somehow allows for the sustained activation of Lck in the absence of CD45. This scenario would account for the presence Lck activity in the CD44-mediated adhesion of $CD45^-$ T cells and introduce a novel method of Lck regulation (Fig. 5.11).

Although this chapter provides novel data that further characterizes the interaction between CD44 and Lck, some questions still remain. Despite the correlation between the 1,10-phenanthroline-induced disruption of CD44-mediated T cell spreading, and the disruption of the CD44:Lck interaction, these separate findings do not address the potential chelation of other cytosolic ions (such as calcium known to be released upon CD44 ligation (273)). Thus, it is unclear whether the 1,10-phenanthroline-mediated inhibition of cell spreading was due to a lack of CD44-associated Lck activity, or to the

chelation of intracellular calcium stores or cations needed for kinase activity. To address these issues, one could ligate CD44 in the presence of 1,10-phenanthroline and analyze the cells for the induction of Lck Tyr 394 phosphorylation (indicating Lck activation), the concentration of cytoplasmic calcium or PKC activity (activated by calcium upon CD44 stimulation (285)), and the overall activity of cellular protein kinases.

Secondly, further experiments with mutations in the Lck zinc binding Cys 20 and Cys 23 residues are required to solidify the evidence that CD44 and Lck interact in the cell via a zinc cation. This work is of particular importance given recent data in our lab showing that although the CD44 C277A association with Lck in T cells is significantly reduced compared to wild type CD44, this weak CD44 C277A:Lck association displays some sensitivity to the presence of 2.5 mM 1,10-phenanthroline (J. Lai, unpublished data). This data suggests that in the cell, either CD44 Cys 277 is not the only residue capable of inducing a zinc-mediated interaction with Lck, or that 1,10-phenanthroline is adversely affecting zinc-independent components of the CD44:Lck interaction.

5.4.4 Summary

Overall this work demonstrates that CD44-mediated T cell spreading and the CD44:Lck interaction specifically, were inhibited by the chelation of divalent cations by 1,10-phenanthroline. Likewise, CD4-mediated cell spreading and the CD4:Lck association were sensitive to 1,10-phenanthroline further supporting a functional relationship between CD44 and CD4 in T cells. Furthermore, the CD44:Lck interaction was shown to be complex, involving an indirect plasma membrane component, as well as a direct cytoplasmic component mediated by a major zinc-dependent interaction and a minor zinc-independent interaction.

CHAPTER 6

General discussion

6.1 Summary of results

The overall goal of my research was to further the understanding of how Lck is regulated in its function in T cells. Thus, I chose to examine the interactions of Lck with three T cell molecules: CD45, CD45AP and CD44. The analysis of these interactions provided insight into the ability of CD45 to regulate Lck via dephosphorylation, the role of CD45AP in this process, and the nature of the Lck association with CD44 during T cell adhesion.

The enzyme–substrate interaction between CD45 and Lck was shown to be complex involving a major catalytic component, and a minor non-catalytic component. Non-catalytic interactions were affected by Lck conformation and favored constructs existing in the open form. CD45 showed a significant preference for dephosphorylating Lck at Tyr 394 as opposed to Tyr 505 in the full-length Lck molecule, suggesting a dominant role for CD45 in the down-regulation of Lck activity. This preference for Tyr 394 was not due to a sequence specificity surrounding the phosphotyrosine residue, but to an interaction with the Lck non-catalytic domains that facilitated dephosphorylation of open "active" Lck, and inhibited that of closed inactive Lck. In contrast to other *in vitro* studies (243), CD45 was shown to dephosphorylate Tyr 505 when Lck is predicted to be in the closed form, and did so in the absence of a high affinity SH2 ligand. Additional work demonstrated that Tyr 505 was indeed bound by its endogenous SH2 domain, indicating a closed Lck conformation. CD45-mediated Lck dephosphorylation was not affected by the prior tyrosine phosphorylation of CD45 by Csk as suggested elsewhere (171). This data reveals the molecular intricacies of the dual roles of CD45 in its ability both to up- and down-regulate Lck activity. Furthermore, these findings demonstrate a

dominant role for CD45 in the dephosphorylation of Lck Tyr 394, supporting the transient activation of Lck in the cell, as well as an independent role for CD45 in the dephosphorylation of the protected inhibitory Lck Tyr 505. Lastly, roles for the elusive regions CD45 D2 and Lck SD10, were elucidated. These regions were important for determining the substrate specificity of CD45 for Lck via a novel non-catalytic binding interaction.

Lck was shown to associate with CD45 and CD45AP to a similar extent in T cells. The CD45AP:Lck association stoichiometry was determined to be 1:10, 10-fold less than the 1:1 CD45AP:CD45 interaction. Interestingly, CD45AP association stoichiometries with Lck and CD45 could both be increased with CD45AP over-expression, suggesting that CD45AP binding to Lck and CD45 is not saturated under endogenous CD45AP levels. This indicates a correlation between CD45AP expression and its ability to associate with Lck and CD45, and suggests that CD45 may be capable of interacting with more than one molecule of CD45AP. Moreover, the CD45AP:Lck association was only slightly affected by the depletion of CD45, suggesting that this association is largely bi-molecular and independent of CD45 in T cells. A direct CD45AP:Lck interaction was confirmed *in vitro* using recombinant proteins. This interaction was localized to the kinase domain of Lck and to the membrane-proximal 115 residues of CD45AP, and was shown to occur independently of Lck phosphorylation and conformation status. Unlike CD45 D2, CD45AP did not bind to the SD10 region of Lck, and D2 competed weakly with CD45AP for binding to the Lck kinase domain. However, CD45AP was found to significantly inhibit the CD45:Lck interaction and in turn, inhibited CD45-mediated Lck dephosphorylation at Tyr 394 specifically. Together, this

data suggests that a direct CD45AP:Lck interaction in the cell serves to protect Lck Tyr 394 from CD45 phosphatase activity, and thereby sustain Lck activation during T cell signaling.

T cell cytoskeletal rearrangement resulting in cell flattening and elongation (or "spreading") was induced by CD44 or CD4 ligation. The cell spreading induced by both molecules was sensitive to the chelation of divalent cations by 1,10-phenanthroline. Consistent with this effect was the disruption of CD44 and CD4 associations with Lck in the presence of 1,10-phenanthroline. The CD44:Fyn association however, was not affected by cation chelation. Using purified recombinant proteins, Lck was shown to interact directly with the cytoplasmic domain of CD44 in a zinc inducible manner. Fyn however, did not bind to CD44 in the presence or absence of zinc. The direct CD44:Lck interaction was shown to involve a major zinc-dependent interaction localized to 13 membrane-proximal amino acids of the cytoplasmic CD44 domain and the non-catalytic domains of Lck, as well as a minor zinc-independent interaction largely mediated by the Lck kinase domain. Finally, a membrane-proximal cysteine residue in CD44 was shown to be critical for the zinc induction of the Lck interaction. This data establishes a direct interaction between CD44 and Lck that is largely mediated by a zinc cation, and suggests that this interaction is critical to mediate cytoskeletal changes upon CD44 ligation.

6.2 The significance of non-catalytic CD45:Lck interactions, and the regulation of Lck in T lymphocytes.

Lck SD10 is an exposed structure at the base of the kinase domain composed of helix G and a random coil connecting to helix H (253). This region is conserved structurally in many tyrosine and serine/threonine kinases, but its amino acid sequence is

highly variable. Although Lck SD10 maintains substantial sequence identity with other Src-family kinases (~60%), there is essentially no identity with a more distantly related kinase, Erk. Likewise, the Erk SD10 is considerably larger than that of Lck and contains two additional small helices adjacent to helix G (Fig. 3.12D). A direct interaction between CD45 D2 and the Lck SD10 was shown here to influence CD45 substrate specificity, a phosphatase property that has remained elusive and been previously attributed to the catalytic pocket or subcellular localization (reviewed in 263). Thus this work has provided evidence for a novel method by which phosphatases determine their substrate specificity. Given the structural conservation and the unknown function of both the SD10 region in kinases and the second domain of two-domain phosphatases, it is tempting to speculate that SD10:D2 interactions provide a global mechanism to dictate the specificity of several phosphatase–kinase interactions in the cell.

Here, CD45 was shown to directly dephosphorylate Lck at both Tyr 394 and Tyr 505. This suggests that one function of CD45 in T cells is to maintain Lck in the dephosphorylated, open and "primed" form (Fig 1.8), thus working to prevent both Lck deactivation (Tyr 505 phosphorylation) and sustained activation (Tyr 394 phosphorylation). Despite the necessity of Tyr 394 phosphorylation for full Lck activation (28, 29, 86), this phosphotyrosine is not readily detectable upon TCR ligation (99), suggesting that the phosphorylation of Tyr 394 may be transient. However, upon immune synapse formation (99) or CD4 cross-linking (286) Lck Tyr 394 phosphorylation is observed, suggesting sustained Lck activation. Interestingly, immune synapse formation involves the coalescence of lipid rafts and the induction of an intimate T cell – APC interface, two events thought to exclude CD45 (85, 122). Given the *in vitro* data

that Lck non-catalytic domains facilitate the rapid dephosphorylation of Lck Tyr 394, we propose that CD45 may rapidly dephosphorylate Tyr 394 upon TCR ligation alone, resulting in the transient activation of Lck. However, in the case of a sophisticated interaction between the T cell and the APC, the formation of the immune synapse allows for sustained Lck activation via Tyr 394 phosphorylation, and subsequent T cell activation due to the exclusion of CD45. Although Lck down-regulation at the immune synapse remains under investigation, events such as the transient CD45 entry into the synapse and the localization of SHP-1 and PEP at the plasma membrane are possible mechanisms of Lck Tyr 394 dephosphorylation after T cell activation.

Given the ability of CD45 to dephosphorylate Lck at both the activating Tyr 394 and inhibitory Tyr 505 residues, we propose that CD45 acts as a "guardian" of Lck activation. In resting cells, CD45 functions to maintain an entirely dephosphorylated form of Lck that is neither inhibited by Tyr 505 phosphorylation nor activated by Tyr 394 phosphorylation. Thus Lck exists in a "primed" state that is readily activated upon receptor-mediated lipid-raft aggregation. Here, studies employed purified recombinant Lck proteins phosphorylated at specific tyrosine residues to establish the direct molecular mechanisms of CD45-mediated Lck regulation. It is important to further establish these findings in T cells, however this can be difficult due to the opposing dual functions of CD45 in regulating Lck. Furthermore, in the cell there are a multitude of other factors to consider in this process such as the presence of CD45AP, Csk, PEP and SHP-1, and the distinct localization of CD45 and Lck with respect to lipid-raft domains and the immune synapse.

The ability of the Lck non-catalytic domains to facilitate CD45-mediated

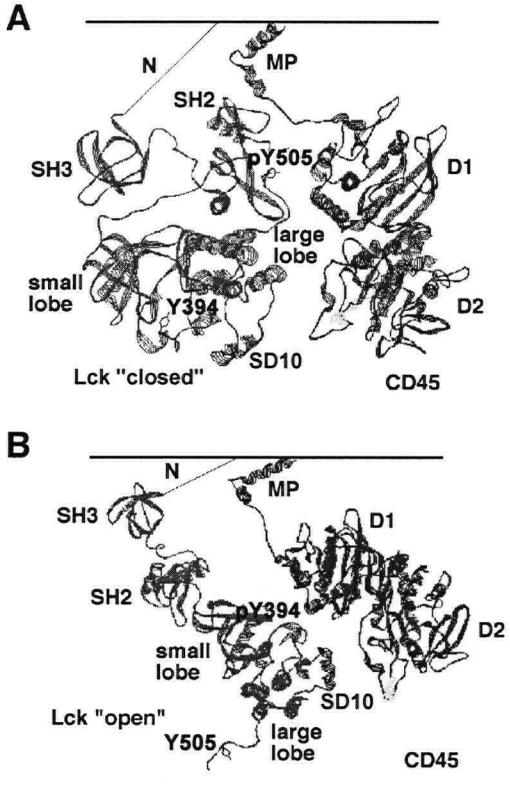

Figure 6.1. Model of the interaction between the cytoplasmic domains of CD45 and the open and closed forms of Lck. Possible interactions between CD45 and (A) the predicted "closed" form of Tyr 505-phosphorylated Lck, and (B) the predicted "open" form of Tyr 394-phosphorylated Lck. The top line indicates the plasma membrane. The unique amino terminal region (N) is shown as a line since recent analysis has revealed the lack of any defined structural elements (27). The small and large lobes of the Lck kinase domain are indicated, as well as the SD10 region present in the large lobe. The model of Lck is based on the 3D structures of other Src-family kinases as well as the Lck kinase domain (45, 46, 51) and CD45 is based on the crystal structure of the two-domain PTP LAR crystal structure (252). These models have been modified from those originally created by J. Felberg using SWISS-MODEL and manipulated using the Swiss-Pdb Viewer program (189).

Tyr 394 dephosphorylation (open Lck), and retard Tyr 505 dephosphorylation (closed Lck), led to a hypothetical model for the interaction between Lck and the cytoplasmic domains of CD45 (Fig 6.1). Two models are provided to indicate the possible interactions of CD45 with the closed and open forms of Lck (representing CD45 roles in the up- and down-regulation of Lck). The co-crystallization of CD45 with Lck is required to validate this model, which is presently based on the *in vitro* analysis from this thesis and the modeling from known structures of Src-family kinases (26, 45, 46, 51) and the two domain phosphatase, LAR (252).

6.3 The effects of CD45AP on CD45-mediated Lck regulation

Established here is a direct interaction between CD45AP and Lck *in vitro*, and an association in T cells that is largely bi-molecular and independent of CD45. Previous work in our laboratory has shown that the over-expression of CD45AP leads to enhanced and sustained intracellular tyrosine phosphorylation in response to CD3 stimulation (D. Wong, unpublished data). In support of this, data here has shown that CD45AP inhibits CD45-mediated Lck dephosphorylation at Tyr 394 as opposed to Tyr 505. Together, this data suggests a role for CD45AP in sustaining TCR-induced cell signaling events by inhibiting the CD45-mediated down-regulation of Lck activity.

This model is in agreement with the data from one strain of CD45AP-knockout mice in which the T cells from these mice exhibited a diminished response to TCR stimuli compared to wild type controls (217). Moreover, another study reported the preferential association of CD45AP with activated forms of Lck (221). Thus, it was suggested that the CD45AP:Lck interaction functions to recruit Lck activity proximal to the TCR, since CD45AP was shown to interact with TCR signaling components. For this

to occur, CD45AP would likely need access to coalesced lipid-raft domains at the immune synapse. Although not shown, studies here with BW5147 T cell lysates in sucrose gradient centrifugation revealed that the low-density sucrose fractions contained substantial amounts of Lck and low levels of CD45AP, but did not contain CD45. These findings suggest that a small percentage of CD45AP is indeed capable of localization in lipid-raft domains like other signaling molecules involved in T cell activation. Moreover, unlike CD45, the extremely short extracellular domain of CD45AP would not preclude its localization at the intimate immune synapse. Thus, it is tempting to speculate that further studies in immune synapse biology will reveal the co-localization of Lck with CD45AP at the c-SMAC, where CD45AP sustains Tyr 394 phosphorylation in concert with CD28 (99). However, the low stoichiometry of the CD45AP:Lck association in resting T cells (1:10) suggests that few Lck molecules would be protected from CD45-mediated Tyr 394 dephosphorylation upon TCR engagement. Thus, perhaps CD45AP becomes concentrated in coalesced lipid rafts during immune synpase formation causing a localized increase in the association with Lck, akin to the stoichiometry increase shown here by the 1:2 (CD45AP:Lck) association produced upon CD45AP over-expression. Future experiments are required to determine if the CD45AP:Lck association is exclusive to lipid-raft domains, and if this association is enhanced upon TCR ligation.

Here, a major caveat to the CD45AP-induced inhibition of CD45-mediated Lck Tyr 394 dephosphorylation *in vitro* is the artificial absence of the CD45:CD45AP interaction, since only the cytoplasmic domains of these proteins were used. Thus in T cells, it is possible that CD45AP recruits Lck to CD45 by a combination of the CD45AP:Lck cytoplasmic interaction and the CD45AP:CD45 transmembrane

interaction, as suggested previously (221). However, this suggests the formation of a tri-molecular comlex (CD45AP:CD45AP:Lck) that is likely transient in nature or induced upon T cell activation, since data here suggests that the CD45AP:Lck association occurs independently from CD45 in resting T cells.

Since Lck Tyr 394 phosphorylation induces kinase activity and causes oncogenic transformation if unregulated (29, 148, 149), the ability of CD45AP to retard CD45-mediated Tyr 394 phosphorylation must be only transient. This is supported by data showing the complete disappearance of pTyr 394 10 minutes after CD4 crosslinking and 45 minutes after immune synapse formation (99). Thus it is possible that CD45AP is degraded at the immune synapse once activated Lck has initiated proximal TCR signaling events. The absence of CD45AP could potentially grant CD45 access to pTyr 394 resulting in dephosphorylation and Lck down-regulation. Although largely excluded from the immune synapse, small amounts of CD45 have been detected at the c-SMAC, possibly to dephosphorylate activated Lck (124, 125). This theory is supported by the fact that CD45AP protein is rapidly degraded in the absence of CD45 (212, 213).

6.4 Implications of the zinc-induced CD44:Lck interaction

Previous work highlighted the importance of Src-family kinase activity in CD44-induced T cell spreading by blocking this process with Src-family kinase inhibitor, PP2 (68). However this data provides no indication of the relative contributions of Lck or Fyn to CD44 function. Here, purified recombinant proteins were assayed *in vitro* to establish a direct, zinc inducible, protein-protein interaction between the cytoplasmic domain of CD44 and Lck, but not Fyn. Fyn did co-immunoprecipitate with CD44 in T cells as reported elsewhere (106, 142), however the inability of Fyn to interact with the

cytoplasmic domain of CD44 *in vitro* suggests that the CD44:Fyn association in T cells is indirect. The chelation of cations with 1,10-phenanthroline was shown to disrupt the CD44:Lck interaction specifically in T cells, and prevent CD44-induced T cell spreading. This, together with the evidence of a direct interaction between CD44 and Lck, but not Fyn, suggests that Lck is the major contributor of Src-family kinase activity required in CD44 signaling. Thus, the contribution of Fyn to CD44 signaling remains unclear. One possible role for Fyn in this process is the amplification of tyrosine phosphorylation initiated by Lck. This model is supported a study showing that the coaggregation of TCR and CD4 leads to immediate Lck activation and recruitment to lipid rafts where it activates Fyn (288).

The induction of protein-protein interactions with a zinc molecule is not unprecedented. Zinc-finger regions common to transcription factors chelate zinc with cysteine residues (and histidine residues in some cases) for structural purposes (282). Likewise, Lck mediates its interactions with the cytoplasmic tails of CD4 and CD8α via the zinc binding tandem cysteine residues of each molecule (38, 39). Interestingly, Lck is the only Src-family kinase that contains these cysteine residues (positions 20 and 23) perhaps explaining why Fyn was not induced to bind CD44 in the presence of zinc. CD44 however, has not previously been shown to employ zinc in any of its known interactions. Despite expressing a conserved, membrane-proximal cysteine residue (Cys 277), CD44 noticeably lacks a second Cys in the C-X-X-C zinc-binding motif. Thus, in chapter 5 it was suggested that the source of the second cysteine could be from a second CD44 molecule, given the evidence of CD44 dimerization in ligand binding (281). Alternatively, a downstream histidine residue (His 312) could be involved, in a

mechanism similar to CHCC zinc fingers, however it is unknown if this histidine exists in close proximity to Cys 277 due to a lack of cytoplasmic CD44 structural data. Thus, further experiments are required to unveil the underlying molecular intricacies of the zinc-mediated CD44:Lck interaction.

Interestingly, CD44 Cys 277 is located in the center of a conserved 9-amino acid region of basic residues implicated in the association of CD44 with ERM proteins (ezrin, radixin, moesin) (289, 290). ERMs commonly act as linker proteins between the cytoplasmic tails of transmembrane receptors and the actin cytoskeleton (291, 292). The CD44:ERM association in T cells was previously characterized, and maximal association (as detected by co-immunoprecipitation) was shown require the presence of EDTA and low ionic strength (R. Li, Ph.D. thesis). Thus the requirement of a cation chelator (EDTA) for the CD44:ERM association is in stark contrast to the disruption of the CD44:Lck interaction by the chelation of cations by 1,10-phenanthroline. Together, these data suggest that the disruption of the zinc-dependent CD44:Lck interaction may enhance the CD44:ERM association, consistent with the presence of overlapping CD44 epitopes for ERM and Lck binding. However, given that CD44 is known to associate with both Lck and ERMs in resting T cells (R. Li, unpublished data), it is likely that these interactions occur simultaneously, possibly via separate individual CD44 molecules. Moreover, a recent study has reported constitutive Lck-mediated ezrin tyrosine phosphorylation in T cells (67). Although the relevance of ezrin tyrosine phosphorylation is not understood, it is tempting to speculate that the co-localization of these two molecules with CD44 in resting T cells provides a platform for ezrin phosphorylation.

Another study has reported that CD44 undergoes PKC-mediated serine phosphorylation at the membrane-proximal Ser 273 in response to PMA stimulation (CD44 numbering as in 231), directly adjacent to the ERM binding region and 4 residues from Cys 277 (285). This phosphorylation occurred only upon PKC activation with phorbol ester and was shown to significantly reduce the CD44:ezrin interaction, a process that was critical for CD44-mediated cell chemotaxis. It remains unknown however, if Ser 273 effects the CD44:Lck interaction, although the presence of a negatively charged phosphate group adjacent to the Cys 277 residue is likely to effect the ability of CD44 to bind a zinc cation.

Together, these data suggest that CD44 is constitutively and independently associated with both ERMs and Lck in resting T cells (where Lck may tyrosine phosphorylate ezrin). At sites of inflammation, CD44 ligation by its natural ligand HA would then induce PKC activation (293) resulting in CD44 Ser 273 phosphorylation and the loss of the ezrin association, possibly freeing zinc inducible binding sites for Lck. However, the regulation of zinc molecules in the cell remains elusive, and it is not known if an influx occurs to influence or regulate the interaction between CD44 and Lck.

Despite the abundance of zinc in cellular proteins, it is estimated that the amount of freely available zinc in mammalian cells is sparse, at picomolar amounts (reviewed in 294). Interestingly, T cells are known to produce a heavy metal binding protein, metallothionein (MT), in response to micromolar concentrations of extracellular cadmium and zinc (295, 296). MT is a cysteine-rich stress-response protein that is generally thought to chelate toxic levels of extracellular heavy metals (reviewed in 297), and thus can be released into the environment upon its induction. In regards to

immunology, a recent study with MT null mice revealed the poor proliferation of isolated splenic T cells in response to CD3 stimulation (298), suggesting a role for heavy metal ions in T cell signaling. Recent *in vitro* data has shown that all 20 MT cysteine residues can be oxidized in the presence of a mild oxidant, dithiopyridine, resulting in the release of all 7 MT bound zinc atoms (299). Moreover, MT has been shown to transfer zinc ions to apoenzymes *in vitro* (300). Thus, given the fact that TCR crosslinking can induce the rapid generation of oxidizing agents hydrogen peroxide and superoxide anion (301), it is possible that these oxidants induce the release of bound zinc ions from MT creating a transient wave of free cytosolic zinc that could induce Lck interactions with CD44, CD4 and CD8.

The correlation between cell redox state and thiol zinc chelation potentially creates a novel system to control the interactions of Lck with molecules such as CD44. Perhaps by controlling redox, cells also control the ability of Lck to participate in T cell signaling events during TCR-mediated T cell activation, or CD44-mediated T cell adhesion. Clearly, further experiments are required to investigate these possibilities.

6.5 Summary and model

The data presented in this thesis provides insight into the regulation of Lck activity by CD45 and characterizes the association of Lck with T cell proteins CD45AP and CD44. Given this data, and that of previous work in the field, I propose the following model for Lck regulation and its interactions with other molecules in the context of T cell activation or adhesion.

In resting T cells, lipid rafts are small and dispersed allowing CD45 access to Lck where it opposes the action of PAG-associated Csk and dephosphorylates Lck at Tyr 505.

This is a continual process, and represents an equilibrium in favour of CD45 since a consistent finding in $CD45^+$ cells is the presence of mainly dephosphorylated Lck (reviewed in 152). CD45-mediated Tyr 505 dephosphorylation does not require the presence of a high affinity ligand as suggested (302), although this process is retarded by Lck non-catalytic domains that create a closed Lck conformation via the pTyr 505:SH2 intramolecular bond. The activity of CD45 creates an Lck intermediate that remains inactive due to a lack of Tyr 394 phosphorylation, but is open and "primed" for activation upon receptor stimulation due to a lack of phosphorylation at Tyr 505. Thus, in resting T cells, the majority of Lck exists in a dephosphorylated primed state and interacts with cytoplasmic CD44, CD4 and CD8 via a single zinc cation, in lipid-raft domains (68, and reviewed in 85).

Lck also maintains a direct bi-molecular interaction with CD45AP in resting T cells. This interaction could be enhanced upon TCR or CD44 clustering during T cell activation or adhesion respectively, due to ability of both Lck and CD45AP to localize to lipid-raft domains. Receptor clustering results in the coalescence of lipid rafts and the induction of Lck auto-phosphorylation at Tyr 394 (99) required for full kinase activation (28, 86). Although largely excluded from the immune synapse and coalesced lipid rafts, small amounts of CD45 have been reported to transiently enter the TCR – APC interface (125), possibly to regulate Lck function. However, the presence of the CD45AP:Lck bi-molecular association could sustain Tyr 394 phosphorylation even in the presence of CD45 as shown here *in vitro* in chapter 4. Lck activation is further sustained by the release of Csk from lipid rafts during T cell signaling (208), thus maintaining the dephosphorylated state of Tyr 505. Sustained Lck activation then results in the

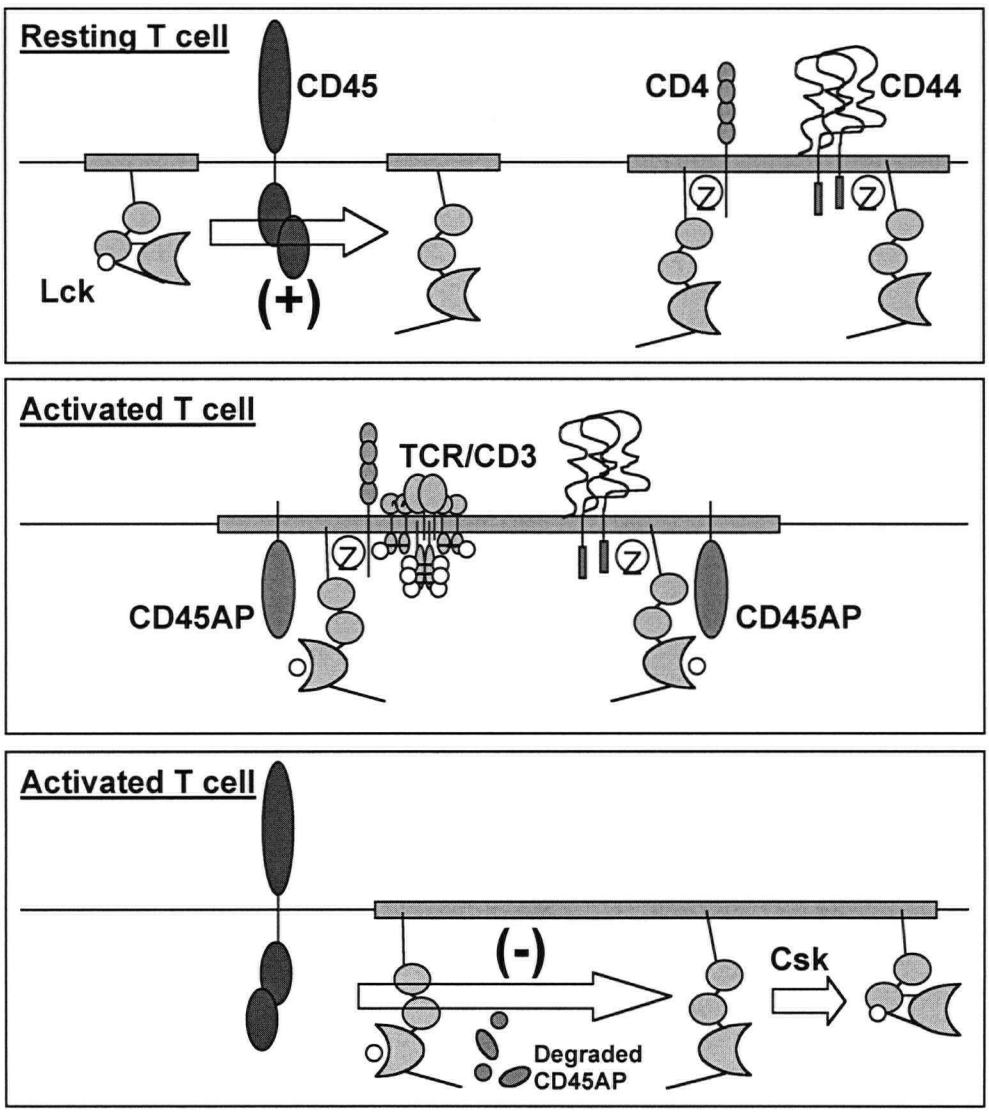

Figure 6.2. Model for Lck regulation and interactions during T cell signaling. Details are provided in the text. Lck is represented in three forms, closed/inactive, dephosphorylated/primed, and open/active. A single zinc atom is represented with "Z". Gray bars represent lipid-raft domains, and tyrosine phosphorylation is represented by white dots. Positive (+) and negative (-) roles of CD45 in Lck regulation are indicated. Upper, middle and lower panels are designed chronologically to represent different stages of Lck function in T cell signaling from initiation to completion, respectively.

phosphorylation and activation of downstream signaling components resulting in T cell activation via TCR stimulation or T cell adhesion via CD44 stimulation.

In the absence of CD45, CD45AP is rapidly degraded (212, 213), likely via its putative PEST sequences. The loss of CD45AP would then expose Lck pTyr 394 to CD45 phosphatase activity. This results in the rapid dephosphorylation of Tyr 394, which is facilitated by Lck non-catalytic domains, and Lck is subsequently down regulated to its intermediate "primed" state. The re-association of Csk with plasma membrane lipid rafts several minutes after TCR stimulation (207) would then provide a means of further de-activating Lck via Tyr 505 phosphorylation, restoring Lck to its closed inactive form. An illustration of this model is provided in figure 6.2. This model is supported in part by the data of this thesis, and by the findings of others in the field. However, many of the details in this model are speculatory and thus require further investigation. Key questions remain such as the localization of CD45AP during immune synapse formation and its ability to maintain Lck activation during T cell signaling. Also, the ability of zinc to mediate the interactions of Lck with TCR co-receptors and CD44 suggests a novel method of Lck regulation if signaling events are found to alter cytoplasmic zinc concentrations. Lastly, novel findings shown in this thesis including the influence of the Lck SD10:CD45 D2 interaction on CD45 substrate specificity, the CD45AP-mediated inhibition of Lck Tyr 394 dephosphorylation, and the direct zinc-mediated CD44:Lck interaction, provide new insights into potential therapies for T cell-induced pathology.

CHAPTER 7

References

1. Chan, A. C., Desai, D. M. and Weiss, A., The role of protein tyrosine kinases and protein tyrosine phosphatases in T cell antigen receptor signal transduction, *Annu. Rev. Immunol.* **12** (1994) 555-592.

2. Mustelin, T. and Tasken, K., Positive and negative regulation of T-cell activation through kinases and phosphatases, *Biochem. J.* **371** (2003) 15-27.

3. Stehelin, D., Varmus, H. E., Bishop, J. M. and Vogt, P. K., DNA related to the transforming gene(s) of avian sarcoma viruses is present in normal avian DNA, *Nature* **260** (1976) 170-3.

4. Brown, M. T. and Cooper, J. A., Regulation, substrates and functions of Src, *Biochim. Biophys. Acta* **1287** (1996) 121-149.

5. Williams, J. C., Wierenga, R. K. and Saraste, M., Insights into Src kinase functions - structural comparisons, *Trends Biochem. Sci.* **23** (1998) 179-184.

6. Pawson, T., New impressions of Src and Hck, *Nature* **385** (1997) 582-5.

7. Perlmutter, R. M., Marth, J. D., Ziegler, S. F., Garvin, A. M., Pawar, S., Cooke, M. P. and Abraham, K. M., Specialized protein tyrosine kinase proto-oncogenes in hematopoietic cells, *Biochim. Biophys. Acta* **948** (1988) 245-62.

8. Ziegler, S. F., Marth, J. D., Lewis, D. B. and Perlmutter, R. M., A novel protein tyrosine kinase gene (Hck) preferentially expressed in cells of hematopoietic origin, *Mol. Cell. Biol.* **7** (1987) 2276-2285.

9. Voronova, A. F., Buss, J. E., Patschinsky, T., Hunter, T. and Sefton, B. M., Characterization of the protein apparently responsible for the elevated tyrosine protein kinase activity in LSTRA cells, *Mol. Cell. Biol.* **4** (1984) 2705-13.

10. Voronova, A. F. and Sefton, B. M., Expression of a new tyrosine protein kinase is stimulated by retrovirus promoter insertion, *Nature* **319** (1986) 682-5.

11. Marth, J. D., Peet, R., Krebs, E. G. and Perlmutter, R. M., A lymphocyte-specific protein-tyrosine kinase gene is rearranged and overexpressed in the murine T cell lymphoma LSTRA, *Cell* **43** (1985) 393-404.

12. Marth, J. D., Disteche, C., Pravtcheva, D., Ruddle, F., Krebs, E. G. and Perlmutter, R. M., Localization of a lymphocyte-specific protein tyrosine kinase gene (Lck) at a site of frequent chromosomal abnormalities in human lymphomas, *Proc. Natl. Acad. Sci. U. S. A.* **83** (1986) 7400-4.

13. Weil, R. and Veillette, A., Signal transduction by the lymphocyte-specific tyrosine protein kinase p56lck, *Curr. Top. Microbiol. Immunol.* **205** (1996) 63-87.

14. Reynolds, P. J., Lesley, J., Trotter, J., Schulte, R., Hyman, R. and Sefton, B. M., Changes in the relative abundance of type I and type II Lck mRNA transcripts suggest differential promoter usage during T-cell development, *Mol. Cell. Biol.* **10** (1990) 4266-70.

15. Wildin, R. S., Garvin, A. M., Pawar, S., Lewis, D. B., Abraham, K. M., Forbush, K. A., Ziegler, S. F., Allen, J. M. and Perlmutter, R. M., Developmental regulation of Lck gene expression in T lymphocytes, *J. Exp. Med.* **173** (1991) 383-93.

16. Marth, J. D., Overell, R. W., Meier, K. E., Krebs, E. G. and Perlmutter, R. M., Translational activation of the Lck proto-oncogene, *Nature* **332** (1988) 171-3.

17. Veillette, A., Zuniga-Pflucker, J. C., Bolen, J. B. and Kruisbeek, A. M., Engagement of CD4 and CD8 expressed on immature thymocytes induces

activation of intracellular tyrosine phosphorylation pathways, *J. Exp. Med.* **170** (1989) 1671-80.

18. Perlmutter, R. M., Marth, J. D., Lewis, D. B., Peet, R., Ziegler, S. F. and Wilson, C. B., Structure and expression of lck transcripts in human lymphoid cells, *J. Cell. Biochem.* **38** (1988) 117-26.

19. Einspahr, K. J., Abraham, R. T., Dick, C. J. and Leibson, P. J., Protein tyrosine phosphorylation and p56lck modification in IL-2 or phorbol ester-activated human natural killer cells, *J. Immunol.* **145** (1990) 1490-7.

20. Taieb, J., Vitte-Mony, I., Auffredou, M. T., Dorseuil, O., Gacon, G., Bertoglio, J. and Vazquez, A., Regulation of p56lck kinase expression and control of DNA synthesis in activated human B lymphocytes, *J. Biol. Chem.* **268** (1993) 9169-71.

21. Ping, P., Zhang, J., Zheng, Y. T., Li, R. C., Dawn, B., Tang, X. L., Takano, H., Balafanova, Z. and Bolli, R., Demonstration of selective protein kinase C-dependent activation of Src and Lck tyrosine kinases during ischemic preconditioning in conscious rabbits, *Circ. Res.* **85** (1999) 542-50.

22. Molina, T. J., Kishihara, K., Siderovski, D. P., van Ewijk, W., Narendran, A., Timms, E., Wakeham, A., Paige, C. J., Hartmann, K.-U., Veillette, A., Davidson, D. and Mak, T. W., Profound block in thymocyte development in mice lacking p56lck, *Nature* **357** (1992) 161-164.

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

24. Karnitz, L., Sutor, S. L., Torigoe, T., Reed, J. C., Bell, M. P., McKean, D. J., Leibson, P. J. and Abraham, R. T., Effects of p56lck deficiency on the growth and cytolytic effector function of an interleukin-2-dependent cytotoxic T-cell line, *Mol. Cell. Biol.* **12** (1992) 4521-4530.

25. Luo, K. and Sefton, B. M., Activated lck tyrosine protein kinase stimulates antigen-independant Interleukin-2 production in T cells, *Mol. Cell. Biol.* **12** (1992) 4724-4732.

26. Xu, W., Doshi, A., Lei, M., Eck, M. J. and Harrison, S. C., Crystal structures of c-Src reveal features of its autoinhibitory mechanism, *Mol. Cell.* **3** (1999) 629-638.

27. Briese, L. and Willbold, D., Structure determination of human Lck unique and SH3 domains by nuclear magnetic resonance spectroscopy, *BMC Struct. Biol.* **3** (2003) 3.

28. Abraham, N. and Veillette, A., Activiation of p56lck through mutation of a regulatory carboxyterminal tyrosine residue requires intact sites of myristylation and autophosphorylation., *Mol. Cell. Biol.* **10** (1990) 5197-5206.

29. Caron, L., Abraham, N., Pawson, T. and Veillette, A., Structural requirements for enhancement of T cell responsiveness by the lymphocyte specific tyrosine protein kinase, p56lck, *Mol. Cell. Biol.* **12** (1992) 2720-2729.

30. Paige, L. A., Nadler, M. J., Harrison, M. L., Cassady, J. M. and Geahlen, R. L., Reversible palmitoylation of the protein-tyrosine kinase p56lck, *J. Biol. Chem.* **268** (1993) 8669-74.

31. Rodgers, W., Crise, B. and Rose, J. K., Signals determining protein tyrosine kinase and glycosyl-phosphatidylinositol-anchored protein targeting to a glycolipid-enriched membrane fraction, *Mol. Cell. Biol.* **14** (1994) 5384-5391.

32. Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A. and Magee, A. I., Palmitoylation of multiple Src-family kinases at a homologous N- terminal motif, *Biochem. J.* **303** (1994) 749-53.

33. Yurchak, L. K. and Sefton, B. M., Palmitoylation of either cys-3 or cys-5 is required for the biological activity of the lck tyrosine protein kinase, *Mol. Cell. Biol.* **15** (1995) 6914-6922.

34. Kosugi, A., Hayashi, F., Liddicoat, D. R., Yasuda, K., Saitoh, S. and Hamaoka, T., A pivotal role of cysteine 3 of Lck tyrosine kinase for localization to glycolipid-enriched microdomains and T cell activation, *Immunol. Lett.* **76** (2001) 133-138.

35. Kabouridis, P. S., Magee, A. I. and Ley, S. C., S-acylation of lck protein tyrosine kinase is essential for its signalling function in t lymphocytes, *EMBO J.* **16** (1997) 4983-4998.

36. Shenoy-Scaria, A. M., Dietzen, D. J., Kwong, J., Link, D. C. and Lublin, D. M., Cysteine^3 of src family protein tyrosine kinases determines palmitoylation and localization in caveolae, *J. Cell. Biol.* **126** (1994) 353-363.

37. Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. D., Perlmutter, R. M. and Littman, D. R., Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs, *Cell* **60** (1990) 755-765.

38. Huse, M., Eck, M. J. and Harrison, S. C., A Zn^{2+} ion links the cytoplasmic tail of CD4 and the N-terminal region of Lck, *J. Biol. Chem.* **273** (1998) 18729-18733.

39. Lin, R. S., Rodriguez, C., Veillette, A. and Lodish, H. F., Zinc is essential for binding of p56(lck) to CD4 and CD8 alpha, *J. Biol. Chem.* **273** (1998) 32878-32882.

40. Gervais, F. G. and Veillette, A., The unique amino terminal domain of p56lck regulates interactions with tyrosine protein phosphatases in T lymphocytes, *Mol. Cell. Biol.* **15** (1995) 2393-2401.

41. Mayer, B. J., SH3 domains: complexity in moderation, *J. Cell. Sci.* **114** (2001) 1253-63.

42. Schlessinger, J., SH2/SH3 signaling proteins, *Curr. Opin. Genet. Dev.* **4** (1994) 25-30.

43. Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. L., Neel, B. G., Birge, R. B., Fajardo, J. E., Chou, M. M., Hanafusa, H., Schaffhausen, B. and Cantley, L. C., SH2 domains recognize specific phosphopeptide sequences, *Cell* **72** (1993) 767-778.

44. Waksman, G., Shoelson, S. E., Pant, N., Cowburn, D. and Kuriyan, J., Binding of a high affinity phosphotyrosyl peptide to the src SH2 odmain: crystal structures of the complexed and peptide-free forms, *Cell* **72** (1993) 779-790.

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

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

47. Peri, K. G., Gervais, F. G., Weil, R., Davidson, D., Gish, G. D. and Veillette, A., Interactions of the SH2 domain of lymphocyte-specific tyrosine protein kinase p56lck with phosphotyrosine containing proteins., *Oncogene* **8** (1993) 2765-2772.

48. Sieh, M., Bolen, J. B. and Weiss, A., CD45 specifically modulates binding of Lck to a phosphopeptide encompassing the negative regulatory tyrosine of Lck, *EMBO J.* **12** (1993) 315-321.

49. Nada, S., Okada, M., MacAuley, A., Cooper, J. A. and Nakagawa, H., Cloning of a complementary DNA for a protein-tyrosine kinase that specifically phosphorylates a negative regulatory site of p60c-src, *Nature* **351** (1991) 69-72.

50. Bergman, M., Mustelin, T., Oetken, C., Partanen, J., Flint, N. A., Amrein, K. E., Autero, M., Burn, P. and Alitalo, K., The human p50csk tyrosine kinase phosphorylates p56lck at Tyr-505 and down regulates its catalytic activity, *EMBO J.* **11** (1992) 2919-2924.

51. Yamaguchi, H. and Hendrickson, W. A., Structural basis for activation of human lymphocyte kinase Lck upon tyrosine phosphorylation, *Nature* **384** (1996) 484-489.

52. Wang, D. X., Huang, X. Y. and Cole, P. A., Molecular determinants for Csk-catalyzed tyrosine phosphorylation of the Src tail, *Biochemistry* **40** (2001) 2004-2010.

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

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

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

56. Kanda, H., Mimura, T., Hamasaki, K., Yamamoto, K., Yazaki, Y., Hirai, H. and Nojima, Y., Fyn and Lck tyrosine kinases regulate tyrosine phosphorylation of p105(CasL), a member of the p130(Cas) docking protein family, in T-cell receptor-mediated signalling, *Immunology.* **97** (1999) 56-61.

57. Walk, S. F., March, M. E. and Ravichandran, K. S., Roles of Lck, Syk and Zap-70 Tyrosine Kinases in Tcr-Mediated Phosphorylation of the Adapter Protein Shc, *Eur. J. Immunol.* **28** (1998) 2265-2275.

58. Heyeck, S. D., Wilcox, H. M., Bunnell, S. C. and Berg, L. J., Lck phosphorylates the activation loop tyrosine of the itk kinase domain and activates itk kinase activity, *J. Biol. Chem.* **272** (1997) 25401-25408.

59. Carrera, A. C., Rodriguezborlado, L., Martinezalonso, C. and Merida, I., T cell receptor-associated alpha-phosphatidylinositol 3-kinase becomes activated by T

cell receptor cross-linking and requires pp56(lck), *J. Biol. Chem.* **269** (1994) 19435-19440.

60. Crespo, P., Schuebel, K. E., Ostrom, A. A., Gutkind, J. S. and Bustelo, X. R., Phosphotyrosine-dependent activation of Rac-1 GDP/GTP exchange by the vav proto-oncogene product, *Nature* **385** (1997) 169-72.

61. Yabana, N. and Shibuya, M., Adaptor protein APS binds the NH2-terminal autoinhibitory domain of guanine nucleotide exchange factor Vav3 and augments its activity, *Oncogene* **21** (2002) 7720-9.

62. King, P. D., Sadra, A., Teng, J. M. C., Liu, X. R., Han, A., Selvakumar, A., August, A. and Dupont, B., Analysis of CD28 cytoplasmic tail tyrosine residues as regulators and substrates for the protein tyrosine kinases, Emt and Lck, *J. Immunol.* **158** (1997) 580-590.

63. Chuang, E., Lee, K. M., Robbins, M. D., Duerr, J. M., Alegre, M. L., Hambor, J. E., Neveu, M. J., Bluestone, J. A. and Thompson, C. B., Regulation of cytotoxic T lymphocyte-associated molecule-4 by Src kinases, *J. Immunol.* **162** (1999) 1270-7.

64. Miyatake, S., Nakaseko, C., Umemori, H., Yamamoto, T. and Saito, T., Src family tyrosine kinases associate with and phosphorylate CTLA-4 (CD152), *Biochem. Biophys. Res. Commun.* **249** (1998) 444-8.

65. Brdicka, T., Pavlistova, D., Leo, A., Bruyns, E., Korinek, V., Angelisova, P., Scherer, J., Shevchenko, A., Hilgert, I., Cerny, J., Drbal, K., Kuramitsu, Y., Kornacker, B., Horejsi, V. and Schraven, B., Phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG), a novel ubiquitously expressed

transmembrane adaptor protein, binds the protein tyrosine kinase Csk and is involved in regulation of T cell activation, *J. Exp. Med.* **191** (2000) 1591-1604.

66. Goldmann, W. H., p56(lck) Controls phosphorylation of filamin (ABP-280) and regulates focal adhesion kinase (pp125(FAK)), *Cell. Biol. Int.* **26** (2002) 567-71.

67. Autero, M., Heiska, L., Ronnstrand, L., Vaheri, A., Gahmberg, C. G. and Carpen, O., Ezrin is a substrate for Lck in T cells, *FEBS Lett.* **535** (2003) 82-6.

68. Li, R. H., Wong, N., Jabali, M. D. and Johnson, P., CD44-initiated cell spreading induces Pyk2 phosphorylation, is mediated by Src family kinases, and is negatively regulated by CD45, *J. Biol. Chem.* **276** (2001) 28767-28773.

69. Pelosi, M., Di Bartolo, V., Mounier, V., Mege, D., Pascussi, J. M., Dufour, E., Blondel, A. and Acuto, O., Tyrosine 319 in the interdomain B of ZAP-70 is a binding site for the Src homology 2 domain of Lck, *J. Biol. Chem.* **274** (1999) 14229-14237.

70. August, A. and Dupont, B., Association between mitogen-activated protein kinase and the zeta chain of the T cell receptor (TCR) with the SH2,3 domain of p56lck. Differential regulation by TcR cross-linking, *J. Biol. Chem.* **271** (1996) 10054-9.

71. Choi, Y. B., Kim, C. K. and Yun, Y. D., Lad, an adapter protein interacting with the SH2 domain of p56(lck), is required for T cell activation, *J. Immunol.* **163** (1999) 5242-5249.

72. Nemorin, J. G. and Duplay, P., Evidence that Lck-mediated phosphorylation of p56(dok) and p62(dok) may play a role in CD2 signaling, *J. Biol. Chem.* **275** (2000) 14590-14597.

73. Berg, N. N. and Ostergaard, H. L., T cell receptor engagement induces tyrosine phosphorylation of Fak and Pyk2 and their association with Lck, *J. Immunol.* **159** (1997) 1753-1757.

74. Ostergaard, H. L., Lou, O., Arendt, C. W. and Berg, N. N., Paxillin phosphorylation and association with Lck and Pyk2 in anti-CD3- or anti-CD45-stimulated t cells, *J. Biol. Chem.* **273** (1998) 5692-5696.

75. Ng, D. H. W., Watts, J. D., Aebersold, R. and Johnson, P., Demonstration of a direct interaction between $p56^{lck}$ and the cytoplasmic domain of CD45 in vitro, *J. Biol. Chem.* **271** (1996) 1295-1300.

76. Holdorf, A. D., Green, J. M., Levin, S. D., Denny, M. F., Straus, D. B., Link, V., Changelian, P. S., Allen, P. M. and Shaw, A. D., Proline residues in CD28 and the Src homology (SH)3 domain of Lck are required for T cell costimulation, *J. Exp. Med.* **190** (1999) 375-384.

77. Rao, N., Miyake, S., Reddi, A. L., Douillard, P., Ghosh, A. K., Dodge, I. L., Zhou, P., Fernandes, N. D. and Band, H., Negative regulation of Lck by Cbl ubiquitin ligase, *Proc. Natl. Acad. Sci. U. S. A.* **99** (2002) 3794-9.

78. Bell, G. M., Fargnoli, J., Bolen, J. B., Kish, L. and Imboden, J. B., The SH3 domain of p56lck binds to proline-rich sequences in the cytoplasmic domain of CD2, *J. Exp. Med.* **183** (1996) 169-78.

79. Hogquist, K. A., Signal strength in thymic selection and lineage commitment, *Curr. Opin. Immunol.* **13** (2001) 225-31.

80. Seavitt, J. R., White, L. S., Murphy, K. M., Loh, D. Y., Perlmutter, R. M. and Thomas, M. L., Expression of the $p56^{lck}$ Y505F mutation in CD45-deficient mice rescues thymocyte development, *Mol. Cell. Biol.* **19** (1999) 4200-4208.

81. Basson, M. A. and Zamoyska, R., The CD4/CD8 lineage decision: integration of signalling pathways, *Immunol. Today.* **21** (2000) 509-14.

82. Zamoyska, R., The CD8 coreceptor revisited: one chain good, two chains better, *Immunity* **1** (1994) 243-6.

83. Veillette, A., Bookman, M. A., Horak, E. M. and Bolen, J. B., The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase, p56lck, *Cell* **55** (1988) 301-308.

84. Barber, E. K., Dasgupta, J. D., Schlossman, S. F., Trevillyan, J. M. and Rudd, C. E., The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase (p56lck) that phosphorylates the CD3 complex., *Proc. Natl. Acad. Sci. U.S.A.* **86** (1989) 3277-3281.

85. Janes, P. W., Ley, S. C., Magee, A. I. and Kabouridis, P. S., The role of lipid rafts in T cell antigen receptor (TCR) signalling, *Semin. Immunol.* **12** (2000) 23-34.

86. D'Oro, U., Sakaguchi, K., Appella, E. and Ashwell, J. D., Mutational analysis of lck in CD45-negative T cells - dominant role of tyrosine 394 phosphorylation in kinase activity, *Mol. Cell. Biol.* **16** (1996) 4996-5003.

87. Liu, K. Q., Bunnell, S. C., Gurniak, C. B. and Berg, L. J., T Cell Receptor-Initiated Calcium Release Is Uncoupled From Capacitative Calcium Entry in Itk-Deficient T Cells, *J. Exp. Med.* **187** (1998) 1721-1727.

88. Perez-Villar, J. J. and Kanner, S. B., Regulated association between the tyrosine kinase Emt/Itk/Tsk and phospholipase-C gamma 1 in human T lymphocytes, *J. Immunol.* **163** (1999) 6435-6441.

89. al-Ramadi, B. K., Nakamura, T., Leitenberg, D. and Bothwell, A. L., Deficient expression of p56(lck) in Th2 cells leads to partial TCR signaling and a dysregulation in lymphokine mRNA levels, *J. Immunol.* **157** (1996) 4751-61.

90. Gupta, S., Weiss, A., Kumar, G., Wang, S. and Nel, A., The T-cell antigen receptor utilizes Lck, Raf-1, and MEK-1 for activating mitogen-activated protein kinase - Evidence for the existence of a second protein kinase C-dependent pathway in an Lck-negative Jurkat cell mutant, *J. Biol. Chem.* **269** (1994) 17349-17357.

91. Zhang, W., Sommers, C. L., Burshtyn, D. N., Stebbins, C. C., DeJarnette, J. B., Trible, R. P., Grinberg, A., Tsay, H. C., Jacobs, H. M., Kessler, C. M., Long, E. O., Love, P. E. and Samelson, L. E., Essential role of LAT in T cell development, *Immunity* **10** (1999) 323-32.

92. Pivniouk, V., Tsitsikov, E., Swinton, P., Rathbun, G., Alt, F. W. and Geha, R. S., Impaired viability and profound block in thymocyte development in mice lacking the adaptor protein SLP-76, *Cell* **94** (1998) 229-38.

93. Myung, P. S., Boerthe, N. J. and Koretzky, G. A., Adapter proteins in lymphocyte antigen-receptor signaling, *Curr. Opin. Immunol.* **12** (2000) 256-66.

94. Chambers, C. A. and Allison, J. P., Co-stimulation in T cell responses, *Curr. Opin. Immunol.* **9** (1997) 396-404.

95. Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H. and Allison, J. P., CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones, *Nature* **356** (1992) 607-9.

96. Schwartz, R. H., Costimulation of T lymphocytes: the role of CD28, CTLA-4, and B7/BB1 in interleukin-2 production and immunotherapy, *Cell* **71** (1992) 1065-8.

97. Tuosto, L. and Acuto, O., CD28 affects the earliest signaling events generated by TCR engagement, *Eur. J. Immunol.* **28** (1998) 2131-42.

98. August, A. and Dupont, B., Activation of Src family kinase Lck Following CD28 Crosslinking in the Jurkat leukemic cell line, *Biochem. Biophys. Res. Commun.* **199** (1994) 1466-1473.

99. Holdorf, A. D., Lee, K. H., Burack, W. R., Allen, P. M. and Shaw, A. S., Regulation of Lck activity by CD4 and CD28 in the immunological synapse, *Nat. Immunol.* **4** (2002) 4.

100. Davis, S. J., Ikemizu, S., Evans, E. J., Fugger, L., Bakker, T. R. and van der Merwe, P. A., The nature of molecular recognition by T cells, *Nat. Immunol.* **4** (2003) 217-24.

101. Guntermann, C. and Alexander, D. R., CTLA-4 suppresses proximal TCR signaling in resting human CD4(+) T cells by inhibiting ZAP-70 Tyr(319) phosphorylation: a potential role for tyrosine phosphatases, *J. Immunol.* **168** (2002) 4420-9.

102. Lee, K. M., Chuang, E., Griffin, M., Khattri, R., Hong, D. K., Zhang, W. G., Straus, D., Samelson, L. E., Thompson, C. B. and Bluestone, J. A., Molecular basis of T cell inactivation by CTLA-4, *Science* **282** (1998) 2263-2266.

103. Brown, D. A. and London, E., Functions of lipid rafts in biological membranes, *Ann. Rev. Cell Dev. Biol.* **14** (1998) 111-136.

104. Fragoso, R., Ren, D., Zhang, X., Su, M. W., Burakoff, S. J. and Jin, Y. J., Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling, *J. Immunol.* **170** (2003) 913-21.

105. Zhang, W. G., Trible, R. P. and Samelson, L. E., LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation, *Immunity* **9** (1998) 239-246.

106. Foger, N., Marhaba, R. and Zoller, M., Involvement of CD44 in cytoskeleton rearrangement and raft reorganization in T cells, *J. Cell Sci.* **114** (2001) 1169-1178.

107. Guo, Y. J., Lin, S. C., Wang, J. H., Bigby, M. and Sy, M. S., Palmitoylation of CD44 interferes with CD3-mediated signaling in human T lymphocytes, *Int. Immunol.* **6** (1994) 213-21.

108. Shan, X. and Wange, R. L., Itk/Emt/Tsk activation in response to CD3 cross-linking in Jurkat T cells requires ZAP-70 and Lat and is independent of membrane recruitment, *J. Biol. Chem.* **274** (1999) 29323-30.

109. Montixi, C., Langlet, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Pierres, M. and He, H. T., Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains, *EMBO J.* **17** (1998) 5334-5348.

110. Xavier, R., Brennan, T., Li, Q., McCormack, C. and Seed, B., Membrane compartmentation is required for efficient T cell activation, *Immunity* **8** (1998) 723-732.

111. Brdicka, T., Cerny, J. and Horejsi, V., T cell receptor signalling results in rapid tyrosine phosphorylation of the linker protein LAT present in detergent-resistant membrane microdomains, *Biochem. Biophys. Res. Comm.* **248** (1998) 356-360.

112. Janes, P. W., Ley, S. C. and Magee, A. I., Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor, *J. Cell. Biol.* **147** (1999) 447-461.

113. Rodgers, W. and Rose, J. K., Exclusion of CD45 inhibits activity of $p56^{lck}$ associated with glycolipid-enriched membrane domains, *J. Cell. Biol.* **135** (1996) 1515-1523.

114. Thomas, M. L. and Brown, E. J., Positive and negative regulation of Src-family membrane kinases by CD45, *Immunol. Today* **20** (1999) 406-411.

115. Varma, R. and Mayor, S., GPI-anchored proteins are organized in submicron domains at the cell surface, *Nature* **394** (1998) 798-801.

116. Burack, W. R., Lee, K. H., Holdorf, A. D., Dustin, M. L. and Shaw, A. S., Cutting edge: quantitative imaging of raft accumulation in the immunological synapse, *J. Immunol.* **169** (2002) 2837-41.

117. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. and Kupfer, A., Three-dimensional segregation of supramolecular activation clusters in T cells, *Nature* **395** (1998) 82-86.

118. Ehrlich, L. I., Ebert, P. J., Krummel, M. F., Weiss, A. and Davis, M. M., Dynamics of p56lck translocation to the T cell immunological synapse following agonist and antagonist stimulation, *Immunity* **17** (2002) 809-22.

119. Viola, A., Schroeder, S., Sakakibara, Y. and Lanzavecchia, A., T lymphocyte costimulation mediated by reorganization of membrane microdomains, *Science* **283** (1999) 680-682.

120. Woollett, G. R., Williams, A. F. and Shotton, D. M., Visualisation by low-angle shadowing of the leucocyte-common antigen. A major cell surface glycoprotein of lymphocytes., *EMBO J.* **4** (1985) 2827-2830.

121. McCall, M. N., Shotton, D. M. and Barclay, A. N., Expression of soluble isoforms of rat CD45. Analysis by electron microscopy and use in epitope mapping of anti-CD45R monoclonal antibodies, *Immunol.* **76** (1992) 310-317.

122. Shaw, A. S. and Dustin, M. L., Making the T cell receptor go the distance: a topological view of T cell activation, *Immunity* **6** (1997) 361-9.

123. Leupin, O., Zaru, R., Laroche, T., Muller, S. and Valitutti, S., Exclusion of CD45 from the T-cell receptor signaling area in antigen-stimulated T lymphocytes, *Curr. Biol.* **10** (2000) 277-280.

124. Johnson, K. G., Bromley, S. K., Dustin, M. L. and Thomas, M. L., A supramolecular basis for CD45 tyrosine phosphatase regulation in sustained T cell activation, *Proc. Natl. Acad. Sci. U.S.A.* **97** (2000) 10138-10143.

125. Freiberg, B. A., Kupfer, H., Maslanik, W., Delli, J., Kappler, J., Zaller, D. M. and Kupfer, A., Staging and resetting T cell activation in SMACs, *Nat. Immunol.* **3** (2002) 911-917.

126. Dustin, M. L. and Springer, T. A., Role of lymphocyte adhesion receptors in transient interactions and cell locomotion, *Annu. Rev. Immunol* **9** (1991) 27-66.

127. Cowin, P. and Burke, B., Cytoskeleton-membrane interactions, *Curr. Opin. Cell. Biol.* **8** (1996) 56-65.

128. Johnson, P., Maiti, A., Brown, K. L. and Li, R., A role for the cell adhesion molecule CD44 and sulfation in leukocyte-endothelial cell adhesion during an inflammatory response?, *Biochem. Pharm.* **59** (2000) 455-465.

129. Camp, R. L., Scheynius, A., Johansson, C. and Pure, E., CD44 is neccessary for optimal contact allergic responses but is not required for normal leukocyte extravasation, *J. Exp. Med.* **178** (1993) 497-507.

130. DeGrendele, H. C., Estess, P., Picker, L. J. and Siegelman, M. H., CD44 and its ligand hyaluronate mediate rolling under physiologic flow - a novel lymphocyte-endothelial cell primary adhesion pathway, *J. Exp. Med.* **183** (1996) 1119-1130.

131. DeGrendele, H. C., Kosfiszer, M., Estess, P. and Siegelman, M. H., CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation, *J. Immunol.* **159** (1997) 2549-2553.

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

133. Dustin, M. L., Ferguson, L. M., Chan, P. Y., Springer, T. A. and Golan, D. E., Visualization of CD2 interaction with LFA-3 and determination of the two-dimensional dissociation constant for adhesion receptors in a contact area, *J. Cell. Biol.* **132** (1996) 465-74.

134. Dustin, M. L., Miller, J. M., Ranganath, S., Vignali, D. A., Viner, N. J., Nelson, C. A. and Unanue, E. R., TCR-mediated adhesion of T cell hybridomas to planar bilayers containing purified MHC class II/peptide complexes and receptor shedding during detachment, *J. Immunol.* **157** (1996) 2014-21.

135. Monks, C. R., Freiberg, B. A., Kupfer, H., Sciaky, N. and Kupfer, A., Three-dimensional segregation of supramolecular activation clusters in T cells, *Nature* **395** (1998) 82-6.

136. Funaro, A., Spagnoli, G. C., Momo, M., Knapp, W. and Malavasi, F., Stimulation of T cells via CD44 requires leukocyte-function-associated antigen interactions and interleukin-2 production, *Hum. Immunol.* **40** (1994) 267-278.

137. Sommer, F., Huber, M., Rollinghoff, M. and Lohoff, M., Cd44 plays a co-stimulatory role in murine t cell activation - ligation of cd44 selectively co-stimulates il-2 production, but not proliferation in tcr-stimulated murine t(h)1 cells, *Int. Immunol.* **7** (1995) 1779-1786.

138. Schlaepfer, D. D. and Hunter, T., Integrin signalling and tyrosine phosphorylation - just the faks, *Trend. Cell Biol.* **8** (1998) 151-157.

139. Clark, E. A. and Brugge, J., Integrins and signal transduction pathways: the road taken, *Science* **268** (1995) 233-239.

140. Giancotti, F. G. and Ruoslahti, E., Integrin signaling, *Science* **285** (1999) 1028-32.

141. Taher, T. E. I., Smit, L., Griffioen, A. W., Schilder-Tol, E. J. M., Borst, J. and Pals, S. T., Signaling through CD44 is mediated by tyrosine kinases: association with p56lck in T lymphocytes, *J. Biol. Chem.* **271** (1996) 2863-2867.

142. Ilangumaran, S., Briol, A. and Hoessli, D. C., CD44 selectively associates with active Src-family protein tyrosine kinases Lck and Fyn in glycosphingolipid-rich plasma membrane domains of human peripheral blood lymphocytes, *Blood* **91** (1998) 3901-3908.

143. Bourguignon, L. Y. W., Zhu, H. B., Shao, L. J. and Chen, Y. W., CD44 interaction with c-Src kinase promotes cortactin-mediated cytoskeleton function and hyaluronic acid-dependent ovarian tumor cell migration, *J. Biol. Chem.* **276** (2001) 7327-7336.

144. Morgan, M. M., Labno, C. M., Van Seventer, G. A., Denny, M. F., Straus, D. B. and Burkhardt, J. K., Superantigen-induced T cell:B cell conjugation is mediated by LFA-1 and requires signaling through Lck, but not ZAP-70, *J. Immunol.* **167** (2001) 5708-18.

145. Calalb, M. B., Polte, T. R. and Hanks, S. K., Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases, *Mol. Cell. Biol.* **15** (1995) 954-63.

146. Schlaepfer, D. D., Hauck, C. R. and Sieg, D. J., Signaling through focal adhesion kinase, *Prog. Biophys. Mol. Biol.* **71** (1999) 435-78.

147. Roach, T., Slater, S., Koval, M., White, L., Cahir McFarland, E., Okumura, M., Thomas, M. and Brown, E., CD45 regulates Src family member kinase activity associated with macrophage integrin-mediated adhesion, *Curr. Biol.* **7** (1997) 408-417.

148. Marth, J. D., Cooper, J. A., King, C. S., Ziegler, S. F., Tinker, D. A., Overell, R. W., Krebs, E. G. and Perlmutter, R. M., Neoplastic transformation induced by an

activated lymphocyte-specific protein tyrosine kinase (pp56lck), *Mol. Cell. Biol.* **8** (1988) 540-50.

149. Amrein, K. E. and Sefton, B. M., Mutation of a site of tyrosine phosphorylation in the lymphocyte-specific tyrosine protein kinase p56lck, reveals its oncogenic potential in fibroblasts, *Proc. Natl. Acad. Sci. U.S.A.* **85** (1988) 4247-4251.

150. Veillette, A., Caron, L., Fournel, M. and Pawson, T., Regulation of the enzymatic function of the lymphocyte specific tyrosine protein kinase, p56lck by the non-catalytic SH2 and SH3 domains, *Oncogene* **7** (1992) 971-980.

151. Amrein, K. E., Panholzer, B., Flint, N. A., Bannwarth, W. and Burn, P., The Src homology 2 domain of the protein-tyrosine kinase p56lck mediates both intermolecular and intramolecular interactions, *Proc. Natl. Acad. Sci. U. S. A.* **90** (1993) 10285-9.

152. Johnson, P. and Felberg, J., CD45: A key regulator of lck and T cell activation, *Mod. Asp. Immunobiol.* **1** (2000) 156-159.

153. Liu, X., Brodeur, S. R., Gish, G., Songyang, Z., Cantley, L. C., Laudano, A. P. and Pawson, T., Regulation of c-Src tyrosine kinase activity by the Src SH2 domain, *Oncogene* **8** (1993) 1119-26.

154. Moarefi, I., Lafevrebernt, M., Sicheri, F., Huse, M., Lee, C. H., Kuriyan, J. and Miller, W. T., Activation of the Src-family tyrosine kinase hck by sh3 domain displacement, *Nature* **385** (1997) 650-653.

155. Couture, C., Baier, G., Oetken, C., Williams, S., Telford, D., Mariecardine, A., Baierbitterlich, G., Fischer, S., Burn, P., Altman, A. and Mustelin, T., Activation

of p56(lck) by p72(syk) through physical association and N-terminal tyrosine phosphorylation, *Mol. Cell. Biol.* **14** (1994) 5249-5258.

156. Veillette, A., Horak, I. D., Horak, E. M., Bookman, M. A. and Bolen, J. B., Alterations of the lymphocyte-specific protein tyrosine kinase (p56lck) during T-cell activation, *Mol. Cell. Biol.* **8** (1988) 4353-61.

157. Marth, J. D., Lewis, D. B., Cooke, M. P., Mellins, E. D., Gearn, M. E., Samelson, L. E., Wilson, C. B., Miller, A. D. and Perlmutter, R. M., Lymphocyte activation provokes modification of a lymphocyte-specific protein tyrosine kinase (p56lck), *J. Immunol.* **142** (1989) 2430-7.

158. Watts, J. D., Sanghera, J. S., Pelech, S. L. and Aebersold, R., Phosphorylation of serine 59 of p56lck in activated T cells, *J. Biol. Chem.* **268** (1993) 23275-82.

159. Lee, J. E., Cossoy, M. B., Chau, L. A., Singh, B. and Madrenas, J., Inactivation of Lck and loss of TCR-mediated signaling upon persistent engagement with complexes of peptide:MHC molecules, *J. Immunol.* **159** (1997) 61-9.

160. Horak, I. D., Gress, R. E., Lucas, P. J., Horak, E. M., Waldmann, T. A. and Bolen, J. B., T-lymphocyte interleukin 2-dependent tyrosine protein kinase signal transduction involves the activation of p56lck, *Proc. Natl. Acad. Sci. U. S. A.* **88** (1991) 1996-2000.

161. Luo, K. X. and Sefton, B. M., Analysis of the sites in p56lck whose phosphorylation is induced by tetradecanoyl phorbol acetate, *Oncogene* **5** (1990) 803-8.

162. Galron, D., Ansotegui, I. J. and Isakov, N., Posttranslational regulation of Lck and a p36-38 protein by activators of protein kinase C: differential effects of the tumor

promoter, PMA, and the non-tumor-promoter, bryostatin, *Cell. Immunol.* **178** (1997) 141-51.

163. Winkler, D. G., Park, I., Kim, T., Payne, N. S., Walsh, C. T., Strominger, J. L. and Shin, J., Phosphorylation of Ser42 and Ser59 in the N-terminal region of the tyrosine kinase p56lck, *Proc. Natl. Acad. Sci. U. S. A.* **90** (1993) 5176-5180.

164. Schroder, A. J., Quehl, P., Muller, J. and Samstag, Y., Conversion of p56(lck) to p60(lck) in human peripheral blood T lymphocytes is dependent on co-stimulation through accessory receptors: involvement of phospholipase C, protein kinase C and MAP-kinases in vivo, *Eur. J. Immunol.* **30** (2000) 635-643.

165. Kesavan, K. P., Isaacson, C. C., Ashendel, C. L., Geahlen, R. L. and Harrison, M. L., Characterization of the *in vivo* sites of serine phosphorylation on Lck identifying serine 59 as a site of mitotic phosphorylation, *J. Biol. Chem.* **277** (2002) 14666-73.

166. Stefanova, I., Hemmer, B., Vergelli, M., Martin, R., Biddison, W. E. and Germain, R. N., TCR ligand discrimination is enforced by competing ERK positive and SHP- 1 negative feedback pathways, *Nat. Immunol.* **4** (2003) 248-54.

167. Johnson, P., Maiti, A. and Ng, D. H. W., CD45: A family of leukocyte specific cell surface glycoproteins, in *Weir's Handbook of Exp. Immunol.*, Herzenberg, L. A., Weir, D. M., Herzenberg, L. A., and Blackwell, C., Editor. 1997, Blackwell Science, Inc. Malden, MA, U.S.A. p. 62.1-62.16.

168. Streuli, M., Krueger, N. X., Thai, T., Tang, M. and Saito, H., Distinct functional roles of the two intracellular phosphatase like domains of the receptor-linked protein tyrosine phosphatases LCA and LAR, *EMBO J.* **9** (1990) 2399-2407.

169. Felberg, J. and Johnson, P., Characterization of recombinant CD45 cytoplasmic domain proteins - Evidence for intramolecular and intermolecular interactions, *J. Biol. Chem.* **273** (1998) 17839-17845.

170. Felberg, J. and Johnson, P., Stable interdomain interaction within the cytoplasmic domain of CD45 increases enzyme stability, *Biochem. Biophys. Res. Comm.* **271** (2000) 292-298.

171. Autero, M., Saharinen, J., Pessa-Morikawa, T., Soularothhut, M., Oetken, C., Gassmann, M., Bergman, M., Alitalo, K., Burn, P., Gahmberg, C. G. and Mustelin, T., Tyrosine phosphorylation of CD45 phosphotyrosine phosphatase by $p50^{csk}$ kinase creates a binding site for $p56^{lck}$ tyrosine kinase and activates the phosphatase, *Mol. Cell. Biol.* **14** (1994) 1308-1321.

172. Stover, D. R. and Walsh, K. A., Protein-tyrosine phosphatase activity of CD45 is activated by sequential phosphorylation by two kinases, *Mol. Cell. Biol.* **14** (1994) 5523-5532.

173. Ostergaard, H. L. and Trowbridge, I. S., Negative regulation of CD45 protein tyrosine phosphatase activity by ionomycin in T cells, *Science* **253** (1991) 1423-1425.

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

175. Mustelin, T., Coggeshall, K. M. and Altman, A., Rapid activation of the T cell tyrosine kinase pp56^{lck} by the CD45 phosphotyrosine phosphatase, *Proc. Natl. Acad. Sci. U.S.A.* **86** (1989) 6302-6306.

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

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

178. Bork, P. and Doolittle, R. F., Fibronectin type-III modules in the receptor phosphatase CD45 and tapeworm antigens, *Protein Sci.* **2** (1993) 1185-1187.

179. Furukawa, T., Itoh, M., Krueger, N. X., Streuli, M. and Saito, H., Specific interaction of the CD45 protein-tyrosine phosphatase with tyrosine-phosphorylated CD3 ζ chain, *Proc. Natl. Acad. Sci. U.S.A.* **91** (1994) 10928-10932.

180. Mustelin, T., Williams, S., Tailor, P., Couture, C., Zenner, G., Burn, P., Ashwell, J. D. and Altman, A., Regulation of the p70^{ZAP} tyrosine protein kinase in T cells by the CD45 phosphotyrosine phosphatase, *Eur. J. Immunol.* **25** (1995) 942-946.

181. Penninger, J. M., Irie-Sasaki, J., Sasaki, T. and Oliveira-dos-Santos, A. J., CD45: new jobs for an old acquaintance, *Nat. Immunol.* **2** (2001) 389-96.

182. Edmonds, S. D. and Ostergaard, H. L., Dynamic association of CD45 with detergent-insoluble microdomains in T lymphocytes, *J. Immunol.* **169** (2002) 5036-42.

183. Kishihara, K., Penninger, J., Wallace, V. A., Kundig, T. M., Kawai, K., Wakeham, A., Timms, E., Pfeffer, K., Ohashi, P. S., Thomas, M. L., Furlonger, C., Paige, C. J. and Mak, T. W., Normal B-lymphocyte development but impaired T-cell maturation in CD45-exon6 protein tyrosine phosphatase-deficient mice, *Cell* **74** (1993) 143-156.

184. Ng, D. H. W., Jabali, M. D., Maiti, A., Borodchak, P., Harder, K., Brocker, T., Malissen, B., Jirik, F. R. and Johnson, P., CD45 and RPTPα display different protein tyrosine phosphatase activities in T lymphocytes, *Biochem. J.* **327** (1997) 867-876.

185. Mustelin, T. and Altman, A., Dephosphorylation and activation of the T cell tyrosine kinase $pp56^{lck}$ by the leukocyte common antigen (CD45), *Oncogene* **5** (1990) 809-13.

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

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

188. Burns, C. M., Sakaguchi, K., Appella, E. and Ashwell, J. D., CD45 regulation of tyrosine phosphorylation and enzyme activity of Src family kinases, *J. Biol. Chem.* **269** (1994) 13594 - 13600.

189. Guex, N. and Peitsch, M. C., SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling, *Electrophoresis* **18** (1997) 2714-23.

190. Schindler, T., Sicheri, F., Pico, A., Gazit, A., Levitzki, A. and Kuriyan, J., Crystal structure of Hck in complex with a Src family-selective tyrosine kinase inhibitor, *Mol. Cell.* **3** (1999) 639-648.

191. Biffen, M., McMichael-Phillips, D., Larson, T., Venkitaraman, A. and Alexander, D., The CD45 tyrosine phosphatase regulates specific pools of antigen receptor-associated p59fyn and CD4-associated p56Lck tyrosine kinases in human T-cells, *EMBO J.* **13** (1994) 1920-1929.

192. Imbert, V., Rupec, R. A., Livolsi, A., Pahl, H. L., Traenckner, E. B. M., Muellerdieckmann, C., Farahifar, D., Rossi, B., Auberger, P., Baeuerle, P. A. and Peyron, J. F., Tyrosine phosphorylation of i-kappa-b-alpha activates nf-kappa-b without proteolytic degradation of i-kappa-b-alpha, *Cell* **86** (1996) 787-798.

193. Ashwell, J. D. and D'Oro, U., CD45 and Src-family kinases: and now for something completely different, *Immunol. Today* **20** (1999) 412-416.

194. Baker, M., Gamble, J., Tooze, R., Higgins, D., Yang, F. T., O'Brien, P. C., Coleman, N., Pingel, S., Turner, M. and Alexander, D. R., Development of T-leukaemias in CD45 tyrosine phosphatase-deficient mutant lck mice, *EMBO J.* **19** (2000) 4644-4654.

195. Neel, B. G. and Tonks, N. K., Protein tyrosine phosphatases in signal transduction, *Curr. Opin. Cell. Biol.* **9** (1997) 193-204.

196. Neel, B. G., Role of phosphatases in lymphocyte activation, *Curr. Opin. Immunol.* **9** (1997) 405-20.

197. Binstadt, B. A., Billadeau, D. D., Jevremovic, D., Williams, B. L., Fang, N., Yi, T. L., Koretzky, G. A., Abraham, R. T. and Leibson, P. J., Slp-76 Is a Direct Substrate of Shp-1 Recruited to Killer Cell Inhibitory Receptors, *J. Biol. Chem.* **273** (1998) 27518-27523.

198. Plas, D. R., Johnson, R., Pingel, J. T., Matthews, R. J., Dalton, M., Roy, G., Chan, A. C. and Thomas, M. L., Direct regulation of zap-70 by shp-1 in t cell antigen receptor signaling, *Science* **272** (1996) 1173-1176.

199. Lorenz, U., Ravichandran, K. S., Burakoff, S. J. and Neel, B. G., Lack of shptp1 results in Src-family kinase hyperactivation and thymocyte hyperresponsiveness, *Proc. Nat. Acad. Sci. U.S.A.* **93** (1996) 9624-9629.

200. Chiang, G. G. and Sefton, B. M., Specific dephosphorylation of the Lck tyrosine protein kinase at Tyr-394 by the SHP-1 protein-tyrosine phosphatase, *J. Biol. Chem.* **276** (2001) 23173-23178.

201. Cloutier, J. F. and Veillette, A., Association of inhibitory tyrosine protein kinase p50(csk) with protein tyrosine phosphatase PEP in T cells and other hemopoietic cells, *EMBO J.* **15** (1996) 4909-4918.

202. Gjorloff-Wingren, A., Saxena, M., Williams, S., Hammi, D. and Mustelin, T., Characterization of TCR-induced receptor-proximal signaling events negatively

regulated by the protein tyrosine phosphatase PEP, *Eur. J. Immunol.* **29** (1999) 3845-3854.

203. Mustelin, T. and Burn, P., Regulation of Src family tyrosine kinases in lymphocytes, *Trend. Biol. Sci.* **18** (1993) 215-220.

204. Chow, L. M. L., Fournel, M., Davidson, D. and Veillette, A., Negative regulation of T cell receptor signalling by tyrosine protein kinase p50csk, *Nature* **365** (1993) 156-159.

205. Nada, S., Yagi, T., Takeda, H., Tokunaga, T., Nakagawa, H., Ikawa, Y., Okada, M. and Aizawa, S., Constitutive activation of Src family kinases in mouse embryos that lack Csk, *Cell* **73** (1993) 1125-1135.

206. Davidson, D., Cloutier, J. F., Gregorieff, A. and Veillette, A., Inhibitory tyrosine protein kinase p50(csk) is associated with protein-tyrosine phosphatase ptp-pest in hemopoietic and non-hemopoietic cells, *J. Biol. Chem.* **272** (1997) 23455-23462.

207. Torgersen, K. M., Vang, T., Abrahamsen, H., Yaqub, S., Horejsi, V., Schraven, B., Rolstad, B., Mustelin, T. and Tasken, K., Release from tonic inhibition of T cell activation through transient displacement of C-terminal Src kinase (Csk) from lipid rafts, *J. Biol. Chem.* **276** (2001) 29313-29318.

208. Davidson, D., Bakinowski, M., Thomas, M. L., Horejsi, V. and Veillette, A., Phosphorylation-dependent regulation of T-cell activation by PAG/Cbp, a lipid raft-associated transmembrane adaptor, *Mol. Cell. Biol.* **23** (2003) 2017-28.

209. Takeda, A., Wu, J. J. and Maizel, A. L., Evidence for monomeric and dimeric forms of CD45 associated with a 30-kDa phosphorylated protein, *J. Biol. Chem.* **267** (1992) 16651-16659.

210. Schraven, B., Schirren, A., Kirchgessner, H., Siebert, B. and Meuer, S. C., Four CD45/p56^{lck}-associated phosphoproteins (pp29-pp32) undergo alterations in human T-cell activation, *Eur. J. Immunol.* **22** (1992) 1857-1863.

211. Takeda, A., Maizel, A. L., Kitamura, K., Ohta, T. and Kimura, S., Molecular cloning of the CD45-associated 30-kDa protein, *J. Biol. Chem.* **269** (1994) 2357-2360.

212. Schraven, B., Schoenhaut, D., Bruyns, E., Koretzky, G., Eckerskorn, C., Wallich, R., Kirchgessner, H., Sakorafas, P., Labkovsky, B., Ratnofsky, S. and Meuer, S., LPAP, a novel 32-kDa phosphoprotein that interacts with CD45 in human lymphocytes, *J. Biol. Chem.* **269** (1994) 29102-29111.

213. Kitamura, K., Matsuda, A., Motoya, S. and Takeda, A., CD45-associated protein is a lymphocyte-specific membrane protein expressed in two distinct forms, *Eur. J. Immunol.* **27** (1997) 383-388.

214. Cahir McFarland, E. D. and Thomas, M. L., CD45 protein-tyrosine phosphatase associates with the WW domain-containing protein, CD45AP, through the transmembrane region, *J. Biol. Chem.* **270** (1995) 28103-28107.

215. Kitamura, K., Maiti, A., Ng, D. H. W., Johnson, P., Maizel, A. L. and Takeda, A., Characterization of the interaction between CD45 and CD45-AP, *J. Biol. Chem.* **270** (1995) 21151-21157.

216. Bruyns, E., Hendricks-Taylor, L. R., Meuer, S., Koretzky, G. A. and Schraven, B., Identification of the sites of interaction between lymphocyte phosphatase-associated phosphoprotein (LPAP) and CD45, *J. Biol. Chem.* **270** (1995) 31372-31376.

217. Matsuda, A., Motoya, S., Kimura, S., McInnis, R., Maizel, A. L., and Takeda, A., Disruption of lymphocyte function and signaling in CD45-associated protein null mice, *J. Exp. Med.* **187** (1998) 1863-1870.

218. Kung, C., Okumura, M., Seavitt, J. R., Noll, M. E., White, L. S., Pingel, J. T. and Thomas, M. L., CD45-associated protein is not essential for the regulation of antigen receptor-mediated signal transduction, *Eur. J. Immunol.* **29** (1999) 3951-3955.

219. Ding, I., Bruyns, E., Li, P., Magada, D., Paskind, M., Rodman, L., Seshadri, T., Alexander, D., Giese, T. and Schraven, B., Biochemical and functional analysis of mice deficient in expression of the CD45-associated phosphoprotein LPAP, *Eur. J. Immunol.* **29** (1999) 3956-3961.

220. Motoya, S., Kitamura, K., Matsuda, A., Maizel, A. L., Yamamoto, H. and Takeda, A., Interaction between CD45-AP and protein-tyrosine kinases involved in T cell receptor signaling, *J. Biol. Chem.* **274** (1999) 1407-1414.

221. Veillette, A., Soussou, D., Latour, S., Davidson, D. and Gervais, F. G., Interactions of CD45-associated protein with the antigen receptor signaling machinery in T-lymphocytes, *J. Biol. Chem.* **274** (1999) 14392-14399.

222. Abraham, K. M., Levin, S. D., Marth, J. D., Forbush, K. A. and Perlmutter, R. M., Thymic tumorigenesis induced by overexpression of p56lck, *Proc. Natl. Acad. Sci. U. S. A.* **88** (1991) 3977-81.

223. Majolini, M. B., Boncristiano, M. and Baldari, C. T., Dysregulation of the protein tyrosine kinase LCK in lymphoproliferative disorders and in other neoplasias, *Leuk. Lymphoma* **35** (1999) 245-54.

224. Goldman, F. D., Ballas, Z. K., Schutte, B. C., Kemp, J., Hollenback, C., Noraz, N. and Taylor, N., Defective expression of p56lck in an infant with severe combined immunodeficiency, *J. Clin. Invest.* **102** (1998) 421-9.

225. Briggs, S. D., Lerner, E. C. and Smithgall, T. E., Affinity of Src family kinase SH3 domains for HIV Nef in vitro does not predict kinase activation by Nef in vivo, *Biochemistry* **39** (2000) 489-95.

226. Cheng, H., Hoxie, J. P. and Parks, W. P., The conserved core of human immunodeficiency virus type 1 Nef is essential for association with Lck and for enhanced viral replication in T-lymphocytes, *Virology* **264** (1999) 5-15.

227. Nervi, S., Atlan-Gepner, C., Kahn-Perles, B., Lecine, P., Vialettes, B., Imbert, J. and Naquet, P., Specific deficiency of p56lck expression in T lymphocytes from type 1 diabetic patients, *J. Immunol.* **165** (2000) 5874-83.

228. Matache, C., Stefanescu, M., Onu, A., Tanaseanu, S., Matei, I., Frade, R. and Szegli, G., p56lck activity and expression in peripheral blood lymphocytes from patients with systemic lupus erythematosus, *Autoimmunity* **29** (1999) 111-20.

229. Wegener, A. M. K., Letourneur, F., Hoeveler, A., Brocker, T., Luton, F. and Malissen, B., The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules, *Cell* **68** (1992) 83-95.

230. Lesley, J., He, Q., Miyake, K., Hamann, A., Hyman, R. and Kincade, P. W., Requirements for hyaluronic acid binding by CD44: a role for the cytoplasmic domain and activation by antibody, *J. Exp. Med.* **175** (1992) 257-266.

231. Nottenburg, C., Rees, G. and St. John, T., Isolation of mouse CD44 cDNA: structural features are distinct from the primate cDNA, *Proc. Natl. Acad. Sci. (U.S.A.)* **86** (1989) 8521-8525.

232. Miyake, K., Medina, K. L., Hayashi, S.-I., Ono, S., Hamaoka, T. and Kincade, P. W., Monoclonal antibodies to Pgp-1/CD44 block lympho-hemopoiesis in long-term bone marrow cultures, *J. Exp. Med.* **171** (1990) 477-488.

233. Trowbridge, I. S., Lesley, J., Schulte, R., Hyman, R. and Trotter, J., Biochemical characterization and cellular distribution of a polymorphic, murine cell-surface glycoprotein expressed on lymphoid tissues, *Immunogenetics* **15** (1982) 299-312.

234. Trowbridge, I. S., Interspecies spleen-myeloma hybrid producing monoclonal antibodies against mouse lymphocyte surface glycoprotein, T200, *J. Exp. Med.* **148** (1978) 313-323.

235. Davidson, D., Chow, L. M. L., Fournel, M. and Veillette, A., Differential regulation of the T cell antigen responsiveness by isoforms of the src related tyrosine protein kinase, p59fyn, *J. Exp. Med.* **175** (1992) 1483-1492.

236. Studier, F. W., Rosenberg, A. H., Dunn, J. J. and Dubendorff, J. W., Use of a T7 RNA polymerase to direct expression of cloned genes, *Methods Enzymol.* **185** (1990) 60-89.

237. Yasukawa, T., Kanei-Ishii, C., Maekawa, T., Fujimoto, J., Yamamoto, T. and Ishii, S., Increase of solubility of foreign proteins in *Escherichia coli* by coproduction of the bacterial thioredoxin, *J. Biol. Chem.* **270** (1995) 25328-25331.

238. Ng, D. H. W., Maiti, A. and Johnson, P., Point mutation in the second phosphatase domain of CD45 abrogates tyrosine phosphatase activity, *Biochem. Biophys. Res. Comm.* **206** (1995) 302-309.

239. Karasuyama, H., Kudo, A. and Melchers, F., The proteins encoded by the VpreB and 5 pre-B cell specific genes can associate with each other and with m heavy chain, *J. Exp. Med.* **172** (1990) 969-972.

240. Pelicci, G., Lanfrancone, L., Grignani, F., McGlade, J., Cavallo, F., Forni, G., Nicoletti, I., Grignani, F., Pawson, T. and Pelicci, P. G., A Novel Transforming Protein (SHC) with an SH2 Domain Is Implicated in Mitogenic Signal Transduction, *Cell* **70** (1992) 93-104.

241. Suen, K. L., Bustelo, X. R., Pawson, T. and Barbacid, M., Molecular cloning of the mouse grb2 gene: differential interaction of the Grb2 adaptor protein with epidermal growth factor and nerve growth factor receptors, *Mol. Cell. Biol.* **13** (1993) 5500-12.

242. Charest, D., Mordret, G., Harder, K., Jirik, F. and Pelech, S., Molecular cloning, expression, and characterization of the human mitogen-activated protein kinase p44erk1, *Mol. Cell. Biol.* **13** (1993) 4679-4690.

243. Gervais, F. G., Chow, L. M., Lee, J. M., Branton, P. E. and Veillette, A., The SH2 domain is required for stable phosphorylation of p56lck at tyrosine 505, the negative regulatory site, *Mol. Cell. Biol.* **13** (1993) 7112-21.

244. Xu, H. and Littman, D. R., A kinase-independent function of lck in potentiating antigen-specific T cell activation, *Cell* **74** (1993) 633-643.

245. Thomas, M. L., Reynolds, P. J., Chain, A., Ben-Neriah, Y. and Trowbridge, I. S., B-cell variant of mouse T200 (Ly-5): Evidence for alternative mRNA splicing., *Proc. Natl. Acad. Sci. U.S.A.* **84** (1987) 5360-5363.

246. Hartley, D. A., Amdjadi, K., Hurley, T. R., Lund, T. C., Medveczky, P. G. and Sefton, B. M., Activation of the Lck tyrosine protein kinase by the Herpesvirus saimiri tip protein involves two binding interactions, *Virology* **276** (2000) 339-348.

247. D'Oro, U. and Ashwell, J. D., Cutting edge: The CD45 tyrosine phosphatase is an inhibitor of Lck activity in thymocytes, *J. Immunol.* **162** (1999) 1879-1883.

248. Schraven, B., Kirchgessner, H., Gaber, B., Samstag, Y. and Meuer, S., A functional complex is formed in human T lymphocytes between the protein tyrosine phosphatase, CD45, the protein tyrosine kinase, $p56^{lck}$ and pp32, a possible common substrate, *Eur. J. Immunol.* **21** (1991) 2469-2477.

249. Watts, J. D., Wilson, G. M., Ettehadieh, E., Clark-Lewis, I., Kubanek, C. A., Astell, C. R., Marth, J. D. and Aebersold, R., Purification and Initial characterization of the lymphocyte-specific protein-tyrosyl kinase p56lck froma baculovirus expression system, *J. Biol. Chem.* **267** (1992) 901-907.

250. Denu, J. M., Lohse, D. L., Vijayalakshmi, J., Saper, M. A. and Dixon, J. E., Visualization of intermediate and transition-state structures in protein-tyrosine phosphatase catalysis, *Proc. Natl. Acad. Sci. U.S.A.* **93** (1996) 2493-2498.

251. Mayer, B. J., Jackson, P. K., Van Etten, R. A. and Baltimore, D., Point mutations in the abl SH2 domain coordinately impair phosphotyrosine binding in vitro and transforming activity in vivo, *Mol. Cell. Biol.* **12** (1992) 609-18.

252. Nam, H. J., Poy, F., Krueger, N. X., Saito, H. and Frederick, C. A., Crystal structure of the tandem phosphatase domains of RPTP LAR, *Cell* **97** (1999) 449-457.

253. Hanks, S. K. and Hunter, T., The eukaryotic protein kinase superfamily. The protein kinase facts book: protein tyrosine kinases, ed. Hardie, G. and Hanks, S. K. (1995): Academic Press Ltd. 7-47.

254. Hanks, S. K., Quinn, A. M. and Hunter, T., The protein kinase family: conserved features & deduced phylogeny of the catalytic domains, *Science* **241** (1988) 42-51.

255. Crews, C. M., Alessandrini, A. A. and Erikson, R. L., Mouse Erk-1 gene product is a serine/threonine protein kinase that has the potential to phosphorylate tyrosine, *Proc. Natl. Acad. Sci. U.S.A.* **88** (1991) 8845-9.

256. Flint, A. J., Tiganis, T., Barford, D. and Tonks, N. K., Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases, *Proc. Natl. Acad. Sci. U.S.A.* **94** (1997) 1680-5.

257. Gervais, F. G. and Veillette, A., Reconstitution of interactions between protein-tyrosine phosphatase CD45 and tyrosine-protein kinase $p56^{lck}$ in nonlymphoid cells, *J. Biol. Chem.* **272** (1997) 12754-12761.

258. Weil, R. and Veillette, A., Intramolecular and extramolecular mechanisms repress the catalytic function of p56lck in resting T lymphocytes, *J. Biol. Chem.* **269** (1994) 22830-22838.

259. Barford, D., Jia, Z. C. and Tonks, N. K., Protein tyrosine phosphatases take off, *Nature Struct. Biol.* **2** (1995) 1043-1053.

260. Song, H., Hanlon, N., Brown, N. R., Noble, M. E., Johnson, L. N. and Barford, D., Phosphoprotein-protein interactions revealed by the crystal structure of kinase-associated phosphatase in complex with phosphoCDK2, *Mol. Cell* **7** (2001) 615-26.

261. Camps, M., Nichols, A., Gillieron, C., Antonsson, B., Muda, M., Chabert, C., Boschert, U. and Arkinstall, S., Catalytic Activation of the Phosphatase Mkp-3 By Erk2 Mitogen-Activated Protein Kinase, *Science* **280** (1998) 1262-1265.

262. Dadke, S., Kusari, J. and Chernoff, J., Down-regulation of insulin signaling by protein-tyrosine phosphatase 1B is mediated by an N-terminal binding region, *J. Biol. Chem.* **275** (2000) 23642-7.

263. Tonks, N. K. and Neel, B. G., Combinatorial control of the specificity of protein tyrosine phosphatases, *Curr. Opin. Cell Biol.* **13** (2001) 182-195.

264. Zheng, X. M., Wang, Y. and Pallen, C. J., Cell transformation and activation of pp60c-src by overexpression of a protein tyrosine phosphatase, *Nature* **359** (1992) 336-339.

265. den Hertog, J., Pals, C. E. G. M., Peppelenbosch, M. P., Tertoolen, L. G. J., Delaat, S. W. and Kruijer, W., Receptor Protein Tyrosine Phosphatase-alpha Activates pp60(c-src) and Is Involved in Neuronal Differentiation, *EMBO J.* **12** (1993) 3789-3798.

266. den Hertog, J., Tracy, S. and Hunter, T., Phosphorylation of receptor protein-tyrosine phosphatase α on Tyr789, a binding site for the SH3-SH2-SH3 adaptor protein GRB-2 in vivo, *EMBO J.* **13** (1994) 3020-3032.

267. Zheng, X. M., Resnick, R. J. and Shalloway, D., A phosphotyrosine displacement mechanism for activation of Src by PTP α, *EMBO J.* **19** (2000) 964-978.

268. Cahir McFarland, E. D., Pingel, J. and Thomas, M. L., Definition of amino acids sufficient for plasma membrane association of CD45 and CD45-associated protein, *Biochem.* **36** (1997) 7169-7175.

269. Bruyns, E., Kirchgessner, H., Meuer, S. and Schraven, B., Biochemical analysis of the CD45-p56^{lck} complex in Jurkat T cells lacking expression of lymphocyte phosphatase-associated phosphoprotein, *Int. Immunol.* **10** (1998) 185-194.

270. Matsuda, A., Motoya, S., Kimura, S., McInnis, R., Maizel, A. L. and Takeda, A., Disruption of Lymphocyte Function and Signaling in CD45-Associated Protein-Null Mice, *J. Exp. Med.* **187** (1998) 1863-1870.

271. Schmits, R., Filmus, J., Gerwin, N., Senaldi, G., Kiefer, F., Kundig, T., Wakeham, A., Shahinian, A., Catzavelos, C., Rak, J., Furlonger, C., Zakaran, A., Simard, J. J. L., Ohashi, P. S., Paige, C. J., Gutierrezramos, J. C. and Mak, T. W., CD44 regulates hematopoietic progenitor distribution, granuloma formation and tumorigenicity, *Blood* **90** (1997) 2217-2233.

272. Ponta, H., Sherman, L. and Herrlich, P. A., CD44: from adhesion molecules to signalling regulators, *Nat. Rev. Mol. Cell Biol.* **4** (2003) 33-45.

273. Dianzani, U., Bragardo, M., Tosti, A., Ruggeri, L., Volpi, I., Casucci, M., Bottarel, F., Feito, M. J., Bonissoni, S. and Velardi, A., CD44 signaling through p56(lck) involves lateral association with CD4 in human CD4(+) T cells, *Int. Immunol.* **11** (1999) 1085-1092.

274. Turner, J. M., Brodsky, M. H., Irving, B. A., Levin, S. D., Perlmutter, R. M. and Littman, D. R., Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs, *Cell* **60** (1990) 755-65.

275. Glaichenhaus, N., Shastri, N., Littman, D. R. and Turner, J. M., Requirement for association of $p56^{lck}$ with CD4 in antigen-specific signal transduction in T cells, *Cell* **64** (1991) 511-520.

276. Collins, T. L., Uniyal, S., Shin, J., Strominger, J. L., Mittler, R. S. and Burakoff, S. J., p56lck association with CD4 is required for the interaction between CD4 and the TCR/CD3 complex and for optimal antigen stimulation, *J. Immunol.* **148** (1992) 2159-62.

277. Trowbridge, I. S. and Thomas, M. L., CD45: An emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development, *Annu. Rev. Immunol.* **12** (1994) 85-116.

278. Parolini, I., Sargiacomo, M., Lisanti, M. P. and Peschle, C., Signal transduction and glycophosphatidylinositol-linked proteins (lyn, lck, CD4, CD45, G proteins, and CD55) selectively localize in Triton-insoluble plasma membrane domains of human leukemic cell lines and normal granulocytes, *Blood* **87** (1996) 3783-3794.

279. Parolini, I., Topa, S., Sorice, M., Pace, A., Ceddia, P., Montesoro, E., Pavan, A., Lisanti, M. P., Peschle, C. and Sargiacomo, M., Phorbol ester-induced disruption of the CD4-Lck complex occurs within a detergent-resistant microdomain of the plasma membrane - Involvement of the translocation of activated protein kinase C isoforms, *J. Biol. Chem.* **274** (1999) 14176-14187.

280. Rozsnyay, Z., Signaling complex formation of CD44 with src-related kinases, *Immunol. Lett.* **68** (1999) 101-108.

281. Li, R. H., Walker, J. R. and Johnson, P., Chimeric CD4/CD44 molecules associate with CD44 via the transmembrane region and reduce hyaluronan binding in T cell lines, *Eur. J. Immunol.* **28** (1998) 1745-1754.

282. Mackay, J. P. and Crossley, M., Zinc fingers are sticking together, *Trends Biochem. Sci.* **23** (1998) 1-4.

283. Matthews, J. M., Kowalski, K., Liew, C. K., Sharpe, B. K., Fox, A. H., Crossley, M. and MacKay, J. P., A class of zinc fingers involved in protein-protein interactions biophysical characterization of CCHC fingers from fog and U-shaped, *Eur. J. Biochem.* **267** (2000) 1030-8.

284. Shenoi, H., Seavitt, J., Zheleznyak, A., Thomas, M. L. and Brown, E. J., Regulation of integrin-mediated T cell adhesion by the transmembrane protein tyrosine phosphatase CD45, *J. Immunol.* **162** (1999) 7120-7127.

285. Legg, J. W., Lewis, C. A., Parsons, M., Ng, T. and Isacke, C. M., A novel PKC-regulated mechanism controls CD44 ezrin association and directional cell motility, *Nat. Cell. Biol.* **4** (2002) 399-407.

286. Luo, K. X. and Sefton, B. M., Cross-linking of T-cell surface molecules CD4 and CD8 stimulates phosphorylation of the lck tyrosine protein kinase at the autophosphorylation site, *Mol. Cell. Biol.* **10** (1990) 5305-13.

287. Ilangumaran, S., Borisch, B. and Hoessli, D. C., Signal transduction via CD44: Role of plasma membrane microdomains, *Leuk. Lymphoma.* **35** (1999) 455-469.

288. Filipp, D., Zhang, J., Leung, B. L., Shaw, A., Levin, S. D., Veillette, A. and Julius, M., Regulation of Fyn through translocation of activated Lck into lipid rafts, *J. Exp. Med.* **197** (2003) 1221-7.

289. Legg, J. W. and Isacke, C. M., Identification and functional analysis of the ezrin-binding site in the hyaluronan receptor, CD44, *Curr. Biol.* **8** (1998) 705-708.

290. Yonemura, S., Hirao, M., Doi, Y., Takahashi, N., Kondo, T., Tsukita, S. and Tsukita, S., Ezrin/radixin/moesin (erm) proteins bind to a positively charged amino acid cluster in the juxta-membrane cytoplasmic domain of cd44, cd43, and icam-2, *J. Cell Biol.* **140** (1998) 885-895.

291. Bretscher, A., Reczek, D. and Berryman, M., Ezrin - a Protein Requiring Conformational Activation to Link Microfilaments to the Plasma Membrane in the Assembly of Cell Surface Structures, *J. Cell Sci.* **110** (1997) 3011-3018.

292. Bretscher, A., Regulation of cortical structure by the ezrin-radixin-moesin protein family, *Curr. Opin. Cell Biol.* **11** (1999) 109-116.

293. Slevin, M., Krupinski, J., Kumar, S. and Gaffney, J., Angiogenic oligosaccharides of hyaluronan induce protein tyrosine kinase activity in endothelial cells and activate a cytoplasmic signal transduction pathway resulting in proliferation, *Lab. Invest.* **78** (1998) 987-1003.

294. Maret, W., Crosstalk of the group IIa and IIb metals calcium and zinc in cellular signaling, *Proc. Natl. Acad. Sci. U. S. A.* **98** (2001) 12325-7.

295. Yamada, H., Minoshima, S., Koizumi, S., Kimura, M. and Shimizu, N., Cadmium-induced synthesis of metallothioneins in human T and B cell purified by a fluorescence activated cell sorter, *Chem. Biol. Interact.* **70** (1989) 117-26.

296. Mesna, O. J., Steffensen, I. L., Hjertholm, H. and Andersen, R. A., Accumulation of metallothionein and its multiple forms by zinc, cadmium and dexamethasone in human peripheral T and B lymphocytes and monocytes, *Chem. Biol. Interact.* **94** (1995) 225-42.

297. Lazo, J. S. and Pitt, B. R., Metallothioneins and cell death by anticancer drugs, *Annu. Rev. Pharmacol. Toxicol.* **35** (1995) 635-53.

298. Mita, M., Imura, N., Kumazawa, Y. and Himeno, S., Suppressed proliferative response of spleen T cells from metallothionein null mice, *Microbiol. Immunol.* **46** (2002) 101-7.

299. Maret, W. and Vallee, B. L., Thiolate ligands in metallothionein confer redox activity on zinc clusters, *Proc. Natl. Acad. Sci. U. S. A.* **95** (1998) 3478-82.

300. Jacob, C., Maret, W. and Vallee, B. L., Control of zinc transfer between thionein, metallothionein, and zinc proteins, *Proc. Natl. Acad. Sci. U. S. A.* **95** (1998) 3489-94.

301. Devadas, S., Zaritskaya, L., Rhee, S. G., Oberley, L. and Williams, M. S., Discrete generation of superoxide and hydrogen peroxide by T cell receptor stimulation: selective regulation of mitogen-activated protein kinase activation and fas ligand expression, *J. Exp. Med.* **195** (2002) 59-70.

302. Thomas, M. L., The regulation of antigen-receptor signaling by protein tyrosine phosphatases: a hole in the story, *Curr. Opin. Immunol.* **11** (1999) 270-276.