FUNCTIONAL AND ENZYMATIC CHARACTERIZATION OF THE
RECEPTOR PROTEIN TYROSINE PHOSPHATASE, CD45

by

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ABSTRACT

CD45 is expressed exclusively on hematopoietic cells and is a major component of the lymphocyte cell surface. CD45 is required both in the development and in the antigen induced activation of lymphoid cells. Despite the demonstration of several *in vitro* substrates, only the src family kinases, $p56^{lck}$ and $p59^{fyn}$ have been clearly identified as potential *in vivo* subtrates. How the substrate specificity of CD45 is achieved *in vivo* is not known. CD45 isolated directly from cells and recombinant cytoplasmic CD45 did not discriminate between a variety of src-family phosphopeptides - indicating no substrate specificity at the peptide level. However, a direct interaction between $p56^{lck}$ and cytoplasmic CD45 was demonstrated *in vitro*. Several regions of $p56^{lck}$ could mediate this interaction, which was further modulated by the tyrosine phosphorylation state of $p56^{lck}$ and by the protein tyrosine phosphatase (PTP) activity of CD45. Overall, results demonstrated a complex interaction that provides insight into the enzyme/substrate relationship. To evaluate the substrate specificity and function of CD45 under cellular conditions, another receptor PTP, RPTPα was expressed in a CD45$^-$, T cell receptor (TCR)$^+$, BW5147 T lymphoma cell for comparative studies. Unlike CD45, high levels of RPTPα did not fully restore either proximal or distal TCR mediated signaling events. Furthermore, immunoprecipitated RPTPα was approximately one-seventh to one-tenth as active as CD45 when tested against artificial substrates. This difference in activity was also observed using recombinant enzymes. Therefore, CD45 is intrinsically a more efficient phosphatase, providing one reason why RPTPα is not able to substitute for CD45. This work reveals possible mechanisms

that may account for CD45 substrate specificity under cellular environments. Ultimately, this information should assist in both the understanding of lymphoid cell biology and in the assessment of experimental and clinical targets towards modulation of lymphoid cell function.

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LIST OF ABBREVATIONS

Ab	antibody
ATP	adenosine 5′-triphosphate
BCA	bicinchoninic acid
BCR	B cell receptor
BSA	bovine serum albumin
CD	cluster of differentiation
CIP	calf intestinal phosphatase
DMEM	Dulbecco's Modified Eagle Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine-tetraactetic acid
ELISA	enzyme linked immunosorbent assay
EGF	epidermal growth factor
FACS	fluorescence activated cell sorter
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
FPLC	fast protein, peptide and polynucleotide liquid chromatography
GSH	glutathione
GST	glutathione S transferase
HEL	hen egg lysozyme
HIV	human immunodeficiency virus
HLA	human histocompatibility leukocyte antigen
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IL	interleukin
IPTG	isopropyl-β-D-thiogalactosidase
ITAM	immunoreceptor tyrosine-based activation motif
LCMV	Lymphocytic choriomeningitis virus
MHC	major histocompatibility complex
NK	natural killer
O.D.	optical density
PBS	phosphate buffered saline
PMSF	phenylmethylsulfonyl fluoride
pNPP	p-nitrophenylphosphate
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride
RIPA	rapid immunoprecipitation assay

RPTP	receptor protein tyrosine phosphatase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	src homology domain 2
SH3	src homology domain 3
TBS	tris buffered saline
TCA	trichloroacetic acid
TCR	T cell receptor
TTBS	tween-tris buffered saline

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CHAPTER 1

Introduction

It is clear that in living systems, the relevance of the phosphate ester bond is seen in a number of situations. It plays a crucial role as the principle chemical bond providing energy for the living cell, and is also a key component in the long and continuous molecular structures of DNA and RNA. Moreover, and particularily important for the subjects dealt within this thesis, the phosphate ester bond, in chemically modifying certain amino acids, grants living systems an efficient mechanism for the regulation of protein function.

This process of protein phosphorylation is a reversible, post translational modification and involves two classes of enzymes which act to phosphorylate (kinases) and dephosphorylate (phosphatases) protein substrates (reviewed in Bishop, 1991; Cantley et al., 1991b). In eukaryotes, the principle amino acids that, to date, have been demonstrated to be phosphorylated are serine, threonine and tyrosine; all of which contain a hydroxyl group capable of accepting the phosphoester. There exists a careful balance between the actions of these protein kinases and phosphatases which is essential for the efficient orchestration of several cellular processes such as cell signaling, division, differentiation, survival and cell death. Furthermore, the control of phosphorylation processes is crucial in the propagation of cellular responses to external stimuli. This notion is particularly prevalent in transformed cells, where abnormal proliferation can, in some cases, be attributed to aberrant amounts of tyrosine phosphorylation resulting from the expression of activated, oncogene-encoded protein tyrosine kinases.

This thesis introduction will predominantly focus on the function and structure of the protein tyrosine phosphatase, CD45 (also known as the leukocyte common antigen (LCA) because of its expression on cells of

the hematopoietic origin). Since CD45 has often been termed the "prototypic" receptor protein tyrosine phosphatase (PTP), both its role in the signal transduction of immune cells and its enzymatic properties will be reviewed.

1.1 The identification of CD45

The identification of many T cell surface molecules was largely due to a genetic approach devised in the 1960s by Boyse and Old (1969). This involved the production of congenic mice strains to allow the isolation of polymorphic determinants present on the lymphocyte cell surface. CD45 was first observed in the early seventies using allogenic and xenogenic antisera in both rat and mouse (Lubaroff, 1973; Komuro et al., 1975; Trowbridge et al., 1975; Fabre and Williams, 1977). However, with the advent of monoclonal antibodies (Kohler and Milstein, 1975), as well as the development of fluorescence activated cell sorter (FACS) techniques, the systematic characterization of lymphocyte cell surface molecules became a reality (Williams et al., 1977; Milstein and Lennox, 1980).

Monoclonal antibodies have been raised against mouse (Trowbridge, 1978), rat (Sunderland et al., 1979) and human CD45 (Andersson et al., 1980; Dalchau et al., 1980; Omary et al., 1980), and initial characterization of this molecule showed that it was highly abundant on the lymphocyte cell surface and that it had heterogeneity in its apparent molecular weight (Williams and Barclay, 1985; Thomas, 1989). The resolution of this heterogeneity was achieved when CD45 was subsequently cloned (Thomas et al., 1985; Saga et al., 1986; Barclay et al., 1987; Ralph et al., 1987; Raschke, 1987; Saga et al., 1987; Streuli et al., 1987; Thomas et al., 1987), and its genomic sequence determined (Hall et al.,

1988; Johnson et al., 1989). Here, it was noted that alternative splicing of three different exons could occur and that the regulated expression of these exons resulted in additional protein and carbohydrate structures found near the amino-terminal ends of the molecule, thereby creating new antigenic determinants (Jackson and Barclay, 1989). These differences in the expression of exons 4, 5, and 6 (designated A, B, and C, respectively), essentially accounted for the heterogeneity observed earlier. Furthermore, a standard method of nomenclature was adopted for the different isoforms, where the CD45 isoform expressing all three alternative spliced exons is termed CD45RABC, and the CD45 isoform expressing none of the exons is termed CD45RO. At present six different CD45 isoforms have been identified, although alternative splicing makes it possible for the existence of eight different CD45 isoforms. While the expression of the CD45 isoforms appears to follow tightly regulated patterns, it is important to note that such patterns are invariably dependant on the cell type, the species type, as well as the stage of development for the aforementioned cell (Johnson et al., 1997). Given this complication in variance, the functional relevance of the different isoforms has remained largely elusive. Presently, research interest in the extracellular domain of CD45 is strong, particularily with efforts to find possible ligand interactions. However, as the work presented in this thesis will predominantly center around the enzymatic activity of CD45, this introduction will place an emphasis on the cytoplasmic domain of CD45. Further information regarding the extracellular region of CD45 has been reviewed (Johnson et al., 1997).

In the early 1980's, knowledge on CD45 dictated that it was a cell surface molecule restricted to cells of the hematopoietic lineage.

Furthermore, it was both a relatively large and abundant molecule in these cells. The structural details of CD45 were first characterized with the establishment of a purification procedure, that led to the determination of the primary sequence of rat CD45 in 1985 (Thomas et al., 1985). Using a monoclonal antibody and affinity chromatography techniques, CD45 was purified in sufficient amounts such that partial amino acid sequence could be obtained from tryptic peptides. This partial sequence subsequently allowed the determination of the full sequence by probing a cDNA library. The cloning of this first rat CD45, quickly led to the subsequent cloning of cDNA's from various lymphoid populations obtained from rat, mouse and human species (Thomas et al., 1985; Saga et al., 1986; Barclay et al., 1987; Ralph et al., 1987; Raschke, 1987; Saga et al., 1987; Streuli et al., 1987; Thomas et al., 1987). Analysis of all CD45 sequences revealed the presence of an extracellular domain, a single spanning transmembrane domain, and an unusually large cytoplasmic domain containing two regions of internal homology.

However, the first insight into the functional properties of CD45 appeared in 1988 when the partial amino acid sequence for a protein tyrosine phosphatase was determined (Tonks, 1988; Tonks et al., 1988). It was observed that there was sequence identity between human placental PTP1B and each of the internally homologous cytoplasmic regions of CD45 (Charbonneau et al., 1988). A 157 amino acid segment, derived from PTP1B, was found to have 40% identity with the first region of homology in CD45 and appproximately 30% identity with the second region of homology. The extent of this sequence identity strongly indicated that the polypeptide chains of these domains may fold in the same manner and produce similar three dimensional structures. This led to the proposal

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that the CD45 family of proteins were catalytically active PTPs, and that CD45 may represent a set of cell-surface receptors involved in signal transduction. Ultimately, further work by Tonks et al. (1990) demonstrated that endogenously purified CD45 did indeed have PTP activity.

1.2 Regulation of cell signaling by CD45

It is interesting to note that the role of CD45 as a signal transduction molecule was first suggested less than 10 years ago. This pays tribute to the efforts and speed of many scientists as today, there is a wealth of research information implicating CD45 in signal transduction pathways.

Initial observations relating to the importance of tyrosine phosphorylation was obtained from work on transformed cells, where levels of tyrosine phosphorylation were noticeably elevated (Cantley et al., 1991b). In some instances, this was attributed to the expression of activated tyrosine kinases. Consequently researchers were led to believe that the identification of protein tyrosine phosphatases may give important information in discerning the relationship between tyrosine phosphorylation, signal transduction, and possible ways of controlling transformed phenotypes. Surprisingly, the functional role of CD45 has never been equated to a tumor suppressor status. Instead, considerable evidence from a number of laboratories suggested that surface expression of this molecule is required for the occurrence of an effective activation signal in immune cells (reviewed in Trowbridge and Thomas, 1994). The following section of the introduction will therefore focus and summarize the data supporting CD45's role in lymphoid cell signal transduction.

The role of CD45 in T cell activation

First, direct evidence for CD45's involvement in signal transduction, and in T cell signaling in particular, was obtained from the analyses of CD45-deficient cell lines. Pingel and Thomas were the first to characterize a T cell clone that was deficient in the cell surface expression of CD45 (Pingel and Thomas, 1989). This mutant $CD4^+$ clone was generated using chemical mutagenesis techniques and selected for by complement mediated cytolysis. Upon receptor stimulation either with antigen or with anti-CD3 antibody crosslinking, these cells were unable to respond efficiently as assessed by proliferation assays. These cells, however, were still able to proliferate in response to IL2 stimulation, implying that the overall cellular machinary for cell cycle and growth was functional. Similar trends were also observed with analyses performed on a CD45 deficient murine $CD8^+$ cytotoxic T cell clone (Weaver et al., 1991). Here, antigen receptor mediated stimulation did not result in cell proliferation or in γ-interferon production. Furthermore, these $CD45^-$ cells were unable to proceed with targeted cell lysis. Since cell line revertants, which had restored CD45 surface expression, could regain their ability to respond normally to TCR stimulation, there was now a strong indication that CD45 played a major role in TCR mediated signaling.

The second experimental strategy involved the use of monoclonal antibodies to co-ligate cell surface signaling molecules. Generally, studies were performed to crosslink CD45 molecules with other signaling molecules (Ostergaard and Trowbridge, 1990; Ledbetter et al., 1991), or were carried out to co-ligate molecules of CD45 with each other (Yakura, 1983; Lefrancois and Bevan, 1985; Mittler et al., 1987; Ledbetter et al., 1988; Kiener and Mittler, 1989; Marvel et al., 1989; Schraven et al., 1989; Hasegawa et al.,

1990; Smeland et al., 1990; Goldman et al., 1992). Although, results definitely implicated CD45's role in both T cell receptor (TCR) and B cell receptor (BCR) mediated signaling processes, the observations yielded conflicting conclusions. The fact that similar experimental strategies would give both positive and negative responses, was often attributed to differences in the cell system studied and the specific antibodies used.

The demonstration that CD45 was important in TCR-mediated cell activation was especially interesting in light of concurrent research on the proximal events involved in TCR signal transduction. Here, a number of groups had suggested that the first biochemically observable event upon TCR ligation was the activation of protein tyrosine kinases and the concomitant increase in cellular tyrosine phosphorylation levels (Samelson et al., 1986; Patel et al., 1987; June et al., 1990). This process apparently took place before the following signaling events: the generation of phosphatidylinositol-derived second messengers through the activation of phospholipase enzymes, the advent of intracellular calcium oscillations, and the activation of other important signaling molecules such as protein kinase C, and the p21^{ras} GTPase. Detailed characterization of CD45 deficient cell lines showed that all of the processes mentioned above did not occur or were severely hindered upon stimulation through the T cell receptor (Koretzky et al., 1990; Koretzky et al., 1991; Weaver et al., 1991; Pingel et al., 1994). Importantly, the CD45 deficiency affected the ability to generate increased tyrosine phosphorylation. Tyrosine phosphorylation levels in the cell lysate could be conveniently monitored using a monoclonal antibody specific for phosphotyrosine. Under these circumstances and using normal (CD45$^+$) T cells, the increase in the number of phosphotyrosine containing bands was prevalent within 90

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seconds after stimulation. By comparison, T cells devoid of CD45 expression, were unable to induce the increase in phosphotyrosine containing proteins. Consequently, this data demonstrated that CD45 was crucial for the efficient execution of these early activation events and strongly indicated a positive and proximal role for CD45 in the TCR signaling cascade.

However, it should be cautioned that the loss of CD45 expression did not always result in identical phenotypes. In fact, several CD45 negative T cell lines show observable, albeit greatly diminished, induction of tyrosine phosphorylation levels after stimulation. This observation has been shown using a CD45 negative variant of the Jurkat cell line, which can be partially activated (Peyron et al., 1991). TCR ligation of this cell line demonstrated a partial response with only a small but detectable increase in intracellular free calcium. Volarevic et al. (1992) have also described a similar cautionary note with a CD45-deficient variant of YAC-1 T cells. These cells generated weakened but observable increases in intracellular free calcium, and interestingly appeared to have constitutively high levels of tyrosine phosphorylated proteins that were not altered during the signaling response. These data, therefore, indicate that CD45 affects signaling thresholds, whereby the differing readouts from cell line to cell line are a direct consequence of subtle differences in the expression levels of other signaling molecules (Appleby et al., 1992; Stein et al., 1992). In addition, the existence of CD45 dependant and independant pathways has also been addressed and lately given precedence to the discovery of a possible T cell activation pathway that did not require CD45 (Chu et al., 1996). The molecular basis for some of these ideas will be discussed in further detail in later sections of this introduction.

The role of CD45 in B cell activation

The success of determining CD45's functional signaling role in T cells by using CD45 deficient T cell lines has prompted other investigators to use a similar genetic approach to examine the role of CD45 in B cell receptor signaling. However, in this regard, the results are not as clear and appear to be strongly dependant on the cell system examined. For example, it was found that immature B cell mutants lacking CD45 expression had a high constitutive level of basal tyrosine phosphorylation that could not be increased upon membrane bound IgM ligation (Ogimoto et al., 1994). Despite this lack of additional tyrosine phosphorylation, there coincided a delayed but prolonged rise in intracellular calcium levels. These observable trends are in stark contrast to data obtained from a mature CD45-deficient cell line, BAL 17 (Ogimoto et al., 1995). Here, BCR-induced activation displayed a profile of tyrosine phosphorylation that was almost identical to that observed with the parental cells, yet the production of calcium fluxes was markably decreased. Furthermore, in these same BAL17 cells, it was determined that CD45 was also required for receptor induced growth inhibition. Analysis of CD45's role in differentiated B cells, was conducted on the plasmocytoma cell line J558Lµm3 that was transfected with cDNA for surface IgM (Justement et al., 1991). In this instance, the calcium response upon BCR stimulation was completely abrogated in the CD45 negative cells. This provides yet another example illustrating the variation of CD45's role in B cell signal transduction. Taken together, these data suggest that the different responses observed may be in accordance to the stage of maturation or differentiation of the B cell.

CD45 deficient mice

The use of homologous recombination and gene targeting techniques to generate mice with specific gene disruptions has proven to be an invaluable procedure in delineating the functions of many genes. CD45 knockout mice were produced by replacing exon 6 with a neomycin-resistance cassette (Kishihara et al., 1993). Despite the ability of exon 6 to be alternatively spliced, this strategy resulted in a mouse that lacked CD45 surface expression on all B cells and on 97% of thymocytes. Consequently, investigators have used these mice as powerful tools to explore the role of CD45 in B and T cell development.

The nuances of lymphocyte development revolve around the ability of the animal to permit the maturation of useful lymphoid cells, those that express specific receptor molecules capable of recognizing foreign antigens, and concurrently delete or negatively select cells that may harbour dangerous self reactive receptors. Work reported using the CD45 knockout mice, has played a major role in elucidating some of the finer points in this process.

When examining the effect of disrupted CD45 expression in B cell development, it was observed that CD45 had a subtle role in this cellular process (Kishihara et al., 1993). Surprisingly, there appeared to be wild type numbers of B cells present, and these cells were able to proliferate normally in response to lipopolysaccharide stimulation. However, signaling via surface IgM ligation was severely abrogated, providing further evidence for CD45 involvement in BCR signal transduction processes. More recently, CD45's role in B cell development was further defined by Cyster et al. (1996). In this experimental system, CD45 deficient

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mice were crossed with mice carrying the immunoglobulin transgene specific for hen egg lysozyme (HEL), and B cell development was monitored in the absence and presence of circulating HEL autoantigen. In the absence of the HEL autoantigen, it was observed that significantly fewer CD45 negative B cells accumulated, although wild type levels of immature B cells were still present. In contrast, the presence of the HEL autoantigen, which would normally negatively select for B cells expressing the immunoglobin transgene, appeared to positively select for the CD45$^{-/-}$ HEL binding B cells. This data, therefore, clearly establishes a role for CD45 in defining signal thresholds for the proper development of self-reacting and non self-reacting B cells.

Analysis on the CD45 exon 6 knockout mice by dual staining FACS analysis showed a profound block in thymocyte development (Kishihara et al., 1993). More specifically, there were elevated numbers of CD4$^-$ CD8$^-$ cells and a substantial decrease in single positive CD4$^+$ and CD8$^+$ cells, suggesting an inability for the developing thymocytes to proceed beyond the double positive CD4$^+$ CD8$^+$ stage. In effect, this resulted in the presence of very few peripheral T cells, and these cells were defective in both signaling via TCR ligation and in facilitating a cytotoxic response after TCR stimulation.

Furthermore, in a recent study where comparisons were made between mice that were homozygous or heterozygous for the disrupted CD45 gene, and were also carrying a TCR transgene (P14) specific for the lymphocytic choriomeningistis virus (LCMV) (Wallace et al., 1997). Again, it was noted that the level of CD45 expression and resultant PTP activity, as determined by the differences between the WT, CD45$^{-/-}$ and CD45$^{+/-}$ mice, could affect the outcomes of thymocyte development. In

particular, maturation of thymocytes expressing the P14 TCR is enhanced in CD45$^{+/-}$ mice, as assessed by transgenic TCR intensity on single positive CD4$^+$ or CD8$^+$ thymocytes and by peripheral T cell counts. Furthermore, negative selection is induced more efficiently in P14 CD45$^{+/-}$ mice than in P14 CD45$^{+/+}$ controls, when mice were neonatally infected with a LCMV viral variant. Despite discrepancies in the trends observed in this system and HEL system (Cyster et al., 1996), these studies further implicate the importance of CD45 expression levels in affecting developmental outcome. Moreover, these studies provide good evidence that the mechanistics of signaling thresholds can exist, and also suggest that autoimmune diseases could generally arise from the altered expression of signaling molecules, leading to improper lymphoid development.

It should also be noted that the use of CD45 knockout mice has implicated CD45's role in natural killer (NK) cell development and in mast cell IgE receptor signaling. Yamada et al. (1996), have shown that although the cytotoxic functions of NK cells are normal in CD45 deficient mice, there exists a significant increase in splenic NK cell numbers. Mast cell degranulation is a central process in the acute allergic response and typically occurs as a result of crosslinking high affinity IgE receptors on the mast cell surface. This activation process leads to the release of granules containing potent inflammatory mediators such as histamine. Unlike wild type cells, crosslinking of surface bound IgE in mast cells deficient for CD45 expression did not induce degranulation (Berger et al., 1994).

In summary, data from work performed on cell lines and on genetically manipulated mice clearly outline a prominent role for CD45 in developmental and antigen mediated immune cell responses. These observations have therefore provided a framework with which detailed

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molecular studies can be based upon. In particular, for the purpose of brevity and because the majority of research has been done on T cells, the following section of the introduction will focus on the molecular aspects of signaling in T cells.

1.3 A molecular model for the role of CD45 in TCR mediated signal transduction

There are three important observations which dictate guidelines for deciphering CD45's role in signal transduction. First, it was generally seen that the induction of global tyrosine kinase activity is significantly less efficient in CD45 negative cells, as assessed by Western blot analysis. This was largely thought to be paradoxical as initially, investigators had predicted that a loss of tyrosine phosphatase activity would result in elevated levels of PTK activity. Second, it was noted early on that differences in the phosphorylation state of immunoprecipitated $p56^{lck}$, a src family tyrosine kinase, could be attributed to the expression of CD45 (Ostergaard et al., 1989). This provided a possible link with another signaling molecule that could be responsible for the observed changes in tyrosine phosphorylation levels. Third, in transfection studies, whereby wildtype, mutant or chimeric forms of CD45 were expressed in CD45 deficient cells, it became clear that the membrane localization and the catalytic activity were crucial factors in restoring T cell signaling events (Koretzky et al., 1992; Desai et al., 1993; Volarevic et al., 1993; Hovis et al., 1993; Niklinska et al., 1994; Desai et al., 1994). These observations reinforced the idea that the catalytic properties of CD45 were implicated in the TCR mediated signal transduction cascade and also demonstrated the

importance of cellular localization of CD45. With these guidelines in mind, it is now important to provide a short summary of TCR signaling.

Proximal events in TCR signaling

The TCR is a polypeptide complex containing protein subunits that are responsible for antigen-binding, as well as protein subunits comprising the signal transduction modules (reviewed in Weiss, 1993; Weiss and Littman, 1994; Chan and Shaw, 1996). On the T cell surface, the TCR complex consists of the TCRαβ heterodimer and the invariant CD3 chains which include the ζ-chain homodimer. Peptide antigen presented by the appropriate MHC molecule binds directly to the TCRαβ chains, whereby the cytoplasmic domains of the CD3 chains subsequently become a central aspect for the continuation of the signal.

All CD3 chains contain the immunoreceptor tyrosine-based activation motif (ITAM) following consensus to the sequence $YXXL/I(X)_{6-8}YXXL/I$ (Romeo et al., 1992; Wegener et al., 1992). The phosphorylation state of this activation sequence consititutes an early focal point in the signal transduction cascade. Antigen receptor signaling is generally thought to adhere to a two step sequential model, whereby two sets of distinct protein tyrosine kinases (PTKs) are activated in a coordinated manner (Kolanus et al., 1993; Iwashima et al., 1994). On ligation of either the TCR or BCR, the signal is believed to be initiated by activation of src family PTKs. Examples of these cytoplasmic non-receptor PTKs that are expressed in hematopoietic cells, include $p56^{lck}$, $p59^{fyn}$, $p60^{c-src}$, $p53/56^{lyn}$ and $p62^{blk}$ (Marth et al., 1985; Hunter and Cooper, 1985; Cooke and Perlmutter, 1989; Cambier et al., 1993; Chan et al., 1994). These src family tyrosine kinases have been shown to be capable of phosphorylating

numerous substrates including the aforementioned ITAM motifs in the antigen receptor complexes. This in turn allows the recruitment and eventual activation of downstream PTKs belonging to a distinct family, ZAP70 and p72^{syk}. Hence, these newly activated PTKs will phosphorylate and activate other signaling molecules to further propogate the activation signal. In particular, ZAP70 has been recently found to phosphorylate a 36-38 kDa protein called LAT, which has been demonstrated to associate with several signaling proteins including GRB2, Phospholipase C-γ1, and the p85 subunit of phosphainositidyl 3-kinase (Zhang et al., 1998).

Relationship between CD45 and src-family tyrosine kinases

The Src-family kinases are a group of closely related non-receptor protein tyrosine kinases that have been implicated in numerous eukaryotic signal transduction pathways (reviewed in Bolen, 1993). The first src kinase was characterized from studies examining the transforming potential of the Rous sarcoma virus (reviewed in Brown and Cooper, 1996). Here, both viral and cellular forms of the *src* gene, *v-src* and *c-src*, were characterized and eventually led to the discovery of the first oncogene-proto-oncogene pair. By examination of primary sequences, functional differences between the two forms of src could be attributed to a truncation in the viral src protein and the accompanying loss of a regulatory tyrosine residue that is located in the carboxylterminal tail of c-src (Tyr-527). The phosphorylation of Tyr-527 was shown to correlate with the reduction of p60^{c-src} tyrosine kinase activity, and it is believed that without this inhibitory control, the v-src protein is constitutively active, resulting in elevated levels of tyrosine phosphorylation and progression into a transforming phenotype.

To date, nine src-family tyrosine kinases have been identified (src, lck, hck, fyn, fgr, yes, blk, lyn, and yrk), and all appear to share this common regulatory mechanism, despite significant differences in cellular expression and localization (reviewed in Brown and Cooper, 1996). For example, $p60^{c-src}$ is expressed in a wide range of different tissues and has also been demonstrated to phosphorylate a wide range of substrates . In contrast, $p56^{lck}$, to date, has been demonstrated to play a more restricted albeit crucial role in lymphoid cell and, particularly, T cell signaling. The physiological roles of several src family kinases have been addressed by generating knockout mice. This approach has been especially fruitful with regards to immune cell regulation and mice lacking expression of $p56^{lck}$ (Molina et al., 1992), $p59^{fyn}$ (Appleby et al., 1992; Stein et al., 1992) and $p53/56^{lyn}$ (Hibbs et al., 1995) have been characterized. Not surprisingly, in these three cases, the phenotypes clearly show involvement of each src kinase in regulating immune function.

The family's sequence similarities extend over all but the first 60 to 80 residues out of a total length of around 500 to 530 residues. This area of disimiliarity is often refered to as the unique amino terminal region. Continuing towards the carboxyl-tail of the molecule, are two highly conserved peptide binding modules, which form the basis of the regulatory apparatus of the src kinases. Termed SH3 and SH2 domains, these modules bind to targets containing polyproline type II helices and phosphotyrosines respectively, and are responsible for mediating the formation of protein-protein complexes during cell signaling (reviewed in Cooper and Howell, 1993).

For several years, it was speculated that the formation of an intramolecular interaction between the SH2 domain and the

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carboxylterminal tail regulatory tyrosine residue would result in the repression of kinase activity. The recent crystalization of sequences from p60^{c-src} and hck has elegantly provided three dimensional structures consistent with the intramolecular interaction model (Sicheri et al., 1997; Xu et al., 1997). Furthermore, these crystal structures as well as SH3 displacement strategies have shown that the SH3 domain can also provide additional inhibitory interactions (Sicheri et al., 1997; Xu et al., 1997; Moarefi et al., 1997). Under this model, it is clear that phosphorylation of the src protein is important in the regulation of src protein function. The phosphorylation at the carboxyl-tail of c-src (Tyr-527) and resultant inhibitory action is mediated by a distinct tyrosine kinase, p50^{csk}, whereas autophosphorylation at another tyrosine residue (Tyr-416 in c-src), located within the 'activation segment' of the catalytic domain, is required for catalytic activity (Brown and Cooper, 1996).

The catalytic domain on its own is functional as a tyrosine kinase, but the SH2 and SH3 domains appear to be required for normal biological activity. This has been largely demonstrated by using deletion mutants or point mutations in these protein binding domains which arise in various host cell dependant phenotypes (Caron et al., 1992; Veillette et al., 1992; Luo and Sefton, 1992; Reynolds et al., 1992; Peri et al., 1993; Xu and Littman, 1993; Leefruman et al., 1996; Straus et al., 1996; Yamasaki et al., 1996). Consequently, the SH2 and SH3 domains have a dual role, where they are required for the regulation of the enzyme's catalytic activity, but once released also function to target the kinase to specific substrates.

The dynamics in src-kinase function, therefore, sets up a possible explanation for the requirement of both tyrosine kinase and tyrosine phosphatase activity in T cell activation, in that CD45 is crucial for efficient

activation because it's primary role is to regulate src kinase function. Data supporting this hypothesis was evident in studies using CD45 deficient cell lines where the phosphorylation states of various src related kinases was monitored (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Hurley et al., 1993; Burns et al., 1994). These reports have suggested that loss of CD45 increases the phosphorylation of Tyr-505, the putative negative regulatory site in p56^{lck}. As assessed by immunoprecipitation, western blot analysis and cyanogen bromide cleavage profiles, the increase in phosphorylation of Tyr-505 has ranged from 2 to 8 fold when compared to wild type cells. A similar, although less dramatic, trend is also observed with p59^{fyn}, a src-family kinase that has been shown to interact with the CD3ζ chains of the TCR complex. Conversely, in cell lines that expressed p60^{c-src}, it was noted that CD45 had virtually no effect on the tyrosine phosphorylation state of this src related kinase. These observations, which will be discussed later, have become a point of contention regarding the substrate preferences of CD45.

Despite a clear correlation between the phosphorylation states of p56^{lck}/p59^{fyn} and the expression of CD45, the underlying effect on the catalytic activity of these kinases is still unclear. A number of studies addressing this issue have reached the conclusion that CD45 expression upregulates kinase activity. Work illustrating this trend includes a study where CD4 immunoprecipitates were mixed with purified CD45, and then assayed for associated p56^{lck} PTK activity (Mustelin and Altman, 1990). In this case, it was shown that CD45 decreased the phosphorylation levels of Tyr-505 and further caused a 2 to 2.5 fold increase in catalytic activity. Other studies that continued the characterization of the src related kinases, p56^{lck} and p59^{fyn}, in a CD45 deficient CD8$^+$ T cell clone,

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demonstrated that the hyperphosphorylation of the carboxyl-tail regulatory tyrosine residue strongly correlated with a decrease in kinase activity (Cahir McFarland et al., 1993). However, there has also been data suggesting the opposite effect to be true. Arguing in favor of CD45 negatively regulating p56^{lck} catalytic activity, is the observation that CD45 effectively decreased p56^{lck} autophosphorylation and CD4-associated p56^{lck} PTK activity when antibodies were used to co-cluster these cell surface molecules (Ostergaard and Trowbridge, 1990), and that the loss of CD45 expression could enhance p56^{lck} activity (Deans et al., 1992). Moreover, data on other CD45 deficient cell lines have seen elevation of p56^{lck} and p59^{fyn} kinase activity despite the concomitant increase in carboxylterminal tail tyrosine phosphorylation (Burns et al., 1994). Taken together, it would appear that there is no simple interpretation of the relationship between CD45 loss, tyrosine phosphorylation and enzymatic activity of Src-family kinases.

However, attention has been recently raised as to the possible reason for this discrepancy in data. D'Oro et al. (1996) have demonstrated that the appearance of the hyperphosphorylated forms of p56^{lck} in YAC-1 CD45 negative cells, could be attributed to increases in phosphorylation levels at Tyr-505 and at Tyr-394. Tyr-394 represents the autophosphorylation site in p56^{lck} and is analogous in both sequence position and function to Tyr-416 in p60^{c-src} (Hardwick and Sefton, 1995; Doro et al., 1996; Boerner et al., 1996). Nestled within the catalytic domain of src-kinases, the autophosphorylation site has been shown to be a positive regulator of src-kinase catalytic activity when phosphorylated. Thus, the *in vivo* possibility of CD45 acting on the autophosphorylation site of src-kinases further complicates the scenario. However, this

additional information suggests that the inconsistencies in $p56^{lck}$ kinase activity that were observed in the previously mentioned studies could be attributed to differences in the phosphorylation state of Tyr-394.

Another point to take into consideration when regarding regulation of src family kinases is the involvement of $p50^{csk}$. This tyrosine kinase has similar overall domain structure to the src-kinases but does not have any lipid anchor mechanisms and does not have a carboxyl-tail negative regulatory tyrosine residue. Importantly, it has been shown to be the kinase responsible for the phosphorylation of the carboxylterminal tails of src-family kinases (Bergman et al., 1992; Chow et al., 1993). Consequently, any model of catalytic regulation must heed the participation of $p50^{csk}$. Serine phosphorylation of $p56^{lck}$ may also be a complicating factor. Studies have shown that $p56^{lck}$ phosphorylation of Ser-42 and Ser-59 on $p56^{lck}$ occurs upon T cell activation and is responsible for a striking change in SDS-PAGE mobility (Winkler et al., 1993). Furthermore, this same phosphorylation event has been demonstrated to alter SH2 domain specificity (Joung et al., 1995).

The emphasis on work examining CD45's ability to alter the phosphorylation states of $p56^{lck}$ and $p59^{fyn}$ has served a useful purpose in demonstrating that this molecular relationship can modulate the conformation of $p56^{lck}/p59^{fyn}$ in a manner consistent with the intramolecular model of regulation of the Src-family kinases. Therefore, this dephosphorylation of $p56^{lck}$ at the carboxylterminal tail tyrosine can also be envisioned to release the regulatory peptide binding domains and consequently CD45 may control their subsequent involvement in both protein-protein interactions and in the formation of signaling complexes.

However, this possibility has only been indirectly addressed with data showing that the presence of CD45 can influence binding of the p56^{lck} SH2 domain to a phosphopeptide (Sieh et al., 1993). This gap in research is surprising given that there is abundant data demonstrating the importance of the SH2 and SH3 domain of p56^{lck} in facilitating T cell signaling and in neoplastic transformation (Caron et al., 1992; Luo and Sefton, 1992; Reynolds et al., 1992; Veillette et al., 1992; Xu and Littman, 1993; Peri et al., 1993; Yamasaki et al., 1996; Leefruman et al., 1996; Straus et al., 1996). Clearly, the effect of CD45 on regulating the ability of p56^{lck} to interact with other proteins needs to be more actively pursued.

Relationship between CD45 and syk-family tyrosine kinases

The neccessity of phosphorylation of the CD3 chains by activated src-kinases is thought to precede the recruitment of syk family tyrosine kinases such as ZAP70 and p72^{syk} (Kolanus et al., 1993; vanOers et al., 1993; Straus and Weiss, 1993; Iwashima et al., 1994). These tyrosine kinases have two tandem SH2 domains followed by a catalytic domain (Taniguchi et al., 1991; Chan et al., 1992). The tandem arrangement of these binding modules on ZAP-70 has been demonstrated to accept the dual phosphotyrosine residues found in the CD3ζ ITAM sequences (Hatada et al., 1995). This recruitment process is believe to allow for the subsequent propogation of the signaling cascade, whereby the re-location of the catalytic domains of ZAP70 and p72^{syk}, permits their subsequent activation and phosphorylation of preferred substrates.

In comparison to src-family kinases, the role of CD45 in the regulation of ZAP70 and p72^{syk} is less understood. Observations on the CD45 deficient YAC-1 T cells, have demonstrated that ZAP70 and CD3ζ are

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both constitutively hyperphosphorylated on tyrosine residues (Mustelin et al., 1995). CD45, therefore, may play a direct or indirect role in modulating the phosphorylation states of both of these signaling molecules. The treatment of cells with an anti-CD45 antibody resulted in the co-capping of ZAP70 and CD45, and provides some evidence of a functional relationship between these two molecules (Mustelin et al., 1995). This is further strengthened with the demonstration that ZAP70 is an *in vitro* substrate for CD45 (Mustelin et al., 1995). CD45 also interacts with the CD3ζ chain in activated Jurkat T cells, suggesting the interesting scenario of CD45 terminating the activation response by dephosphorylating the CD3ζ chain (Furukawa et al., 1994).

1.4 Biochemistry of protein tyrosine phosphatases

Currently, the protein tyrosine phosphatases family consists of over 40 different enzymes (reviewed in Denu et al., 1996b; Tonks and Neel, 1996). As a distinct group, these enzymes have no structural similarity with the serine/threonine phosphatases nor do they have any structural similarity with other phosphatases of no protein specificity (i.e alkaline phosphatases and acid phosphatases). PTPs, like protein tyrosine kinases, can exist as soluble cytoplasmic phosphatases, as membrane associated phosphatases, or as transmembrane, receptor-like phosphatases. The intracellular PTPs generally harbour a single catalytic domain and other regulatory or targeting sequences that extend from the amino- or carboxyl-terminal ends. The receptor-like PTPs typically include an extracellular domain of variable length and composition, a single transmembrane spanning region, and two discrete domains in the cytoplasm, which share homology to the PTP catalytic domain. In every PTP, there exists an active

site signature motif, (I/V)HCXAGXGR(S/T)G, containing important residues necessary for full enzymatic activity. In particular, this motif harbours the catalytic cysteine residue which is involved in the formation of phosphoenzyme reaction intermediate. Given the scope of research involved with CD45 and cell signaling, the remainder of this introduction will focus on the catalytic properties of CD45 and of PTPs in general. In this respect, the biochemical processes involved in PTP function will be reviewed, as well as potential mechanisms in regulating this catalytic activity.

Structural characterization of protein tyrosine phosphatases
Studies using site directed mutagenesis and the subsequent analysis of phosphatase activity provided the first steps in elucidating the enzymatic mechanisms of PTPs. In particular, a number of reports were published analysing the requirements of certain residues that were found within the active site motif. Overall, these studies were able to show that a cysteine residue played a crucial role as a conserved point mutation to serine totally abolished activity (Streuli et al., 1990; Johnson et al., 1992). These data were in agreement with previous studies that implicated the importance of the cysteinyl thiol groups in playing a key role in catalytic function (Tonks et al., 1990). Similarily, considerable data was attained regarding other residues, within the signature motif, that could modulate enzymatic efficiency (Streuli et al., 1990; Johnson et al., 1992). These observations provided clues as to the mechanisms involved but could not preclude effects caused by inadvertant structural changes to the active site geometry.

Understandably, with the resolution of the crystal structure of PTP1B (Barford et al., 1994a; Barford et al., 1994b), the effectiveness of studying the biochemical mechanisms behind tyrosine phosphate hydrolysis increased dramatically. Since then, three other PTP crystal structures, the *Yersinia* PTP (Stuckey et al., 1994), the first PTP domain of RPTPα (Bilwes et al., 1996), and the first PTP domain of RPTPμ (Hoffmann et al., 1997) have been resolved. In addition, the crystal structure of the dual specificity phosphatase VHR (Vaccinia H1-related) has also been resolved (Yuvaniyama et al., 1996). Amazingly, despite sequence identity scores as low as 5%, all four proteins exhibit strikingly similar structural folds. This structural similarity, on its own, enabled investigators to propose the minimal structural characteristics of the PTP catalytic domain.

PTPs exhibit a single α+β type domain consisting of four central parallel β-strands that are flanked by α-helices. Amino acids within the PTP signature motif, (I/V)HCXAGXGR(S/T), form a phosphate binding pocket, that is nestled between the β-strand ending with HC and the α-helix starting with RS of the signature motif. Within this region, at the center of the active site cleft, sits the cysteine residue which acts as a catalytic nucleophile. The crystal structure of PTP1B also showed that adjacent to the cysteine residue, the guanidinium group of the arginine residue is positioned directly towards the active site. However, perhaps the most striking finding from examination of crystal structure data was the elucidation of an allosteric mechanism of action that is involved in the catalytic process. Using purified protein complexed with tetrahedral-like molecules (such as tungstate), it was resolved that approximately 30 to 40 residues amino terminal of the nucleophilic cysteine, there exists a flexible loop structure that is able to undergo a significant conformational

change when substrate is bound to enzyme (Stuckey et al., 1994; Jia et al., 1995). Within this loop is a catalytically important aspartic acid residue that is brought into the vicinity of the active site after the conformational change occurs. It is believed that the presence of these two structural elements described above constitute the minimum PTP domain.

Areas of the molecule not included within this minimal catalytic framework are particularily striking when comparing the structures of the PTPs with the dual-specificity phosphatase. Major differences are evident at insertion loops between the secondary structural elements and have been postulated to play roles in substrate specificity or targeting. In agreement, crystal studies have already implicated that sequences along some of these loop structures directly contact the putative peptide backbone of a phosphotyrosine containing peptide substrate. Furthermore, these insertional loop folds also contribute to the overall depth of the active site cleft, which has been suggested to contribute to differences in amino acid specificity between PTPs and dual specificity phosphatases (Yuvaniyama et al., 1996).

Sequence analysis of the low molecular weight phosphatases show that these enzymes contain the crucial cysteine and arginine residues within an imperfect PTP signature motif (Zhang et al., 1994). Interestingly, despite no other sequence homology, x-ray structure of the bovine low molecular weight phosphatase revealed that the active site motif formed a similar active site architecture (Zhang et al., 1994; Su et al., 1994). However, since the overall topology of the full structure is completely different, investigators have suggested that the low molecular weight phosphatases and the PTPs represent examples of convergent evolution. From a biochemical viewpoint, the commonality of the active site motif

leads to the suggestion that these two classes of phosphatases share the same catalytic strategy. In all cases, a nucleophilic cysteine, arginine and an aspartic acid are employed in the enzymatic mechanism. Consequently, numerous studies have been done where mutations in all aforementioned phosphatases are analysed kinetically and/or structurally in an effort to determine the molecular reaction mechanism (Zhang et al., 1992; Zhang, 1995; Zhang, 1995; Dunn et al., 1996; Evans et al., 1996; Wu and Zhang, 1996; Burke Jr. et al., 1996; Fauman et al., 1996; Denu et al., 1996a; Zhang et al., 1997; Zhang and Wu, 1997).

The molecular reaction mechanism of protein tyrosine phosphatases

The elucidation of the biochemistry behind the tyrosine phosphatase reactions is crucial for the proper interpretation of any PTP activity data where regulation of substrate preference is being assessed. As a result of this, it is important to understand the enzymatic mechanism involved in this catalytic event under the context of the three dimensional structure.

Efforts to comprehend this process have been greatly aided with the demonstration that PTP activity proceeds through a phosphoenzyme intermediate (Cho et al., 1992; Denu et al., 1996a; Zhang et al., 1997). This piece of information has allowed investigators to divide the catalytic event into two distinct steps: 1) the phosphoryl transfer to the enzyme accompanied by a rapid release of the dephosphorylated product; and 2) the hydrolysis of the thiol-phosphatase intermediate concomitant with a rapid release of phosphate (see Figure 1.1). Given that these two reactions encompass different biochemical attributes, the individual rates of reaction for each step are likely different as well. This allows the

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determination and assignment of a particular step as being the rate limiting step. These facts have been utilized by a number of groups who have performed site directed mutagenesis in an effort to observe whether changes to any of these properties occur (Zhang, 1995; Denu et al., 1996a; Evans et al., 1996; Wu and Zhang, 1996). By virtue of this two step process, in depth kinetic analyses is therefore able to discern whether a residue plays a key role in step 1 or in step 2. These kinetic analyses, in conjunction with observations made from x-ray structures, have afforded a working molecular model of catalysis for these enzymes. The following text will provide a summary of this molecular reaction.

The formation of the phosphoenzyme intermediate is congruent with the binding of the dianion phosphate group to the enzyme. For this reaction to proceed, the cysteinyl nucleophile must be unprotonated, while the conserved aspartic acid located on the flexible loop must be protonated (Denu et al., 1996a; Zhang, 1995). Examination of various x-ray structures has greatly aided the visualization of residues important for this catalytic step. For example, the structure of the *Yersinia* PTP complexed with vanadate is a good representation of the enzyme in a transition state (Denu et al., 1996a). Since vanadate is able to covalently interact with the enzyme and also conforms to a trigonal bipyramidal geometry, this structure has been extremely useful in the visualization of crucial residues in the transition state. The guanidinium group of arginine and the backbone amide groups within the active site form bidentate hydrogen bonds that coordinate the three nonbridging oxygens of the phosphoryl group. This orientation forces the thiolate anion of the nucleophilic cysteine to be situated directly in line with the P-O bond for efficient attack on the leaving group (Jia et al., 1995). As previously mentioned, substrate

Figure 1.1: <u>Schematic representation of the two step protein tyrosine phosphatase reaction</u>. The protein tyrosine phosphatase reaction proceeds in two distinct steps. First, the phosphate is transferred from tyrosine (R) to a functional group on the enzyme (I) and then the phosphoenzyme intermediate is hydrolysed (II).

binding also results in conformational change on the flexible loop structure, allowing the aspartic acid to move 8 - 12 angstroms into the vicinity of the active site (Stuckey et al., 1994; Jia et al., 1995). This residue is then able to act as a general acid to protonate the leaving group phenolic oxygen.

Step two of the reaction mechanism can proceed once the phosphoenzyme intermediate is formed and consists of a hydrolysis reaction whereby the enzyme turnover can be completed. This hydrolysis step is therefore dependant on the activation of a water molecule. In this light, mutagenesis and kinetic studies have indicated that the same conserved aspartic acid on the flexible loop structure may act as a general base by proton extraction from the water molecule (Denu et al., 1996a). Structural data using *Yersinia* PTP complexed with vanadate also supports the aspartic acid as having a role as both a general acid and general base (Denu et al., 1996a).

In summary, the in depth study of the catalytic mechanisms of PTP1B, *Yersinia* PTP, VHR-1 and low molecular weight phosphatases, have provided a suitable framework in which to base the molecular reactions of other PTPs.

1.5 CD45 protein tyrosine phosphatase activity

Strict biochemical characterization of both endogenously purified and homogenous recombinant protein has been performed. In these instances, the substrates used have included small phosphoryl containing compounds such as *p*-nitrophenol phosphate, radioactively labeled protein substrates such as RCM-lysozyme and Myelin Basic Protein, as well as synthetically prepared phosphopeptides encompassing amino acid

sequences derived from relevant and irrelevant protein substrates (Streuli et al., 1990; Tonks et al., 1990; Cho et al., 1992; Itoh et al., 1992; Johnson et al., 1992; Cho et al., 1993; Iida et al., 1994; Pacitti et al., 1994).   With the possible exception of radioactively labelled substrates, these reagents can be conveniently and easily used to obtained useful kinetic information about the PTP. Recombinant cytoplasmic CD45 K_m values for pNPP have been reported at 5.5 mM and 4.8 mM (Cho et al., 1993; Pacitti et al., 1994), whereas K_m values for phosphotyrosine containing peptides have generally fallen between 50 μM and 300 μM (Cho et al., 1992; Cho et al., 1993; Pacitti et al., 1994). V_{max} values have been demonstrated at 36 μmol/min/mg and 87.5 μmol/min/mg for pNPP (Cho et al., 1993; Pacitti et al., 1994) and between 70 to 200 μmol/min/mg for a variety of phosphopeptides (Cho et al., 1992; Cho et al., 1993; Pacitti et al., 1994). Interestingly, kinetic data for phosphopeptides falls within a relatively small range of values, and has presented a conundrum in the subject of substrate specificity for CD45 and PTPs in general. Researchers had originally assumed that in a manner similar to the specificity governed by SH2 domains, the substrate specificity for PTPs would be predominantly contained in the amino acid sequences that are immediately adjacent to the tyrosine residue (Cantley et al., 1991a). However, the overwhelming majority of data has shown that this is not the case and is perhaps best illustrated with CD45 PTP activity determined using peptides corresponding to the carboxylterminal tail of the src family kinases, p56[lck], p59[fyn], and p60[c-src] (Cho et al., 1992; Cho et al., 1993; Pacitti et al., 1994).

Despite clear substrate preferences for src-family kinases observed in lymphoma derived cell lines and in transgenic mice (Hurley et al., 1993), the V_{max} and K_m values for the corresponding src family peptides are

very similar (see Table I). Consequently, it is quite likely that substrate specificity is dictated by the cellular location or by the physiological protein in its entirety. Some evidence of the importance of an intact protein in determining substrate specificity had been previously seen in an early report showing that the use of intact radioactively labelled proteins as substrates had resulted in K_m values between 1.2 μM and 35 μM (Tonks et al., 1990). However, it should be noted that this data was obtained using physiologically irrelevant proteins and that values were assessed using endogenously purified full length enzyme. In summary, this difference between *in vivo* and *in vitro* substrate specificity presents an interesting question that needs to be further addressed.

The presence of two PTP domains found within the cytoplasmic domain of many receptor-like PTPs (including CD45) creates an additional dimension of complexity. This is especially relevant, given that studies in ascertaining the molecular reaction of the phosphotyrosine hydrolysis have all been done with cytoplasmic single domain phosphatases. However to date, the crystal structures of the first PTP domain of RPTPα and RPTPμ have been resolved and were found to contain all of the structural hallmarks found in the other PTP x-ray structures (Bilwes et al., 1996; Hoffmann et al., 1997). In light of these features, numerous mutagenesis studies have been performed to characterise the PTP activity associated with each of CD45's PTP domains individually (Streuli et al., 1990; Johnson et al., 1992). Questions regarding the function of CD45 PTP domain 2 were particularly relevant as a deviated signature active site motif (HCRDGSQQTGK) is found within this domain. Although this sequence still contains the nucleophilic cysteine residue, a point mutation of this cysteine residue in PTP domain 2 to a serine did not alter the PTP

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Substrate	K_m (mM)	V_{max} ($\mu mol/min/mg$)
lck505 (TEGQpYQPQP)[a]	0.13	66
src527 (TEPQpYQPGE)[a]	0.16	186
fyn531 (FTATEPQpYQPGENL)[b]	0.17	185

Table I: <u>Previously determined kinetic values for recombinant cytoplasmic CD45 using src phosphopeptides</u>. K_m and V_{max} values expressed in (mM) and ($\mu mol/min/mg$) respectively and were obtained from [a] Cho et al., 1992 and Cho et al., 1993; and [b] Pacitti et al., 1994.

activity of recombinant cytoplasmic CD45. This is in stark contrast to the analogous point mutation on the cysteinyl nucleophile residue in PTP domain 1. In this situation, CD45 PTP activity was completely abrogated. These types of alterations indicated that only the N-terminal domain is catalytically active. To further delineate the involvement of either PTP domain, the individual domains of CD45 were prepared and assayed for PTP activity (Johnson et al., 1992). Initial reports showed that domain 2 of CD45 had insignificant PTP activity which was consistent with the previous observation that mutation of its conserved cysteine residue had no noticable effect on full length cytoplasmic CD45 activity. However, there has since been a report which has suggested that there is enzymatic activity in the second domain (Tan et al., 1993). Although this notion was indirectly shown by analyses of tryptic fragments and mutant constructs of CD45, the question of activity residing in the second catalytic domain of CD45 is still controversial and will require further work to fully elucidate a definitive answer.

Initially, constructs containing domain 1 alone also showed little or no activity when tested against a variety of artificial protein substrates (Streuli et al., 1990; Johnson et al., 1992). However, a more recent report has determined that PTP domain 1 does exhibit significant catalytic activity although to a far less efficient degree than full length cytoplasmic domain (Iida et al., 1994). These contradictory observations may be due, in part, to differences in the N-terminal regions of the construct as significant changes in activity can be dictated by where the PTP domain 1 sequences start (Wang and Pallen, 1992) or may be due to the experimental systems used to produce the recombinant proteins. Overall, these studies attest to the requirement of both PTP domains for CD45 activity to be at its most

efficient. This trend is seen in another receptor PTP, RPTPα, which also harbours dual PTP domains (Wang and Pallen, 1991; Wu et al., 1997). Although different from CD45 by its unambiguous accounts of activity in its first PTP domain, this PTP exhibits optimal enzymatic activity when both catalytic domains are present (Wang and Pallen, 1991; Lim et al., 1997; Wu et al., 1997). Because of the large discrepancies between wild type CD45 activity and activity in domain 2 versus domain 1, several speculations as the putative role of domain 2 in CD45 function have been put forth. Suggestions have included roles in substrate specificity to roles in cellular localization. It is still quite possible that activity of domain 2 is distinct or that the activity may only be observed under certain circumstances which investigators have not yet approached.

1.6 Regulation of CD45 PTP activity

Chemical factors that regulate CD45 PTP activity

Efforts to test factors that may regulate CD45 PTP activity have been in effect since the realization that CD45 contained sequence identity with the PTP1B molecule. Initial characterization focused around the assaying of PTP activity under various buffer conditions. Work in this format has shown that the presence of a reducing agent such as DTT greatly elevates CD45 activity, implying either the formation of unwanted disulfide bonds in the purification process or that activity is sensitive to the redox environment of the assay. The influence of divalent cations is varied and depending on the report, has been shown to have little effect, no effect, or contribute to both significant inhibitory and stimulatory outcomes. Tonks et al. (Tonks et al., 1990), when examining purified endogenous CD45,

showed that the presence of 1 mM of Mg^{2+}actetate, $MnCl_2$, or $CaCl_2$ can have stimulatory effects. Interestingly, similar studies with recombinant protein have shown that the same compounds at identical concentrations had no effect on CD45 PTP activity; whereas the presence of these chemicals at higher concentrations (10 mM) led to inhibitory effects (Itoh et al., 1992). Studies on purified endogenous CD45 protein have also shown that the addition of EDTA stimulates the PTP activity (Tonks et al., 1990). Characterization of both recombinant and purified endogenous CD45 have indicated the protein exhibiting a narrow pH optima at pH 7.2 (Tonks et al., 1990; Itoh et al., 1992). This, suggests that CD45 PTP activity under physiological conditions could be greatly affected by its surrounding cellular environment should the pH be altered. Overall, it is important to point out that these observations regarding reducing agents, cations, EDTA and pH are consistently seen using a number of different substrates, therefore implying that these variations in conditions contribute to CD45 PTP activity in general, and do not contribute to CD45 substrate specificity.

Role of the extracellular domain of CD45 in regulating CD45 catalytic activity

The apparent differences described in the previous section between recombinant cytoplasmic CD45 domain and purified endogenous CD45 suggest that the extracellular region plays an integral part in the control of catalytic activity. This idea of ligand mediated control of CD45 activity is attractive in that it would follow similar principles exhibited by receptor protein tyrosine kinases. Reports focusing on the use of monoclonal antibodies specific for the extracellular elements of CD45 have highlighted

the importance of this region in CD45 activity (Goldman et al., 1992). In particular, this approach has been interesting in recent reports, with the discovery of a monoclonal antibody specific for the human CD45RB isoform having the ability to prevent and reverse the rejection of a renal allografts (Lazarovits et al., 1996). Perhaps a more direct demonstration of ligand mediated control and resultant changes in PTP activity was published in 1993. Here, Desai et al. (1993) reported the use of a chimera molecule, in which the extracellular and transmembrane domains of CD45 were replaced with those of the epidermal growth factor (EGF)-receptor. This chimeric molecule when expressed on CD45 deficient cells was able to reconstitute antigen receptor signaling. More striking, however, the investigators were able to show that addition of EGF, during antigen stimulation, greatly affected the outcome of T cell signaling assays. In effect, this indicated that the dimerization of cell surface CD45 could influence CD45 catalytic activity.

Studies directed at individual isoforms of CD45 with regards to T cell signaling have provided evidence that specific isoforms may also have differential functions. However, such reports show a discrepancy in results dependant on the use of cell lines or transgenic mice. Work performed on lymphoma cell lines have implicated the smallest isoform (CD45R0) as being able to enhance signaling most efficiently (Novak et al., 1994). Conversely, studies done using transgenic mice showed that the increased expression of the largest isoform (CD45RABC) was capable of increasing the efficiency of the signal, whilst the increased expression of the CD45R0 isoform had no noticeable enhancement (Chui et al., 1994). How the distinct extracellular regions are responsible for these differences is currently unknown. However, given that CD45 is heavily glycosylated

and accounts for up to 10% of the surface glycosylation on T cells (Thomas, 1989), it has been previously postulated that cell surface receptor/ligand interactions may be inhibited by charge repulsions created by the sugar moieties (Springer, 1990). Clearly, the role of the extracellular region still needs to addressed and will probably involve the full characterization of putative ligands that can interact with the isoforms of CD45. Presently, there are a few suspected ligands which have been reported to interact with the CD45 molecule via specific carbohydrate structures. These include, the B cell surface molecule CD22 (Stamenkovic et al., 1991) and a thymic epithelial cell surface marker galectin (Perillo et al., 1995). However, the studies on these putative ligands have not examined the relationship between CD45 binding and CD45 PTP activity. It should also be pointed out that with the exception of some reports performed with the monoclonal antibodies, the studies reviewed in this section did not directly assess CD45 PTP activity. Instead, only CD45's ability to function in the context of T cell activation was examined. It is therefore possible, that the differential effects conferred by the extracellular region may have little to do with regulating PTP activity per se.

Cellular mechanisms of regulation of CD45 PTP activity

Early reports had shown the serine phosphorylation of CD45 after cells were treated with phorbol esters (Autero and Gahmberg, 1987). CD45 was also later shown to be serine phosphorylated after stimulation with interleukin 2 (Valentine et al., 1991). Furthermore, within the same year, a report was published that outlined the negative regulation of CD45 PTP activity through the use of ionomycin to artificially increase intracellular Ca^{2+} levels (Ostergaard and Trowbridge, 1991). This change in calcium

levels correlated with a decrease in serine phosphorylation of CD45, and the investigators argued for a regulatory mechanism defined by the phosphorylation state of CD45. Since protein serine kinases generally phosphorylate defined consensus motifs (reviewed in Kennelly and Krebs, 1991), the primary amino acid sequence of CD45 could be scanned for putative serine phosphorylation sites. Upon evaluation of possible phosphorylation sites, CD45 was used as a putative substrate in protein kinase assays. In this regards, CD45 can be phosphorylated to high stoichiometry by casein kinase 2 (~1.5 mol/mol), glycogen synthase kinase 3 (~0.3 mol/mol) and protein kinase C (~0.1 mol/mol) (Tonks et al., 1990). However, in all three cases, no alteration in enzyme activity was observed. Recently, the physiological serine phosphorylation sites of CD45 isolated from 70Z/3.12 cells were identified using a variety of techniques (Kang et al., 1997). The authors suggest that since one of these sites resides near what would be the active cleft in domain 2, the phosphorylation of this residue may affect domain 2 function.

Tyrosine phosphorylation of CD45 has also been observed, although generally to a much lesser degree. When Jurkat T cells are activated using phytohemagglutinin or anti-CD3 antibodies in the presence of a PTP inhibitor, a transient phosphorylation of CD45 on tyrosines is observed (Stover et al., 1991). Furthermore, p50[csk] has been implicated in the *in vitro* tyrosine phosphorylation of CD45 on a residue within PTP domain 2 (Autero et al., 1994). *In vitro* characterization of this event has also indicated that this phosphotyrosine residue acts as a binding site for the src kinase, p56[lck], and also results in an enhancement in catalytic activity. However, the effect of tyrosine phosphorylation on CD45 is arguably

difficult to ascertain, as the event is difficult to observe except under stringent phosphatase inhibition conditions.

There have also been published accounts of differential CD45 PTP activities in cellular systems. Of particular note, are articles assessing CD45 PTP activity during cell cycle progression and during neutrophil activation (Melkersonwatson et al., 1994; Fialkow et al., 1997). In the first case, CD45 PTP activity was examined in a pre-B lymphoma cell line and in a cytolytic T lymphoma cell line after fractionation by counterflow centrifugal elutriation in cell-cycle stage enriched subpopulations (Melkersonwatson et al., 1994). In both cell types, the specific activity of CD45 was elevated as much as 10 fold during mitosis. CD45 catalytic activity has also been assessed during neutrophil activation (Fialkow et al., 1997). Here, it was observed that CD45 PTP activity was significantly downregulated and that this negative regulation of activity could be attributed to an oxidant-mediated effect. This finding is particularily interesting, as the requirement for reducing agents in efficient CD45 PTP activity has been known since the initial characterization of the CD45 enzyme. Furthermore, this report emphasizes the possible importance of localization, where a particular cellular locale may have a particular redox environment.

Proteins that are able to interact with CD45

The importance of protein-protein interactions in the signaling field has been exemplified on many occasions, particularly with the characterization of numerous discrete protein binding domains. Therefore, the possibility of CD45 function being controlled by what it interacts with cannot be ignored. CD45 has been documented to associate

with a variety of cell surface molecules including Thy-1, TCR, CD2, CD3, CD4, CD7, CD8, CD26, CD28, LFA and the α and β subunit of alpha-glucosidase II (Dianzani et al., 1990; Schraven et al., 1990; Volarevic et al., 1990; Torimoto et al., 1991; Mittler et al., 1991; Zocchi et al., 1992; Dianzani et al., 1992; Furukawa et al., 1994; Lazarovits et al., 1994; Brown et al., 1994; Arendt and Ostergaard, 1997). Furthermore, some of these associations have been reported to be isoform specific. However, as these associations were generally determined by chemical cross-linking, co-capping, co-immunoprecipitation, and fluorescence energy transfer techniques, there is no confirmation of a direct or indirect interaction. There have also been several reports indicating an interaction between CD45 and a 30 kDa protein (Schraven et al., 1991; Takeda et al., 1992; Koretzky et al., 1993). This protein, called CD45-AP (or LPAP in humans), has been cloned and characterized (Schraven et al., 1994; Takeda et al., 1994). The interaction was demonstrated to occur with the transmembrane region of cell surface CD45 at very high stoichiometry (approximately 1mol/mol ratio) (Schraven et al., 1994; Cahir McFarland and Thomas, 1995; Kitamura et al., 1995; Cahir McFarland et al., 1997). Despite no definitive function attributed to CD45-AP, the high stoichiometry of the interaction suggests the potential to modulate CD45 function, and one group has speculated its involvement in co-localizing CD45 with p56^{lck} (Cahir McFarland and Thomas, 1995). Overall, this interaction, as well as others, could conceivably dictate or alter cellular locale, by moving CD45 to a new location or by simply acting as bridges between CD45 and substrates of physiological relevance.

Interactions with the cytoplasmic domain of CD45 have been especially interesting, given that the PTP activity is contained within this

region of the protein, and that most signaling complexes occur due to interactions in the cytoplasm. For example, there are reports suggesting an interaction with the cytoskeletal component, fodrin (Bourguignon et al., 1985; Lokeshwar and Bourguignon, 1992; Iida et al., 1994). This molecule is a heterodimer containing 235 kDa and 240 kDa polypeptides, and is structurally and functionally related to spectrin. Subsequently, the *in vitro* demonstration of a specific and direct association between CD45 and fodrin provided a link between CD45 and the actin cytoskeletal matrix. Furthermore, this association caused a significant increase in specific catalytic activity (Iida et al., 1994). There are also reports which examine protein-protein interactions between CD45 and the src-family kinases, $p53/p56^{lyn}$ and $p56^{lck}$. These studies are particularily intriguing in light of the substrate preferences observed between CD45 and src-kinases that were previously outlined in cell lines and in genetically manipulated mice. $p53/p56^{lyn}$ has been shown to co-preciptate with CD45 in B cells under mild lysis conditions using a 1% digitonin buffer (Brown et al., 1994). There is, however, more evidence for an association between CD45 and $p56^{lck}$ in T cells. In Jurkat T cells, the co-precipitation of *in vitro* kinase activity and CD45, has been demonstrated to be dependant on the expression of $p56^{lck}$ (Koretzky et al., 1993). In addition, this association did not require expression of CD4 and CD8, and was independant of TCR stimulation (Ross et al., 1994). Similar reports using pervanadate treated cells, have also immunoprecipitated complexes containing both CD45 and $p56^{lck}$ molecules (Lee et al., 1996). More recent reports have shown that in a heterologous system, the unique amino terminal region of $p56^{lck}$ is particularily important for CD45 to dephosphorylate $p56^{lck}$ (Gervais and Veillette, 1995). The importance of this region of $p56^{lck}$ was further

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verified in a non-lymphoid system, whereby cDNAs for both CD45 and p56^{lck} were transfected into NIH 3T3 mouse fibroblasts (Gervais and Veillette, 1997). These same experiments also suggest that CD45-AP is not required for CD45 to dephosphorylate p56^{lck}, since CD45-AP tissue expression is restricted to cells of lymphoid origin.

Finally, there is growing evidence that dimerization of CD45 may play a role in CD45 function. This possibility has been tantalizingly put forward with results obtained using the EGF receptor/CD45 chimera molecule (Desai et al., 1993). Furthermore, studies using sucrose gradients and ultracentrifugation have shown the existence of CD45 dimers in endogenous protein samples (Takeda et al., 1992). The possible effect of dimerization is still not clearly understood, although the data obtained using the chimeras have implicated a negative effect whereby PTP activity is downregulated. Crystallography data on the first PTP domain of RPTPα, offers a possible explanation where the protein crystalized as a homodimer; in particular the structure resolved a novel mechanism of dimerization whereby the membrane proximal region of RPTPα adopted a helix-turn-helix motif, and lodged itself within the active site of the other PTP molecule (Bilwes et al., 1996). This observation predicts a steric hindrance of the active cleft, so that it is conceivable that there is a downregulation of PTP activity. Despite the fact that crystal topology is often obtained under extreme conditions and is not indicative of physiological circumstances, recent evidence using a mutant form of the EGF receptor/CD45 chimera molecule expressed in a CD45 deficient T cell line, has indicated that the same mechanism may occur under cellular circumstances. In this study, a glutamic acid residue in the membrane proximal region of CD45 was mutated and the cells characterized. This

amino acid was carefully chosen by examination of the RPTPα crystal structure and was predicted to play a crucial role in dimer formation (Majeti et al., 1998). Essentially, unlike the previous study (Desai et al., 1993), this mutant was unable to downregulate CD45's ability to participate in T cell signaling after addition of EGF.

1.7 Experimental objectives of this thesis

The principle aim of this work was to ascertain the mechanisms behind cellular CD45 substrate specificity. To do this, it was first necessary to develop an *in vitro* PTP assay which would allow the simple determination of PTP activity from both recombinant PTPs and immunoprecipitated PTPs. Furthermore, a system to generate purified recombinant cytoplasmic CD45 protein was required and subsequently developed. With these tools in hand, one could then examine CD45 substrate specificity and a possible mechanism of substrate specificity by characterizing the *in vitro* association between the cytoplasmic domain of CD45 and the T cell substrate, $p56^{lck}$. Finally, substrate preferences were also addressed within a cellular context by determining whether CD45 has a specific and unique role in T cells, or whether it could be substituded for by equivalent expression levels of another similar transmembrane PTP, RPTPα.

CHAPTER 2

Materials and Methods.

2.1 Materials

Antibodies

The I3/2 rat antibody which is reactive against all isoforms of mouse CD45 provided by I. S. Trowbridge (Trowbridge, 1978) and rabbit antiserum specific for the cytoplasmic domain of CD45 (R01.1) (J. Felberg, W. Schoorl, W. Jefferies and P. Johnson, unpublished) were used to immunoprecipitate CD45. A rabbit antiserum (131) raised against a common extracellular CD45 epitope (kindly provided by J. Marth) was used for immunoblotting. The 4G10 anti-phosphotyrosine monoclonal antibody was purchased from Upstate Biotechnology Inc (Lake Placid, New York). SFR8-B6 is specific for HLA-Bw6 (Radka et al., 1982) and was kindly provided by W. Jefferies. Anti-mouse CD3ϵ (145-2C11), and TCR β (H57-597) hamster monoclonal antibodies were from the American Type Culture Collection (Rockville, Maryland); the mouse monoclonal antibody G3, specific for the mouse CD3ζ chain, was a gift from H-S. The (Van oers et al., 1994). Biotinylated anti-mouse early activation marker (CD69) hamster antibody was from Pharmingen (San Diego, California). Anti-GST antisera was obtained from S. Robbins, G. W. Hooper Foundation, University of California, San Francisco. Rabbit antisera (R-49 & R-54) specific for p56^{lck} were raised against a purified TrpE-p56^{lck} fusion protein containing residues 34-150 of p56^{lck} (Ng et al., 1996). p59^{fyn} antisera was a gift from R. Perlmutter (Cooke and Perlmutter, 1989) and A. Veillette (Davidson et al., 1992). Rabbit antiserum specific for the extracellular domain of RPTPα (PTPα-ext) was used for FACS analysis and rabbit antiserum specific for the cytoplasmic domain of RPTPα (PTPα-2) was a gift from F. Jirik and used for immunoprecipitation. Protein A Sepharose was purchased from Pharmacia Biotech Inc. (Baie D'Urfé,

Quebec) and Repligen (Cambridge, Massachusetts). Fluoresceinated (FITC-labeled) goat anti-rat immunoglobulin G (IgG) was from Pierce (Rockford, Illinois). FITC-labeled goat anti-hamster, mouse and rabbit IgG; FITC-labeled Streptavidin; and horse-radish peroxidase (HRP) conjugated goat anti-mouse IgG were purchased from Southern Biotechnology Associates Inc. (Birmingham, Alabama). HRP-protein A was bought from Biorad Laboratories (Mississauga, Ontario).

Cells

The T cell lines, BW5147$^-$ (CD45$^-$, CD3$^+$, TCR$^+$), BW5147$^+$ (CD45$^+$, CD3$^+$, TCR$^+$) (Wegener et al., 1992), American Type Culture Collection (ATCC) and kindly provided by B. Malissen), EL4 (ATCC), and two B lymphoid cell lines, RAW 253.1, and A20 (ATCC) were used in experiments concerning the initial characterization of the malachite green assay (Chapter 3). Splenic dendritic cells obtained by I. Haidl from BALB/c and CBA male and female mice were purified after overnight culture and prepared as a cell lysate sample for analysis (Crowley et al., 1989; Haidl et al., 1995). In addition, in experiments comparing CD45 PTP activity in naive and proliferating lymph node T lymphocytes, purified cells were provided by P. Orchansky and were taken from H-2^b H-Y TCR transgenic female mice as previously described (Orchansky et al., 1996). In brief, these lymph node T cells were manipulated in RPMI 1640 medium supplemented with 2% FCS and naive CD4$^-$CD8$^+$ T lymphocytes were purified by depleting CD4$^+$ and B cells with the anti-CD4 mAb and sheep-anti-mouse Ig coated magnetic beads (Dynabeads, Dynal Inc., Lake Success, NY). Proliferating cells were activated using immobilized anti-TCRα mAb and grown for 7-10 days in Iscove modified Dulbecco's medium

supplemented with 10% FCS, 5 x 10^{-5} M β-mercaptoethanol (I medium) and murine rIL-2 at 20 units/ml. Cells were split every 2 to 3 days until used for experiments. At this time cells were exclusively transgenic TCRα^+, TCRβ^+, and CD8$^+$ as determined by three color staining.

BW5147 CD45$^+$ and BW5147 CD45$^-$ mouse T lymphoma cells were also studied in more detail in the RPTPα transfection studies (Chapter 6). These cells were previously transfected with CD3δ and CD3ζ cDNAs resulting in the expression of surface TCR/CD3 (Wegener et al., 1992). These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% horse serum supplemented with 100 units/ml of penicillin, 100 µg/ml streptomycin, 0.25 mg/ml amphotericin B and 3 mM histidinol to maintain the expression of the transfected plasmids. Cells were checked routinely by FACS analysis to ensure similar levels of TCR/CD3 levels were expressed in the CD45$^+$ and CD45$^-$ cell lines. It was observed that whilst the TCR/CD3 expression in the CD45$^-$ BW5147 was quite stable, expression in the CD45$^+$ cells would decline over time. Work done by Mojgan Jabali and Peter Borodchak resulted in the production of RPTPα expressing BW5147 CD45$^-$ cells, which were created by transfection of the expression vector, pBCMGSneo (Karasuyama et al., 1990) containing human RPTPα cDNA (Jirik et al., 1990)(which shares 99% sequence identity with the cytoplasmic domain of mouse RPTPα). These RPTPα^+ cell lines were maintained in a similar manner to their parental cell lines, except that all media was supplemented with 1mg/ml G418.

Special reagents; phosphopeptides, plasmids and recombinant proteins

One 11mer and three 13mer phosphopeptides containing sequences from either the negative regulatory site or the auto-phosphorylation site of three src kinase family members were provided by I. Clark-Lewis (Ng et al., 1995). The peptides used in these studies were: fyn pY531 (TATEPQpYQPGENL), src pY416 (TSTEPQpYQPGENL), and lck pY505 (TATEGQpYQPQP) which encompass the negative regulatory sites of murine p59^{fyn} ,p60^{src} and p56^{lck} respectively; and src pY416 (LIEDNEpYTARQGA) which encompasses the autophosphorylation site of murine p60^{src}.

Escherichia coli strains used were CJ236 and DH5αF' (Biorad), XL-1 Blue (Stratagene) and BL21(DE3) obtained from F. Studier (Studier et al., 1990). The bacterial expression pET-3D vector, also obtained from F. Studier (Studier et al., 1990) was previously modified by removing a Nco I-Bam HI fragment and replacing it with linker oligonucleotides encoding a six histidine tag and the Factor Xa cleavage site, IEGR (pET-3D-6HisIEGR). This linker contained multiple cloning sites including a BamHI site. Work by Arpita Maiti entailed the use of the cytoplasmic domain of murine CD45 in pBluescript SK+ vector (Stratagene) for mutagenesis (Ostergaard et al., 1989). cDNA for wild type, C817S, S573G/S574A, Q1180G and Δ876-931 cytoplasmic domains were then subcloned into a shuttle vector containing BglII restriction sites flanking the cytoplasmic domain and then subsequently subcloned into the Bam HI site of the pET-3D-6HisIEGR vector (Ng et al., 1995).

Wild type recombinant GST-p56^{lck} protein was constructed using a StuI cut fragment from mouse p56^{lck} cDNA (Marth et al., 1985), ligated in frame into the SmaI site of pGEX-2T (Pharmacia). GST-p56^{lck} Kinase domain (residues 234 to 505) was constructed using a NcoI - StuI fragment

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from mouse p56^{lck} cDNA, klenow filled in and ligated in frame into the SmaI site of pGEX-4T-3 (Pharmacia). The unique amino terminal region of p56^{lck} was excised as a Stu1-BsmA1 fragment from mouse p56^{lck} cDNA, blunt ended and ligated in frame into the SmaI site of pGEX 2T. This generated a GST fusion protein containing residues -8 to 76 of p56^{lck}. GST-p56^{lck} SH2 and GST-p56^{lck} SH3 domain constructs (encoding residues 67 to 122 and 122 to 234 respectively) were provided by A. Veillette, produced in *E. coli* and purified as described (Peri et al., 1993). The cDNA for the following full length p56^{lck} mutants in pGEM vectors were also kindly provided by A. Veillette: mutation of the regulatory Tyr-505 to Phe (Y505F), mutation of the autophosphorylation Tyr-394 to Phe (Y394F), and the kinase dead form of p56^{lck} (K273R) (Abraham and Veillette, 1990). The mutant versions of p56^{lck} were also subcloned into the SmaI site of pGEX-4T-2 as StuI fragments . Recombinant p56^{lck} purified from a baculovirus expression system was generously provided by J. Watts (Watts et al., 1992). The TrpE-p56^{lck} fusion protein was produced in *E. coli* and purified as described previously (Spindler et al., 1984).

Purified GST-RPTPα fusion protein containing the entire cytoplasmic domain of human RPTPα was a gift from K. Harder (Jirik et al., 1990). Purified GST-SH2 domain proteins from the bovine p85α subunit of phosphatidylinositol 3-kinase (PI 3-kinase) (residues 612 - 722) (Reedijk et al., 1992) and from human SHC (residues 366 - 473) (Pelicci et al., 1992) were provided by S. Barbazuk and M. Gold. pGEX-2TK p60^{c-src}, containing the cDNA for murine p60^{c-src} was modified by removal of the 6 unique amino acids found in the neuronal form of src and kindly provided by Tony Hunter (Martinez et al., 1987).

2.2 Characterization of materials

Phosphotyrosine peptide quantitation

Quantitation of phosphopeptide amounts was achieved by determining phosphate concentrations rather than by dry weight measurements. Lyophilized peptide was dissolved in HPLC grade water and phosphopeptide concentration was quantitated as in (Hasegawa et al., 1982). Briefly, aliquoted amounts of peptide solution were dried by heating at 100°C. The remaining solid peptide was resuspended in 30 µl of a mixture of 1 part 50% sulfuric acid and 1 part 60% perchloric acid and placed in reacti-vials (Pierce) to be superheated at 160°C for 4 to 5 hours. The acid-peptide solutions were cooled, neutralized with equivalent volumes of 10 M NaOH and then aliquoted into a well containing 80 µl of filtered sterilized malachite green reagent (see below). Addition of malachite green reagent resulted in a colorimetric response that was directly related to the amount of free inorganic phosphate present, which was determined using a standard curve.

Protein amount determination

Amounts of protein were determined by running purified proteins on SDS PAGE gels and comparing them to known BSA protein amounts (100 ng to 2 µg) on adjacent lanes. Quantitative values were then obtained by densiometric scanning of the Coomassie Blue stained bands. Amounts of recombinant protein were also measured using the BCA protein assay (Pierce, Rockford, Illnois).

Stimulation of BW5147 cells with anti-CD3ε antibody

Approximately 1×10^7 cells were resuspended in 100 µl

DMEM/0.25% fetal calf serum (FCS) and equilibriated at 37°C. 10 µg of

purified anti-CD3ε (145-2C11) monoclonal antibody was added at time 0 to

stimulate the cells. For detection of phosphotyrosine containing proteins

in whole cell lysates, cells were incubated at 37°C for the appropriate

amount of time and then lysed by adding ice cold 10x TNE lysis buffer

(10% Triton-X-100, 1.5 M NaCl, 0.2 M Tris-HCl pH 7.5, 20mM EDTA, 5 mM

sodium ortho-vanadate, 2 mM sodium molybdate, 2 mM PMSF, 10 µg/ml

leupeptin, 10 µg/ml aprotinin and 10µg/ml pepstatin). For CD3ζ

immunoprecipitations, cells were stimulated for 30 minutes at 37°C

(stimulated samples) or on ice (unstimulated samples). Cells were washed

one time in PBS, and then lysed in 0.5 ml 1x TNE lysis buffer. For

experiments monitoring the expression of the early activation marker

(CD69), 1×10^5 BW5147 cells, resuspended in 200 µl of DMEM/10% fetal

calf serum, were stimulated with 0.1 µg of 145-2C11. These cells were then

taken at 0 hrs, 24 hrs, and 48 hrs and analysed for CD69 expression by FACS

analysis.

FACS analysis

Approximately 2×10^5 cells were incubated with 100 µl of one the

following: 145-2C11 anti-CD3ε tissue culture supernatant; H57-597, anti

TCRβ tissue culture supernatant; a 1/10 dilution of I3/2 anti-CD45 tissue

culture supernatant; a 1/100 dilution of RPTPα antisera (PTPα-ext); or a

1/200 dilution of anti-CD69 monoclonal antibody. Incubation time was

approximately 20 minutes on ice. Cells were subsequently washed with

phosphate buffered saline (PBS) containing 2.5% horse serum and

incubated for 20 minutes on ice with 100 µl of a 1/100 dilution of the appropriate FITC labeled secondary antibody or FITC labeled Streptavidin. Cells were then washed with PBS without horse serum, resuspended in PBS containing 5 µg/ml propidium iodide, and analyzed on a FACSCAN (Becton Dickinson, Mississauga, Ontario) using LYSIS™ II software (Becton Dickinson, Mississauga, Ontario).

2.3 Immunoprecipitation and immunoblotting procedures

Immunoprecipitation of CD45 for phosphatase assays

Approximately 1.0×10^6 cells were lysed in 0.5% Triton-X100, 20 mM Tris pH 7.5, 150 mM NaCl, 2.0 mM EDTA, 0.2 mM PMSF, 1.0 µg/ml pepstatin, 1.0 µg/ml leupeptin, 1.0 µg/ml aprotinin and incubated on ice for 10 minutes. Immunoprecipitates from CD45⁻ T cells were used as negative controls. Lysates were then centrifuged at 14000g for 10 minutes. The supernatant was then precleared with 10µl of a 50% slurry of Sepharose CL-4B (Pharmacia) at 4ºC for 1 hour. This lysate solution was then centrifuged and the supernatant was transfered to a fresh tube where 10µl of a 50% slurry of I3/2 coupled Sepharose CL-4B (4.0 mg antibody conjugated/ml packed beads) were added, and allowed to incubate end over end for 2 hours at 4ºC. The immunoprecipitate was subsequently washed three times in 0.2% Triton-X100, 20 mM Tris pH 7.5, 150 mM NaCl, 2.0 mM EDTA, plus protease inhibitors, and washed twice in non-reducing PTP buffer (50 mM Imidazole-Cl pH 7.2 or 50 mM Tris-Cl pH7.2, 1.0 mM EDTA). Beads were then resuspended in a volume of PTP buffer (50 mM Imidazole-Cl pH 7.2 or 50 mM Tris-Cl pH7.2, 1.0 mM EDTA, 0.1% β-mecaptoethanol) such that 5 µl of bead suspension contained CD45 immunoprecipitated from the appropiate cell equivalents to be tested. In

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studies using the various lymphoid cell lines, 2.5×10^4 cell equivalents per 5.0 µl were used. For studies involving splenic dendritic cells, 2×10^5 cell equivalents per sample were purified after overnight culture and lysed (Crowley et al., 1989; Haidl et al., 1995). I3/2 or SFR8-B6-conjugated beads were used to immunoprecipitate CD45 or the control protein HLA-Bw6, respectively. For studies involving naive and proliferating lymph node T cells, CD45 PTP activity was tested from a 5.0 µl bead suspension containing material immunoprecipitated from 2×10^7 cell equivalents.

Immunoprecipitation of surface CD3ζ

Stimulated and unstimulated cell lysates were placed on ice, centrifuged at 12,000g for 10 minutes to remove the insoluble pellet. 10 µl of protein A Sepharose beads were then added to each 0.5 ml lysate sample, and left to rotate at 4°C for 1hr. Beads were then washed three times in 1 x TNE lysis buffer, and 5×10^6 cell equivalents were loaded onto a 15% SDS polyacrylamide gel. Proteins were subsequently transferred onto a PVDF membrane and Western blot analysis was performed using a 1/2000 dilution of anti-phosphotyrosine antibody (4G10) or a 1/500 dilution of anti CD3ζ antibody (G3).

Cell labeling and immunoprecipitation of CD45 and RPTPα

Approximately 5×10^6 cells were incubated for 30 mins in methionine and cysteine free media prior to incubation for 8 hrs with 200 µCi of [^{35}S] methionine/cysteine (1175 Ci/mmol, DuPont NEN, Boston, Massachusetts) in 5.0 mls of DMEM (Methionine and Cysteine free) supplemented with 10% dialyzed fetal calf serum. Cells were washed and

then lysed in 1.0 ml lysis buffer (0.5% Triton X-100, 10 mM Tris pH 7.5, 0.15 M NaCl, 1 mM EDTA, with protease inhibitors) and the amount of radioactivity incorporated determined after TCA precipitation of a 50 μl aliquot of the lysate. CD45 and RPTPα were then precipitated from cell lysates containing equal amounts of radioactivity. Lysates from approximately 2 x 10^6 cells were precleared with 20 μl Sepharose beads and used to immunoprecipitate CD45 and RPTPα using 20 μl of protein A Sepharose beads precoupled with either 2 μl CD45 antisera (R01.1) or 10 μl of RPTPα antisera (PTPα-2). These amounts had previously been optimized to ensure maximum immunoprecipitation of the relevant proteins. After 2 hrs incubation at 4°C, the immunoprecipitates were then washed 2 times with lysis buffer containing 0.2% Triton X-100 and then subjected to SDS-PAGE where [^{35}S]-methionine and cysteine labeled proteins were observed upon autoradiography of the dried gel after treatment with Amplify (Amersham Canada Ltd., Mississauga Ontario). Alternatively, unlabeled immunoprecipitates were washed 2 times further in PTP buffer (20 mM Tris pH 7.4, 1.0 mM EDTA, 0.1% β-mercaptoethanol, and protease inhibitors) and used in PTP assays. Relative amounts of the [^{35}S]-methionine and cysteine labeled proteins were measured using densitometry scanning (PDI Systems, New York) and quantitative phosphoimaging techniques (Molecular Devices, Sunnyvale, California).

Immunoblotting of immunoprecipitated CD45 or RPTPα

Immunoprecipitates were electrophoresed on 7.5% SDS gels and proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Canada Ltd.) according to the manufacturers instructions (Bio-Rad). The membranes were blocked in 5% skim milk

protein (Lucerne Foods Ltd., Vancouver, British Columbia) in TBS (10
mM Tris-HCl, pH 7.5, 150 mM NaCl) for approximately 1.5 hours at room
temperature and then washed 3 times in TBS containing 0.05% Tween-20
(TTBS). The blots were then incubated with 1/2000 anti CD45 antiserum
(131 or R01.1) or 1/300 anti RPTPα antiserum (PTPα-2) in 5% skim milk
protein-TTBS for 1.5 hours at room temperature, washed and then
incubated with protein A conjugated to horseradish peroxidase (Biorad,
1/10,000 dilution in 5% skim milk protein-TTBS). The blot was washed
and developed using enhanced chemiluminesence (ECL kit, Amersham
Canada Ltd.).

Detection of phosphotyrosine proteins from cell lysates

Approximately 1.5×10^6 cells were lysed in 1 x TNE lysis buffer and
the cell lysates were subjected to electrophoresis on a 10% SDS
polyacrylamide gel. Proteins were subsequently transferred to PVDF
membrane (Millipore Canada Ltd., Mississauga, Ontario) in a transblot
apparatus (Bio-Rad Laboratories, Mississauga, Ontario) according to
manufacturers instructions and blotted with 4G10 antibody at 1/2000
dilution in 5% BSA-TTBS (0.1% Tween 20, 0.15M NaCl , 20mM Tris-HCl,
pH 7.5, with protease and phosphatase inhibitors) for 45 mins after first
blocking the membrane for 1 hour in the same buffer, but lacking
antibody. The blots were washed in 0.1% Tween 20, 0.15M NaCl, 20mM
Tris-HCl, pH 7.5, and incubated with 1/10,000 HRP conjugated goat anti-
mouse IgG in 5% BSA-TTBS for 45 minutes, washed thoroughly, and then
developed using the enhanced chemiluminescence (ECL) assay according
to the manufacturers instructions (ECL kit; Amersham Canada Ltd.,
Oakville, Ontario). In all cases, prestained molecular weight markers

(New England Biolabs, Mississauga, Ontario) were electrophoresed on the gel and transferred to the PVDF membrane.

Western blot analysis of recombinant proteins

Immunoblots performed on SDS-PAGE gels containing recombinant 6HIS CD45 proteins, p56^{lck} proteins, or GST-fusion proteins were probed using a 'quick method' Western blot protocol (Mansfield, 1993). This method specifically requires the use of Immobilon-P (Millipore Canada Ltd., Mississauga, Ontario) PVDF membrane and takes advantage of the membrane's high hydrophobicity so that a blocking step is not required and so that the overall surface area being probed with antibody is significantly decreased. Briefly, proteins were transferred to PVDF membrane and allowed to air dry for a minimum of 1 hr. With the exception of antiphosphotyrosine blots, the following protocol was followed. Membranes were immersed for 1 hour in the primary antibody solution containing the primary antibody (1/2000 anti CD45 antiserum (R01.1); 1/2000 anti-p56^{lck} antiserum (R49); 1/300 anti RPTPα antiserum (PTPα-2); or 1/2500 anti-GST antiserum) in 5% skim milk protein-TTBS. After this step, membanes were washed three times, for 10 seconds each, in TTBS, and then incubated in the secondary antibody solution (1/10000 protein A HRP conjugated in 5% skim milk protein-TTBS) for 30 minutes. Upon three more 10 second washes and a final 30 second wash in TTBS, membranes were developed using the ECL chemiluminescence as previously described. For quick method immunoblotting using the 4G10 antiphosphophotyrosine monoclonal antibody (used at 1/2500 in 5% BSA-TTBS), the procedure was as above except for the inclusion of a 30 minute blocking step (in 5% BSA-TTBS) followed by three 10 second

washes in TTBS. The rest of the procedure was then essentially the same except that the secondary antibody was Goat anti-mouse HRP antibody used at 1/10000 in 5% BSA-TTBS.

2.4 Recombinant protein production

Buffers for purification of recombinant cytoplasmic domain of CD45

The original protocol involved lysing bacterial pellets in buffer 1 (0.1%Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl, 20 mM Imidazole pH 7.2, 0.025% β-mercaptoethanol). Elution buffer (buffer 2) for the Nickel column was 1 M Imidazole pH 7.2, 150 mM NaCl and equilibration buffer (buffer 3) for the PD10 column was 0.1% Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl. Buffers A and B for the FPLC (Pharmacia) were 0.1% Triton X-100, 20 mM Tris pH 7.5 and 0.1% Triton X-100, 20 mM Tris pH 7.5, 2.0 M NaCl. All above buffers involved in the purification process contained 0.2 mM PMSF, 1.0 µg/ml pepstatin, 1.0 µg/ml leupeptin, and 1.0 µg/ml aprotinin. As the procedure was optimized over time (Glen Dibble and Jackie Felberg, pers. comm.), the buffer recipes were altered to the following: buffers 1 and 2 (0.5% Triton X-100, 20 mM Tris pH 7.5, 150 mM NaCl, 20 mM Imidazole pH 7.2, 0.025% β-mercaptoethanol), buffer A (0.1% Triton X-100, 20 mM Tris pH 7.5, 0.025% β-mercaptoethanol), buffer B (as buffer A but with 1 M NaCl), and buffer 3 (90% buffer A and 10% buffer B).

Expression and purification of recombinant 6 histidine cytoplasmic domain of CD45

500 ml of Luria broth supplemented with 100 µg/ml ampicillin was inoculated with a fresh culture of BL21(DE3) containing the appropriate pET vector and grown at 37ºC, 180 rpm. When the O.D.at 600nm of the

culture was ~0.6 to 0.8, the culture was induced with 0.1mM IPTG and left to grow for ~12 hours at 26°C, 180 rpm. The culture was then centrifuged at 4000g for 10 min at 4°C, and the pellet resuspended in 10 ml of buffer 1. The solution was frozen in a dry ice/ethanol bath and thawed slowly at room temperature; this step was repeated twice more. 100 µg DNase I was added and the solution kept at 37°C under constant agitation until no longer viscous. All subsequent steps, except for the PD10 column, were performed on ice or at 4°C. The solution was then spun at 10000g for 20 min and the supernatant was filtered using a syringe apparatus and a low protein binding 0.2 µm filter. This sample was loaded onto a column containing nickel chelate beads (Ni^{2+}-NTA agarose, 1.0 ml bed volume, Qiagen). The column was washed with buffer 1 until the O.D. of the eluate was zero. Bound proteins were eluted with buffer 2 and passed through a PD10 column previously equilibriated with buffer 3. The PD10 eluent was filtered using a low protein binding 0.2 µm filter and loaded onto a Mono Q column for ionic exchange chromatography. FPLC was performed using buffers A and B under a linear gradient ranging from 7.5% to 30% buffer B. In the optimized protocol using the newer buffer A and buffer B recipes, a linear gradient from 10% to 40% was used. Protein fractions were electrophoresed on an SDS-PAGE gel and were also tested for PTP activity. Elution profiles indicated that the recombinant 6HIS cytoplasmic CD45 generally eluted at approximately 150 mM to 200 mM salt concentration. Active elutes were then aliquoted and stored in 50% glycerol at -80°C.

Purification of recombinant GST fusion proteins

Bacterial culture handling and expression of GST-fusion proteins used the same protocol as that described above for the expression of the

6HIS-CD45 recombinant protein. Purification of GST-fusion proteins is essentially as described (Pharmacia, 1996). Briefly, glutathione beads (Pharmacia) were added to the soluble bacterial lysate and incubated for 1 hour at 4°C. Beads were then washed three times using the lysis buffer and eluted with a 10 mM glutathione. In general, 500 ml bacterial cultures were prepared to which 200 µl of glutathione beads were added. For poor expressing GST fusion proteins (full length p56^{lck} proteins, kinase domain proteins, and p60^{c-src} proteins), 1.0 L cultures were prepared and approximately 100 µl of 50% slurry glutathione beads were used. However, to increase yields for GST-full length p56^{lck} proteins, p56^{lck} full length mutants, as well as for the GST-p56^{lck} kinase domain protein, the following changes were incorporated (Frangioni and Neel, 1993). The lysis buffer for the purification of these low expressing GST-proteins was 20 mM Tris pH 7.5, 150 mM NaCl, 1.0% Triton X-100, 1.0% N-laurylsarcosine, 0.025% β-ME, 1 mM EDTA, and protease inhibitors. Furthermore, a sonication step was done after the lysis procedure using a sonicating water bath at 35% power output for 2 minutes (Fisher, sonic dismembrator - model 300). All purified GST-fusion proteins in both bound (in lysis buffer) and eluted (in elution buffer, 20 mM Tris pH 7.5, 150 mM NaCl, 5 mM glutathione) forms were stored in 50% glycerol at -80°C. For removal of the GST portion of the fusion protein, thrombin cleavage was performed. In brief, approximately 2 NIH units of thrombin (Sigma-Aldrich Canada Ltd., Mississauga, Ontario) was added per 10 µl of bound recombinant protein/GSH Sepharose beads, after the immobilized protein was washed three times in ice cold 20 mM Tris pH 7.5, 150 mM NaCl plus no protease inhibitors. Although the original protocol called for the cleavage protocol being performed in phosphate buffered saline (PBS), the

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cleavage was done in a Tris buffer to avoid problems associated with the presence of phosphate and subsequent analysis done with the malachite green assay (described later). In general, the thrombin cleavage was performed for 2 hours at 4°C in a rotating apparatus; approximately 70% of the recombinant protein could be cleaved using these guidelines, although the yield varied significantly depending on amount of expressed recombinant protein and on the particular recombinant protein.

2.5 In vitro enzymatic assays

In vitro phosphatase assays

1.   Malachite Green Micro Assay: All solutions were pre-equilibrated to 30°C. For CD45 immunoprecipitated directly from cells, 5.0 µl of CD45 bead suspension was added directly to 100 µl microtitre wells (Half-Area, Tissue Culture Treated, flat bottomed 96 well microtitre plates, Costar Corporation) for each timepoint tested. The reaction was initiated by the addition of 10.0 µl PTP buffer (50 mM Imidazole-Cl pH 7.2 or 50 mM Tris-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol) containing phospho-peptide at the defined concentration to be tested. Saturating amounts of peptides were used in all cases except when determining V_{max} and K_m values for specific phosphopeptides. During the reaction, the microtitre plate was agitated at 120 rpm. Timepoints were taken at 0 min, and two other timepoints (generally at 1 min, and 3 min). The reaction was stopped by the addition of filtered malachite green reagent (1 part 0.135% malachite green - oxalate salt in distilled water, 1 part 4.2% ammonium molybdate in 4.0 M HCl, 2 parts distilled water, and Tween-20 to a final concentration of 0.01%) to a total reaction volume of 100 µl. Release of inorganic phosphate was measured by determining the absorbance at 650

nm using a multiwell microtitre plate reader (Molecular Devices, Sunnyvale, California) and compared to a standard curve for inorganic phosphate detection obtained using potassium dihydrogen phosphate (KH_2PO_4).

Alternatively, for studies involving determination of PTP activity from purified recombinant phosphatases, an aliquot of 20 ng of purified protein was diluted in 10 µl PTP buffer (50 mM Imidazole-Cl or 50 mM Tris-Cl, 1.0% EDTA, 0.1% β-mercaptoethanol) and was added directly to 100 µl microtitre wells (Half-Area, Tissue Culture Treated, flat bottomed 96 well microtitre plates, Costar Corporation) for each timepoint tested. Addition of peptide substrate and malachite green reagent was as above.

2.   *p*-nitrophenol phosphate (pNPP) Assay: Phosphatase assays using p-NPP as substrate in 100 µl PTP buffer (50 mM Tris-Cl, 1.0 mM EDTA, 0.1% β-mercaptoethanol) were performed in half area 96 well microtitre plates at 30°C. Again saturating amounts of pNPP were used unless K_m and V_{max} values were being determined. Rate of absorbance at 405nm was monitored continuously using Softmax software on the Molecular Devices multiwell plate reader. A molar extinction coefficient of 1.78×10^4 $M^{-1}cm^{-1}$ and a path length of 0.5 cm was then used to calculate the concentration of the *p*-nitrophenolate ion produced in the reaction.

3.   PTP Assay on recombinant protein: For phosphatase assays on intact recombinant p56^{lck} or p60^{c-src} protein, approximately equimolar amounts of recombinant enzymes (50 ng for CD45 and 44 ng for RPTPα) was added to 200 ng of tyrosine autophosphorylated recombinant GST-p56^{lck} protein or GST-p60^{c-src} protein in a total volume of 10 µl PTP

buffer (50 mM Tris-Cl, 1.0 mM EDTA, 0.1% β-mercaptoethanol). Reactions were performed at 30°C and stopped at various timepoints from 0 to 32 mins by immersion in a dry-ice ethanol bath. These samples were then boiled in SDS sample buffer before being subjected to SDS-PAGE and transferred to PVDF membrane. The tyrosine phosphorylation state of GST-p56^{lck} was then analyzed by blotting with the antiphosphotyrosine monoclonal antibody, 4G10. It should be noted that for PTP assays done on recombinant material, the 50 mM Imidazole pH 7.2 in the PTP buffer was substituted with 50 mM Tris pH 7.2 with no observable differences in the efficiency of the malachite green PTP assay.

Dephosphorylation of GST-p56^{lck} proteins with alkaline phosphatase

Recombinant GST-p56^{lck}, or domain constructs were dephosphorylated using calf intestinal alkaline phosphatase (CIP), according to manufacturer's instructions (New Engand Biolabs, Mississauga, Ontario). Briefly, 2.0 µg recombinant GST protein, bound to GSH beads were washed twice in 1 x CIP buffer (50 mM NaCl, 10 mM Tris-HCl pH 7.9, 10 mM MgCl$_2$, 1 mM dithiothreitol). The beads are then suspended in 100 µl of 1 x CIP buffer to which 1.0 µl of alkaline phosphatase (10000 units/µl) was added. The reaction was left to proceed for 60 minutes at 37°C. The tyrosine phosphorylation states of the recombinant proteins were then checked by Western blotting with an anti-phosphotyrosine mAb (4G10).

In vitro kinase assays

For experiments determining the ability of p56^{lck} or p50^{csk} to phosphorylate the recombinant cytoplasmic domain of CD45 or the

inactive C817S mutant of CD45, 300 ng of baculovirus purified p56^{lck} or 300 ng thrombin-cleaved GST-p50^{csk} was resuspended in 20 µl kinase buffer 1 (20 mM PIPES pH 7.2, 10 mM MnCl$_2$) containing 10 µCi [γ^{32}P]-ATP (~3000 Ci/mmol, Amersham Canada Ltd., Mississauga, Ontario), 1 µM ATP (Pharmacia Biotech, Baie D'Urfé, Quebec). Included in the reaction was 5µg of acid-denatured enolase (Boehringer Mannheim, Laval, Quebec); 5µg of recombinant cytoplasmic domain of CD45; 5.0 µg of the C817S CD45 protein; or 5.0 µg of recombinant protein containing sequences from PTP domain 2 of RPTPα. *In vitro* kinase reactions were incubated at 30°C for 10 minutes. The reactions were stopped with 25 mM EDTA pH 8.0 and samples electrophoresed on 10% SDS-PAGE, the gel dried down and bands visualized by autoradiography with Kodak BioMax film (InterScience, Markham, Ontario).

For studies involving the phosphorylation of recombinant RPTPα with thrombin-cleaved p50^{csk} the following procedure was used. 2.0 µg of GST-RPTPα attached to GSH sepharose beads was washed and resuspended in 50 µl of kinase buffer 1 with 1.0 µg of recombinant kinase. Samples were then supplemented with or without unlabeled ATP to a final concentration of 10 µM. Phosphorylated and unphosphorylated samples were then tested for PTP activity, both in the presence and absence of 1.0 µg of recombinant thrombin-cleaved GST-GRB2 protein.

2.6 Binding assays

Binding assay

5 mgs of purified, recombinant cytoplasmic domain of CD45 was covalently coupled to 1 ml of packed CNBr activated Sepharose CL-4B beads according

to manufacturer's instructions (Pharmacia). An aliquot of beads containing approximately 2.5 μg of recombinant CD45 cytoplasmic domain was used in the binding assays. Beads with equivalent amounts of the monoclonal antibodies I3/2 or Ly 5.2 (Shen, 1981) were used as controls. 2.5 μg of GST fusion protein containing the cytoplasmic domain of RPTPα bound to glutathione Sepharose 4B beads was also used as a control in the binding assays. Approximately 200 ng of p56[lck], 200 ng of TrpE-p56[lck] fusion protein, or 2.0 μg of GST fusion protein was incubated for two hours at 4°C with immobilized recombinant CD45 cytoplasmic domain in a total volume of 40 μl of 20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, containing 0.2 mM PMSF, 1 μg/ml of aprotinin, leupeptin and pepstatin protease inhibitors and 0.5 mM sodium ortho-vanadate and 0.2 mM sodium molybdate tyrosine phosphatase inhibitors. Alternatively, the binding assay was performed using 2.0 μg of the appropriate GST-fusion protein immobilized to glutathione (GSH) beads. 1.0 μg of soluble recombinant cytoplasmic CD45 or soluble thrombin cleaved GST-cytoplasmic RPTPα is then added to the GSH beads in solutions and under conditions as described above. The beads were then washed and vortexed vigorously three times in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 0.025% β-mercaptoethanol, plus protease and protein tyrosine phosphatase inhibitors). The beads were then boiled in reducing SDS sample buffer and proteins separated by SDS-PAGE. Proteins were then stained with Coomassie blue or transferred to PVDF membrane (Immobilon P, Millipore Canada Ltd., Mississauga, Ontario) and detected by Western blotting with the appropriate antibody.

Equilibrium binding assay and analysis

Recombinant p56^{lck} was radioactively labeled by autophosphorylation using [γ^{32}P]-ATP (~3000 Ci/mmol, Amersham) in kinase buffer (20 mM PIPES pH 7.2, 10 mM MnCl$_2$) at 30°C for 15 minutes. The free [γ^{32}P]-ATP was separated from the ^{32}P-labeled p56^{lck} protein using a Sephadex G25 spin column equilibrated with binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, plus protease and phosphatase inhibitors) and the specific activity determined. It ranged from 50 cpm/ng to 457 cpm/ng. An aliquot of purified cytoplasmic CD45 domain protein coupled to sepharose beads (approximately 300 ng of protein) was incubated in 100 μl of binding buffer containing various concentrations (up to 4.0 x 10^{-7}M) of ^{32}P-labeled p56^{lck} at 4°C for 5 hours to ensure that equilibrium binding had been reached. Beads were then centrifuged and unbound ^{32}P-labeled p56^{lck} was measured by counting an aliquot of the supernatant. The amount of radioactively labeled p56^{lck} specifically bound to the beads was calculated from ([total amount of radioactive p56^{lck} added - amount remaining in supernatant] - non-specifically bound radioactivity). Non-specific binding was determined as the amount of radioactivity remaining bound to the beads after incubation in the presence of 50 fold molar excess of unlabeled p56^{lck} protein and was measured after washing the beads three times in binding buffer. The stoichiometry of the interaction was determined by double reciprocal plot analysis of the amount of p56^{lck} specifically bound (cpm) against the total amount of p56^{lck} added - non-specifically bound material (cpm). The 1/y-intercept was equivalent to the total amount of p56^{lck} bound under saturating conditions. The molar stoichiometry could then be calculated knowing the molar amount of CD45 present in the assay and the specific activity of p56^{lck} (cpm/ng).

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Competition binding assays

2.0 µg of ^{32}P-labeled p56^{lck} (2 x 10^5 cpm) was prepared as described above and incubated at 4°C for 8 hours with 15 µg of immobilized recombinant CD45 and then washed three times in binding buffer. Aliquots of beads containing 300 ng of CD45 and ~2000 cpm of bound p56^{lck} were then incubated in the presence of 1 x 10^{-13} M to 2 x 10^{-5} M (total volume 45 µl) of competing unlabeled GST-fusion protein at 4°C for 12 hours. The beads were then centrifuged, the supernatants removed, and the amount of ^{32}P-labeled p56^{lck} remaining bound to the beads determined using a scintillation counter. Nonspecific p56^{lck} binding was determined as described above and subtracted from each sample.

CHAPTER 3

Development of a non-radioactive protein tyrosine phosphatase assay for the analysis of protein tyrosine phosphatase activity from CD45 isolated directly from cells.

Related Publications:

Ng, D. H. W., Harder, K., Clark-Lewis, I., Jirik, F., Johnson, P. (1995) Non-radioactive method to measure protein tyrosine phosphatase activity isolated directly from cells. *J. Immunol. Methods* **179**, 177-185.

Haidl, I. D., **Ng, D. H. W.**, Rothenberger, S., Johnson, P., Jefferies, W. A. (1995) Detection of restricted isoform expression and tyrosine phosphatase activity of CD45 in murine dendritic cells. *Eur. J. Immunol.* **25**, 3370-3374.

Orchansky, P. L., **Ng, D. H. W.**, Johnson, P., Teh, H. S. (1996) Increase in the specific activity of $p50^{csk}$ in proliferating T cells correlates with decreased specific activity of $p56^{lck}$ and $p59^{fyn}$ and reduced phosphorylation of CD3 subunits. *Molec. Immunol.* **33**, 531-540.

3.1 Introduction

The cloning of CD45 and subsequent sequence analysis led to the realization that there was sequence identity between regions of the cytoplasmic domain of CD45 and the protein tyrosine phosphatase PTP1B (Tonks, 1988; Tonks et al., 1988; Charbonneau et al., 1988). Consequently, a large part of the appeal in studying CD45 concerns its use as a prototype for kinetic and biochemical analysis of protein tyrosine phosphatases (PTPs). Moreover, as CD45's role in signal transduction is more clearly defined than other PTPs, these biochemical characterizations become important in permitting investigators to coalesce information from both the catalytic and signaling traits of the molecule. Consequently, a working assay that can effectively and efficiently monitor and measure PTP activity is an invaluable tool. When work described in this thesis was first initiated, the standard protocol of measuring PTP activity entailed the use of radioactively phosphorylated substrates (Ostergaard et al., 1989; Streuli et al., 1990). However, preparation of this type of substrate is a laborious process resulting in limiting yields of substrate that is further hindered by the isotope's [^{32}P] short half life. In addition, available phosphorylation procedures generally resulted in a low percentage of phosphorylated product which then has to be separated from non-phosphorylated material. As proper kinetic analysis often requires large amount of substrate, the factors described above prohibit the usefulness of radioactively phosphorylated substrates in comprehensive kinetic assays.

In this chapter, an alternative method for the kinetic analysis of purified or recombinant soluble phosphatases using the malachite green reagent (Itaya and Ui, 1966; Lanzetta et al., 1979; Harder et al., 1994) is described. This reagent can detect picomoles of phosphate product

released from chemically synthesized phosphorylated peptides (Hudson, 1988; Cho et al., 1991; Clark-Lewis et al., 1991). Overall, the protocol is rapid, sensitive, non-radioactive and can be used to measure PTP activities of both transmembrane and soluble phosphatases isolated directly from cells. This colorimetric microassay is performed in 96 well plates using a standard Microplate reader and can reliably detect 100 pmoles of free phosphate. The phosphatase activity of CD45, a transmembrane PTP, was determined from as few as 1×10^4 lymphoma cells. We also examined CD45 PTP activity from lymph node T cells as well as splenic dendritic cells. The development of this colorimetric assay to measure immunoprecipitated CD45 PTP activity isolated from very small numbers of cells has general applicability for other PTP's and will help identify the cellular situations and conditions that result in changes in PTP activity. Furthermore, this malachite assay can also be utilized for analysis of soluble recombinant enzymes capable of generating free phosphate.

3.2 Results

Optimum conditions for CD45 PTP assay

The enzyme reaction was performed in a total volume of 15 µl in one well of a 96 well microtitre plate (well capacity is 100 µl). Malachite green reagent was added to terminate the reaction and produced a final volume of 100 µl. The color was allowed to develop and stabilize for 10 minutes, and was read at 650 nm in a microplate reader, the absorption at 650 nm being proportional to the amount of free phosphate generated over a range of 0 to 4 nmoles (Figure 3.1). The sensitivity of the assay was such that 100 pmol phosphate generated an absorbance reading that was significantly above background levels.
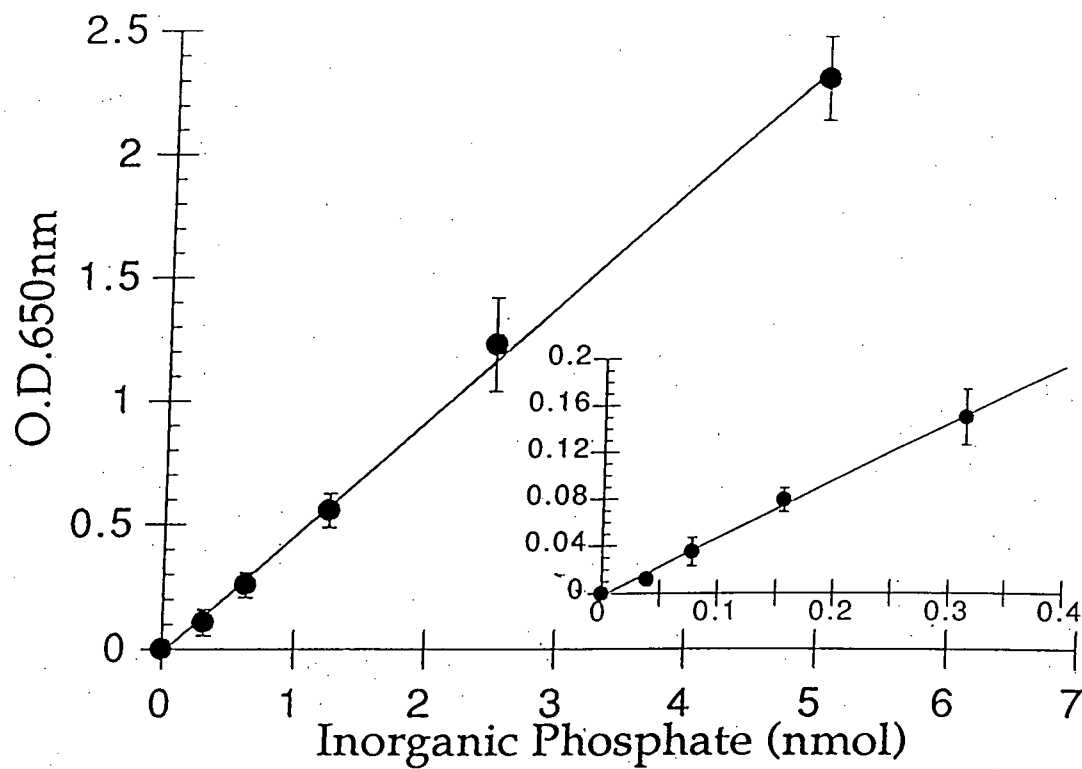
Figure 3.1: <u>Standard curve of O.D. at 650 nm vs inorganic phosphate</u>
<u>concentration</u> Absorbance readings (●) at a wavelength of 650 nm were

taken at different concentrations of potassium dihydrogen phosphate

(KH_2PO_4)

To determine the optimum enzyme concentration for this range, enzyme concentration was varied and the initial rates of reaction determined. CD45 immunoprecipated from 2×10^3, 5×10^3, 1×10^4, 2×10^4, 5×10^4 and 1×10^5 BW5147 CD45$^+$ cells was tested (Figure 3.2). Initial rates of activity could be measured from CD45 isolated from as little as 1×10^4 cells. CD45 isolated from 2.5×10^4 cells was used for all subsequent experiments. Identical immunoprecipitation experiments were done with 1×10^5 BW5147 CD45$^-$ cells and showed no observable PTP activity (Figure 3.2), illustrating that the generation of inorganic phosphate was likely due solely to the PTP activity of CD45.

It had previously been shown that both purified endogenous and recombinant CD45 PTP activity reach an optimum at pH 7.2 (Tonks et al., 1990; Itoh et al., 1992). Given that at alkaline pH's, the malachite green reagent becomes more strongly absorptive at 650 nm, it was important to determine the optimal pH of CD45 activity under our immunoprecipitation scheme such that subsequent experiments could be performed without compromising the effectiveness of the colorimetric capabilities of the reagent. Results indicated that like previously demonstrated, immunoprecipitated CD45 had a pH optimum of 7.2 and a narrow pH range, being virtually inactive below pH 6 and above pH 8, when determined using a 50 mM imidazole, 1 mM EDTA, 0.1% β-mercaptoethanol buffer system (Figure 3.3).

Effect of Sepharose beads in malachite green PTP assay

Since the amount of phosphate released is measured by optical density at 650 nm, it was possible that the presence of the Sepharose beads used to immunoprecipitate the PTP may scatter the incident beam of light
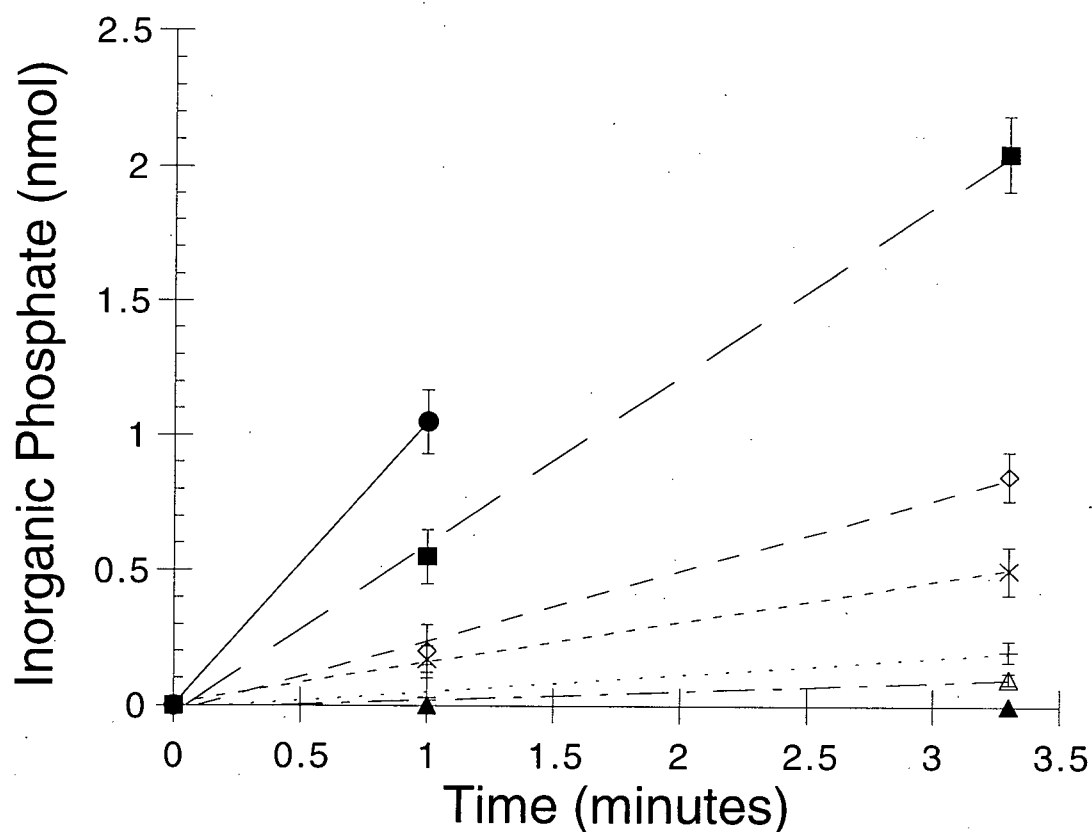
Figure 3.2: Effect of enzyme concentration on CD45 PTP activity using saturating amounts of substrate (10 mM fyn pY531). CD45 was immunoprecipitated from BW5147 CD45$^+$ cells at the following cell equivalents: ($\bullet$) 1×10^5, ($\blacksquare$) 5×10^4, ( ) 2×10^4, (x) 1×10^4, (+) 5×10^3, and (Δ) 2×10^3. CD45 was also immunoprecipitated from 1×10^5 BW5147 CD45$^-$ cells and tested for PTP activity ($\blacktriangle$). Assay readings were taken at 0 min, 1 min and 3 min 20 sec using a 50mM Imidazole-Cl pH7.2, 1.0mM EDTA, 0.1% β-mercaptoethanol buffer.
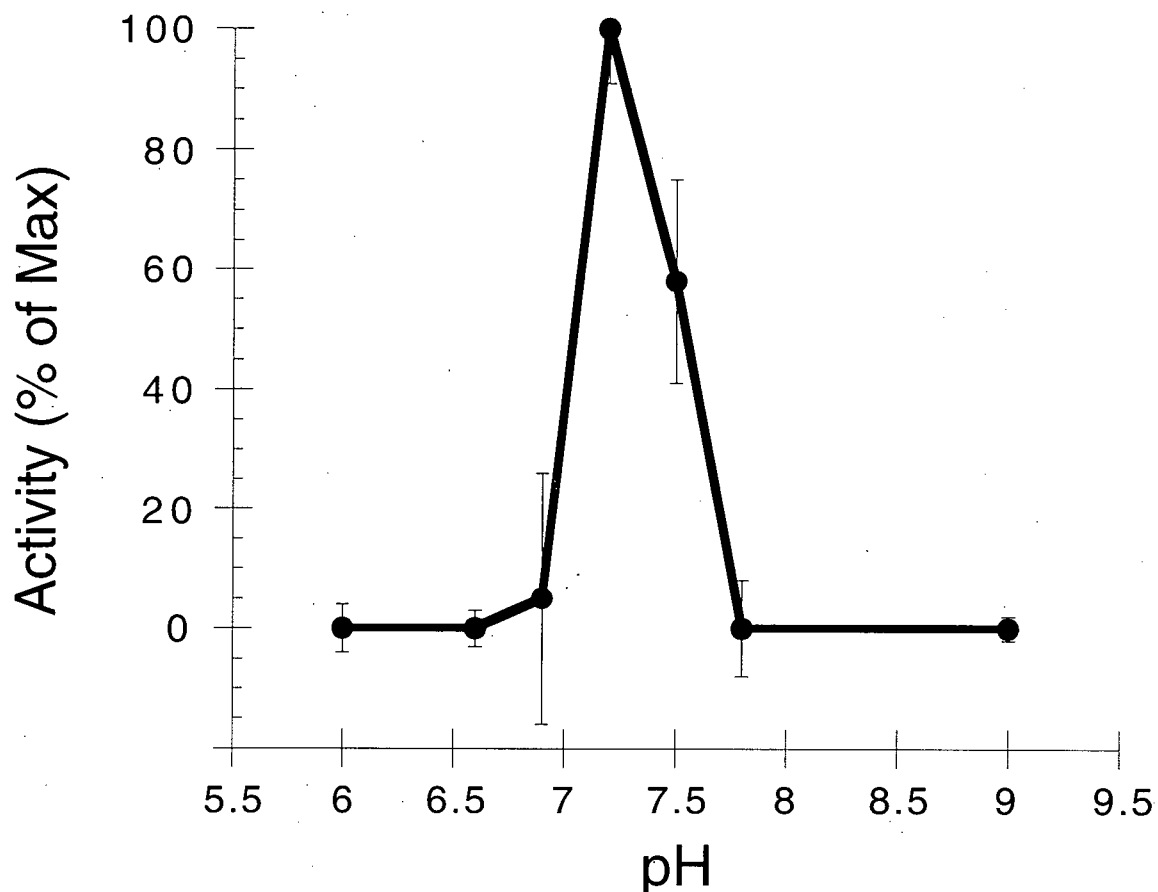
Figure. 3.3: <u>pH Optimum for immunoprecipitated CD45.</u> PTPase activity was measured using standard assay conditions, 3.0 mM fyn pY531 phosphopeptide substrate and with the following buffers: 50 mM Imidazole-Cl, 1mM EDTA, 0.1% β-mercaptoethanol at pH 6.0, 6.6, 6.9, 7.2, 7.5, 7.8 and 9.0. (100% PTP activity is equivalent to approximately 0.50 nmol/min/2.5 x 10^4 cells)

and interfere with the spectrophotometric readings. In a PTP assay of CD45 immunoprecipitated from 2.5×10^4 cells, an estimated volume of 0.125 µl of packed antibody conjugated Sepharose beads would be present in the assay. This volume of antibody conjugated beads was previously found to be sufficient to precipitate all CD45 from the cells. Experiments titrating Sepharose bead amounts into the malachite green reagent indicated that bead volumes up to 0.625 µl were found to have no significant effect on optical density readings at 650 nm (data not shown).

A further concern regarding the use of immobilized CD45 was the possibility of substrate limiting conditions. If the immunoprecipitated CD45 was concentrated and compacted in a small area of the reaction, then the local substrate concentration may be used up too quickly within the confines of the experiment. To address this concern, the effect of agitating the plate during the incubation was examined. It was generally seen that at lower peptide concentrations (concentrations well below the K_m of the substrate) a linear reaction rate was only observed with agitation of the microtitre plate at 120 rpm or higher. If agitation was not initiated, then reactions were not linear and instead were indicative of possible substrate limiting conditions. However, at higher peptide concentrations, agitation did not have a significant effect (data not shown).

Use of assay to compare CD45 PTP activity isolated from different lymphoid cell lines

CD45 immunoprecipitated from four different T or B lymphoma cell lines (BW5147, EL4, RAW 253.1, and A20) was tested for its PTP activity. SDS-PAGE was performed on immunoprecipitated CD45 followed by Western blot analysis using an antiserum raised against a

common extracellular CD45 epitope (Figure 3.4). Differences in the apparent molecular weight of CD45 were due to the differential expression of CD45 isoforms. Using equivalent cell numbers (5×10^4 cells), no appreciable CD45 was immunoprecipitated from the RAW 253 cell line and consequently the specific PTP activity was not determined. Relative protein amounts of CD45 were calculated by comparing the intensity of the CD45 bands on the Western blot using densitometry scanning (PDI systems, New York). Amounts of CD45 were expressed as a function of the optical density of the band and the area which the band occupied. Table II shows the PTP activity of CD45 isolated from each cell line, comparing PTP activity obtained from equal cell numbers and from equivalent amounts of CD45 protein.

Use of assay to compare CD45 PTP activity isolated from naive and proliferating lymph node T cells

To further evaluate the usefulness of the PTP assay, CD45 from lymph node T cells was also assessed. The expression of CD45 in purified murine lymph node T cells was determined by immunoblotting total cell lysates or anti-CD45 immunoprecipitates of cell lysates with a pan-specific anti-CD45 antiserum. Figure 3.5 demonstrates that CD45 has 3 to 6 fold higher expression levels in proliferating cells than in naive cells. Using the malachite green assay with 3.0 mM of the fyn pY531 phosphopeptide, it was found that when lysates corresponding to 2.5×10^4 cell equivalents were immunoprecipitated with anti-CD45 mAb, the PTP activity was 3-4 fold higher in proliferating than in naive cells. However, when the same amount of immunoprecipitated CD45 was tested, the same PTP activity was observed indicating that the PTP activity of CD45 parallels the

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Figure 3.4: <u>Immunoblot of CD45 immunoprecipitated from two T and two B cell lines.</u> CD45 was immunoprecipitated from 1 x 10^6 cells of BW5147 and EL-4 T lymphoma cell lines and from RAW 253 and A20 B cell lines. The immunoprecipitate was subjected to a 7.5% SDS-PAGE and the protein transferred to PVDF membrane. CD45 protein was detected by Western blot analysis using an anti-CD45 antisera specific for an extracellular epitope common to all CD45 isoforms.

Cell Line	Cell Type	PTP activity/ 5×10^4 cells (nmol/min)	Relative amounts of CD45 (O.D. x mm^2)	PTP activity/ Equiv. amts of CD45 (nmol,min^{-1}, O.D.$^{-1}$,mm^{-2})
BW5147	T lymphoma	0.77	4.86	0.16
EL4	T lymphoma	0.57	3.61	0.16
RAW 253.1	B lymphoma	0.00	n/d	n/d
A20	B lymphoma	0.19	1.20	0.16

Table II: <u>Comparison of CD45 PTP activity isolated from various lymphoma cell lines</u>. Amount of CD45 immunoprecipitated from each cell line was compared by Western blot analysis. Undetectable levels of CD45 were immunoprecipitated from RAW 253 cells, hence a comparative rate could not be determined (n/d). Reaction rate was measured in nmol phosphate released per min per 5×10^4 cell equivalents using saturating amounts of the src phosphopeptide (1.6 mM src pY416) as substrate and a 50 mM Imidazole-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer. The adjusted reaction rate of CD45 from equivalent amounts of CD45 was obtained by dividing the PTP activity by the relative amount of CD45 present.
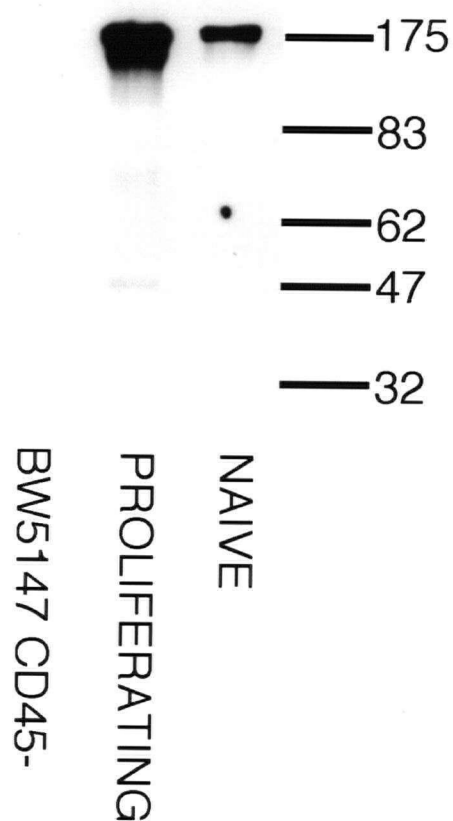
Figure 3.5: <u>CD45 expression in naive and proliferating murine lymph node T cells.</u> Approximately 1×10^6 cell equivalence of BW5147 CD45⁻, naive and proliferating T lymph node cells were lysed and lysates were immunoprecipitated with anti-CD45 mAb (I3/2) and resolved on 7.5% SDS-PAGE. Proteins were immunoblotted with anti-CD45 antiserum.

expression level (Figure 3.6). Consequently, the specific activity of CD45 in naive and proliferating cells is equivalent.

Use of assay to compare CD45 PTP activity isolated from purified splenic dendritic cells

To determine whether CD45 from dendritic cells could be active and therefore possibly function in a signal transduction pathway, CD45 was isolated from splenic dendritic cells and assayed for its PTP activity (Haidl et al., 1995). As seen in Figure 3.7, the CD45 immunoprecipitates from dendritic cells exhibited tyrosine phosphatase activity when tested using 3.0 mM fyn pY531 phosphopeptide. Western blotting of the immunoprecipitated material used in the tyrosine phosphatase assay clearly showed that CD45 is present in dendritic cell lysates immunoprecipitated with I3/2 antibody but absent in control immunoprecipitations (data not shown). Work performed by I. Haidl demonstrated that macrophages, which comprise 5-10% of the splenic dendritic cell preparation, exhibited a similar level of CD45 activity per cell (data not shown). This, therefore, indicates that the majority of CD45 activity isolated from purified splenic dendritic cells was due to dendritic cells themselves.

Use of malachite green PTP assay to make kinetic measurements

Initial rates of phosphate hydrolysis were determined for CD45 immunoprecipitated from 2.5×10^4 BW5147 cells using four peptide substrates (fyn pY531, src pY527, lck pY505 and src pY416) derived from the carboxyl terminal region of three src-family kinases, $p59^{fyn}$, $p60^{src}$ and $p56^{lck}$ and the autophosphorylation site of $p60^{src}$. By varying peptide
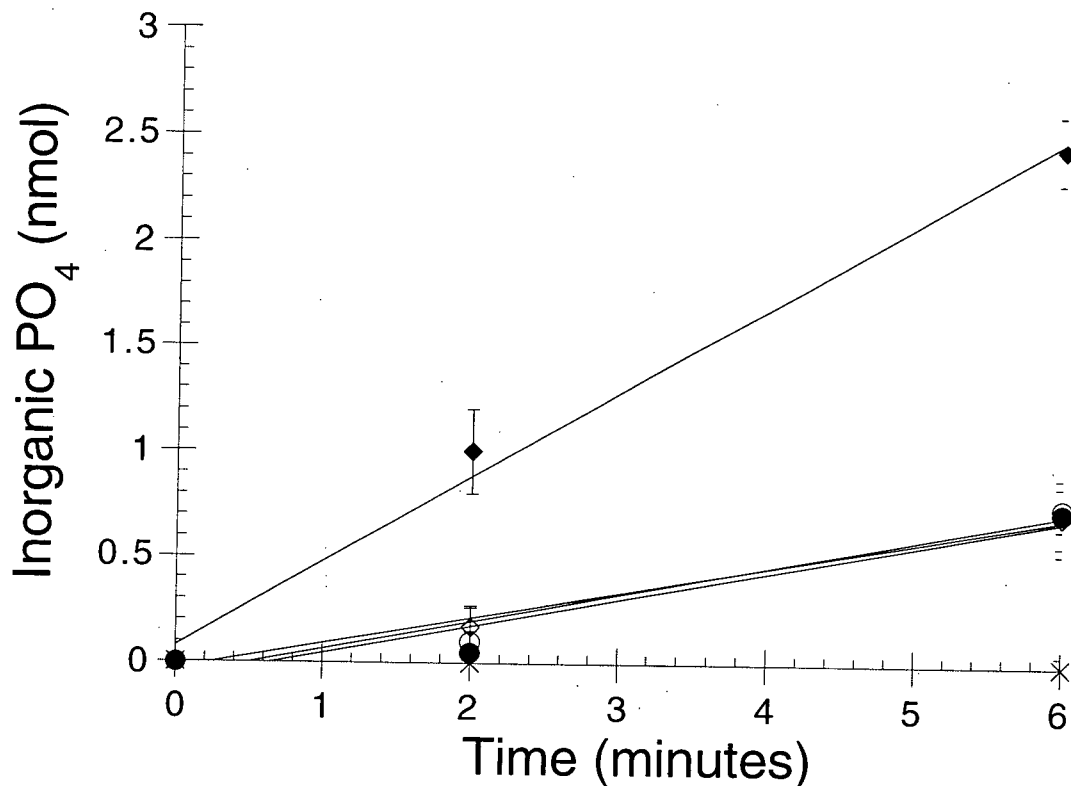
Figure 3.6: <u>CD45 phosphatase activity in naive and proliferating murine lymph node T cells.</u> CD45 was immunoprecipitated from naive and proliferating T cells and phosphatase activity was assayed with 3.0 mM phosphorylated fyn pY531 peptide and a 50 mM Imidazole-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer. Time points were taken at 0, 2, and 6 min and nmoles of inorganic phosphate released were calculated using the malachite green phosphate assay. Rates of reaction for CD45 immunoprecipitated from naive T cells (o, ●), proliferating T cells

(,◆), or BW5147 cells deficient in CD45 (X) are shown. The open signs are values derived from equivalent amounts of CD45 (as assessed by densiometric scanning) whereas the closed signs denote CD45 isolated from equal cell numbers (2.5×10^4 cells). Results are representative of three independant experiments.
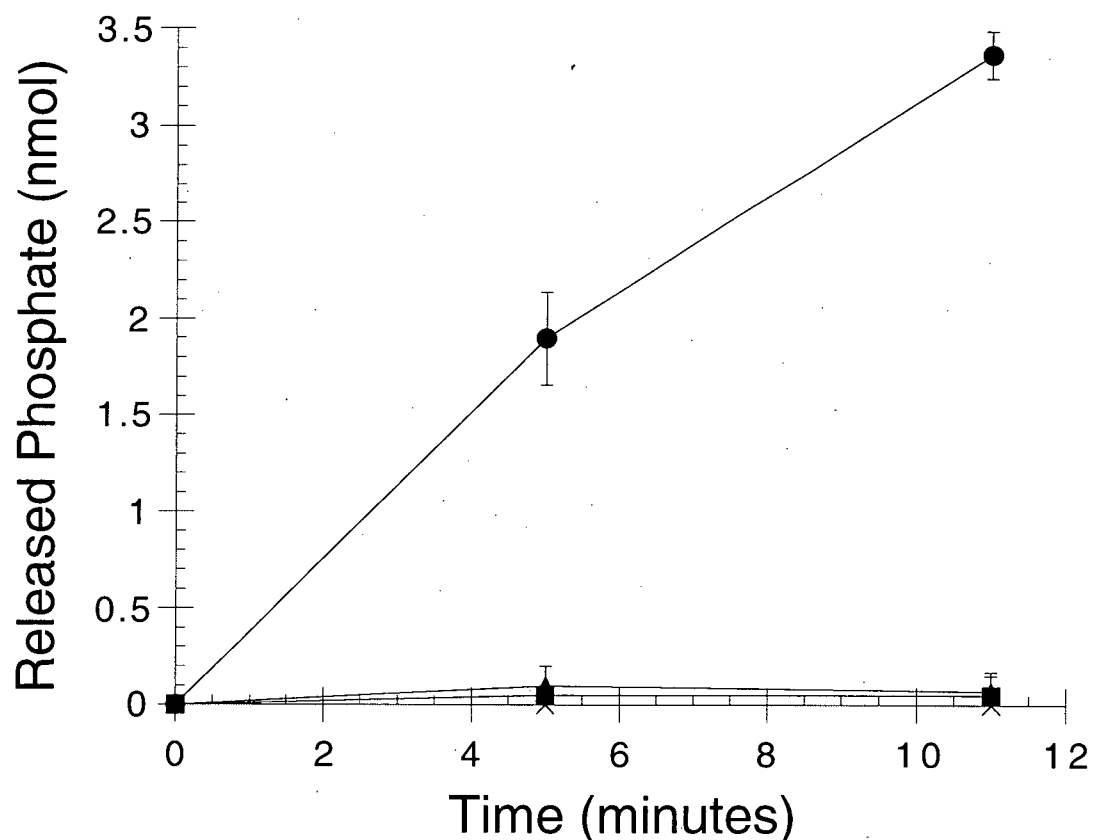
Figure 3.7: <u>Tyrosine phosphatase activity of dendritic cell CD45.</u> Splenic

dendritic cells were purified by overnight culture and lysed. L cells with

no CD45 expression were used as control cell type. Immunopreciptations
were carried out using anti-CD45 mAb (I3/2) in both dendritic cells (●)

and L cells (▲); or using an isotype control in both dendritic cells (■) and L

cells (X). The tyrosine phosphatase activity was then assayed in a 50 mM

Imidazole-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer,

using 2×10^5 cell equivalents for each timepoint.

concentration between 0mM and approximately 3.0 mM, conditions of substrate saturation and maximum velocity values could be obtained (Table III). K_m values and V_{max} values were then derived from Lineweaver Burk (Figure 3.8) and Eadie Hofstee analyses .

Additional parameters to improve CD45 PTP activity.

Although the PTP assay experiments described in this chapter entailed the use of no detergent in PTP buffer solutions, it was noted that addition of detergent in particular buffers could affect the specific PTP activity measured. Consequently, detergent conditions in the malachite PTP assay were varied and tested while PTP activity from CD45 immunoprecipitated from 5×10^4 CD45$^+$ BW5147 T cells, using 3.0 mM fyn pY531, was ascertained. In particular, changes in the lysis procedure whereby the non-ionic detergent Triton X-100 was substituted with the milder Brij 96 revealed significant differences in immunoprecipitated CD45 PTP activity (Figure 3.9). Furthermore, it was found that the inclusion of various detergents in the PTP buffer composition could profoundly effect rates of dephosphorylation (Figure 3.9). Importantly, the KH_2PO_4 standard curve when prepared in PTP buffers containing detergent were unaltered, suggesting that the effects seen with detergent addition coincide with an increase in PTP activity, rather than an increase in reagent sensitivity.

3.3 Discussion

This work demonstrates the feasibility of using immunoprecipitated CD45 isolated from as few as 10,000 lymphoid cells in a non-radioactive,

Substrate	Km (mM)	Vmax (nmol/min/2.5 x 10^4 cells)
fyn pY531 (TATEPQpYQPGENL)	0.9 ± 0.06	0.54 ± 0.05
src pY527 (TSTEPQpYQPGENL)	0.9 ± 0.05	0.53 ± 0.07
lck pY505 (TATEGQpYQPQP)	0.8 ± 0.04	0.57 ± 0.09
src pY416 (LIEDNEpYTARQGA)	0.6 ± 0.06	0.53 ± 0.03

Table III: <u>Kinetic measurements of CD45 immunoprecipitated from BW5147 T cells using four different src family phospho-peptides as substrates</u>. Enzyme reactions were performed in PTP buffer (50 mM Imidazole-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol) at 30°C. K_m values are given in mM and comparative V_{max} values are given in nmol phosphate per min per 2.5 x 10^4 cell equivalents of immunoprecipitated CD45.
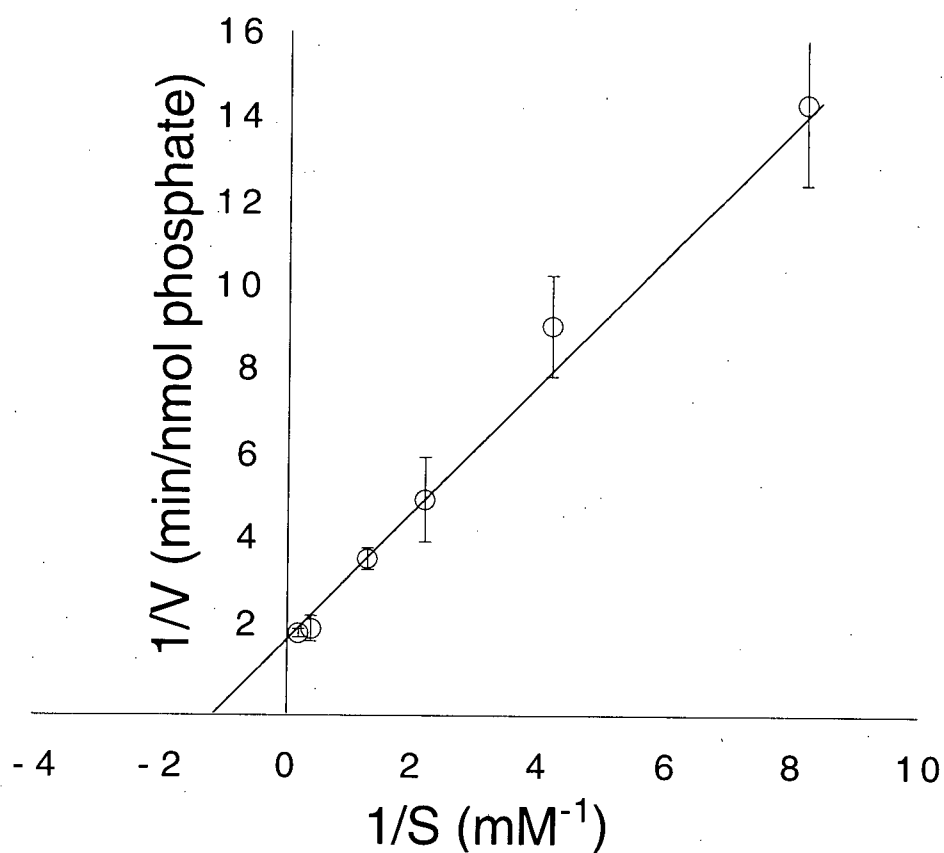
Figure 3.8: <u>Lineweaver Burk analysis on kinetic data obtained from CD45 PTP assays using lck pY505 peptide substrate</u>. Lineweaver Burk plot for immunoprecipitated CD45, comparing the initial rates of reaction (V) in nmol of inorganic phosphate produced per min per 2.5×10^4 cell equivalents versus lck pY505 substrate concentration (S) in mM.
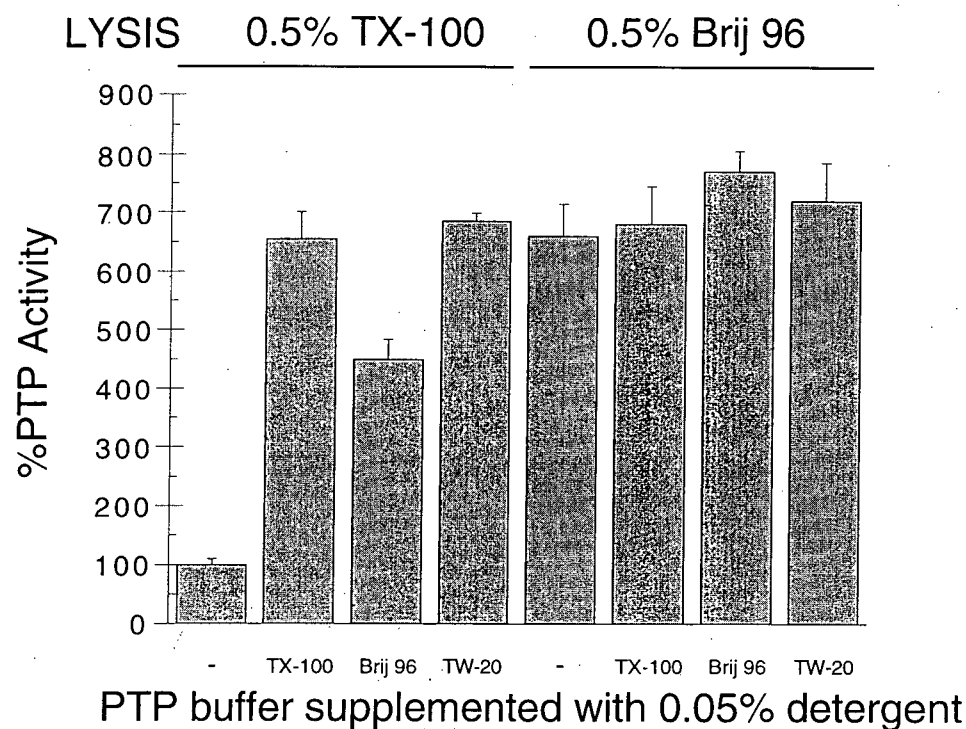
Figure 3.9: Test of different detergent conditions in malachite green PTP assay. Rates of activity are expressed in relation to activity under standard conditions (set at 100% which is equivalent to approximately 0.50 nmol/min/2.5 x 10^4 cells). Parameters tested include varying detergent conditions in the cell lysis procedure (top of the figure) and in the 50 mM Imidazole pH 7.2, 1 mM EDTA, 0.1% β-mercaptoethanol PTP buffer (bottom of figure). Results shown are representative of three separate experiments.

colorimetric, micro assay to quantitate enzymatic activity by measuring the release of inorganic phosphate. It demonstrates that the Sepharose beads used to immunoprecipitate the PTP do not interfere with the assay and that comparative enzymatic studies can be performed on CD45 isolated from different cell types. In addition, kinetic analysis can be used to determine and compare substrate affinity (K_m) and maximum rates of reaction (V_{max}) values.

The advantages of this method over the existing radioactive method are that it is quick, easy to perform and sensitive. No preparation of substrate is required after initial synthesis and enzymatic activity can be determined by measuring the amount of free phosphate present without prior separation from phosphorylated substrate. The assay is very sensitive allowing CD45 PTP activity to be determined from as few as 1×10^4 cells and can easily detect 100 pmol of free phosphate. Use of a phosphorylated peptide as a substrate instead of simple phosphotyrosine analogs such as *p*-nitrophenyl phosphate and O-phosphotyrosine is preferred as phosphorylated peptides have been shown to have a higher affinity for CD45 (Cho et al., 1993).

Other colorimetric methods for measuring PTP activity have all used purified, soluble recombinant PTP's. In general, these methods have tended to use larger assay volumes (0.4 - 1 ml) and large amounts of substrate, but have also provided useful kinetic analysis and insights into PTP substrate specificity (Daum et al., 1991; Cho et al., 1993; Zhang et al., 1994). Recently, we and others (Fisher and Higgins, 1994; Harder et al., 1994) have scaled down the malachite green assay such that lower volumes (40-100 μl) could be used in microtitre wells for the measurement of PTP activity using an ELISA plate reader. Robotization of

this method allows one to screen thousands of chemicals that may act as either phosphatase inhibitors or activators (Fisher and Higgins, 1994). These assays using recombinant soluble protein and phosphopeptide do not, however, provide information on how these PTP enzymes may be regulated in the cell, as physiological post translational modifications cannot occur under these conditions. Therefore, the PTP assay described in this chapter allows the option of assessing possible *in vivo* modifications since the material is isolated directly from cells. It is important, however, to stress that there are still limitations with this protocol, particularly with transmembrane PTP's, since these molecules are anchored in the cell membrane and may be further regulated by cellular locale or extracellular interactions.

In this chapter, CD45 was immunoprecipitated from T and B cell lines and their phosphatase activities compared. It was shown that CD45 from the B cell line, A20 or the T cell lines, EL-4 and BW5147, had similar specific phosphatase activity when immunoprecipitated protein amounts were isolated and normalized using densitometric scanning. Furthermore, the electrophorectic mobility of immunoblotted proteins seen in Figure 3.4, suggests that each of these three cell lines had a distinct isoform of CD45. Therefore, the specific activity of the different isoforms of CD45 does not appear to vary. In agreement with earlier data (Ostergaard et al., 1989), this result is also illustrated in the immunoprecipitation of CD45 from naive and proliferating lymph node T cells isolated from mice. There is a discrete difference in electrophoretic mobility between these two samples, that is made clearer when samples were subjected to a 5% SDS-PAGE (Orchansky et al., 1996). This experiment indicated that the naive T cells express higher molecular

weight forms of CD45, whereas the proliferating cells express lower molecular weight forms. Comparison of the specific PTP activity between the different isoforms present in naive versus proliferating lymph node T cells would again suggest no intrinsic difference. Analysis performed on CD45 isolated from splenic dendritic cells illustrate that this protocol can also be easily extended to less defined cell populations. The CD45 tyrosine phosphatase activity from dendritic cells is less than that of the aforementioned T and B cell lines tested, when examining the enzymatic rates on a cell equivalent basis (Table IV). Indeed, the CD45 specific activity from dendritic cells was only three fold less than the values obtained from the naive T cells, so it is still conceivable that CD45 would be fully capable of mediating signaling events in dendritic cells. Overall, this method thus provides a convenient means to assay phosphatase activity of enzymes isolated directly from cells and allows the comparison of enzymatic activities to be made from phosphatases isolated from different cell populations.

This method can also be used to perform comprehensive kinetic analysis on PTP's isolated from cells. Unlike the method that uses radioactively labeled substrate, the availability of large amounts of chemically synthesized phosphorylated substrate allows kinetic measurements to be made and rates of reaction compared. To illustrate this point, K_m and V_{max} values were determined for CD45 isolated from 2.5×10^4 BW5147 T lymphoma cells using four src family phosphotyrosine peptides as substrates. Although we were unable to compare our V_{max} values to previously reported literature (since the mass of protein being isolated in our immunoprecipitations was not ascertained) the affinity of the substrates could still be compared. K_m values (Table III) derived from

Cell Type	Reaction rate (nmol/min/2.5 x 10^4 cells)
naive murine lymph node T cells	0.12 ± 0.02
proliferating murine lymph node T cells	0.42 ± 0.04
splenic dendritic cells	0.04
CD45+ BW5147 T lymphoma cells	0.54 ± 0.05

Table IV: <u>Comparison of Immunoprecipitated CD45 PTP activity from 2.5 x 10^4 cells of different cell populations</u>. Enzyme reactions were performed in phosphatase buffer, pH 7.2 at 30°C. Reaction rate values were derived from previous data for comparison of immunoprecipitated CD45 from different cell populations in nmol phosphate per min per 2.5 x 10^4 cell equivalents.

our studies, were somewhat higher than the reported values for soluble purified CD45 (12 µM for the substrate RCM-lysozyme, 0.15 mM for fyn 531 peptide) and for recombinant cytoplasmic CD45 against phosphopeptide substrates (0.13 mM for lck 505 peptide, 0.17 mM for fyn 531 peptide, 0.21 mM for CD3ζ peptide). However, they were lower than K_m values (4.8 mM) reported using pNPP as a substrate (Cho et al., 1993; Pacitti et al., 1994; Tonks et al., 1990). It is possible that the presence of the anchoring effect of the protein to the beads or the presence of the antibody attached to the extracellular domain may affect K_m values.

Interestingly, one of the phosphopeptides tested had a significantly higher affinity than the other three. This peptide (src pY416) is also distinct from the other three peptides in that it represents sequences from the autophosphorylation site of p60$^{\text{c-src}}$. The other three peptides are all derived from the negative regulatory carboxyl-tail tyrosine site of their respective kinases. That the autophosphorylation site had a K_m value of 0.6 mM, whilst the carboxyl-tail sequences had K_m values of 0.8 mM to 0.9 mM (see Table III) is indicative of CD45 showing a possible substrate preference for the autophosphorylation site over the negative regulatory site. This observation is particularly intriguing given the demonstration of CD45's ability to affect the autophosphorylation site of p56$^{\text{lck}}$ in BW5147 and YAC-1 T cells (Ostergaard et al., 1989; Doro et al., 1996).

Although phosphotyrosine containing peptides may not exactly mimic the intact protein, it has been demonstrated that they do contain key amino acids that determine substrate specificity for certain PTPs (Zhang et al., 1994). Analysis of PTP peptide specificity together with the recent 3D structure of PTP1B (Barford et al., 1994a) suggests that 4-5 residues proximal of the phosphotyrosine influence the efficiency of

binding and hydrolysis of substrate. In light of this information, we were also intent on comparing kinetic values obtained from phosphopeptides to the observations that have been seen in a variety of T cell lines. Despite CD45 displaying clear substrate preferences in cell line experiments (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Hurley et al., 1993), our kinetic data on the fyn pY531, src pY527 and lck pY505 phosphopeptides firmly establish that other structural or kinetic constraints must play a dominant role in determining substrate preferences *in vivo*.

Attempts to improve the sensitivity of the malachite green assay have led to the observation that detergent conditions can significantly alter results obtained. In particular, it was found that lysis in a milder detergent such as Brij 96, resulted in higher PTP rates, when compared to samples lysed in Triton X-100. In addition, simple supplementation of the PTP buffer with small amounts of detergent was shown to greatly enhance PTP activity of cells lysed in Triton X-100. The mechanisms involved that could explain this phenomenom are currently unknown, although there are two possible reasons. First, it is quite possible that in our experimental system the immunoprecipitated CD45 exists in an aggregated manner that may affect activity. Under different detergent conditions the degree of aggregation may be changed resulting in altered PTP rates. Aggregation can be envisioned to decrease activity since immunoprecipitated proteins may be clumped together such that not all active sites are assessible to the reaction environment. This first possibility regarding detergent addition, may also explain why previously reported K_m values using recombinant cytoplasmic protein are significantly lower than the values derived here. It would therefore be interesting to assess K_m and V_{max} values of

immunoprecipitated CD45 under these new detergent conditions. Second, it has been found that the maintenance of protein-protein interactions is much more favoured in gentle detergents such as Brij 96 (Schraven et al., 1991; Koretzky et al., 1993; Takeda et al., 1994). Consequently, the change in PTP activity may be associated with co-precipitating proteins which can upregulate CD45 activity. However, it should be noted that this second option is unlikely, as the enhancement effect is also seen in cell lysis under Triton X-100 conditions with subsequent detergent supplementation in the PTP buffers.

In conclusion, this method has widespread application for measuring and comparing specific PTP activities and for performing kinetic analysis on PTP enzymes isolated directly from cells. In particular, this assay can be used to determine the enzymatic activity of PTP's immunoprecipitated from cells which have undergone specific manipulations or from cells that are at different stages of development or activation.

CHAPTER 4

Expression and purification of recombinant cytoplasmic CD45 proteins.

Related Publication:

4.1 Introduction

In the early 1990s, despite strong interest in CD45 research, it was still clear that the structural and functional properties of the catalytic domain of CD45 were largely undefined. In response to this, a number of groups began utilizing recombinant protein technology to express and purify recombinant CD45 cytoplasmic domain for characterization (Cho et al., 1992; Itoh et al., 1992; Pacitti et al., 1994). Recombinant protein technology offers the simple advantage of high yields. Given large amounts of purified protein, complex biochemical or kinetic analyses can be more conveniently performed. In particular, production of the cytoplasmic domain of CD45 coinciding with a mutagenesis scheme would allow the careful mapping of relevant amino acid residues or relevant protein regions in CD45 that play a role in catalytic activity. Careful analysis of receptor PTPs like CD45 is particularly important since, contrary to soluble cytoplasmic PTPs, the majority of transmembrane phosphatases have two phosphatase domains located in the cytoplasmic region of the protein (reviewed in Denu et al., 1996b; Tonks and Neel, 1996). Previous mutagenesis studies on PTP's LAR and CD45, indicated that mutation of a highly conserved cysteine in domain I, but not in domain II, resulted in the loss of PTP activity, implying that the first PTP domain of CD45 and LAR, but not the second, was catalytically active (Streuli et al., 1989; Streuli et al., 1990). Given that the functional significance of two phosphatase domains remains unknown, continued efforts to explore this trait are crucial in elucidating CD45 function.

The production of recombinant cytoplasmic domain of CD45 would also allow analysis of possible interactions with other signaling molecules. Again, with a mutagenesis scheme in place, any observed interaction with

a putative protein can be further characterized with the use of mutant constructs. The use of binding assays can therefore offer insights into CD45 substrate preferences such as those indicated by comparisons between CD45 expressing and CD45 deficient cell lines. Furthermore, binding assays can be used generally in examining possible signaling pathways involving CD45. Finally, the recombinant cytoplasmic CD45 protein can be used to monitor whether post-translational modifications can alter its enzymatic capabilities. These considerations will be further discussed in proceeding chapters.

Chapter 4, however, will show work accomplished in the development of an expression and purification protocol for the production of a recombinant 6 histidine CD45 cytoplasmic domain fusion protein. The cytoplasmic domain of murine CD45 was expressed in a bacterial expression system, purified using a simple two step procedure, and was determined to be catalytically active. The protocol also provided a model expression system for the production of mutant forms of CD45. Since a long standing goal of this laboratory is to continue characterizing the regions and amino acids in the CD45 cytoplasmic domain that are absolutely required for enzymatic activity, this chapter will also highlight results pertaining to the expression and purification of four mutant forms of cytoplasmic CD45.

4.2 Results

Expression and purification of soluble recombinant CD45 cytoplasmic domain

The cytoplasmic domain of murine CD45 was expressed in *Escherichia coli.* This protein contained residues 564-1268 of murine CD45

with six histidines followed by the Factor Xa recognition sequence, IEGR, incorporated at the amino terminus of the molecule. Expression of the six histidine tag allowed the protein to be recovered from the soluble fraction by attachment to a Nickel affinity column. Elution from this column in 1 M imidazole resulted in substantially purified recombinant protein, and a 30-40 fold purification (see Table V). A second purification step involving ion-exchange chromatography separated the remaining proteins, yielded approximately a 100 fold purification and generated a single protein band of apparent molecular mass of 95 kDa after Coomassie blue staining (figure 4.1). This protein reacted with antisera raised against the cytoplasmic domain of CD45 and had protein tyrosine phosphatase activity. The purified CD45 protein was then used for kinetic studies to demonstrate the feasibility of this type of analysis using the recombinant protein. The specific activity was observed to be 81.0 µmol/min/mg and the K_m value was calculated at 230 µM using a 13 amino acid phosphopeptide derived from the carboxylterminal region of $p59^{fyn}$ as a substrate (fyn pY531). These kinetic values also translate into a turnover number (K_{cat}) of approximately 115 s^{-1}. Table VI summarizes the kinetic values of the fyn and other phosphopeptides, when PTP assays were performed in a 50 mM Imidazole-Cl, 1 mM EDTA, 0.1% β-mercaptoethanol buffer at pH 7.2. The pH optimum for this protein was also obtained indicating that the 6 histidine cytoplasmic domain of CD45 achieved peak activity at approximately pH 7.2 (data not shown). Overall, results show a purification procedure for the production of an active cytoplasmic domain of CD45 yielding approximately 2mg of substantially purified recombinant protein per litre of bacterial culture.
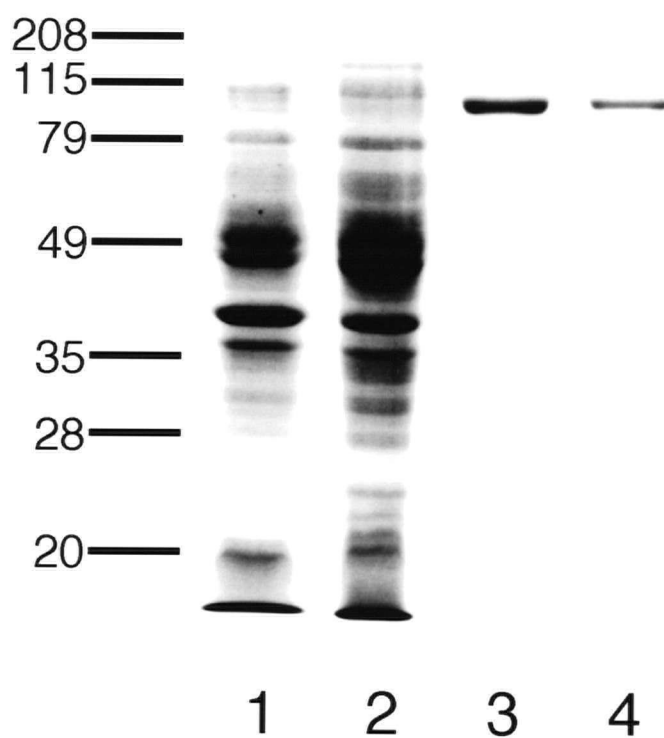
Figure 4.1: <u>SDS-PAGE analysis of the purification of recombinant cytoplasmic CD45</u>. Lane 1: whole cell lysate, lane 2: soluble cell lysate fraction, lane 3: imidazole eluent from nickel-NTA column, and lane 4: post Mono Q fractions containing purified cytoplasmic CD45. Protein samples were fractionated on a 10% SDS polyacrylamide gel and stained with Coomassie Blue.

Sample	Volume (mL)	Protein concentration (mg/mL)	Total protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min/mg)	Fold purification	Yield (%)
Soluble Cell Lysate	12	31.8	382	249	0.65	-	100
Imidazole Eluent	3.0	1.5	4.6	105	22.8	35	42
Mono Q Eluent	5.0	0.21	1.1	62.9	60.0	92	25

Table V: <u>Purification of murine recombinant cytoplasmic CD45 from 500mls of *E. coli* culture</u>. The purification procedure has been repeated several times and the values given in this table are representative values derived from a single experiment.

Substrate	Vmax (μmol/min/mg)	K_m (mM)	K_{cat} (s^{-1})
fyn pY531 (TATEPQpYQPGENL)	81.0±1.0	0.23	115±1
src pY527 (TSTEPQpYQPGENL)	81.4±4.8	0.23±0.01	115±7
src pY416 (LIEDNEpYTARQGA)	79.8±4.3	0.22±0.01	114±6

Table VI: V_{max}, K_m and K_{cat} values for recombinant cytoplasmic CD45 using phosphopeptide substrates. All assays for recombinant CD45 were performed in 50 mM Tris-Cl pH 7.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer. V_{max} and K_m values were determined from Lineweaver-Burk and Eadie-Hofstee plots of the data and expressed in units of (μmol/min/mg) and (mM), respectively.

Expression and purification of mutant forms of recombinant cytoplasmic domain of CD45

In addition to the recombinant murine CD45 cytoplasmic domain, four mutants produced by site directed mutagenesis were expressed in bacteria. A CD45 cytoplasmic domain protein with point mutation in domain II (Gln-1180 to Gly) and a protein with two point mutations in the membrane proximal region (Ser-573 and Ser-574 to a Gly and Ala, respectively) were expressed and purified, as was a CD45 cytoplasmic mutant lacking the protein sequence separating the two phosphatase domains (the spacer region, amino acids 876 to 931) and a CD45 cytoplasmic domain with a point mutation in domain I (Cys-817 to Ser) that had previously been shown to abrogate CD45 phosphatase activity (Figure 4.2). In brief, the rationale for the other point mutations were as follows: the Gln-1180 to Gly mutant was selected since the glutamine residue was seen to be highly conserved between species when looking at CD45 primary sequences; whereas both serine residues were chosen due to their situation within consensus sequences for protein kinase A phosphorylation. Approximately 2-4 mg of purified recombinant protein/litre were obtained for the point mutation proteins and approximately 0.2 mg/litre of purified protein was obtained for the spacer deletion mutant.

Phosphatase activity of CD45 cytoplasmic domain mutants

The amount of purified recombinant CD45 cytoplasmic domain protein was determined by the BCA protein assay. 10 ng of protein was taken for each mutant and the initial rates of phosphatase activity determined using saturating amounts (3.0 mM) of fyn phosphopeptide
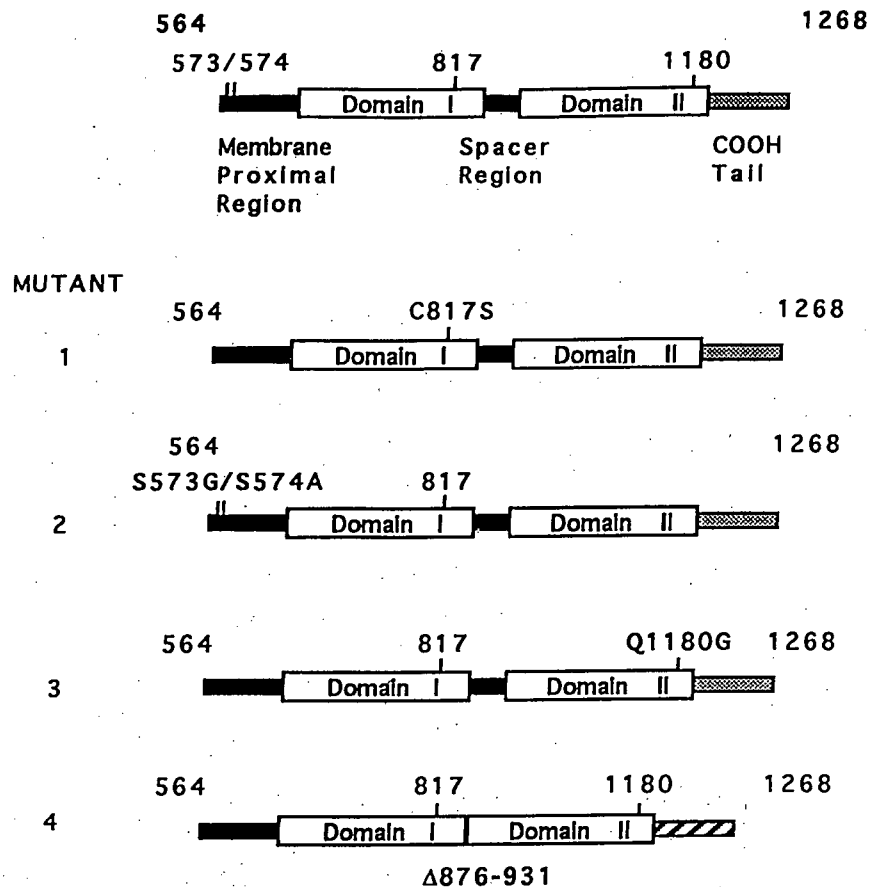
Figure 4.2: <u>Schematic representation of the murine CD45 cytoplasmic</u> <u>domain and the mutant CD45 cytoplasmic domain proteins</u>. The recombinant CD45 protein is shown at the top and is divided into 5 distinct protein domains; the membrane proximal region, phosphatase domain I, the spacer region, phosphatase domain II and carboxyl terminal tail. The amino acids are numbered according to the sequence of the mouse CD45(ABC) isoform (Thomas et al., 1987). Four mutant CD45 proteins (1-4) are shown illustrating the mutation made in each case. Mutant 1 has Cys-817 mutated to Ser (C817S); mutant 2 has Ser-573 and Ser-574 mutated to a Gly and Ala, respectively (SS573,574GA); mutant 3 has Gln-1180 changed to a Gly (Q1180G) and mutant 4 has amino acids 876 to 931 deleted (Δ876-931, spacer region deletion).

substrate. Confirmation that equivalent amounts of protein were used to compare phosphatase activities was demonstrated by running equivalent protein amounts on an SDS gel (Figure 4.3) and by densitometric scanning of the Coomassie blue stained bands (data not shown). Comparison of initial phosphatase activities is shown in Figure 4.4. These results indicate that two point mutations in the membrane proximal region did not affect phosphatase activity illustrating that not all point mutations have a detrimental affect on CD45 phosphatase activity. Second, the mutation of a highly conserved Gln-1180 to a Gly in domain II generated a CD45 protein with undetectable phosphatase activity when tested against the fyn phosphopeptide substrate. The spacer deletion mutant also had undetectable levels of activity. These mutants produced similar results to the cysteine to serine mutation in domain I which, as previously shown, also had undetectable levels of activity.

4.3 Discussion

This purification protocol is a simple two step procedure which generates a single protein band and as such offers a significant improvement over existing methods to purify recombinant CD45 from *E. coli*. Such methods have used multiple steps and have not generated a single protein species (Cho et al., 1992; Itoh et al., 1992). The purified recombinant cytoplasmic domain of CD45 was analysed and its kinetic characteristics were assessed. A V_{max} of 81 μmol/min/mg and a K_m of 230 μM are comparable to previously published values, including those of purified full length protein where a K_m of 149 μM was derived using a similar fyn pY531 peptide (FTATEPQpYQPGENL) as substrate (Pacitti et al., 1994). Furthermore, when the pH optimum of the recombinant protein
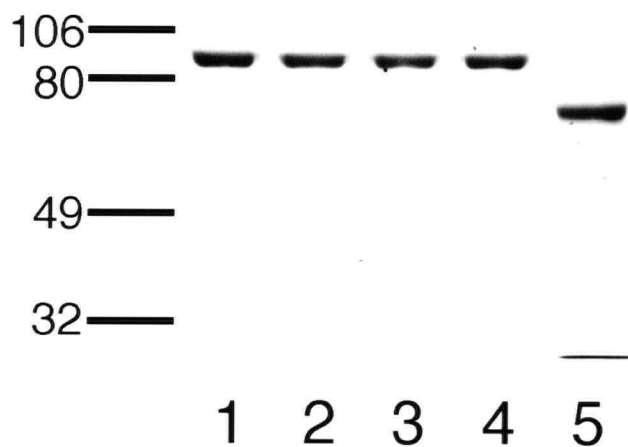
Figure 4.3: <u>10% SDS-polyacrylamide gel of equivalent protein amounts for</u> <u>each recombinant CD45 protein</u>. Bacterially expressed proteins were purified as described and assayed for protein amount using BCA assay. Equivalent protein amounts were loaded and subjected to SDS-PAGE and then stained with Coomassie blue. Lane 1: recombinant cytoplasmic CD45 protein. Lanes 2, 3, 4, and 5 show mutant CD45 cytoplasmic domain proteins, 1-4, respectively (refer to Figure 4.2): 1 (C817S), 2 (SS573,574GA), 3 (Q1180G) and mutant protein 4 (Δ876-931, spacer region deletion).
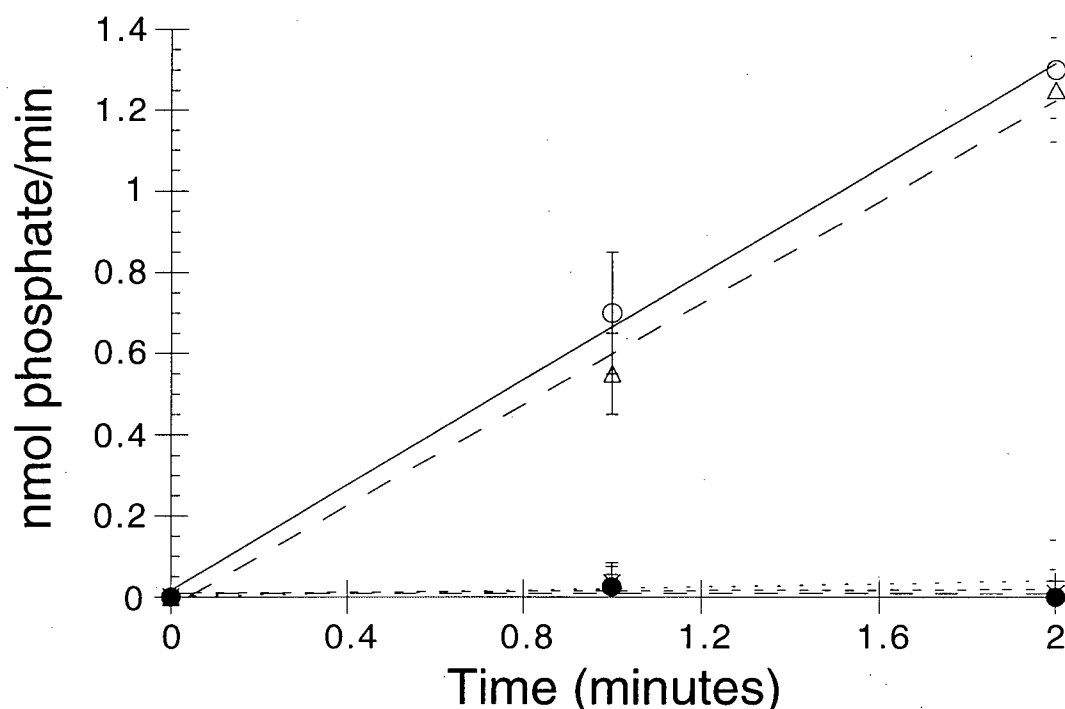
Figure 4.4: <u>Comparison of initial rates of PTP activity for wild type murine</u>

<u>cytoplasmic CD45 and the four mutant CD45 cytoplasmic domain proteins.</u>
The symbols represent the following CD45 cytoplasmic proteins: (o) wild
type, (x) mutant 1: C817S, (Δ) mutant 2: SS573,574GA, (●) mutant 3:

Q1180G, and (+) mutant 4: Δ876-931, spacer region deletion. Reaction rates

of 10 ng of recombinant protein were assayed using fyn peptide at a final

concentration of 3.0 mM in a 50 mM Imidazole-Cl pH 7.2, 1.0 mM EDTA,

0.1% β-mercaptoethanol PTP buffer. Time points were taken at 0, 1, and 2

min and nanomoles of inorganic phosphate (PO_4) released were calculated

using the malachite green phosphate assay (Ng et al., 1995).

was analysed, it was found to be most active at pH 7.2, a value consistent with a pH optimum previously published for wild type CD45 purified from cells (Tonks et al., 1990; Itoh et al., 1992). In general, the characteristics defined indicate that the recombinant cytoplasmic domain of CD45 shows some similarities to wild type CD45 isolated from cells and can therefore be used as a suitable alternative for *in vitro* studies. However, V_{max} values derived from endogenously purified protein was calculated to be 4.8 μmol/min/mg which was ten fold less than obtained for the recombinant protein. This highlights the possibility that despite the usefulness of using recombinant protein, there are still differences between the two circumstances. Most obvious, these include the absence of the extracellular and transmembrane regions of CD45 which possibly play a role in regulating PTP activity, either due to alterations in structure or through anchoring or localization effects created by possible ligand binding or membrane association. Furthermore, it should be noted that the experiments done under *in vitro* systems may never fully mimic physiological conditions.

However, an example of how this recombinant protein can be used is demonstrated with the acquisition of data to ascertain CD45's ability to dephosphorylate several phosphopeptides containing sequences derived from the carboxyltails or autophosphorylation sites of src-related kinases. These results (Table VI) indicate that there was no substrate preference observed between these phosphopeptides. These trends are similar to those observed with immunoprecipitated CD45 except that the recombinant cytoplasmic CD45 did not show any preference for the autophosphorylation site peptide (src pY416). The full analyses of these data, complemented with additional kinetic studies on CD45 and another

two domain PTP, RPTPα, will be presented and discussed in greater detail in Chapter 6.

A further demonstration on the usefulness of this recombinant system is seen with the expression of mutant forms of CD45 in bacteria (Figure 4.2). These recombinant proteins were purified and tested for phosphatase activity. Two point mutations in the membrane proximal region had no deleterious effect on activity. These two serine residues are located in a stretch of amino acids having consensus to sites of protein kinase A phosphorylation (Kennelly and Krebs, 1991). Consequently, the original intent for producing this mutant was to evaluate whether these phosphorylation sites are important for CD45 function. However, it is important to stress that our PTP activity results do not address this possibility of cellular regulation as neither phosphorylation states of wild type cytoplasmic CD45 nor mutant CD45 proteins were monitored. This mutant, therefore, needs to be evaluated in an *in vivo* setting to be fully appreciated.

The Cys-817 to Ser point mutation was observed to have no catalytic activity, as previously demonstrated (Streuli et al., 1989; Streuli et al., 1990; Johnson et al., 1992; Desai et al., 1994). It was also shown that deletion of the region between the two phosphatase domains and a point mutation in domain II both resulted in the total loss of enzymatic activity. These results indicate that the region separating the two phosphatase domains is required to generate an active enzyme. Whether the loss of activity was due to the spacer region containing important residues necessary for catalytic activity, or was due to a general structural perturbation remains to be resolved. However, the latter possibility is

deemed more likely due to the nature of the large deletion. More suprisingly, it was found that a single amino acid change in domain II abolished the phosphatase activity of CD45, despite the fact that the catalytic cysteine residue is thought to be located in domain I (Streuli et al., 1989; Streuli et al., 1990; Johnson et al., 1992; Desai et al., 1994). This would imply that the phosphatase activity of CD45 is dependant on elements from both the PTP domain I and domain II and indicate a possible interplay between these two domains in the formation of the active site of CD45. Evidence for this scenario has been recently generated with data demonstrating an interaction between PTP domain I and domain II of CD45 (Felberg and Johnson, 1998). Since the initial development of the purification protocol, the procedure has been further optimized (Glenn Dibble, and Jackie Felberg, pers. comm.) by small changes in the constituents of the buffers and a general emphasis in performing all steps quickly and at $4^\circ C$ or below (see Materials and Methods). In fact, the most recent purification conditions have been outlined by Jackie Felberg (Felberg and Johnson, 1998) and include the addition of high salt washes (buffer 1 plus 0.5 M and 1.0 M NaCl) after the loading of the nickel column, as well as changes in the FPLC gradient buffers (now at pH 7.2). Curiously, under these newer conditions, the Gln-1180 to Gly CD45 protein resulted in an active protein, which directly contradicts the results presented in this chapter (Felberg and Johnson, 1998) and Jackie Felberg, unpublished data). It is possible that the mutation of the residue glutamine to glycine, may result in a slight alteration of the enzyme's tertiary structure such that it is more sensitive to the subtle environmental changes caused by the modification of the procedures. Overall, this provides a cautionary note regarding the interpretation of

data obtained from *in vitro* conditions using recombinant proteins produced in *E. coli.* Whilst it is obvious that the use of such technologies provides an extremely powerful tool, it is also imperative to emphasize the care that is required when extrapolating *in vitro* results to physiological situations.

CHAPTER 5

Use of recombinant cytoplasmic CD45 to investigate potential protein-protein interactions. Demonstration of an *in vitro* association between cytoplasmic CD45 and p56^{lck}

Related Publications:

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

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

5.1 Introduction

The leukocyte specific transmembrane protein CD45 is an important representative of the phosphatase enzyme family and is also an integral part of the signaling process responsible for the effective activation of immune cells. As such, there are several important biological and biochemical questions that can be addressed using the recombinant cytoplasmic domain of CD45. CD45 has been shown to be able to dephosphorylate several protein substrates and phosphorylated peptides *in vitro* (Cho et al., 1993; Mustelin et al., 1992; Mustelin and Altman, 1990; Tonks et al., 1990) , yet only p56^{lck}, p59^{fyn}, and p53/p56^{lyn} have been clearly identified as potential *in vivo* substrates (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Hurley et al., 1993; Katagiri et al., 1995; Yanaga et al., 1996). In T cells, both p56^{lck} and, to a lesser extent, p59^{fyn} were found to be hyperphosphorylated in the absence of CD45 whereas the tyrosine phosphorylation state of p60^{src} was reported to be unaffected (Hurley et al., 1993). How the substrate specificity of CD45 is achieved *in vivo* is not known, although substrate accessibility may be a factor. There is some evidence indicating that p56^{lck} may be associated with CD45 in T cells (Schraven et al., 1991; Guttinger et al., 1992; Koretzky et al., 1993). p56^{lck} and proteins of approximately 30 kDa can be co-precipitated with CD45 using a mild detergent to lyse the cells (Schraven et al., 1991; Schraven et al., 1994; Takeda et al., 1994). CD4 and CD8 are strongly associated with p56^{lck} (reviewed in Chan et al., 1994) and have been reported to associate with CD45 (Dianzani et al., 1990; Mittler et al., 1991), yet the interaction between p56^{lck} and CD45 was shown to occur independantly of the expression of CD4, CD8 and the TCR (Ross et al.,

1994). Hence in T cells, CD45 may associate directly with p56^{lck} or indirectly via the 30 kDa proteins.

An interaction between p56^{lck} and tyrosine phosphorylated CD45 has also been described in phenylarsine oxide treated T cells (Autero et al., 1994) Here, tyrosine phosphorylation of CD45 *in vitro* by p50^{csk} resulted in the binding of p56^{lck} to CD45. This binding was thought to be mediated by the SH2 domain of p56^{lck} as the interaction was prevented by the addition of excess recombinant p56^{lck} SH2 domain (Autero et al., 1994).

To further investigate the potential interaction of p56^{lck} and CD45, we chose to analyze this association using purified, recombinant forms of p56^{lck} and the cytoplasmic domain of CD45. An *in vitro* interaction was observed between recombinant p56^{lck} and the cytoplasmic domain of CD45 but not with the cytoplasmic domain of another transmembrane two domain phosphatase, RPTPα (LRP). Under equilibrium binding conditions, this binding was found to be saturable and occurred at a stoichiometry of approximately 1:1. Localization of the site of interaction in p56^{lck} indicated that 2 distinct regions in the amino terminal half of the molecule could bind to CD45. Glutathione-S-transferase (GST) fusion proteins of both the unique amino terminal region of p56^{lck} and the SH2 domain of p56^{lck} specifically bound to the cytoplasmic domain of CD45. Interestingly, the interaction involving the SH2 domain of p56^{lck} did not require CD45 to be tyrosine phosphorylated. Further characterization of the interaction demonstrated that the cytoplasmic domain of CD45 can also interact with the kinase domain of p56^{lck}. Interestingly, experiments manipulating the phosphorylation state of p56^{lck} indicate that the interaction is strongly regulated by the phosphorylation state of p56^{lck}.

5.2 Results

Demonstration of a specific and direct interaction between the cytoplasmic domain of CD45 and p56^{lck}

Recombinant, active CD45 cytoplasmic domain protein generated with an amino terminal affinity tag of six histidine residues was produced and purified from bacteria as described previously (Chapter 4 and Ng et al., 1995) Purified, active p56^{lck} was obtained from the baculovirus expression system (Watts et al., 1992).   The recombinant CD45 cytoplasmic domain protein was covalently coupled to cyanogen bromide-activated Sepharose CL-4B beads and approximately 2.5 µg of the immobilized protein was incubated with approximately 200 ng of recombinant p56^{lck} at concentrations of 700 nM and 90 nM respectively. Binding assays were performed using a buffer that mimicked physiological conditions (20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol) Furthermore, under the conditions used (the effects of the immobilization procedure, the presence of phosphatase inhibitors, the absence of ATP and divalent cations, and with CD45 covalently coupled to beads) neither CD45 nor p56^{lck} was enzymatically active. After a two hour incubation at 4°C, the CD45 conjugated beads were washed three times in RIPA buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 0.025% β-mercaptoethanol, plus protease and PTP inhibitors), and any remaining proteins were subjected to SDS-PAGE, transferred to PVDF membrane and probed by Western blot analysis for the presence of p56^{lck}. The results are shown in Figure 5.1 and illustrate that p56^{lck} bound to CD45 coupled beads (lane 2), but did not bind to an equivalent amount of Sepharose beads alone (lane 1) or to equivalent amounts of Sepharose
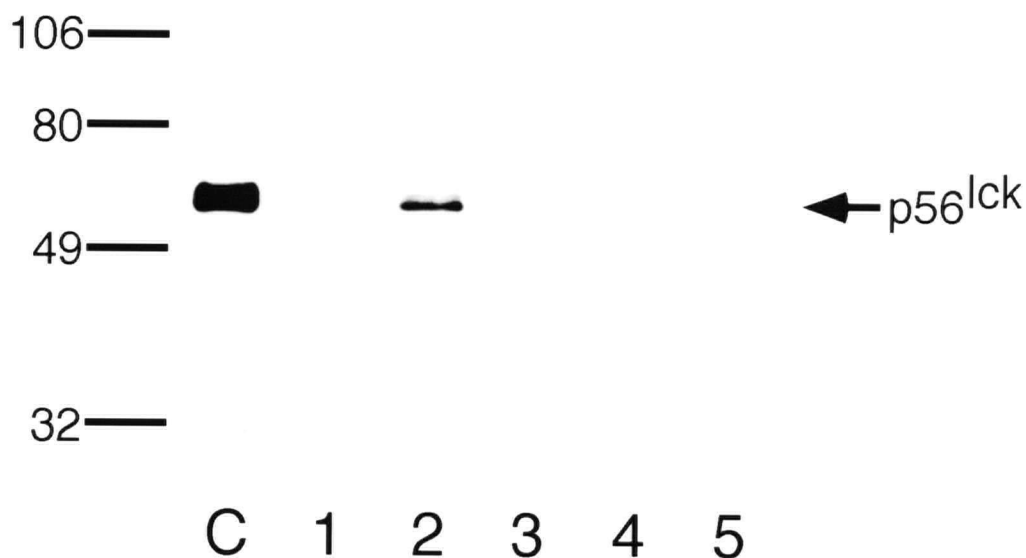
Figure 5.1: Binding of recombinant p56^{lck} to the cytoplasmic domain of CD45. Approximately 200 ng of p56^{lck} was incubated with 2.5 µg of various proteins coupled to beads at 4°C for 2 hours in 40 ml of binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, plus protease and PTP inhibitors). After washing in RIPA buffer, the entire sample was subjected to SDS-PAGE, transferred to PVDF membrane and bound p56^{lck} was detected by immunoblot analysis using anti-p56^{lck} antisera. Lane C is a control containing 25 ng of recombinant p56^{lck}. Lanes 1-5 show the amount of recombinant p56^{lck} remaining bound to 1: Sepharose CL-4B beads alone; 2: recombinant CD45 cytoplasmic domain conjugated to Sepharose beads; 3: recombinant RPTPa cytoplasmic domain GST fusion protein coupled to glutathione Sepharose beads; 4: I3/2 antibody conjugated to Sepharose beads; 5: Ly 5.2 antibody conjugated to Sepharose beads.

beads covalently coupled to two irrelevant antibody proteins (lanes 4 & 5). Furthermore, p56^{lck} did not associate with a recombinant GST fusion protein of the cytoplasmic domain of a related protein tyrosine phosphatase, RPTPα, which had been immobilized on glutathione Sepharose beads (Figure 5.1, lane 3). It was verified by Coomassie blue staining of recombinant CD45 and RPTPα proteins that similar amounts of protein had been taken and coupled to beads (data not shown). From Figure 5.1 it was shown that only a small percentage of total p56^{lck} (approximately 3-4%) bound to CD45. This may be due in part to the use of stringent wash conditions (RIPA buffer) employed to demonstrate the specificity of the interaction.

Recombinant cytoplasmic CD45 does not interact with GST control protein and recombinant p56^{lck} does not interact with immobilized 6HIS-cenA protein

To further establish that the interaction between the cytoplasmic domain of CD45 and p56^{lck} was specific and was not due to non specific interactions with the immobilized CD45 protein, CD45 coupled beads were incubated with 2.0 µg of another recombinant protein, glutathione S-transferase (GST). Incubation of a 20 fold higher molar concentration of recombinant GST protein, but with otherwise similar binding and washing conditions, produced no detectable binding of recombinant GST protein to CD45 coupled beads, whereas, as expected, binding was observed to control glutathione coupled beads (data not shown). In addition, it was shown that p56^{lck} did not bind to another immobilized recombinant 6 histidine fusion protein (endoglucanase, CenA) (Damude et al., 1995)

excluding the possibility that p56^{lck} may be binding to recombinant CD45 through this region (data not shown).

Association between p56^{lck} and the cytoplasmic domain of CD45 exhibits a 1:1 stoichiometry

To determine the stoichiometry of this interaction, p56^{lck} was autophosphorylated in the presence of [γ^{32}P]-ATP, separated from free ATP and its specific activity determined to be 50 cpm/ng. It was then used to quantitate the amount of p56^{lck} that could specifically bind to the cytoplasmic domain of CD45 under equilibrium binding conditions. As seen in Figure 5.2, the specific binding of p56^{lck} to the cytoplasmic domain of CD45 was saturable. By analyzing double reciprocal plots, it was determined that under saturating binding conditions, approximately 1 mole of p56^{lck} bound to 1 mole of the cytoplasmic domain of CD45. The average molar ratio determined from 7 experiments was 1.1 $\pm$ 0.2. Thus under saturation binding conditions, there is a 1:1 interaction between p56^{lck} and the cytoplasmic domain of CD45. Attempts were made to determine the affinity of this interaction, but Scatchard analysis generated a non linear plot indicating a complex interaction from which the affinity constant could not be readily determined.

Characterization of discrete domains within p56^{lck} that can mediate the interaction with CD45

To further demonstrate the specificity of this association, the site of interaction between these two proteins was localized. It was observed that when antisera raised against amino acid residues 34-150 of p56^{lck} was

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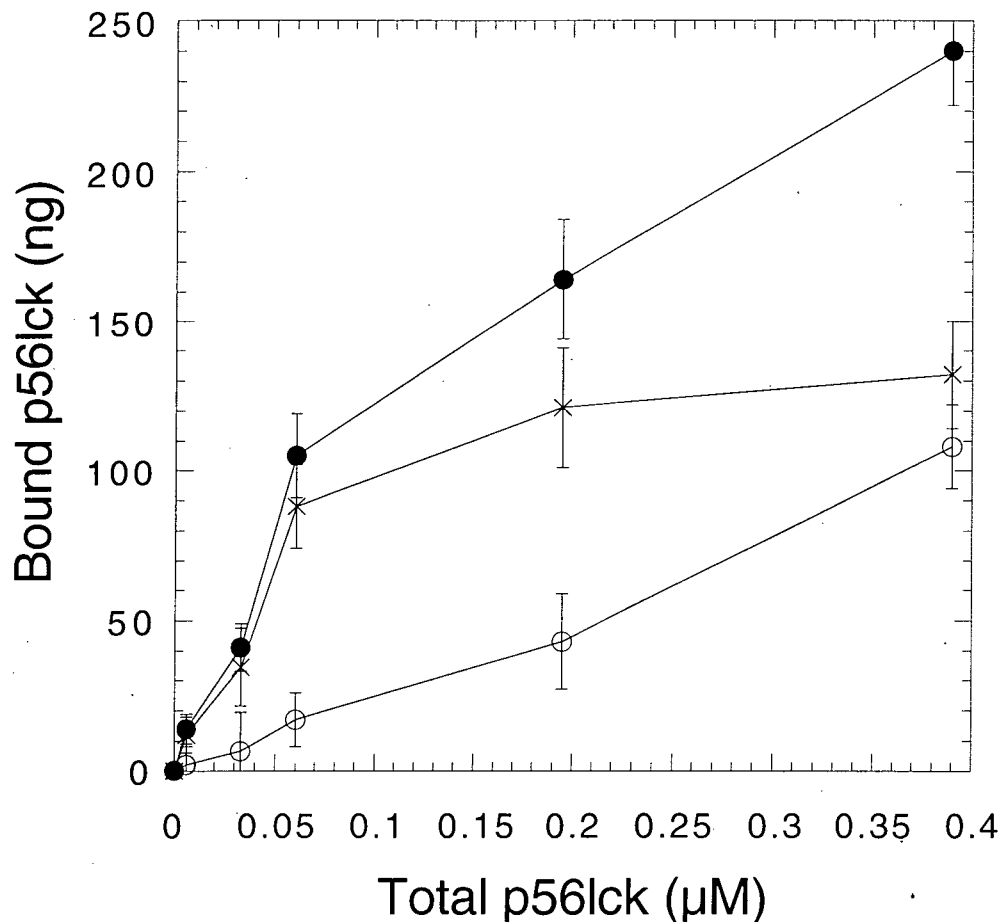
Figure 5.2: <u>Graph showing amount of radioactively labeled p56^{lck} bound to immobilized cytoplasmic domain of CD45 at equilibrium.</u> Graph of total amount of radioactive p56^{lck} (ng) bound to 300 ng of immobilised cytoplasmic domain of CD45 after incubation in increasing concentrations (nanomolar) of radioactively labeled p56^{lck} ($\bullet$) , (o) is non-specifically bound radioactive p56^{lck} (nanomolar) and (X) is the amount of radioactive p56^{lck} that specifically bound. Specifically bound radioactive p56^{lck} was calculated from [(total radioactivity added - amount free) - non-specifically bound radioactivity]. Non specific binding was ascertained by remaining radioactivity after competition with excess cold p56^{lck} protein.

added together with recombinant p56^{lck}, significant inhibition of the association occurred (data not shown). To confirm whether this region of p56^{lck} could mediate the binding to cytoplasmic CD45, approximately 200 ng of a purified TrpE fusion protein containing residues 34-150 of p56^{lck} was incubated with immobilized recombinant CD45 cytoplasmic domain. This TrpE-p56^{lck} fusion protein has a predicted molecular mass of 51 kDa and specifically bound to the recombinant cytoplasmic domain of CD45 immobilized on Sepharose beads (Figure 5.3). The TrpE-p56^{lck} fusion protein contains part of the unique amino terminal region of p56^{lck}, the entire SH3 domain of p56^{lck} and part of the SH2 domain of p56^{lck} (see Figure 5.4). To further determine which specific region of p56^{lck} was mediating this interaction, immobilized CD45 cytoplasmic domain was incubated with 2.0 µg of purified GST fusion proteins containing the unique amino terminal region of p56^{lck}, the SH3 domain of p56^{lck} or the SH2 domain of p56^{lck} (see schematic in Figure 5.4). From the Western blot probed with anti-GST antisera in Figure 5.5A, it can be seen that GST fusion proteins containing the unique amino terminal region and SH2 domain of p56^{lck}, but not the SH3 domain of p56^{lck} or GST alone, specifically bound to the immobilized cytoplasmic domain of CD45. The GST-p56^{lck} unique amino terminal region and SH2 fusion proteins did not associate with an immobilized irrelevant protein, an anti-CD45 monoclonal antibody. Since a similar detection system was employed to identify both the unique amino terminal and SH2-GST fusion proteins, it would appear that a greater amount of the SH2-GST fusion protein bound to recombinant CD45. It was verified by Coomassie blue staining that similar amounts of recombinant GST fusion proteins were incubated with

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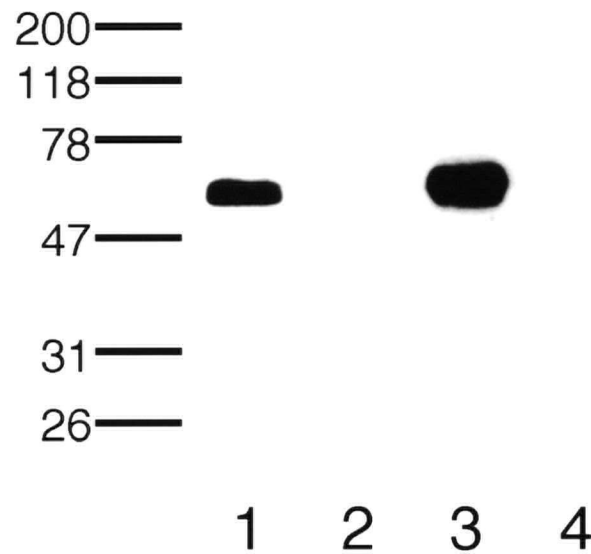
Figure 5.3: <u>Binding of the TrpE- p56^{lck} fusion protein to the cytoplasmic domain of CD45</u>. Approximately 200 ng of p56^{lck} (lanes 1 and 2) or 200 ng of TrpE-p56^{lck} fusion protein containing amino acids 34-150 of p56^{lck} (lanes 3 and 4) were incubated with immobilized recombinant CD45 cytoplasmic domain protein (lanes 1 and 3) or with an irrelevant protein (monoclonal antibody) coupled to beads (lanes 2 and 4). Recombinant p56^{lck} and TrpE-p56^{lck} fusion proteins remaining bound to the immobilized proteins were detected by immunoblot analysis using p56^{lck} antisera specific for the amino terminal region of p56^{lck}.

CD45
Binding

1　67　122　　234
| N |SH3| SH2 | kinase domain |　+

34　　　150
‎SH3‎
trpE　　lck
+

-8　　76
‎N‎
GST　lck
+

67　122
SH3
GST　lck
-

122　　234
SH2
GST　　lck
+

612　　722
-SH2
GST　p85-C
-

366　　473
SH2
GST　SHC
-

GST
-
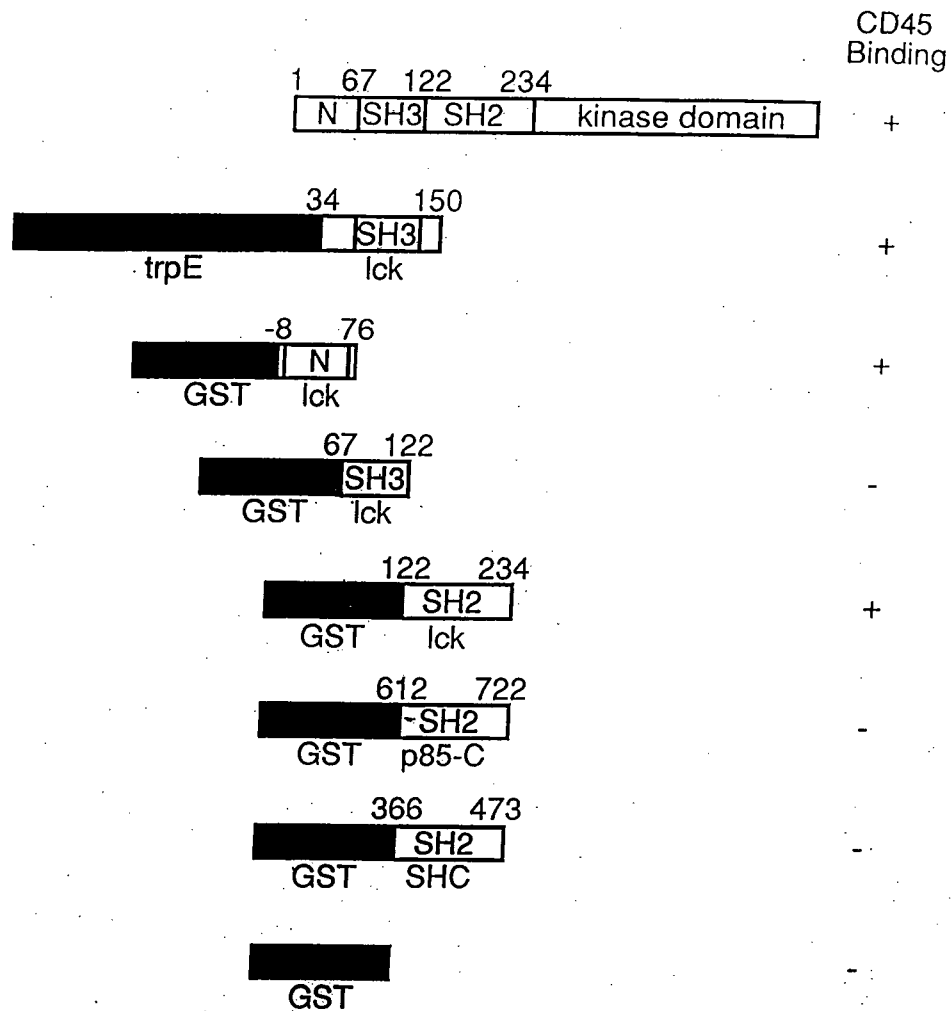
Figure 5.4: <u>Schematic representation of the recombinant proteins used in this study and their ability to associate with the cytoplasmic region of CD45.</u> The numbers above each construct indicate the first and last amino acid residues in murine p56^{lck} that were expressed in each of these constructs. p85-C is the C terminal SH2 domain of the bovine p85α subunit of PI3 kinase the SHC SH2 domain is from human SHC (see Materials and Methods).

Figure 5.5: Binding of recombinant GST fusion proteins to the cytoplasmic domain of CD45. **A**. Western blot analysis of GST fusion proteins remaining bound to the immobilized cytoplasmic domain of CD45 (odd numbered lanes) or to the control I3/2 antibody (even numbered lanes). GST fusion proteins were detected using GST antisera. 2.0 μg of the GST fusion proteins were added to 2.5 μg of immobilised CD45 cytoplasmic domain and incubated for 2 hours at 4°C in 40 ml binding buffer. The beads were washed 3 times in RIPA buffer and subjected to SDS PAGE. Lanes 1 and 2: GST protein alone; lanes 3 and 4: GST p56^{lck} unique amino terminal region; lanes 5 and 6: GST p56^{lck} SH3; lanes 7 and 8: GST p56^{lck} SH2. **B**. SDS-PAGE analysis of the purity of GST fusion proteins used in the binding assay, stained with Coomassie blue. Lane 1: GST protein alone; lane 2: GST p56^{lck} unique amino terminal region; lane 3: GST p56^{lck} SH3; and lane 4: GST p56^{lck} SH2.

recombinant CD45 cytoplasmic domain coupled Sepharose beads (Figure 5.5B). Thus, at least two distinct regions of p56^{lck} can bind to the cytoplasmic domain of CD45.

Competition assays performed using recombinant p56^{lck} proteins and recombinant cytoplasmic CD45

Competition binding assays were performed to determine the role of these two regions in the binding of intact p56^{lck} to CD45. Both the unique amino-terminal region and the SH2-GST fusion protein could compete for the binding of p56^{lck} to CD45 (Figure 5.6). The SH2 domain was approximately 5-fold more efficient at competing with p56^{lck} for binding to CD45 than the unique amino-terminal region. This indicates that the SH2 domain of p56^{lck} binds with higher affinity and supports the earlier observation that more SH2 domain of p56^{lck} bound to CD45 than the unique amino-terminal region. Interestingly, when the two domains were used together, they acted synergistically to compete with p56^{lck} for binding to CD45, but were still not as efficient as the intact p56^{lck}.

Interaction between SH2 domain of p56^{lck} and CD45 is specific

To further confirm the specificity of the interaction with the SH2 domain of p56^{lck}, two other GST-SH2 domain fusion proteins were incubated with the immobilized recombinant CD45 cytoplasmic domain. It was observed that neither the carboxyl terminal SH2 domain from the p85α subunit of PI3 kinase nor the SH2 domain of SHC bound to recombinant cytoplasmic CD45 protein (Figure 5.7).
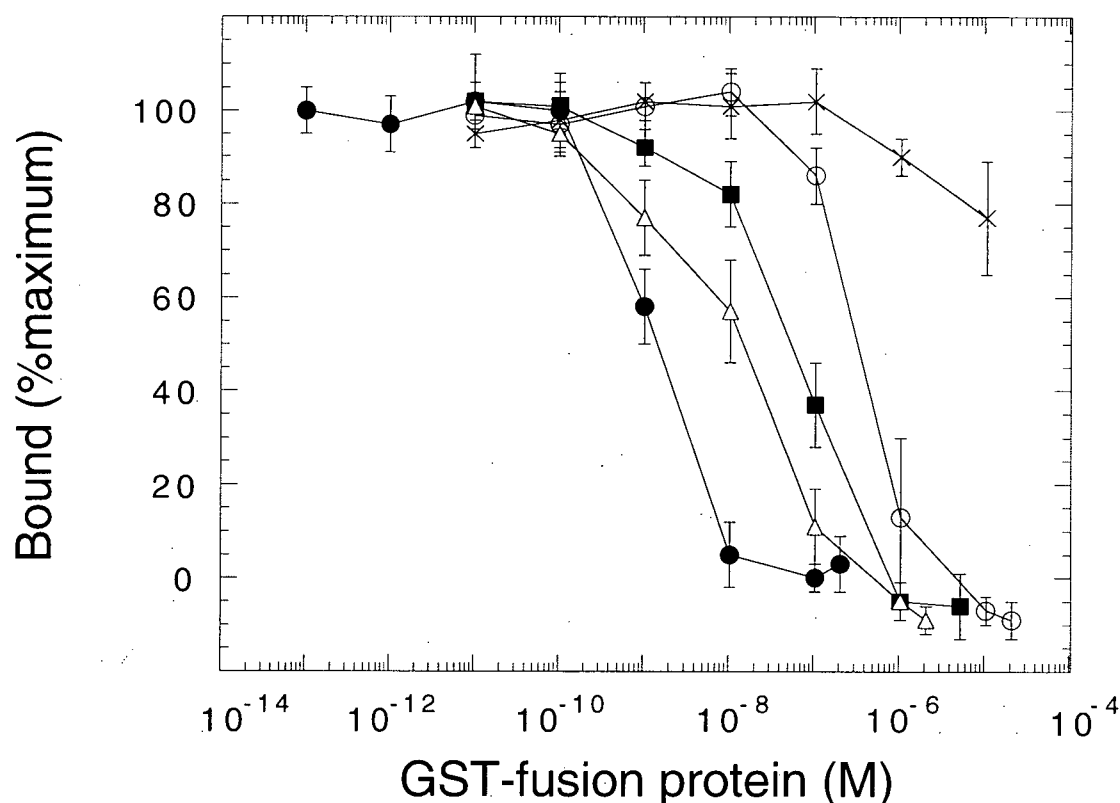
Figure 5.6: <u>Using GST fusion proteins to compete for the binding of p56^{lck}</u>

<u>to the cytoplasmic domain of CD45.</u> 300 ng of immobilized recombinant

cytoplasmic domain of CD45 was mixed with ^{32}P-labeled p56^{lck} for 8 hr,

washed and then incubated with various concentrations of competing

GST fusion protein (●, GST-p56^{lck}; O, GST-unique amino terminal

region; X, GST-SH3 domain; ■ , GST-SH2 domain; Δ, GST unique amino

terminal region and GST-SH2 domain) for 12 hr. The immobilized CD45

was centrifuged, the supernatent removed, and the remaining ^{32}P-labeled

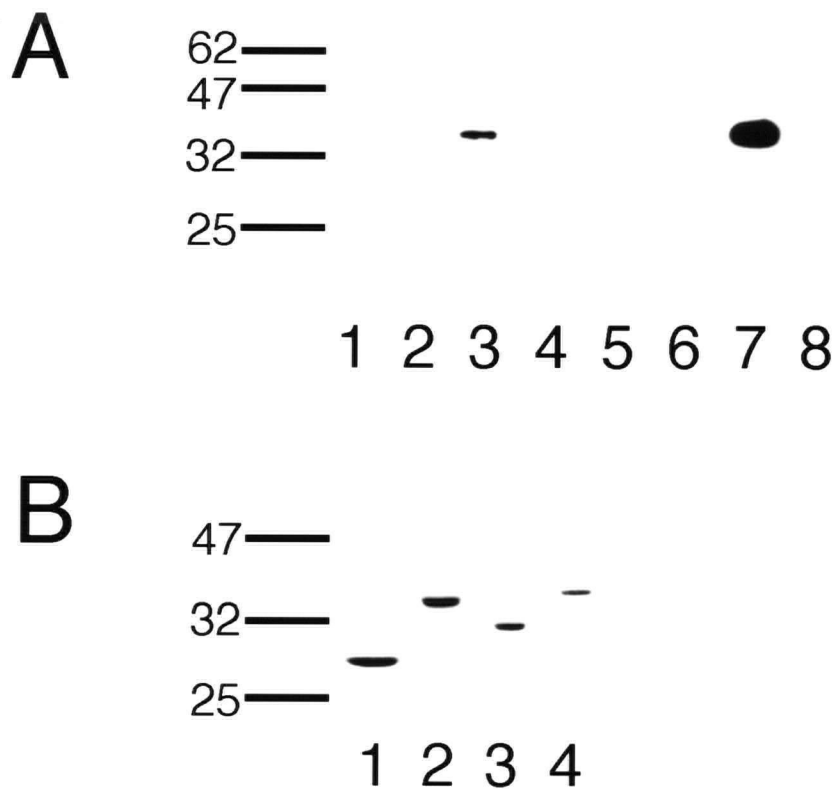p56^{lck} bound to CD45 determined.

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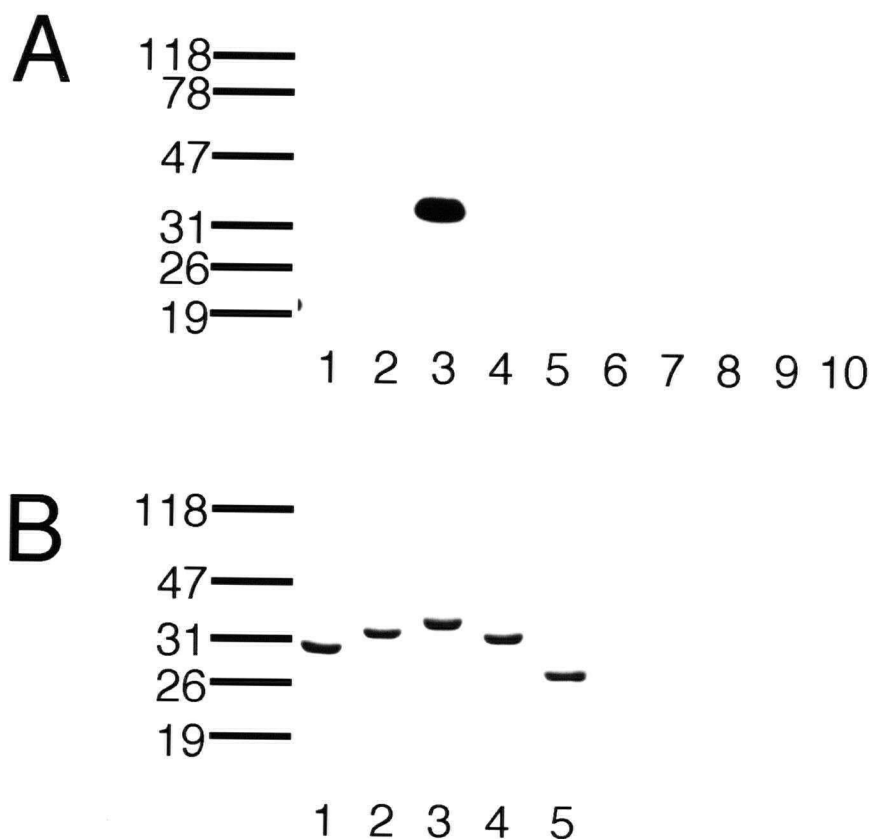
Figure 5.7: <u>Binding of recombinant GST SH2 domain fusion proteins to the cytoplasmic domain of CD45.</u> **A**. Western blot analysis of GST fusion proteins remaining bound to the immobilized cytoplasmic domain of CD45 (odd numbered lanes) or to the control I3/2 antibody (even numbered lanes). GST fusion proteins were detected using GST antisera. 2.0 µg of the GST fusion proteins were added to 2.5 µg of immobilised CD45 cytoplasmic domain and incubated for 2 hours at 4°C in 40 µl binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, plus protease and PTP inhibitors). The beads were washed 3 times in RIPA buffer and subjected to SDS PAGE. Lanes 1 and 2: GST p56^{lck} SH3; lanes 3 and 4: GST p56^{lck} SH2; lanes 5 and 6: GST p85α SH2; lanes 7 and 8: GST SHC SH2; lanes 9 and 10: GST protein alone. **B**. SDS-PAGE analysis of GST fusion proteins used in the binding assay and stained with Coomassie blue. Lane 1: GST p56^{lck} SH3; lane 2: GST p56^{lck} SH2; lane 3: GST p85α SH2; lane 4: GST SHC SH2; and lane 5: GST protein alone.

Interaction between SH2 domain of p56^{lck} and CD45 is specific and independant of tyrosine phosphorylation

Protein - protein interactions involving SH2 domains have previously been shown to be mediated by the binding of the SH2 domain to a region of the protein containing a phosphorylated tyrosine residue (reviewed in Cooper and Howell, 1993). In this situation, the possibility that the SH2 domain of p56^{lck} was binding to a phosphorylated tyrosine residue in CD45 was considered unlikely for two reasons. First, recombinant CD45 had been generated and purified from *E. coli*, which is not known to contain any tyrosine kinase activity and second, no tyrosine kinase or ATP was present in the incubation of recombinant cytoplasmic CD45 with the SH2-GST fusion proteins, which themselves do not possess intrinsic tyrosine kinase activity. However, in order to evaluate this possibility experimentally, 2.5 µg of recombinant CD45 was incubated with approximately 200 ng of p56^{lck} or 2.0 µg of GST-p56^{lck} SH2 domain fusion protein in the binding buffer for 2 hours at 4°C. These proteins and aliquots of purified proteins that had not been incubated were separated by SDS PAGE and then either transferred to PVDF membrane and Western blotted with 4G10, a monoclonal antibody specific for phosphotyrosine residues (Figure 5.8A) or stained with Coomassie blue (Figure 5.8B). As can be seen in Figure 5.8A (lanes 1 and 2) approximately 200 ng of p56^{lck} gave a strong signal with the anti-phosphotyrosine antibody. Even 20 ng of p56^{lck} (lanes 6 and 7) provided a strong signal indicating that recombinant p56^{lck}, produced using a baculovirus expression system, was tyrosine phosphorylated. However, even with 100 times the amount required to give a signal for p56^{lck}, no bands at 95 kDa representing the
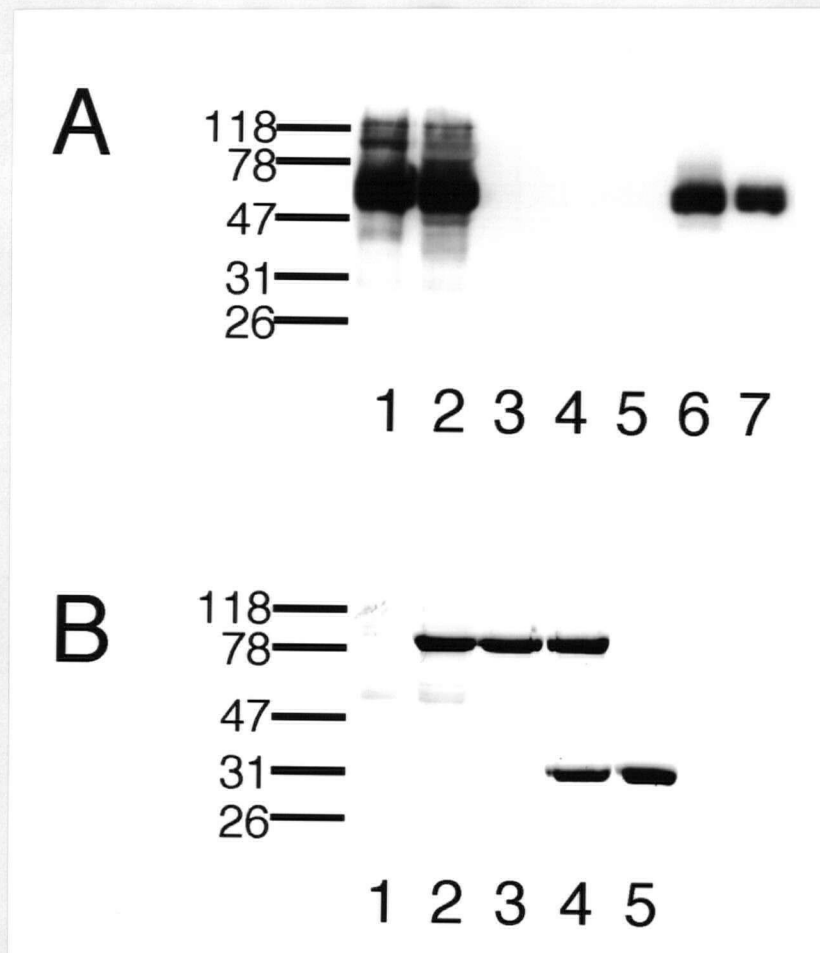
Figure 5.8: <u>Tyrosine phosphorylation state of recombinant p56^{lck} and CD45 cytoplasmic domain proteins</u>. **A.** Western blot of soluble recombinant proteins probed with the 4G10, an anti-phosphotyrosine monoclonal antibody. Proteins were incubated in 40 µl binding buffer (20 mM Tris pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, containing protease and phosphatase inhibitors) for 2 hours at 4°C. Lane 1: approximately 200 ng p56^{lck}; lane 2: approximately 200 ng p56^{lck} and 2.5 µg cytoplasmic CD45; lane 3: 2.5 µg cytoplasmic CD45; lane 4: 2.0 µg GST p56^{lck} SH2 domain and 2.5 µg cytoplasmic CD45; lane 5: 2.0 µg GST p56^{lck} SH2 domain; lane 6: 20 ng p56^{lck} and 2.5 µg cytoplasmic CD45; and lane 7: 20 ng p56^{lck}. **B** SDS-PAGE analysis of recombinant proteins detected by Coomassie blue staining. Lanes 1 - 5 are identical to those in panel A.

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cytoplasmic domain of CD45 (lanes 3, 4 and 6) were observed using the anti-phosphotyrosine antibody. This data demonstrates that neither CD45 nor the GST-p56^{lck} SH2 domain proteins were detectably tyrosine phosphorylated and that the tyrosine phosphorylation state of p56^{lck} and CD45 did not change during the course of the binding assay, indicating that under these conditions both p56^{lck} and CD45 were not catalytically active. Thus, the SH2 domain of p56^{lck} interacts with the cytoplasmic domain of CD45 by a mechanism that does not depend on the tyrosine phosphorylation of CD45.

Optimal conditions for the production of GST-p56^{lck} and GST-p56^{lck} kinase domain

GST fusion proteins of full length p56^{lck} and the kinase domain of p56^{lck} were also created and produced so that the recombinant p56^{lck} protein could be immobilized onto glutathione coated Sepharose beads. This would permit the initiation of binding studies using soluble and therefore, conceivably, active preparations of cytoplasmic CD45. However, whilst this option was being pursued, it was noticed that the yields obtained for these soluble protein constructs were significantly less than those for the GST-SH2 domain fusion proteins. In general, yields for GST-SH2 and GST-SH3 protein were in the milligram range per 500 ml bacterial culture range, whereas a similar protocol would produced less than 10 μg of GST-p56^{lck} and GST-p56^{lck} kinase domain protein per 500 ml bacterial culture. Consequently, several variations on the purification procedure were tested, including the use of sonication and the addition of the detergent N-laurylsarcosine which was previously described to increase soluble protein yields of enzymes such as phosphatases which

were difficult to obtain (Frangioni and Neel, 1993). Figure 5.9 displays a Western blot analysis of whole, insoluble and soluble lysate fractions of IPTG induced *E. coli* cultures, containing pGEX-2T plasmid with murine p56^{lck} cDNA, that were obtained using the different lysis conditions. It is clear that when the lysis procedure includes both the addition of 1.0% N-laurylsarcosine and the sonication step, the resultant soluble yield increased significantly. As a result of this observation, all subsequent GST full length p56^{lck}, and p56^{lck} kinase domain purifications were performed incorporating these changes, and generally enabled the purification of approximately 100 μg of purified protein per 500 ml of bacterial culture. Coomassie blue staining of the purified GST-p56^{lck} and GST-p56^{lck} kinase domain is shown in Figure 5.10.

Binding of recombinant full length p56^{lck} or p56^{lck} kinase domain to recombinant CD45 is affected by the kinase's phosphorylation state

With the production of the GST-full length p56^{lck} protein, binding assays were now performed using p56^{lck} as the immobilized component and the inactive C817S cytoplasmic domain mutant of CD45 as the soluble component. From Figure 5.11, it is clear that under these reversed conditions, CD45 still specifically interacts with full length p56^{lck} and is also demonstrated to specifically interact with the kinase domain of p56^{lck}. Furthermore, it was shown that the GST fusion protein of an SH2 domain derived from SHC, did not interact with CD45. Under the new binding assay conditions, the role of the phosphorylation state of p56^{lck} in the binding of CD45 was investigated. Both the GST-p56^{lck} and the GST-p56^{lck} kinase domain are autophosphorylated in their purified state (Figure 5.11). Consequently, the GST fusion proteins were treated with calf
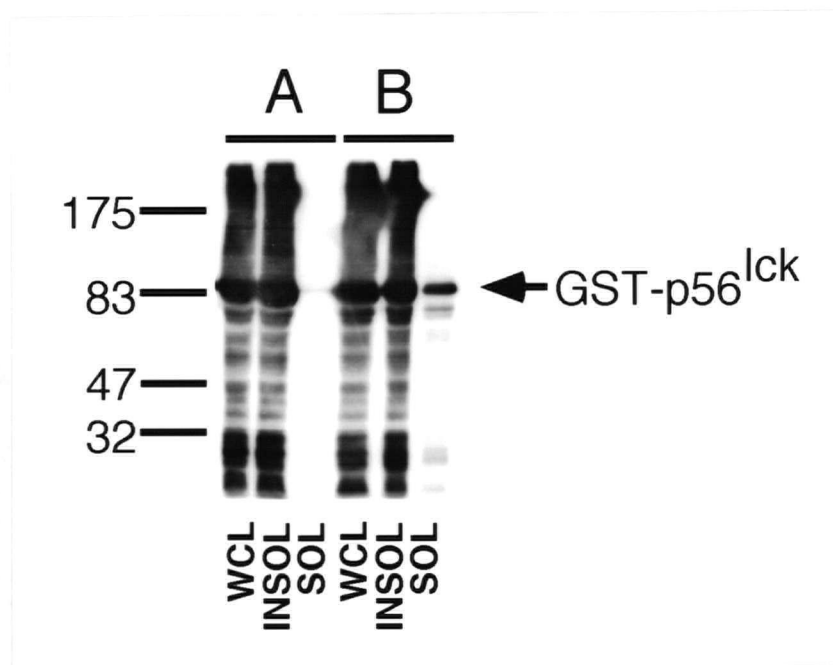
Figure 5.9: <u>Inclusion of 1.0% N-laurylsarcosine and sonication step</u> <u>increase the yield of soluble GST-p56^{lck} recombinant protein</u>. Western blot analysis using anti-GST antisera to assess solubility of recombinant protein. Lysis procedure was as described in Materials and Methods and involved comparisons between lysis with a 1.0% Triton X-100 buffer (A) versus lysis using a 1.0% Triton X-100, 1.0% N-laurylsarcosine buffer and an additional sonication step (B). Whole cell lysates (WCL), insoluble fractions (INSOL), and soluble (SOL) fractions were analysed.

175—
83—
62—
47—
32—
25—

1   2
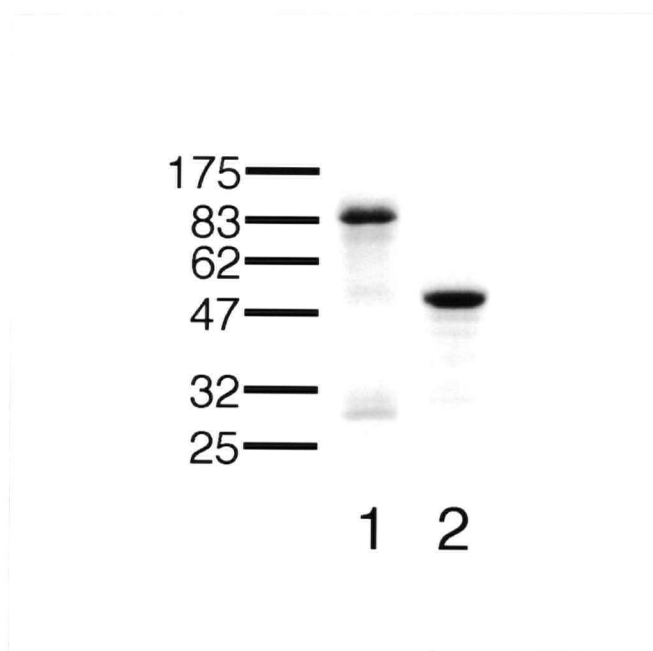
Figure 5.10: <u>Coomassie blue staining of purified GST-p56^{lck} and GST-</u><u>p56^{lck} kinase domain proteins.</u> SDS-PAGE analysis of purified GST-fusion proteins obtained using the lysis procedure that included the use of 1.0% N-laurylsarcosine and sonication. Proteins are stained with Coomassie blue and include 1: GST-full length p56^{lck}, and 2: GST-p56^{lck} kinase domain.

intestinal alkaline phosphatase in an attempt to dephosphorylate any potential phosphorylated residues that may participate in the interaction between these two signaling molecules. From Figure 5.11, it is clear that the p56^{lck} fusion proteins showed a significant loss in phosphorylation after enzymatic treatment, although 100% dephosphorylation was never realized as assessed by anti-phosphotyrosine Western blot analysis. Interestingly, when the dephosphorylated GST fusion proteins were used in the binding assay, it was observed that the interaction between CD45 and the fusion proteins was dramatically reduced under these conditions, implying that phosphorylation of p56^{lck} is an important factor in the overall interaction between CD45 and p56^{lck}.

Interaction between recombinant p56^{lck} proteins and recombinant CD45 is affected by the PTP activity of CD45

The new binding conditions also permitted the determination of whether the PTP activity of CD45 could affect binding of p56^{lck}. Under the previous parameters where CD45 was immobilized via the use of cyanogen bromide, CD45 PTP activity was destroyed by the process. With the use of GST-immobilized p56^{lck}, active forms of soluble CD45 could be used in the assay. Furthermore, comparisons of binding studies using inactive CD45 could be initiated using specific phosphatase inhibitors or by simply performing experiments with an inactive mutant of CD45 (C817S mutant). Figure 5.12 shows a binding assay done with soluble active CD45 and CD45 that has been inactivated by the addition of 0.5 mM sodium orthovanadate to the binding buffer. PTP activity of CD45 appears to affect its ability to bind to full length p56^{lck}, such that under active CD45
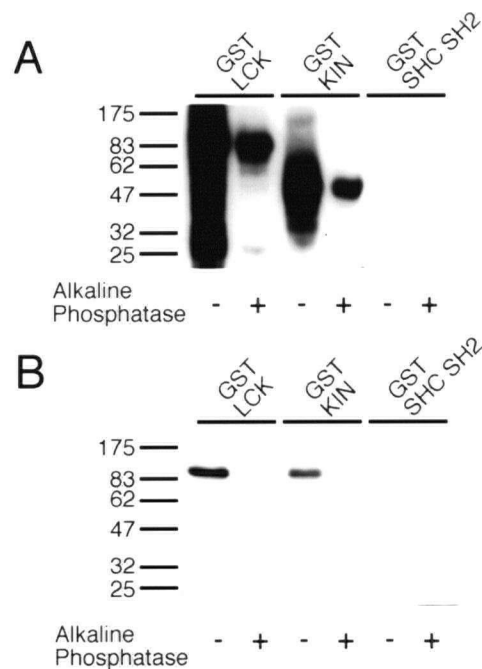
Figure 5.11: <u>Analysis of binding between GST-p56^{lck} fusion proteins and recombinant cytoplasmic CD45 before and after treatment of Alkaline Phosphatase</u>. **A**. Western blot analysis of GST-full length p56^{lck} (lanes 1,2); GST p56^{lck} kinase domain (lanes 3,4); GST-SHC SH2 domain (lanes 5,6) probed with an anti-phosphotyrosine antibody (4G10), before (odd numbered lanes) and after (even numbered lanes) treatment with calf intestinal alkaline phosphatase. GST fusion proteins were immobilized with GSH sepharose beads and treated with calf intestinal alkaline phosphatase (New England Biolabs) in reaction buffer (50 mM NaCl, 10 mM Tris pH 7.9, 10 mM MgCl$_2$, 1 mM dithiothreitol) for 1h at 37°C. Beads were then washed, subjected to SDS-PAGE, and transferred to PVDF. **B**. Western blot analysis of inactive recombinant cytoplasmic CD45 (C817S) remaining bound to immobilized GST-fusion proteins that were treated or untreated with calf intestinal alkaline phosphatase. The CD45 antisera, R01.1 was used.
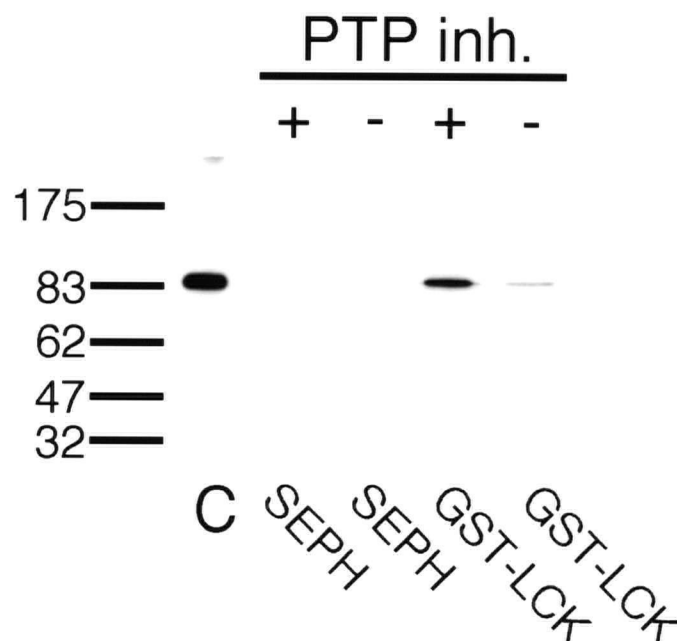
Figure 5.12: <u>Analysis of binding between GST p56^{lck} fusion protein and recombinant active cytoplasmic CD45 in the presence and absence of phosphatase inhibitors.</u> Western blot analysis, using anti-CD45 polyclonal antisera (R01.1), of recombinant cytoplasmic CD45 (wild type) remaining bound to immobilized GST p56^{lck} fusion protein (GST-LCK) or control Sepharose (SEPH) alone beads. Addition of phosphatase inhibitors is indicated by a "+" symbol at the top of the gel. C indicates a control lane with approximately 100 ng of recombinant cytoplasmic CD45 loaded onto the gel. Phosphatase inhibitors used were sodium orthovanadate and sodium molybdate at final concentrations of 0.5 mM and 0.2 mM, respectively.

conditions, the interaction is less prevalent. Surprisingly, there is also preliminary data demonstrating that the ability of the unique amino terminal region and SH2 domain of p56^{lck} to interact with CD45 was also affected by CD45's PTP activity in the same manner as wild type p56^{lck} (data not shown).

Production and purification of p56^{lck} mutants

Mutants of the full length p56^{lck} included the point mutation of Tyr-505 to a Phe (Y505F), which has been previously described as a constitutively active form of p56^{lck}; a point mutation at p56^{lck}'s autophosphorylation site, Tyr-394 being mutated to a Phe (Y394F); and finally a kinase dead mutant which produces an inactive p56^{lck} molecule (Lys-273 mutated to an Arg, K274R). All mutants were received from Dr. A. Veilliette in pGEM vectors, and subcloned into pGEX vectors using the same restriction sites as our wild type construct. Figure 5.13A shows the purified proteins visualized using Coomassie blue staining techniques. Figure 5.13B also shows the tyrosine phosphorylation states of these mutants. As previously shown, except for the kinase dead mutant K273R, all proteins are tyrosine phosphorylated although the Y394F mutant is consistently phosphorylated to a lesser degree.

Interaction of recombinant cytoplasmic CD45 and the recombinant mutant forms of full length p56^{lck}

Binding assays using immobilized p56^{lck} proteins and soluble cytoplasmic CD45 protein were now performed to assess whether the point mutations could affect the interaction. Since, the wild type p56^{lck} is
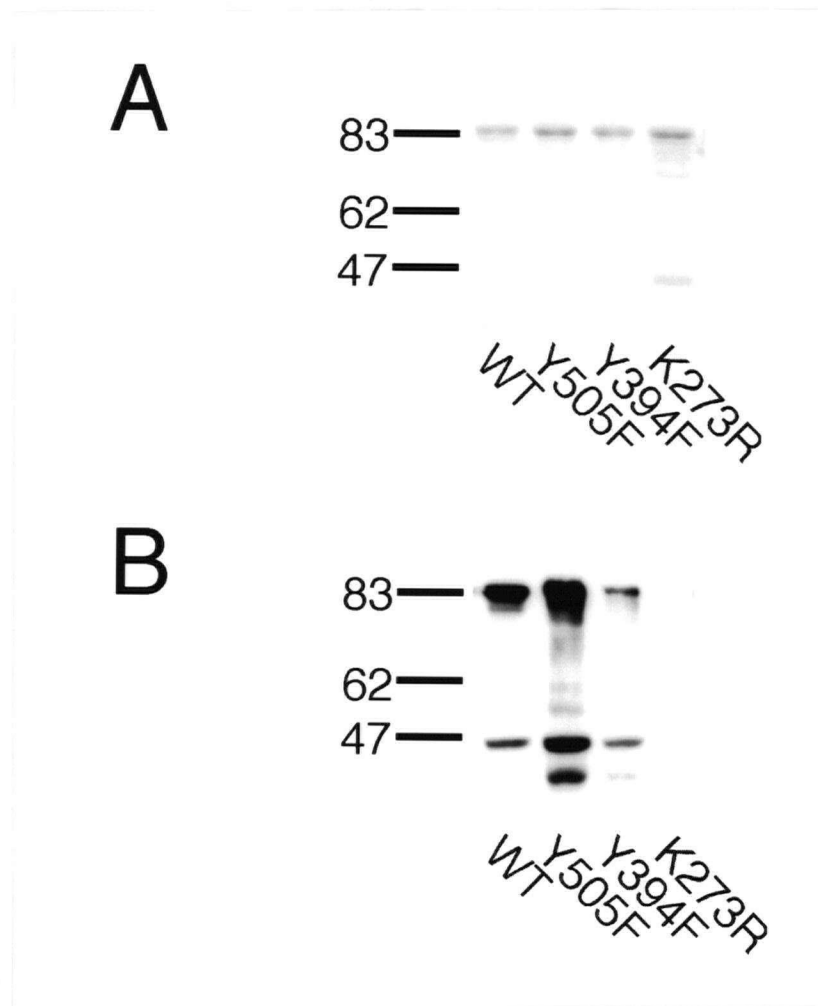
Figure 5.13: <u>Coomassie blue staining and anti-phosphotyrosine Western blot analysis of purified GST full length p56lck fusion proteins</u>. **A**. 1.0 μg of wild type GST p56^{lck} (WT), Y505F GST p56^{lck} (Y505F), Y394F GST p56^{lck}(Y394F), and K273R GST p56^{lck} (K273R) fusion proteins were subjected to SDS-PAGE and stained with Coomassie blue. **B**. 20 ng of the each of the p56^{lck} fusion proteins described above were subjected to SDS-PAGE, transferred to PVDF, and then probed with an antiphosphotyrosine antibody (4G10).

heavily tyrosine phosphorylated and previous results had demonstrated that the interaction could be modulated by the tyrosine phosphorylation state of p56^{lck}, it was envisioned that the use of the Y505F and Y394F mutants would enable the determination of which phosphotyrosine site had a more dominant role. Furthermore, the use of the kinase-dead K273R p56^{lck} mutant would permit characterization of an interaction between CD45 and a p56^{lck} that has no phosphotyrosine content. Binding assays were performed with inactive C817S cytoplasmic domain mutant, with wild type cytoplasmic domain in the presence of sodium vanadate (WT+NaVAN), or with polyclonal antisera known to react with the secondary horseradish peroxidase conjugated antibody. Figure 5.14 shows that wild type p56^{lck} was able to interact specifically with both the C817S mutant as well as the WT+NaVAN preparation. Interestingly, the Y505F mutant could interact with the C817S mutant but displayed significantly less binding to the WT+NaVAN sample. Little binding for either CD45 protein was observed when using immobilized Y394F p56^{lck}, and both CD45 proteins were able to interact with the kinase-dead form of p56^{lck}. No associations were observed when using the control antisera as a negative control.

5.3 Discussion

This work demonstrates that recombinant p56^{lck} can specifically associate *in vitro* with the recombinant cytoplasmic domain of CD45. The specificity of this interaction has been defined on the basis of the following criteria: 1) the interaction did not occur with a structurally related tyrosine phosphatase, RPTPα; 2) the binding was saturable and occurred at a stoichiometry of 1:1; 3) the binding could be localized to distinct protein

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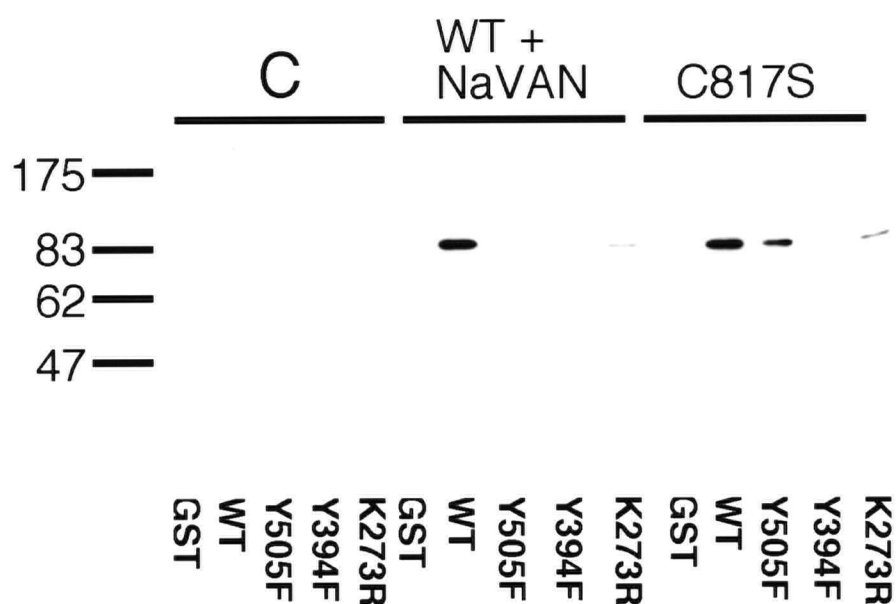
Figure 5.14: <u>Binding of recombinant cytoplasmic CD45 proteins to the wild type and mutant full length GST p56^{lck} fusion proteins.</u> Western blot analysis of soluble control antibody (C), soluble wild type cytoplasmic CD45 (in presence of 0.5 mM sodium vanadate) (WT+NaVAN) protein, or C817S cytoplasmic CD45 (C817S) protein remaining bound to the immobilized wild type GST p56^{lck} (WT), Y505F GST p56^{lck} (Y505F), Y394F GST p56^{lck}(Y394F), and K273R GST p56^{lck} (K273R) fusion proteins. Equivalent amounts of immobilized protein were used (2.0 µg) and 1.0 µg of soluble CD45 protein was added to each binding assay.

domains of p56^{lck}; 4) the association was still detectable after washing in the presence of 0.1%SDS, 1%NP-40 and 0.5% sodium deoxycholate and 150 mM NaCl (RIPA buffer).

The cytoplasmic region of RPTPα has a similar predicted domain structure to CD45, having two tandemly repeated phosphatase domains separated by a unique spacer region and flanked by unique membrane proximal and carboxyl terminal sequences. The PTP domain 1 and domain 2 of CD45 share 47% and 33% sequence identity with PTP domain 1 and domain 2 of RPTPα, respectively (Thomas et al., 1987; Jirik et al., 1990). The observation that p56^{lck} associates with CD45 but not with RPTPα implies a specific interaction. The *in vitro* interaction also occurred in the absence of PTP inhibitors ruling out the possibility that these inhibitors were mediating the association (data not shown). In addition, p56^{lck} also bound to an immobilized recombinant CD45 protein in which the catalytic cysteine had been replaced with a serine (C817S), indicating that a phospho-cysteine intermediate was not responsible for the observed interaction (data not shown). It was clear from the binding assay, that only a small percentage of p56^{lck} bound to recombinant CD45, yet under equilibrium binding conditions and using saturating amounts of p56^{lck}, a 1:1 interaction was indicated. These differences may reflect the fact that in the initial binding experiments, the amount bound was observed after washing 3 times in stringent conditions (RIPA buffer).

In an attempt to determine the affinity of the interaction, Scatchard analysis of the equilibrium binding data was performed. However, a non-linear plot was obtained indicating that it was not a simple single binding site interaction (data not shown). Interpretation of the plot as two distinct

binding sites with different affinities was also precluded as the interaction at saturation resulted in a 1:1 interaction. This indicated that the criteria for Scatchard analysis was not met and thus could not be used to determine the affinity of the interaction. Additional data using GST-fusion proteins indicated that several distinct regions of p56^{lck}, the unique amino terminal region, the SH2 domain, and the kinase domain, could specifically bind to recombinant CD45 suggesting that these regions may contribute to the binding of one molecule of p56^{lck} to CD45. Thus the interaction between p56^{lck} and the cytoplasmic domain of CD45 appears to be quite complex.

It was also shown that the association between p56^{lck} or the GST-p56^{lck} SH2 fusion protein with the cytoplasmic domain of CD45 did not require the tyrosine phosphorylation of CD45. This is in contrast to previous studies by Autero et al. (1994) where an interaction between p56^{lck} and CD45 was observed when CD45 was tyrosine phosphorylated *in vitro* by p50^{csk}. It is possible that p56^{lck} may bind to both non tyrosine phosphorylated and phosphorylated forms of CD45. In support of this notion, another src family kinase, p59^{fyn}, has been reported to associate with both non-tyrosine phosphorylated and phosphorylated forms of Igα. Increased binding of p59^{fyn} was observed upon tyrosine phosphorylation of Igα which resulted in an increase in the kinase activity of p59^{fyn} (Clark et al., 1994).

Before the demonstration of the interaction between CD45 and the p56^{lck} SH2 domain, other tyrosine phosphorylation independent interactions with SH2 domains had been reported for three other tyrosine kinases; ABL, p59^{fyn}, and p60^{src} (Pendergast et al., 1991; Cleghon and Morrison, 1994). However, in these examples, the SH2 domains were

thought to bind to phosphoserine/phosphothreonine residues. More recently, a tyrosine phosphorylation independant interaction between the SH2 domain of p56^{lck} and a 62 kDa protein has been characterized. This interaction was also shown to be independant of protein serine/threonine phosphorylation and the eventual sequence determination of p62 has suggested a role in ubiquitination-mediated protein degradation (Park et al., 1995; Vadlamudi et al., 1996). Presently, whether the interaction between the p56^{lck} SH2 domain and the cytoplasmic domain of CD45 requires protein serine/threonine phosphorylation has not been determined. However, this interaction has been observed to be specific for the SH2 domain of p56^{lck} since two other SH2 domains derived from two other signaling proteins, the p85α subunit of PI3 kinase and SHC, did not interact.

Although a direct interaction between p56^{lck} and the cytoplasmic domain of CD45 has been demonstrated *in vitro*, it still remains to be established whether a direct interaction also occurs *in vivo* and whether such an interaction is regulated by the association of other proteins such as CD45AP. In T cells, p56^{lck} can be detected in immunoprecipitates of CD45 along with the presence of a 30 kDa protein (Schraven et al., 1991; Koretzky et al., 1993; Ross et al., 1994) and can be coprecipitated with CD45 under conditions where CD45 is not detectably tyrosine phosphorylated (P. Borodchak, A. Maiti, and P. Johnson, unpublished data). Since p56^{lck} has not been co-precipitated in the absence of the 30 kDa protein (CD45AP or LPAP), it is not known whether p56^{lck} can directly associate with CD45 in T cells. In contrast, p30 has been shown to co-precipitate with CD45 in the absence of p56^{lck} (Koretzky et al., 1993). Work using the same 6HIS recombinant CD45 demonstrated that this interaction was independant of

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the cytoplasmic domain of CD45. Rather, it was the transmembrane region of CD45 that was shown to be required for the association of p30 (Schraven et al., 1994; Takeda et al., 1994; Cahir McFarland et al., 1995; Cahir McFarland et al., 1997). However, as only a small percentage of p56^{lck} is coprecipitated with CD45, the physiological significance of this association remains to be established.

Initially, in the *in vitro* assay conditions used to observe the binding of p56^{lck} to the cytoplasmic domain of CD45, neither enzyme was catalytically active and p56^{lck}, but not CD45, was detectably tyrosine phosphorylated. Therefore, it is possible that these *in vitro* conditions favour or stabilize the interaction between these proteins whereas conditions in the cell may favour a more transient association. In this respect, efforts were undertaken to establish the possible role of p56^{lck} phosphorylation state as well as CD45 PTP activity in the regulation of the *in vitro* association. The full length recombinant p56^{lck} proteins exhibited high amounts of phosphotyrosine, although the specific sites and exact molar amounts of phosphate were not determined. This phosphorylation was demonstrated to play a significant part in mediating the association between CD45 and p56^{lck} as shown with experiments performed with dephosphorylated forms of the GST p56^{lck} fusion protein. Analysis also revealed that the cytoplasmic domain of CD45 could interact with the autophosphorylated GST p56^{lck} kinase domain. These observations were not surprising since it was assumed that a tyrosine phosphatase must, at some point, directly contact the phosphotyrosine residue. In particular, crystal structures of a number of PTPs have clearly established structural features within the enzyme's active sites that can interact with the phosphotyrosine residue (Barford et al., 1994a; Stuckey et

al., 1994; Bilwes et al., 1996). This notion is further strengthened with the demonstration that CD45 PTP activity can affect the degree of interaction seen between the two signaling molecules. Again, it is not surprising that the active CD45 molecule would bind significantly less than an inactive C817S CD45 molecule, since one can propose that the active enzyme hydrolyses the phosphotyrosine residue, hence destroying an important binding element.

What is surprising, however, is the observation that PTP activity of CD45 also affects interaction with the unique amino terminal region and SH2 domain of p56^{lck}. These recombinant proteins are not tyrosine phosphorylated, and hence this result cannot be accounted for under the same hypothesis. Furthermore, the wild type cytoplasmic CD45 was still able to interact with p56^{lck} even after treatment with 0.5 mM sodium orthovanadate. This phosphatase inhibitor is generally used as a phosphate analogue and is believed to have an inhibitory effect due to its ability to interact with the active site of PTPs. Consequently, the data presented regarding the regulation of the association by phosphorylation or PTP activity may also involve complex allosteric effects that are associated with changes in tertiary protein structure as well as the simple active site to phosphotyrosine interaction. The importance of other interacting elements other than the active site is also exemplified by recent evidence that the aforementioned cysteine to serine conserved point mutation may result in substantial changes to the active site cleft (Zhang and Wu, 1997).

The notion of conformational changes in protein structures is consistent with the elegant demonstration of intramolecular interactions found in the overall structures of the p60^{c-src} and hck kinases (Sicheri et

al., 1997; Xu et al., 1997). Furthermore, it is entirely conceivable that CD45's large cytoplasmic region could exist in open and closed conformations, since it contains two discrete phosphatase domains separated by a spacer region. Hints of this complex possibility are also highlighted in the Scatchard analysis of the interaction between CD45 and p56^{lck}, which exhibited non-linear trends despite a consistent 1:1 molar stoichiometry. Other points include the simple observation that the interaction between CD45 and p56^{lck} can be mediated by three out of the possible four regions of p56^{lck} examined; such complexity is indicative of allosteric effects.

Finally, binding data assessing whether mutant forms of full length p56^{lck} can interact with the C817S CD45 protein, or with the wild type CD45 protein in the presence of 0.5 mM sodium orthovanadate, show that addition of sodium vanadate can inhibit the interaction of CD45 with only the Y505F mutant of p56^{lck}. This effect was not observed with experiments using the wild type GST p56^{lck} fusion protein. The effect was also not seen in the Y394F mutant of p56^{lck}, although significantly less CD45 interacted with this p56^{lck} construct. However, it should be noted that the phosphotyrosine levels of the Y394F mutant were substantially less than phosphotyrosine levels seen in wild type GST p56^{lck} or in Y505F GST p56^{lck}. Efforts to correct this were initiated, but repeated attempts to increase its phosphotyrosine content by using recombinant p56^{csk} in *in vitro* kinase assays were unsuccessful. Furthermore, binding experiments performed with the kinase dead p56^{lck} protein showed that significant binding was observed in the absence or presence of sodium vanadate. Therefore, there appears to be discrete differences in the degree of the

strength of the interaction which are dependant on the mutant proteins used.

Although the data presented in this chapter is intriguing in its complexity, it is victim to one simple caveat, whereby all experiments were performed under an *in vitro* system. However, there is some physiological data that is congruent with some of the observations presented here. First, data from CD45 negative and positive cell lines have indicated that the absence of CD45 has a larger effect on the phosphorylation state of $p56^{lck}$ than $p59^{fyn}$ and that the phosphorylation state of $p60^{src}$ is unaffected (Hurley et al., 1993). Since all these kinases are closely related, particularly at their phosphorylation sites, it is not clear why some members of this family appear to be preferred substrates *in vivo*. Recent data using a $p56^{lck}/p59^{fyn}$ chimera has shown that the unique amino terminal region of $p56^{lck}$, but not that of $p59^{fyn}$, was required for the dephosphorylation of Y505 in $p56^{lck}$, suggesting that the unique amino terminal region of $p56^{lck}$ may influence substrate specificity (Gervais and Veillette, 1995). In addition, this observation has been extended into a non-lymphoid system (Gervais and Veillette, 1997), therefore eliminating the requirement of the 30 kDa CD45-AP protein. In this chapter, I have demonstrated that the unique amino terminal region the SH2 domain and the kinase domain of $p56^{lck}$ can bind to the cytoplasmic domain of CD45 *in vitro*. It is tempting to speculate that an interaction between these non-catalytic domains of $p56^{lck}$ and CD45 may act to facilitate the dephosphorylation of $p56^{lck}$. Curiously, the same report also indicated that a $p56^{lck}$ construct missing its SH2 domain was still dephosphorylated by CD45 implying that the SH2 domain was not required for this event (Gervais and Veillette, 1995).

Another report has examined the structural requirements for the co-localization of $p59^{fyn}$ and CD45 (zur Hausen et al., 1997). $p59^{fyn}$, as previously mentioned, has also been shown to be a potential *in vivo* substrate for CD45 in T cells. This work demonstrates that the unique amino-terminal region, the SH3 domain and the SH2 domain of $p59^{fyn}$ is required for its co-localization with CD45. Furthermore, the authors present data suggesting that the role of the SH3 domain may have more to do with separating the unique amino-terminal region and the SH2 domain at a correct distance, than with a direct interaction via poly-proline motifs. These observations are congruent with the *in vitro* data presented in this chapter.

Overall, the results presented in this chapter indicate a complex interaction between the cytoplasmic domain of CD45 and $p56^{lck}$. Furthermore, it is our belief that the principle purpose of this interaction is to allow the efficient dephosphorylation of the substrate, $p56^{lck}$, by CD45. This chapter, therefore, outlines data obtained from an *in vitro* experimental system where the interaction may be stabilized or enhanced by conditions used in the assays. It is likely that under cellular situations, the enzyme-substrate interaction is transient in nature. However, the observations presented here, still provide useful information on the association between the enzyme and substrate. In this context, I wish to present a working model that would provide a sound basis for the data reported in this chapter as well as *in vivo* data presented from other groups.

To present this model, it is important to begin by setting a number of guidelines. First, one must assume that $p56^{lck}$ is able to exist in a closed conformation that is dependant on the interaction of a phosphorylated

Tyr-505 residue with the SH2 domain of the same molecule. In addition, this conformation is also indicative of the absence of phosphotyrosine at the autophosphorylation site (Y394). The recently crystalized structures of p60$^{\text{c-src}}$ and hck support these considerations (Sicheri et al., 1997; Xu et al., 1997). The second assumption dictates that although the release of the phospho-Tyr-505 from the SH2 domain is an important step in the realization of an open conformation for p56$^{\text{lck}}$, the phosphorylation of Tyr-394 is particularly crucial to maintain this open state whilst Tyr-505 is still phosphorylated. Although there is no direct evidence for this statement, there have been several studies that have determined that the autophosphorylation of src-family kinases represent an important step in permiting these kinases to exhibit maximal catalytic activity (Woods and Verderame, 1994; Sotirellis et al., 1995; Hardwick and Sefton, 1995; Doro et al., 1996; Boerner et al., 1996; Yamaguchi and Hendrickson, 1996). Therefore, it is conceivable that one of the governing features of this post-translational event is to maintain an open kinase conformation. Third, from data presented in this chapter, one must assume that the cytoplasmic domain of CD45 is able to interact with p56$^{\text{lck}}$ through either the unoccupied SH2 domain, the phosphorylated Tyr-505 residue, or the phosphorylated Tyr-394 residue. Consequently, this would explain why the cytoplasmic domain of CD45 appears to interact with both phosphorylated and non-phosphorylated (kinase-dead) forms of p56$^{\text{lck}}$. However, interaction with any of these three elements is dependant on p56$^{\text{lck}}$ existing in an open conformation.

Under these guidelines, the following model is proposed. We predict that the interaction between CD45 and p56$^{\text{lck}}$, within the context of a physiological environment, serves primarily to target a p56$^{\text{lck}}$ molecule

that exists in an unactivated state. In this state, the Tyr-505 is phosphorylated and in contact with the SH2 domain: hence the protein is found in a closed conformation. However, kinetic equilibrium dictates that this particular molecule will also be found in open conformations (perhaps in only a small fraction of cases) where the SH2 domain is no longer in contact with the phosphotyrosine residue. Here, CD45 is able to contact p56^{lck}, and this initial interaction is likely to be dependant on interactions with both the unique amino terminal region and the SH2 domain of p56^{lck}. It is important to emphasize that according to our results, the interaction via the unique amino terminal region is of relatively low affinity, and so it is possible that although it is able to substantially help the direct association between these two molecules, it is unlikely, by itself, to stably mediate the interaction in a physiological setting. It is also important to propose that the primary purpose of CD45 interacting with the unoccupied SH2 domain of p56^{lck}, is to prevent reversion back to the closed conformation. This provides a reason why *in vivo* studies have shown that the SH2 domain of p56^{lck} is not required for CD45 dephosphorylation (Gervais and Veillette, 1995). Here, one can argue that with the elimination of the SH2 domain of p56^{lck}, the interaction of CD45 with the SH2 domain is no longer required since the SH2 deletion mutant of p56^{lck} can exist in an open and unhindered conformation all the time. In this view, one would predict that in a cellular system involving CD45 and the SH2 deletion of p56^{lck}, CD45 would still function to dephosphorylate p56^{lck}.

Again, it is stressed that the interaction with the SH2 domain of p56^{lck} is independant of tyrosine phosphorylation. Recent crystal structure data on domain 1 of RPTPα has shown that the membrane

proximal region of this protein existed in a helix-turn-helix topology (Bilwes et al., 1996). This structural "elbow" was then shown to crystallize within the active site of an adjacent RPTPα molecule providing a possible means of regulating catalytic activity. However, not all PTPs harbour this putative regulatory mechanism as demonstrated with the resolution of the three dimensional structure of PTP domain 1 of RPTPμ (Hoffmann et al., 1997). Whether CD45 has a similar structure in its cytoplasmic region is currently unknown, but given the common binding partners of PTPs and SH2 domains (i.e. the phosphotyrosine residue), it is tempting to speculate that the helix turn helix fold can also interact with specific SH2 domains in a non-tyrosine phosphorylated fashion. Such a mechanism may also provide CD45 with a more active role in determining the conformation of p56^{lck}, in that this helix-turn-helix structure may open the molecule by competitive binding to the SH2 domain.

The extension of the open conformation of p56^{lck} created by the interaction between CD45 and the unoccupied SH2 domain, would then allow for the phosphorylation of Tyr-394, which would further potentiate the open conformation. Although termed the autophosphorylation site, studies performed on p53/p56^{lyn} would suggest that this site is actually a transphosphorylation site (Sotirellis et al., 1995). Moreover, careful scrutiny of the three dimensional structures of various tyrosine kinases, has made it difficult to support the idea that autophosphorylation can actually occur under the structural constraints defined by crystal topology. Therefore, transphosphorylation of Tyr-394 may be enhanced significantly if CD45 plays an active role in keeping the molecule open longer.

Once, the Tyr-394 is phosphorylated, this optimal open state would then permit CD45 to interact with other elements of p56^{lck} such that the

overall interaction now exhibits higher affinity. This higher affinity may result from greater accessibility (due to the open conformation), or from the ability of CD45 to interact with more than one element at a time. In this case, perhaps a higher affinity interaction is observed when CD45 is interacting with p56^{lck} through both the non-phosphotyrosine elements (unique amino terminal region and SH2 domain), as well as through direct contact between the CD45's active site and the phosphorylated Tyr-394. Under this context, it can then be envisioned that the blocking of the active site with a phosphate analogue (such as sodium orthovanadate) could easily lead to profound steric hindrance resulting in the significant loss of the interaction altogether as demonstrated in Figure 5.14.

At this point, it is also important to consider the differences between the interaction observed under *in vitro* conditions to that of an interaction that occurs in the cell. By using recombinant proteins, where CD45 PTP activity is abrogated or non-existent, one could envision that these conditions may enhance or stabilize an enzyme-substrate association that may normally be transient in the cell. In addition, by virtue of the ability to obtain high yields of purified recombinant proteins, the concentrations of p56^{lck} and CD45 in the binding assays are likely to be far greater than physiological concentrations: thereby furthering an enhancement of the interaction. In this context, one must therefore include in the model, the notion that under cellular settings, the interaction between CD45 and p56^{lck} is lost upon the dephosphorylation of p56^{lck}. However, under our *in vitro* system, these same considerations would equate the dephosphorylation event to a significant loss in the affinity of the interaction, that may still be observed under the binding assay conditions described above.

This model, however, does not explain why CD45 PTP activity would affect its ability to interact with the non-tyrosine phosphorylated p56^{lck} domains such as the SH2 domain, or the unique amino-terminal domain. Furthermore, it does not address the complexity that is potentially created by CD45 having two PTP domains which would both contain areas of active site architecture. This additional point can affect both interpretations of interaction via phosphotyrosine residues and can also affect interpretations of data obtained from studies using phosphate analogs such sodium orthovanadate.

To conclude, this chapter demonstrates the power of recombinant protein technology and highlights some of the analyses that can be performed. In particular, this technology allows the careful characterization of an interaction between two cytoplasmic proteins. However, one must also caution that studies entailing the use of recombinant proteins, produced and purified from *E. coli* under controlled settings, may never fully simulate the experimental circumstances found under *in vivo* settings. Consequently, the next chapter will explore the questions of CD45 and p56^{lck} substrate specificity in a cellular system.

CHAPTER 6

Characterization of CD45 and RPTPα protein tyrosine
phosphatase activities in T cells.

Related publication:

Ng, D. H. W., Jabali, M. D., Maiti, A., Borodchak, P., Harder, K. W.,

Brocker, T., Malissen, B., Jirik, F. R., Johnson, P. (1997) CD45 and RPTPα

display different protein tyrosine phosphatase activities in T lymphocytes.

Biochem. J. **327**, 867-876.

6.1 Introduction

Due to the extensive work performed using *in vitro* recombinant materials, efforts were initiated to study CD45 function under an *in vivo* setting. In this context, RPTPα was expressed in a CD45⁻, T cell receptor (TCR)⁺, BW5147 T lymphoma cell, to permit the examination and comparison of two receptor protein tyrosine phosphatases, CD45 and RPTPα. In particular, the role of each phosphatase with regard to substrate specificity, PTP activity, and propagation of the T cell receptor mediated activation signal was investigated.

PTPs can act on a wide range of substrates *in vitro*. However, data indicate that PTPs have a much more restricted substrate specificity in the cell, although little is understood about how this is achieved.

As shown in chapter 5, the existence of a direct interaction between CD45 and p56^{lck} provides a possible reason explaining the substrate specificity observed in cell lines. This observation indicates that the specificity of the association, between these two signaling molecules, is dictated by more than just the few amino acids adjacent to the phosphotyrosine residue. The specificity of this stable protein-protein association was illustrated by the observation that an interaction with p56^{lck} did not occur using the related 2 domain PTP, RPTPα.

RPTPα was chosen as the candidate PTP for our comparative studies, and as such, it is important to provide reasons for why it was selected. RPTPα is a widely expressed transmembrane two domain phosphatase which is expressed at low levels in lymphoid tissues (Jirik et al., 1990; Kaplan et al., 1990; Krueger et al., 1990; Matthews et al., 1990; Sap et al., 1990). The entire cytoplasmic domain of RPTPα shares approximately 37% sequence identity with that of CD45: 47% sequence

identity is shared between the first PTP domains and 33% between the second PTP domains. Overexpression of RPTPα in rat embryo fibroblasts and embryonic carcinoma cells resulted in the activation of pp60^{c-src} and its dephosphorylation at the negative regulatory tyrosine phosphorylation site (Tyr-527) (Zheng et al., 1992; den Hertog et al., 1993). RPTPα has also been shown to dephosphorylate this site *in vitro*, implying that pp60^{c-src} is a substrate for RPTPα (Zheng et al., 1992; den Hertog et al., 1993). This phosphatase thus shares many attributes with CD45; in that they both have similar cytoplasmic domain structures, and significant sequence identity within their cytoplasmic regions (particularly within the catalytic PTP domain I). More importantly, both phosphatases can dephosphorylate the negative regulatory sites of src family kinases.

To understand more about the role of CD45 in T cells and to assess the potential substrate specificities of the protein tyrosine phosphatases CD45 and RPTPα, RPTPα was expressed in the TCR^{+}, CD45^{-} BW5147 T cell line (Hermans and Malissen, 1993). Despite high levels of expression, RPTPα did not fully restore either proximal or distal TCR mediated signaling events. RPTPα was unable to reconstitute the phosphorylation of CD3ζ and did not increase expression of the activation marker, CD69, upon TCR/CD3 stimulation. The discussion will also highlight results indicating that RPTPα did not significantly alter the phosphorylation state or kinase activity of two CD45 substrates, p56^{lck} or p59^{fyn}, suggesting that RPTPα does not have the same specificity or function as CD45 in T cells.

Further comparison of the two phosphatases, indicated that immunoprecipitated RPTPα was approximately 7-10 fold less active than CD45 when tested against artificial substrates. This difference in activity was also observed *in vitro* using purified recombinant enzymes at

physiological pH, and further analysis was provided using enzymes at their respective optimum pH's. Overall, analysis using src family phosphopeptides and recombinant p56^{lck} as substrates indicated that CD45 was consistently more active than RPTPα having both higher V$_{max}$ and lower K$_m$ values. Curiously, the efficiency of CD45 over RPTPα was also observed when using recombinant p60^{c-src} protein as a protein, in spite of discrete differences in data from binding assays. Thus CD45 is intrinsically a much more active phosphatase than RPTPα which provides one reason why RPTPα cannot effectively dephosphorylate p56^{lck} and substitute for CD45 in T cells. This work establishes that these two related protein tyrosine phosphatases are not interchangeable in T cells and that this is due, at least in part, to quantitative differences in phosphatase activity.

6.2 Results

Expression of RPTPα in BW5147 CD45$^-$ T lymphoma cells

RPTPα$^+$, CD45$^-$, TCR$^+$, CD3$^+$ BW5147 T lymphoma cells were created and maintained as described in the Materials and Methods. In order to compare the level of expression of RPTPα in the transfected CD45$^-$ BW5147 cells to that of CD45 in the parental CD45$^+$ BW5147 cells, both cell lines were metabolically labeled with [^{35}S]-methionine and cysteine and the phosphatases immunoprecipitated (Figure 6.1). CD45 and RPTPα (which are predicted to have a molecular mass of 180 kDa and 125 kDa and contain 48 and 33 methionines and cysteines, respectively) were found to be expressed at similar levels in the respective cells. The transfected clones 2-10 and 3-11 expressed slightly higher molar amounts (1.2 times) of RPTPα than CD45 expressed by the parental cell. All cells were also
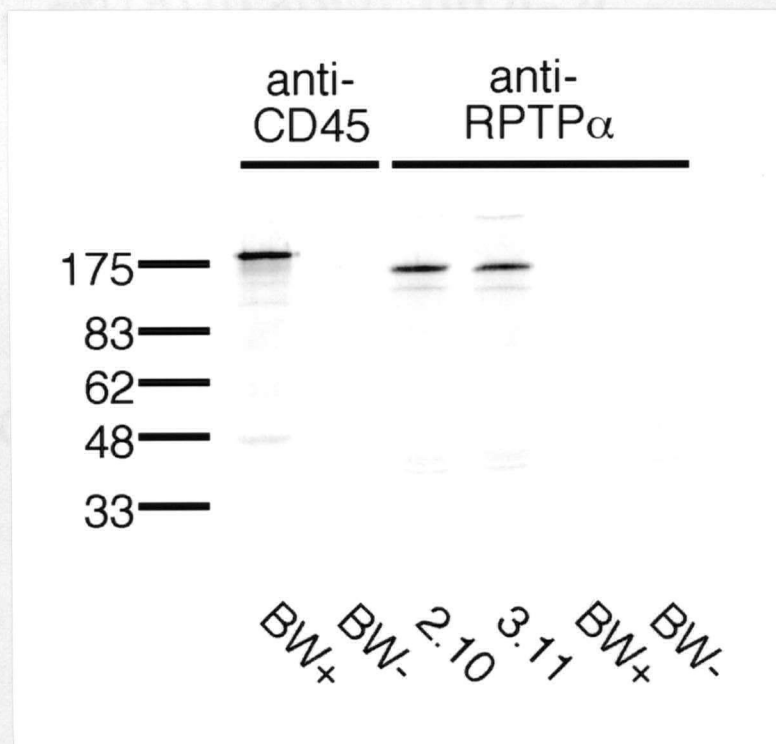
Figure 6.1: <u>Immunoprecipitation of CD45 and RPTPα from BW5147 cells</u>. Cells were metabolically labeled with [^{35}S] methionine and cysteine for 8 hours at 37°C. RPTPα and CD45 proteins were immunoprecipitated from BW5147 CD45$^+$ T cells (BW$^+$), BW5147 CD45$^-$ T cells (BW$^-$) and RPTPα transfected BW5147 CD45$^-$ T cells (2-10 and 3-11) as indicated. Proteins were immunoprecipitated from cell lysates containing equivalent amounts of radioactivity using polyclonal rabbit anti-RPTPα cytoplasmic domain specific antisera (PTPα-2) or polyclonal rabbit anti-CD45 cytoplasmic domain specific antisera (R01.1) (see Methods for details). Immunoprecipitated proteins were analyzed after separation on a 10% SDS-polyacrylamide gel. The position and size (kDa) of the pre-stained molecular weight markers is as indicated.

routinely checked by FACS analysis to ensure that TCR/CD3 expression levels were similar (Mojgan Jabali, data not shown).

Effect of RPTPα on TCR/CD3 mediated stimulation in BW5147 CD45⁻ T cells

Since CD45 has been implicated in the initiation of TCR/CD3 mediated signaling events, TCR/CD3 induced tyrosine phosphorylated proteins were examined in RPTPα transfected cells (clone 2-10) and compared to those observed in the parental CD45⁻ and CD45⁺ BW5147 T cells. Cells were stimulated with an anti-CD3 antibody and tyrosine phosphorylated proteins detected at 0 second and 90 second time points by SDS-PAGE and Western blotting with the anti-phosphotyrosine antibody (4G10). The 90 second timepoint was chosen in these experiments since there is a rapid induction of tyrosine phosphorylation of specific proteins observed in the CD45⁺ cells (Figure 6.2). A major phosphorylated protein was observed at an apparent molecular mass of 38 kDa, and minor bands were observed between approximately 70 and 175 kDa and between 19 and 27 kDa. In contrast, the induction of tyrosine phosphorylated proteins after CD3 stimulation in BW5147 CD45⁻ cells was virtually absent (Figure 6.2). Transfection of RPTPα into the BW5147 CD45⁻ cells partially restored their ability to tyrosine phosphorylate proteins in response to addition of anti-CD3 antibody. Similar results were also observed with the 3-11 RPTPα clone (data not shown). To further determine the effect of RPTPα on proximal TCR/CD3 induced signaling events, the phosphorylation state of CD3ζ was examined at 30 minutes after stimulation with an anti-CD3ε antibody. 30 minutes was chosen as a suitable timepoint since previous work had shown that although the global induction of tyrosine

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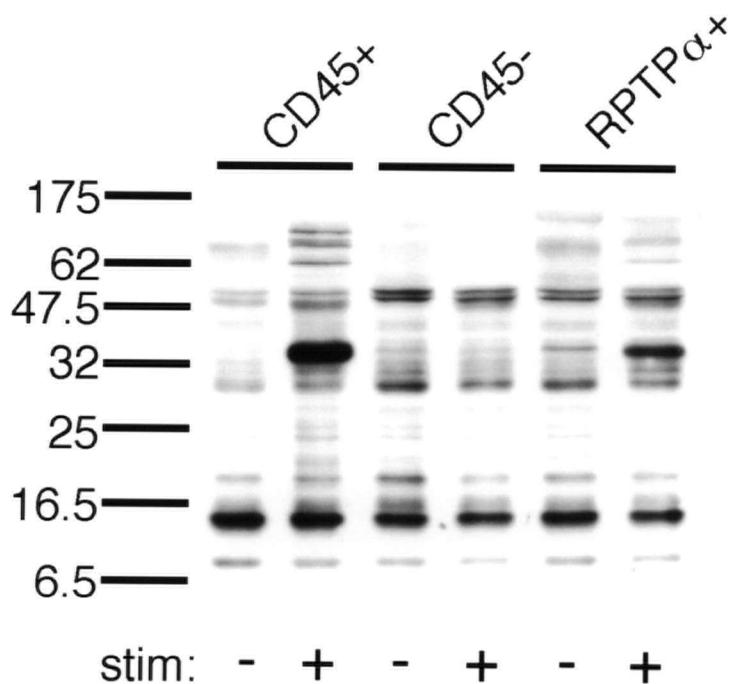
Figure 6.2: Effect of CD45 and RPTPα on TCR/CD3 mediated induction of phosphotyrosine containing proteins in BW5147 cells. BW5147 CD45+ (CD45+), BW5147 CD45- (CD45-), and clone 2.10 CD45-, RPTPα+ (RPTPα+) cells were stimulated by addition of purified anti-CD3ε monoclonal antibody (145-2C11) and incubated at 37°C for 0 seconds (-) or 90 seconds (+) prior to lysis. Approximately 1.5×10^6 cell equivalents were then electrophoresed on a 10% SDS polyacrylamide gel, transferred to PVDF membrane and probed with the anti-phosphotyrosine monoclonal antibody, 4G10.

phosphorylation is a quick and transient event, the phosphorylation of CD3ζ is less transient and is sustained at longer time periods (P. Johnson, P. Borodchak, M. Jabali, unpublished data). In Figure 6.3A and 6.3B, RPTPα expression did not result in the increased phosphorylation of CD3ζ upon TCR/CD3 stimulation. Thus the expression of RPTPα to levels at least equivalent to that observed for CD45 could not restore the proximal T cell signaling events observed in the CD45+ BW5147 cells.

To evaluate whether the partial induction of tyrosine phosphorylation observed in TCR/CD3 stimulated RPTPα+ cells was sufficient to stimulate downstream events, the induction of CD69 expression, a known distal marker of T cell activation (Hara et al., 1986; Testi et al., 1989) was determined by flow cytometry. It was observed that there was a distinct difference in CD69 upregulation between the CD45+ BW5147 T cells when compared to both the CD45- and RPTPα transfected BW5147 T cells (Figure 6.4). This indicates that the presence of RPTPα had no significant effect on the induction of CD69 expression in response to TCR/CD3 stimulation.

Comparison of phosphatase activities of RPTPα and CD45 isolated from BW5147 T cells

To further investigate potential differences between these two PTPs, CD45 and RPTPα were immunoprecipitated from the respective BW5147 T cells and their relative phosphatase activities measured. RPTPα immunoprecipitated from equivalent cell numbers was approximately 6-8 fold less catalytically active than immunoprecipitated CD45, when a phosphorylated fyn peptide (fyn pY531) was used as the substrate in an *in vitro* phosphatase assay (Table VII). When the amount of specific

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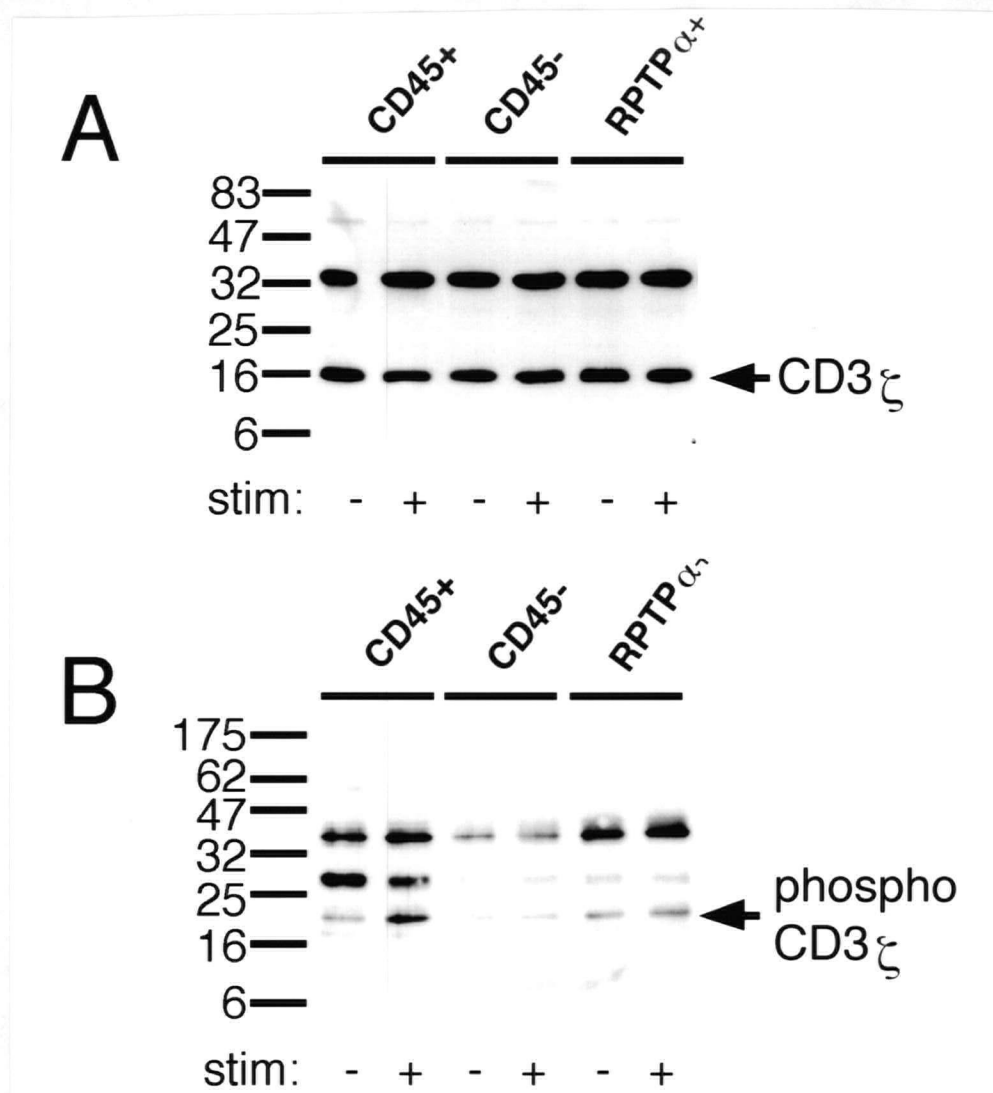
Figure 6.3: <u>Effect of CD45 and RPTPα on TCR/CD3 mediated induction of</u> <u>phospho-CD3ζ in BW5147 cells</u>. Surface CD3ζ was immunoprecipitated, after addition of 145-2C11 to all three cell lines for stimulated (30 minutes at 37°C) or unstimulated (30 minutes on ice) samples, and 5.0 x 10^6 cell equivalents were electrophoresed on a 15% SDS polyacrylamide gel, transferred to PVDF membrane and probed either with the **(A)** anti-CD3ζ monoclonal antibody (G3) or with the **(B)** anti-phosphotyrosine monoclonal antibody (4G10).
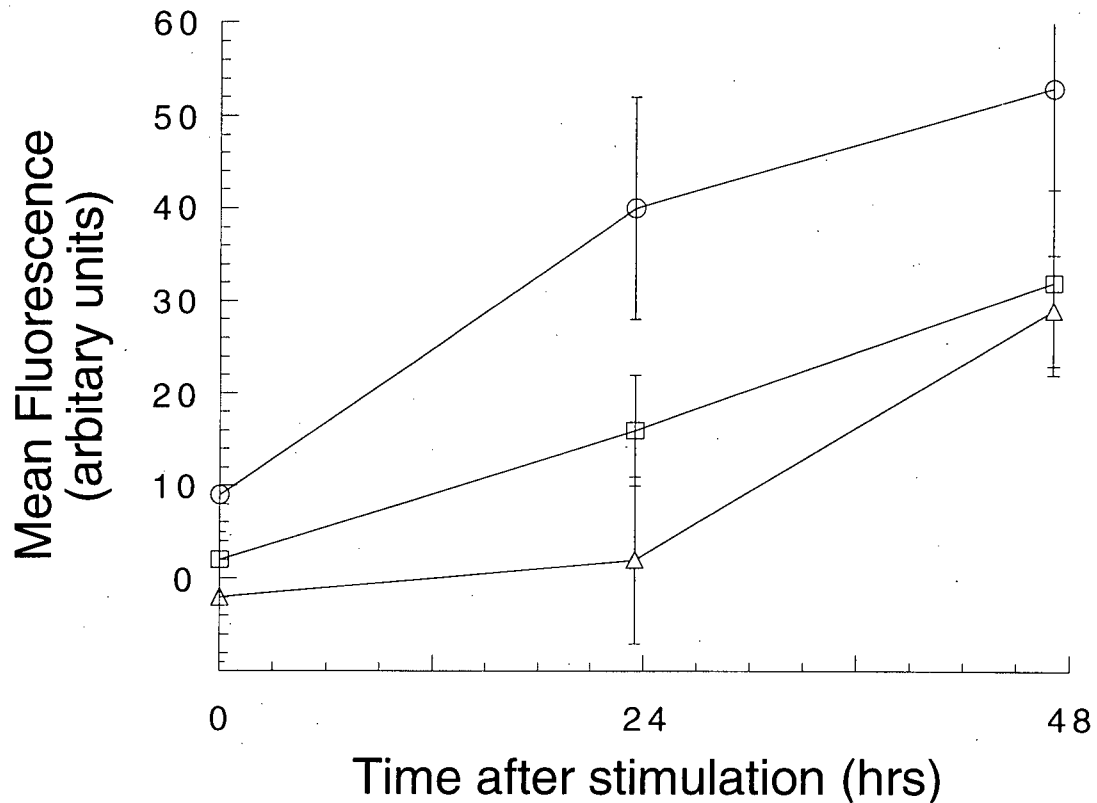
Figure 6.4: Effect of CD45 and RPTPα on TCR/CD3 mediated induction of the late activation marker, CD69, in BW5147 cells. Cells were incubated with the purified 145-2C11 for 0 hr, 24 hrs or 48 hrs and then analysed for CD69 expression by flow cytometry. Graph shows the mean flourescence intensity of CD69 expression over time for BW5147 CD45$^+$ (o), BW5147 CD45$^-$(☐), and clone 2.10 CD45$^-$, RPTPα$^+$ (Δ) cells. This graph represents data from one of 4 separate experiments, and arbitrary units were derived by substracting fluorescence values from experiments performed with secondary antibody alone.

Cell line	Cell description	Precipitating antiserum	PTP activity (nmol/min per 2×10^5 cells)
CD45-	$CD45^-$, $RPTP\alpha^-$	CD45	n/d
CD45+	$CD45^+$, $RPTP\alpha^-$	CD45	0.44 ± 0.03
2.10	$CD45^-$, $RPTP\alpha^+$	$RPTP\alpha$	0.057 ± 0.003
3.11	$CD45^-$, $RPTP\alpha^+$	$RPTP\alpha$	0.073 ± 0.007

TABLE VII: Comparison of the phosphatase activity of CD45 and RPTPα immunoprecipitated from BW5147 T cell lines. Phosphatase activity was measured using saturating amounts of the fyn phosphopeptide (3.5 mM fyn pY531) as substrate in 50 mM Tris-Cl pH 7.4, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer. Data for each cell line was derived from 3 separate experiments. Undetectable levels of CD45 were immunoprecipitated from $CD45^-$ BW5147 cells, hence no phosphatase activity was detected from these samples (n/d).

phosphatase expressed by each cell was taken into account, CD45 was approximately 7-10 fold more active than RPTPα on a molar basis. A similar result was also obtained using p-NPP as the substrate indicating that the fyn peptide was not acting as a preferential substrate for CD45 (data not shown). This observation provided the first suggestion that the reason why RPTPα is unable to fully substitute for the activities of CD45 may be due to its reduced phosphatase activity.

Expression and purification of recombinant RPTPα cytoplasmic domain protein and GST p60^{c-src} fusion protein for subsequent PTP assays

To further investigate whether cellular factors such as post-translational modifications were responsible for these observed differences in phosphatase activity, the activities and specificities of recombinant CD45 and RPTPα proteins were determined. Recombinant proteins used in the *in vitro* phosphatase assays include recombinant 6HIS cytoplasmic CD45 fusion protein (see Chapter 5), recombinant GST p56^{lck} fusion protein (see Chapter 5), recombinant RPTPα cytoplasmic domain protein, and recombinant GST p60^{c-src} fusion protein.

GST-cytoplasmic domain of RPTPα was expressed and purified as described in the Materials and Methods. Recombinant RPTPα used in subsequent tyrosine phosphatase assays had the GST portion removed by thrombin cleavage at a thrombin recognition site present between GST sequences and cytoplasmic RPTPα sequences. GST p60^{c-src} fusion protein was also produced as previously described using the optimized protocol that included sonication and N-laurylsarcosine addition (Materials and Methods). Figure 6.5 shows a Coomassie blue stained SDS-PAGE gel depicting the purity of these recombinant proteins.
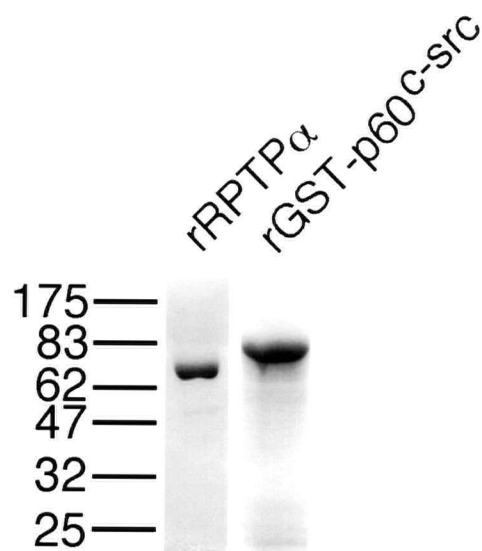
Figure 6.5: <u>Coomassie blue staining of additional purified recombinant proteins used in *in vitro* phosphatase assays</u>. Coomassie blue staining of cytoplasmic RPTPα domain protein (cleaved from GST using thrombin) and GST-p60$^{\text{c-src}}$ protein.

The pH optimum of recombinant GST cytoplasmic domain of RPTPα was also assessed and was found to be within pH 6.0 and 6.2 (see Figure 6.6). This pH optimum agrees with previously obtained data performed on immunoprecipated material (Daum et al., 1991).

In vitro activities and specificities of recombinant RPTPα and CD45 proteins using pNPP or phosphotyrosine containing peptides

To try and distinguish between qualitative and quantitative differences in activity, the V_{max} and K_m values for each phosphatase were determined using src family phosphopeptides and p-NPP as substrates (Table VIII). First, in agreement with the enzymatic activities determined from the immunoprecipated phosphatases, the activity of the recombinant cytoplasmic domain RPTPα was approximately 7-10 fold less active than the recombinant cytoplasmic domain CD45 when either saturating amounts of phosphopeptides or p-NPP were used as substrates at physiological pH, illustrating that RPTPα is intrinsically less active than CD45. The fact that RPTPα was consistently 6-11 fold less active than CD45 with all substrates tested indicated that quantitative differences, not qualitative differences, accounted for its inability to effectively dephosphorylate p56[lck]. Second, although the K_m values for recombinant RPTPα and recombinant CD45 were similar when p-NPP was the substrate, K_m values were 3-5 fold higher for recombinant RPTPα when src family phosphopeptides were used as substrates, indicating that CD45 had a higher affinity for these peptides than RPTPα. As a measure of overall enzyme efficiency, turnover numbers (k_{cat}) values (s^{-1}) were calculated to be approximately 9 for RPTPα and approximately 87 for CD45 using the src family phosphopeptides as substrates, confirming that CD45

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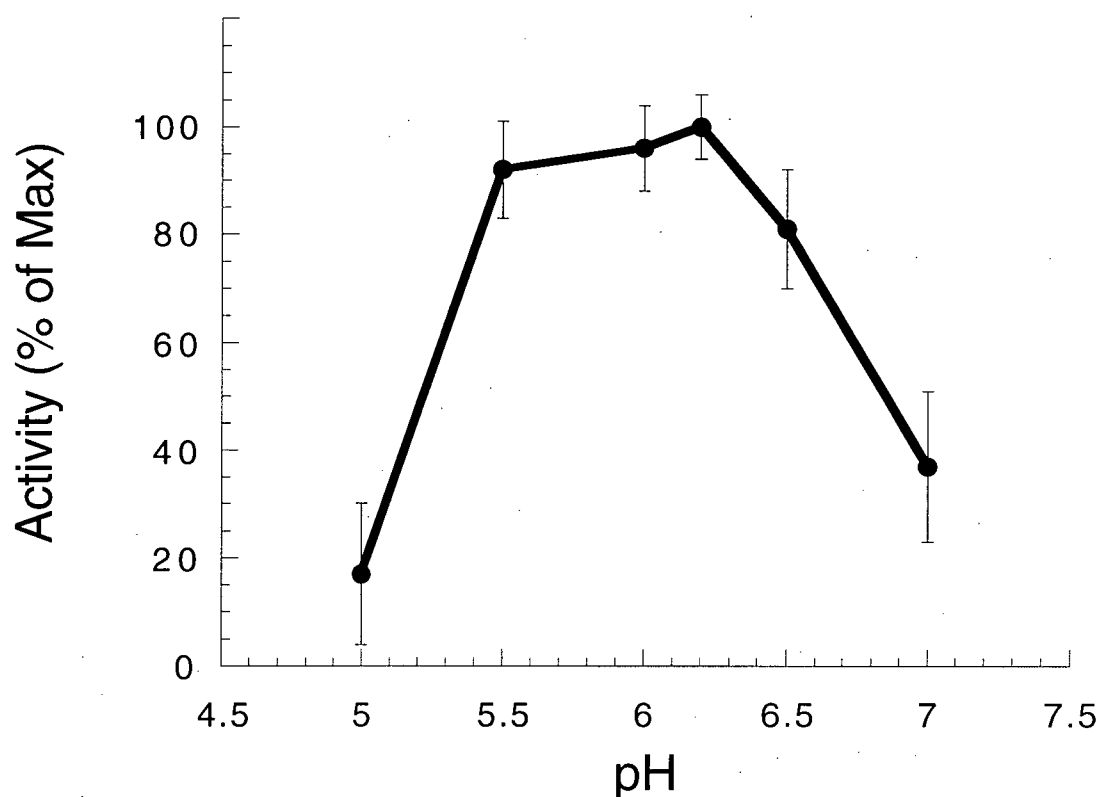
Figure 6.6: pH optimum for recombinant RPTPα cytoplasmic protein:
PTPase activity was measured using standard assay conditions with the
following buffers: 50 mM Mes, 1.0 mM EDTA, 0.1% β -mercaptoethanol at
pH 5.0, 5.5, 6.0, 6.2, 6.5 and 50 mM Tris-Cl pH 7.0, 1.0 mM EDTA, 0.1% β-
mercaptoethanol. (100% PTP activity is equivalent to approximately 18
μmol/min/mg).

Substrate	Vmax (μmol/min/mg)	K_m (mM)	K_{cat} (s^{-1})
Recombinant cytoplasmic CD45:			
pNPP	0.53±0.06	0.22±0.01	0.75±0.1
fyn pY531 (TATEPQpYQPGENL)	58.2±0.8	0.23±0.01	82±1.0
src pY527 (TSTEPQpYQPGENL)	60.6±0.7	0.23	85±0.9
src pY416 (LIEDNEpYTARQGA)	66.0±2.4	0.22±0.01	93±3.3
Recombinant cytoplasmic RPTPα:			
pNPP	0.05	0.22±0.02	0.06
fyn pY531 (TATEPQpYQPGENL)	8.3±0.2	1.07±0.03	10±0.2
src pY527 (TSTEPQpYQPGENL)	7.6±0.4	1.16±0.14	9±0.5
src pY416 (LIEDNEpYTARQGA)	7.1±0.2	0.77±0.02	9±0.2

TABLE VIII: <u>Comparison of kinetic values between recombinant cytoplasmic CD45 and RPTPα enzymes at physiological pH.</u> All assays were performed in 50 mM Tris-Cl pH 7.4, 1.0 mM EDTA, 0.1% β-mercaptoethanol PTP buffer (see Materials and Methods). V_{max}, K_m, and K_{cat} values were determined from Lineweaver-Burke and Eadie-Hofstee plots of the data and are expressed in units of (μmol/ min/ mg), (mM), and (s^{-1}) respectively.

was a more effective enzyme against these peptide substrates. Third, when the PTP activity of either phosphatase was examined individually, both enzymes were more active against src family phosphopeptides than the pNPP substrate. No major substrate preferences were observed for either phosphatase between the src family phosphopeptides tested. Thus recombinant RPTPα dephosphorylated recombinant p56[lck], src and fyn phosphopeptides, and pNPP all less effectively than CD45 at physiological conditions.

The kinetic parameters of each phosphatase were next analysed at their respective pH optima. Recombinant CD45 had exhibited a pH optimum of pH 7.2, whereas recombinant RPTPα display an optimum at pH 6.2. Again, pNPP and phosphopeptides encompassing phosphorylated regions of p60[c-src] or p59[fyn] were used. From Table IX, it is clear that under these conditions, the PTP activity of either phosphatase was increased. CD45 phosphatase activity increased slightly (~1.3 fold) under these conditions. In particular, when assayed at pH 6.2, recombinant cytoplasmic RPTPα PTP activity was increased approximately 2.0 to 2.5 fold for both the pNPP and phosphopeptides as compared to values obtained at pH 7.4. It is important to note, however, that even in these optimal pH conditions, the PTP activity of RPTPα was still 3 to 4 fold less than CD45 at physiological pH. When turnover values (K_{cat}) values were calculated, it was found that RPTPα now had a K_{cat} of approximately 22, whereas CD45 had a K_{cat} value of 115. Perhaps more striking was the change in K_m values for recombinant RPTPα at pH 6.2. The affinity values improved greatly under the optimal pH, exhibiting values of 0.14 mM compared to values obtained under physiological conditions that were approximately 1.0 mM. More significant is the fact that these values were better than

Substrate	Vmax (μmol/min/mg)	K_m (mM)	K_{cat} (s^{-1})
Recombinant cytoplasmic CD45:			
pNPP	0.70	0.20±0.03	1.0
fyn pY531 (TATEPQpYQPGENL)	81.0±1.0	0.23	115±1.4
src pY527 (TSTEPQpYQPGENL)	81.4±4.8	0.23±0.01	115±6.8
src pY416 (LIEDNEpYTARQGA)	79.8±4.3	0.22±0.01	114±6.1
Recombinant cytoplasmic RPTPα:			
pNPP	0.11	0.11±0.01	0.13
fyn pY531 (TATEPQpYQPGENL)	18.9±0.02	0.14±0.01	23±0.02
src pY527 (TSTEPQpYQPGENL)	19.4±0.16	0.14±0.01	24±0.19
src pY416 (LIEDNEpYTARQGA)	16.9±0.08	0.14	21±0.10

TABLE IX: <u>Comparison of kinetic values between recombinant cytoplasmic CD45 and RPTPα enzymes at optimal pH.</u> All assays for recombinant CD45 were performed at pH 7.2 and all assays for recombinant RPTPα were performed at pH 6.2 (see Materials and Methods). V_{max}, K_m, and K_{cat} values were determined from Lineweaver-Burke and Eadie-Hofstee plots of the data and are expressed in units of (μmol/ min/ mg), (mM) and (s^{-1}), respectively.

CD45 values which remained the same at its optimal pH of 7.2.

In vitro   activities and specificities of recombinant RPTPα and CD45 proteins using recombinant p56^{lck} or p60^{c-src}

In vitro phosphatase activities of purified recombinant cytoplasmic CD45 and RPTPα were next determined using GST-p56^{lck} as a substrate (Figure 6.7). At physiological pH, CD45 could dephosphorylate p56^{lck} within a minute, whereas only a small percentage was dephosphorylated by RPTPα after 30 minutes (Figure 6.7). This demonstrates that intrinsic differences in activity or specificity exist between these phosphatases, irrespective of whether the phosphatase was isolated from a T cell or produced as a recombinant protein in *E. coli.* However, when the phosphatase assays were repeated in conditions where the optimal pH of each phosphatase was used (pH 7.2 for CD45, pH 6.2 for RPTPα), it was noted that the ability of RPTPα to dephosphorylate recombinant p56^{lck} increased significantly (Figure 6.8).

In an effort to characterize substrate preference exhibited by either tyrosine phosphatase being examined, recombinant GST p60^{c-src} was utilized as another protein substrate. Figure 6.9 summarizes PTP assays done with recombinant CD45 or RPTPα vs recombinant GST-p56^{lck} or GST-p60^{c-src}. From the values, expressed in densitometric scanning O.D. units, there was no apparent substrate preference for either p56^{lck} or p60^{c-src}. RPTPα appeared to dephosphorylate both GST-p56^{lck} and GST-p60^{c-src} when assessed at either physiological pH or at the optimum pH of 6.2. Likewise, CD45 demonstrated no substrate preference between GST p56^{lck} or GST p60^{c-src} when assayed in physiological pH or at the optimal pH of 7.2.
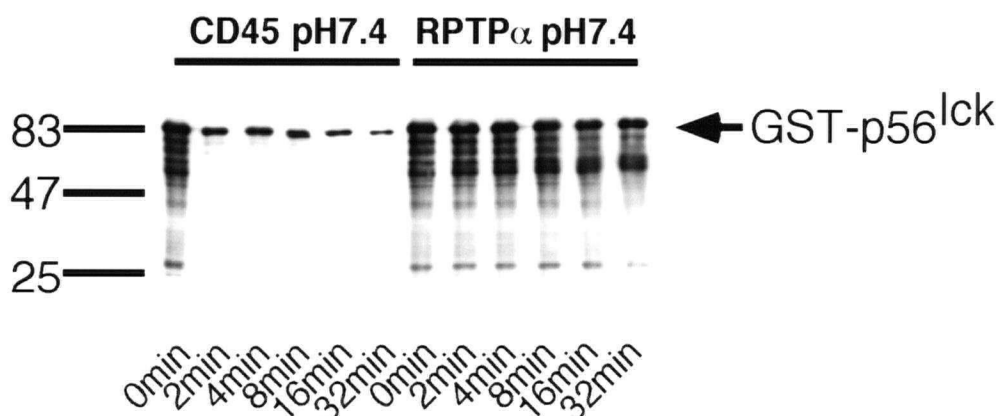
Figure 6.7: _In vitro_ phosphatase assay of recombinant cytoplasmic CD45 and recombinant cytoplasmic RPTPα using recombinant GST-p56[lck] protein as a substrate at physiological pH (pH 7.4). _In vitro_ phosphatase assays were initiated by adding approximately 50 ng of recombinant cytoplasmic CD45 or 44 ng of recombinant cytoplasmic RPTPα to 200 ng of recombinant GST-p56[lck] in a final volume of 10 µl of PTP buffer (see Materials and Methods). Reactions were stopped at various time points (2-32 mins) by immersing samples in a dry-ice ethanol bath. Samples were subsequently electrophoresed on a 10% SDS polyacrylamide gel, transferred to PVDF membrane and the level of phosphotyrosine remaining on GST-p56[lck] monitored by Western blotting with the anti-phosphotyrosine antibody (4G10). The predicted size for the GST-p56[lck] fusion protein is approximately 83 kDa.
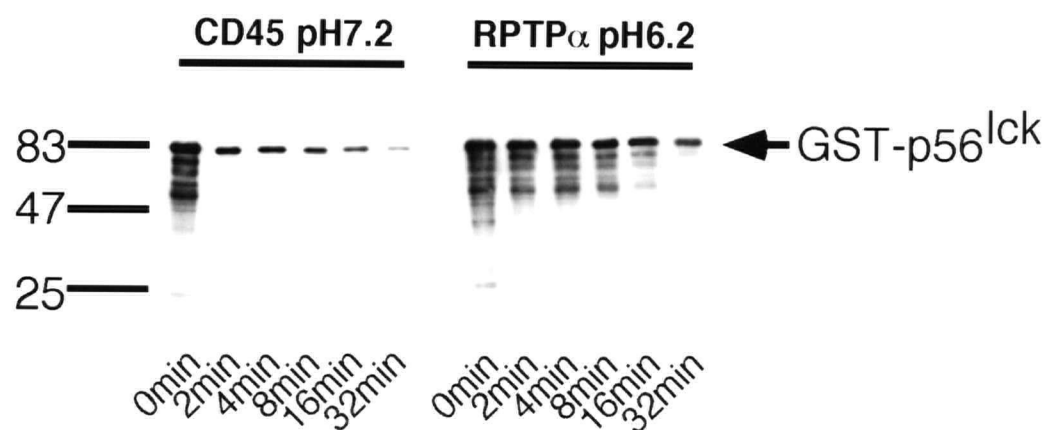
Figure 6.8: *In vitro* phosphatase assay of recombinant cytoplasmic CD45 and recombinant cytoplasmic RPTPα using recombinant GST-p56[lck] protein as a substrate at optimal pH. *In vitro* phosphatase assays were initiated by adding approximately 50 ng of recombinant cytoplasmic CD45 or 44 ng of recombinant cytoplasmic RPTPα to 200 ng of recombinant GST-p56[lck] in a final volume of 10 µl of PTP buffer (at pH 7.2 and pH 6.2 respectively)(see Materials and Methods). Reactions were stopped at various time points (2-32 min) by immersing samples in a dry-ice ethanol bath. Samples were subsequently electrophoresed on a 10% SDS polyacrylamide gel, transferred to PVDF membrane and the level of phosphotyrosine remaining on GST-p56[lck] monitored by Western blotting with the anti-phosphotyrosine antibody (4G10).

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A

Phosphotyrosine Content (%)

100
80
60
40
20
0

0 4 8 12 16 20 24 28 32
Time (minutes)

B

Phosphotyrosine content (%)

100
80
60
40
20
0

0 4 8 12 16 20 24 28 32
Time (minutes)
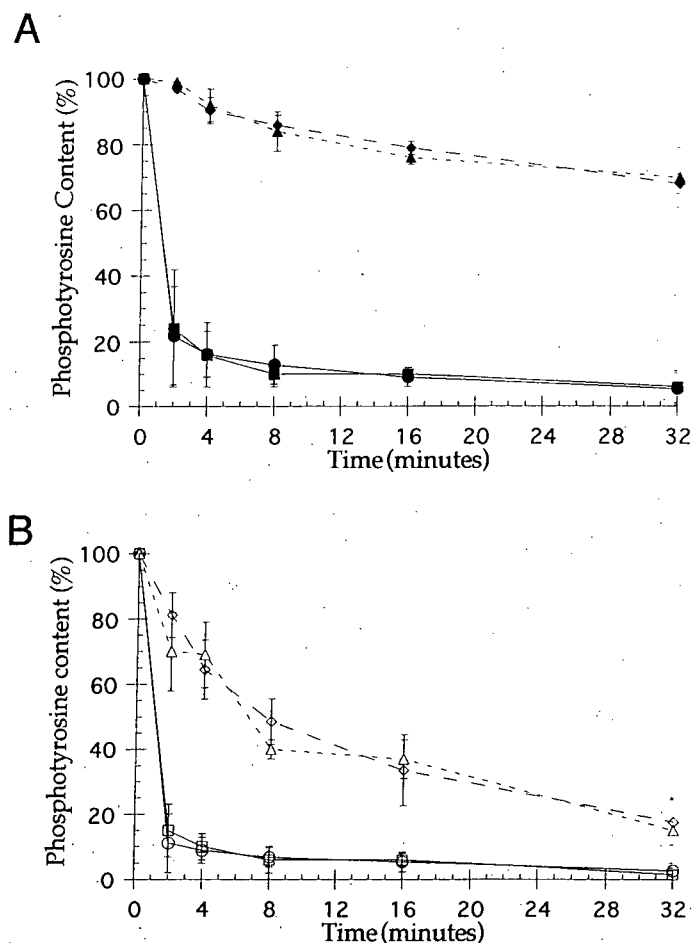
Figure 6.9: PTP assays using recombinant CD45, RPTPα, GST p56^{lck}, and GST p60^{c-src} proteins at physiological or at optimal pHs: *In vitro* phosphatase assays were performed at physiological pH (A) or at optimal pHs (B). Approximately 50 ng of recombinant cytoplasmic CD45 or 44 ng of recombinant cytoplasmic RPTPα to 200 ng of recombinant GST-p56^{lck} or GST-p60^{c-src} protein in a final volume of 10 μl of PTP buffer (50 mM Tris-Cl pH 7.2 or 7.4, 1.0 mM EDTA, 0.1% β-mercaptoethanol)(50 mM MES pH 6.2, 1.0 mM EDTA, 0.1% β-mercaptoethanol). The following combinations were used: ●, CD45 and p56^{lck} at pH 7.4; ■, CD45 and p60^{c-src} at pH 7.4; ◆, RPTPα and p56^{lck} at pH 7.4; ▲, RPTPα and p60^{c-src} at pH 7.4; O, CD45 and p56^{lck} at pH 7.2; □, CD45 and p60^{c-src} at pH 7.2; , RPTPα and p56^{lck} at pH 6.2; Δ, RPTPα and p60^{c-src} at pH 6.2. Reactions were stopped at various time points (2-32 mins) by immersing samples in a dry-ice ethanol bath and SDS-PAGE performed. Western blot analysis and subsequently densitometric scanning of membranes allowed the determination of relative rates of dephosphorylation as assessed by optical density units.

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The ability of recombinant p60^{c-src} to associate with CD45 or RPTPα was ascertained in a binding assay similar to one previously described. Briefly, recombinant GST p60^{c-src} kinase was immobilized and soluble inactive C817S recombinant CD45 or sodium orthovanadate treated RPTPα protein was added. Beads were then washed and association of CD45 or RPTPα was detected in Western blot analysis as shown in Figure 6.10. Here, the data indicated that p60^{c-src} was able to specifically interact with both CD45 and RPTPα. This is in contrast to results obtained with p56^{lck}, whereby p56^{lck} specifically associated with CD45 and not with RPTPα.

6.3 Discussion

Expression of RPTPα in CD45$^-$ BW5147 T cells to equivalent levels observed for CD45 in CD45$^+$ BW5147 T cells resulted in a partial induction of tyrosine phosphorylated proteins upon TCR/CD3 stimulation. Further analysis of the induction of tyrosine phosphorylation in RPTPα$^+$ cells indicated that the induction occurred with both less intensity and slower kinetics when assessed over a thirty minute period (Ng et al., 1997). Consistent with this reduced level of induction, no increase was observed in the tyrosine phosphorylation state of CD3ζ after TCR/CD3 stimulation. Analysis of CD69 upregulation, a distal event associated with T cell activation, indicated that expression of RPTPα in the CD45$^-$ BW5147 cells was also not able to fully restore downstream TCR/CD3 mediated signaling events. Overall, these data indicate that the tyrosine phosphatase CD45 is specifically required for the efficient progression of
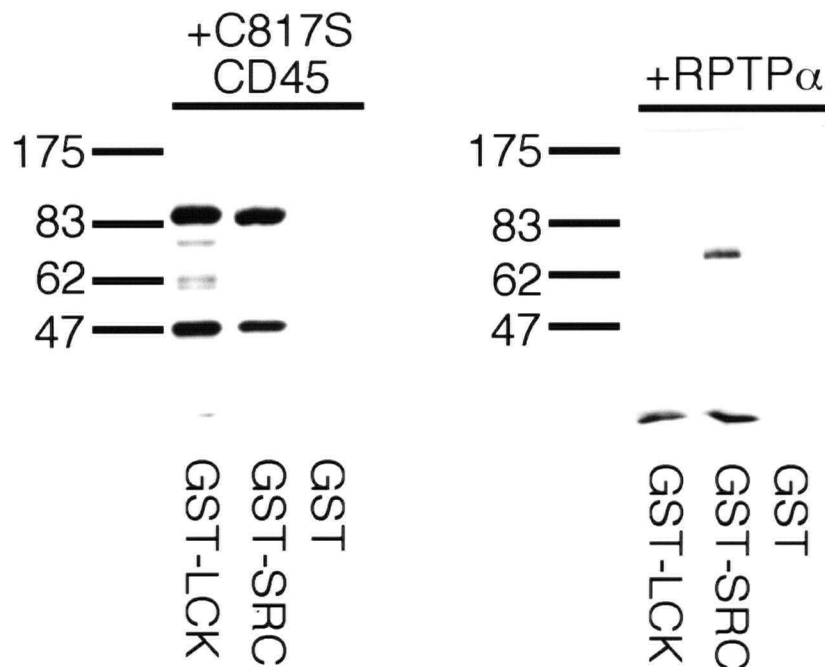
Figure 6.10: <u>Binding of GST p56^{lck} or GST p60^{c-src} fusion proteins to the cytoplasmic domains of CD45 or RPTPα.</u> Western blot analysis of recombinant PTP proteins remaining bound to the immobilized GST p56^{lck} (GST-LCK), GST p60^{c-src} (GST-SRC) proteins or to the control GST (GST) alone protein. Recombinant PTP proteins were detected using anti-CD45 (R01.1) or anti-RPTPα (PTPα-2) antisera. 1.0 μg of the soluble recombinant C817S cytoplasmic CD45 protein (+C817S CD45) or thrombin cleaved RPTPα protein (+RPTPα) were added to 2.0 μg of immobilized GST src-kinase proteins and incubated for 2 hours at 4°C in 40 μl binding buffer (20 mM Tris-Cl pH 7.5, 150 mM NaCl, 0.025% β-mercaptoethanol, plus protease and PTP inhibitors). The beads were washed 3 times in RIPA buffer and subjected to SDS PAGE.

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TCR/CD3 initiated signaling events. CD45, but not RPTPα, is required for the optimal phosphorylation of CD3ζ, the generation of a rapid and strong induction of tyrosine phosphorylation upon TCR/CD3 stimulation, and for the induction of downstream signaling events, as illustrated by the expression of the T cell activation antigen, CD69.

Phosphorylation of CD3ζ is thought to be mediated by p56^{lck}, a tyrosine kinase involved in the initiation of TCR/CD3 signaling events (Iwashima et al., 1994). In order to function effectively in this process, p56^{lck} needs to be dephosphorylated at its negative regulatory tyrosine which occurs in CD45$^+$ cells, but not in CD45$^-$ T cells (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Hurley et al., 1993). Arpita Maiti has characterized p56^{lck} in the RPTPα$^+$, BW5147 T cells (Ng et al., 1997). These results indicated that p56^{lck} remained in its phosphorylated form consistent with it being an inefficient substrate for RPTPα. The inability of phosphorylated p56^{lck} to participate effectively in TCR mediated signal transduction, is consistent with the observed lack of CD3ζ phosphorylation and overall reduced induction of tyrosine phosphorylation.

In the same study, observations demonstrated that CD45 had a distinct effect on p59^{fyn} (Ng et al., 1997). Although no significant difference was observed in the overall phosphorylation state of p59^{fyn} between CD45$^+$ and CD45$^-$ BW5147 T cells, a consistent difference in *in vitro* kinase activity was observed whereby p59^{fyn} kinase activity is upregulated in the presence of CD45. Once again, RPTPα was unable to cause any detectable change in either the phosphorylation state or kinase activity of p59^{fyn} indicating that RPTPα cannot mimic the effect of CD45

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on $p59^{fyn}$ in these cells. However, due to the sensitivity of the assays, it is difficult to exclude the possibility that RPTPα may have had a slight effect on $p56^{lck}$ or $p59^{fyn}$ which may, in turn, have led to the low level of induction of tyrosine phosphorylated proteins seen in Figure 6.2.

By expressing RPTPα at similar levels to CD45 in BW5147 T cells, we were able to directly compare the effects of the two phosphatases. Comparison of the catalytic activities of these two phosphatases immunoprecipitated from BW5147 T cells indicated that, when tested against two artificial substrates, CD45 was approximately 7-10 fold more active than RPTPα. This difference in catalytic efficiency between CD45 and RPTPα was also observed *in vitro* at physiological pH, using recombinant enzymes purified from *E. coli*, indicating that intrinsic factors alone could account for the difference in activity observed in the BW5147 T cells. However, it is possible that additional factors, such as post-translational modifications, cellular locale or cellular interactions, may further contribute towards CD45 specific functions in the T cell. It is also important to point out that expression of transfected CD45 into the CD45 negative cells failed despite repeated attempts (P. Johnson, P. Borodchak, pers. comm.). Therefore, there exists the possibility that RPTPα^{+} cells were unable to fully restore signaling due to an unknown defect present in the CD45^{-} BW5147 cells.

The ability of CD45, but not RPTPα, to significantly dephosphorylate relatively low concentrations (approximately 0.2 mM) of recombinant GST-p56^{lck} was consistent with RPTPα having a lower activity and reduced affinity for $p56^{lck}$ than CD45. Even when 10 fold more RPTPα was used to generate equivalent V_{max} values using phosphopeptide

substrates, RPTPα was still unable to dephosphorylate GST-p56^{lck} as efficiently as CD45 (data not shown). These data indicated that both in T cells and *in vitro*, p56^{lck} was preferentially dephosphorylated by CD45 and not by RPTPα, suggesting differences in substrate specificity. However, further comparison of phosphatase activities with phosphopeptide and pNPP substrates revealed that RPTPα was consistently less active than CD45, implying that quantitative differences in phosphatase activity may account for the differential dephosphorylation of p56^{lck}. Taken together, these results suggest that the high catalytic efficiency of CD45, coupled with its abundance at the cell surface, contributes towards the specific function of CD45 in T cells. Recombinant CD45 has also been shown to be catalytically more active when compared to three other recombinant two domain phosphatases (Pulido et al., 1995), supporting the notion that CD45 is a very efficient phosphatase.

Factors that make one phosphatase more catalytically active than another are presently unknown. Interestingly, RPTPα and CD45 have different pH optima (pH 6.2 for RPTPα and pH 7.2 for CD45) (Daum et al., 1991; Itoh et al., 1992; Ng et al., 1995 and Harder and Jirik, unpublished observations), suggesting that the environment surrounding their catalytic centres may be different. The activity of the two recombinant phosphatases at pH 7.4 was consistent with the observed activity of the two phosphatases isolated from T cells. Comparison of phosphatase activities at their respective pH optima indicated that CD45 was still 4-5 fold more catalytically active than RPTPα on the substrates tested. Having a pH optima of 6.2, which is not particularly close to physiological pH, has led other researchers to propose that RPTPα may exist in two conformations, a

high and low affinity form (Daum et al., 1991). If this is so, then it is possible that in BW5147 T cells, RPTPα is present in its low affinity form. An additional consideration to take in account is the possibility that the lower pH causes the substrates to be in a more favourable conformation or ionization state for dephosphorylation. However, this is unlikely as the work outlined in this Chapter examined six different substrates, which would indicate that the effect was due to the enzyme itself.

Previous reports have demonstrated that when evaluating data from CD45 deficient T cell lines, CD45 appears to exhibit substrate preference for p56^{lck} over other src-family kinases such as p59^{fyn} and p60^{c-src} (Hurley et al., 1993). Furthermore, there have been several studies that indicate p60^{c-src} as a potential *in vivo* substrate for RPTPα (Zheng et al., 1992; den Hertog et al., 1993). Consequently, recombinant GST p60^{c-src} fusion protein was expressed and purified for *in vitro* characterizations. Data presented in this chapter has shown that no substrate preferences are observed for either p56^{lck} or p60^{c-src} proteins, when either recombinant CD45 or recombinant RPTPα proteins are used in a tyrosine phosphatase assay. Taking all data into account, it would appear that the most likely mechanism of substrate specificity observed in cells is due to the general enzymatic efficiency and expression levels of the PTP itself, as well as the expression levels of each potential substrate being assessed. Perhaps, the difference in src-kinase phosphorylation profiles seen in CD45 deficient cells are due to higher levels of expression of p56^{lck} when compared to expression levels of p59^{fyn} and p60^{c-src}. However, it is important to note that regulation of cellular locale or cellular interactions of both the phosphatases and the *in vivo* substrates may provide an alternative explanation.

Interestingly, when binding assays using recombinant CD45, RPTPα, p56^{lck} and p60$^{c\text{-}src}$ were performed, CD45 was shown to associate with both src-family kinases (Figure 6.10) in agreement with the idea of physiological substrate preference being dictated by mechanisms described in the preceeding paragraph. This result also refutes an hypothesis brought up in Chapter 5, that substrate specificity between CD45 and p56^{lck} is due to a specific and direct association between the two molecules. Under the same hypothesis and the binding results presented in Figure 6.10, one would expect p60$^{c\text{-}src}$ phosphorylation levels to be similarily affected in CD45 negative cell lines. The same figure also demonstrates that RPTPα can specifically bind to recombinant p60$^{c\text{-}src}$ but not recombinant p56^{lck}. This contrasts with the *in vitro* phosphatase assays using recombinant proteins, which would indicate that both src-family kinases are acted upon equally. The physiological relevance of this final observation is yet to be determined and also falls into the caveat of being performed in an *in vitro* setting. However, it does provides further evidence that the transmembrane PTPs may differ in how they achieve specificity and in how they function under cellular situations.

To conclude, equivalent levels of RPTPα could not substitute effectively for CD45 in TCR mediated signaling events. The fact that RPTPα immunoprecipitated from BW5147 T cells was found to be 7-10 fold less active than CD45 provided one explanation for the observed effects. *In vitro* studies using purified recombinant enzymes also found a 7-10 fold difference in activity illustrating that the observed difference in activity between the phosphatases isolated from T cells was not due to cellular inactivation or post-translational mechanisms. These results

demonstrate that CD45 and RPTPα do have distinct activities in T cells and that this can be attributed, at least in part, to quantitative differences in phosphatase activity. Furthermore, when substrate preference for phosphopeptides or recombinant proteins was assessed for each phosphatase individually, no concensus for specificity was observed. This highlights the possibility that substrate specificity is strongly dictated by different levels of phosphatase and different levels of substrate expressed in cells. Therefore, we caution the interpretation of studies derived from transfected cell lines where artificially high levels of enzyme and/or substrate are present in the cell.

CHAPTER 7

General Discussion

The work in this thesis focused on the hematopoietic glycoprotein, CD45. This molecule is, perhaps, the best characterized receptor PTP to date (reviewed in Johnson et al., 1997), and as reviewed in the Introduction, has been demonstrated on numerous accounts to play a major role in immune cell antigen triggered signaling pathways. Furthermore, there are many reports that examine the structural and enzymatic traits of this molecule. However, despite the volume of research that has occurred and is occurring, there are still several prevalent questions regarding the function of CD45 that have not been fully addressed.

First, it has been well established that within the context of T cells, CD45 exhibits substrate preferences between molecules of similar structures, such that the dephosphorylation of $p56^{lck}$ appears to be favoured over the dephosphorylation of other src-family kinases, such as $p59^{fyn}$ or $p60^{c-src}$ (Cahir McFarland et al., 1993; Hurley et al., 1993; Burns et al., 1994). However, the exact mechanism that dictates this substrate preference is still unknown. Furthermore, the complete details of how $p56^{lck}$ and CD45 interact and affect one another is also unknown. Second, CD45, like many receptor PTPs, has a cytoplasmic domain that contains two distinct regions of PTP homology (Charbonneau et al., 1988). The dynamic nature of these two discrete domains, their function and their regulation has yet to be determined, but is likely to add a degree of complexity to the mechanism of action of all dual phosphatase domain PTPs. Third, there exists the continued search for proteins that interact with CD45, and the subsequent characterization of these signaling events to ascertain additional features of CD45 function. An example of this, concerns the association between CD45 and the small 30 kDa protein,

CD45-AP or LPAP (Schraven et al., 1994; Takeda et al., 1994). This interaction has been well characterized and has been demonstrated to occur at high stoichiometry (Cahir McFarland and Thomas, 1995; Kitamura et al., 1995; Cahir McFarland et al., 1997). However, the relevance of this lymphoid specific association still remains elusive.

This thesis has presented work that provides further insights on the subject of enzyme/substrate specificity and contributes to the delineation of how substrate preferences are achieved. However at its most rudimentary state, this thesis also describes the development of a non-radioactive PTP assay and of an expression/purification protocol which yields milligram amounts of homogenous recombinant CD45 cytoplasmic domain protein. Both of these tools can be utilized in future attempts to address the questions regarding both the function of dual phosphatase domain PTPs and the characterization of protein interactions with CD45.

A predominant function of CD45 is its ability to dephosphorylate and consequently regulate the function of src-family kinases. However, as previously mentioned, it is apparent that CD45 exhibits clear substrate preferences among members of this kinase family despite the relatively high sequence identity between these proteins. Consequently, a major focus of the work described here pertained to the elucidation of a possible explanation for this substrate specificity. In Chapter 3, data was presented using immunoprecipitated CD45 in a non-radioactive PTP assay. Here, the substrates used were phosphotyrosine containing peptides encompassing sequences derived from the carboxyltail regulatory tyrosine of src family kinases. These peptides were chosen since the same tyrosine residue is also the predominant *in vivo* phosphorylation site of src-family kinases,

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that was affected by the expression of CD45 in T cell lines (Ostergaard et al., 1989; Cahir McFarland et al., 1993; Hurley et al., 1993; Burns et al., 1994). In agreement with other published reports, the *in vitro* determination of PTP activity using recombinant CD45 and src-family phosphopeptides, showed no significant substrate specificity (Cho et al., 1992; Cho et al., 1993; Pacitti et al., 1994). Therefore, these data indicate that the CD45 substrate preferences observed in cells is not determined by the sequences immediately adjacent to the site of dephosphorylation.

Our data highlighting a direct and specific interaction between the cytoplasmic domain of CD45 and p56^{lck} provides a possible explanation for why CD45 substrate specificity is observed in cells lines but not when *in vitro* PTP assays were performed using phosphopeptides as substrates. The fact that several distinct regions of p56^{lck} appeared to contribute to the association with CD45 further argued that the intact protein and not just a small section of the protein was crucial for the enzyme-substrate interaction. Therefore, the specificity observed in the physiological setting of the cell may be a result of a relatively high affinity interaction between CD45 and p56^{lck} that exists by virtue of structural requirements for the association. The regions of p56^{lck} that were shown to interact with CD45 included the unique amino terminal region, the SH2 domain and the kinase domain. In essence, these three domains constitute approximately 90% of the total amino acid sequence, and likewise indicate that the interaction between CD45 and p56^{lck} is quite complex. It is still not clear whether all aforementioned domains partake in the association in unison, or whether each domain has a distinct role and will only interact with CD45 under certain circumstances. However, the three dimensional structure of src-family kinases would suggest that the latter situation is a

possibility (Sicheri et al., 1997; Xu et al., 1997). As src kinases can undergo conformational changes in their tertiary structure, it is conceivable that the role and position of the distinct regions of p56^{lck} could exist in several different forms.

Under this hypothesis, one would then argue that substrate preferences would be observed under *in vitro* conditions, if PTP assays were performed using intact proteins as substrates, rather than the 11 - 13mer phosphopeptides. However, when *in vitro* phosphatase assays were performed using recombinant cytoplasmic CD45 against recombinant GST-p56^{lck} or GST-p60^{c-src} fusion protein, it was apparent that even in these circumstances, no differences in PTP rate were observed. Furthermore, when binding assays were done with immobilized GST-p60^{c-src} protein and soluble recombinant C817S cytoplasmic CD45, an interaction between these two molecules was observed. Therefore, the similarity of *in vitro* PTP activity and binding results when using p60^{c-src} and p56^{lck} suggest that the simple difference in the ability to associate with CD45 is not an acceptable explanation for achieving substrate preferences in cells.

It should be noted however, that analysis of the PTP results using recombinant proteins as substrate must be approached with caution. This is primarily because the phosphorylation states of the recombinant p56^{lck} and of recombinant p60^{c-src} have not been fully characterized. Previous reports had shown that the majority of phosphorylation observed in recombinant GST-p56^{lck} was found on Tyr-394, whereas a small but significant amount of phosphorylation was found on Tyr-505 (Jullien et al., 1994) . The direct result of this information, is that PTP assays performed using intact recombinant proteins as substrates may not truly

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reflect specificity of CD45 against the carboxyltail tyrosine residues found on the src kinases. It is quite possible that the PTP assays simply demonstrate that there is no substrate preference of CD45 when assessing the ability to dephosphorylate the autophosphorylation sites of either p56^{lck} or of p60^{c-src}. Consequently, it may be interesting to reassess these results using the mutant forms of p56^{lck} or p60^{c-src} which contain the appropriate tyrosine to phenylalanine point mutations. These measures would ensure that results observed are restricted to a particular phosphorylation site. Another cautionary point regards the fact that PTP assays using recombinant proteins as substrate, were performed in conditions of extreme substrate limitation. In these circumstances, the concentration of the substrates were approximately 60 nM. Therefore, it becomes very important to know the degree of phosphorylation on both the negative regulatory tyrosine and the autophosphorylation tyrosine, since even small discrepancies can greatly affect the results obtained. Therefore, one must be careful with the perceived results, as they may be due simply to differences in phosphorylation levels rather than in substrate specificity per se. Since it is obviously not feasible to perform these assays at substrate saturating conditions (approximately 2.5 mg of protein per 10.0 μl reaction volume would be required), then the use of tyrosine to phenylalanine mutants and the careful monitoring of phosphotyrosine amounts through Western blot analysis would, again, be a useful method of reassessing substrate specificity.

The concentration of substrates would also provide a contrary argument. Here, the *in vitro* PTP assays that were performed to ascertain kinetic values using phosphopeptides, are probably not indicative of PTP activities seen at substrate concentrations found in a physiological setting.

Consequently, kinetic values such as V_{max} may have no real significance, as the cellular concentrations of $p56^{lck}$ and $p59^{fyn}$ are unlikely to reach levels required for maximal reaction rates.

Withstanding the above caveats, one can now state that the simplest hypothesis concerns the idea that the expression levels of substrates is the dominant factor in dictating substrate preference. Since it is well established that substrate concentration can greatly affect enzymatic rate, then the substrate specificity observed in cell lines may simply be due to $p59^{fyn}$ and $p60^{c-src}$ being expressed at significantly lower levels than $p56^{lck}$. In agreement with this scenario, $p56^{lck}$ protein levels are higher than $p59^{fyn}$ levels in the BW5147 T cells used in this thesis (P. Johnson, unpublished results). It is interesting to note that careful analyses and comparisons of the expression levels of $p56^{lck}$ and $p59^{fyn}$ is not performed in any of the papers examining phosphorylation effects due to CD45 expression in lymphoid cell lines. However, there is one report which analyses the protein expressions of both src-family kinases in thymocytes subsets, peripheral T cells, NK cells, and in certain lymphoid cell lines (Olszoy et al., 1995). It would therefore be useful for researchers to integrate this knowledge when assessing CD45 substrate preferences. Furthermore, examination of the effects of CD45 expression in a B cell line clearly show that CD45 had a greater effect on the phosphorylation state of $p53/p56^{lyn}$ than on $p56^{lck}$ (Katagiri et al., 1995; Yanaga et al., 1996). Although in stark contrast to observations in T cells, the preference for $p53/p56^{lyn}$ could perhaps be attributed to higher protein expression levels. This same hypothesis could extend to PTPs in general. Data from analysis of the substrate specificity of RPTPα exhibited similar trends. However, it

is interesting to point out that RPTPα was shown to interact specifically with p60^{c-src} and not p56^{lck}, thereby suggesting that discrete differences do exist between PTP's in determining specificity.

At this point, one should also consider alternative hypotheses to the notion that protein expression levels dictate substrate preferences seen in cells. Most obvious, is the possibility of substrate specificity being defined by interactions with a third party. This third could be another signaling molecule, that may have adapter-like functions. Under this scenario, the degree of substrate specificity would not necessarily correlate with the expression levels of the substrate, but rather correlate with the expression of other molecules that can affect the association between the enzyme and the substrate. Despite data showing a stable and direct interaction between recombinant cytoplasmic CD45 and recombinant p56^{lck} under the *in vitro* conditions, it is interesting to point out that in all *in vivo* p56^{lck}/CD45 coprecipitation studies to date, there is always the detection of additional proteins in the immunocomplex (Koretzky et al., 1993; Ross et al., 1994; Lee et al., 1996). Indeed, proteins such as CD45-AP or LPAP have already been postulated to function as adaptor-like proteins in the physiological setting (Cahir McFarland and Thomas, 1995). It is therefore quite possible that the presence of proteins like CD45-AP may increase the affinity of p56^{lck} towards CD45. At present, there is no direct evidence for this phenomenon. Indeed, some evidence exists that CD45-AP is not required for CD45 to dephosphorylate p56^{lck}, as shown in a report using a reconstitution system to drive expression of CD45 and p56^{lck} in a non-lymphoid cell line (Gervais and Veillette, 1997). However,

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this same study did not address whether the affinity values between CD45 and p56^{lck} were altered in any way by the presence or absence of CD45-AP.

Cellular locale of either CD45 or its putative substrates may also provide an explanation for the substrate preferences observed. Under this system, the restricted localization or compartmentalization of the different src-family kinases would dictate the accessibility of these substrates for CD45. In agreement with this, there is some evidence suggesting that p56^{lck}, p59^{fyn} and p60^{c-src} reside in prefered cellular compartments. For example, reports have demonstrated that p59^{fyn} may localize to microtubule elements, whereas p60^{c-src} can associate with endosomal structures (Ley et al., 1994).

Another possibility is that a post-translational modification of CD45 may alter the activity of CD45 in such a way that substrate preference is achieved. Evidence for this has been seen in studies where phosphorylation of CD45 appears to modulate its PTP activity (Autero et al., 1994; Stover and Walsh, 1994). Under the context of the experiments described in this thesis, it is quite possible that the conditions required for this putative phenomenon have not been met. This is particularly relevant as p56^{lck}, and therefore perhaps other kinases, can efficiently phosphorylate CD45 (D. Ng, unpublished data).

In summary, the data presented in this thesis indicate that the continued delineation of the association between CD45 and p56^{lck} will require detailed and carefully controlled analysis. The data also revealed that the structures of these two signaling molecules entail a high degree of complexity. This is further exacerbated since the results presented in this thesis have not even addressed the possibility that CD45, itself, may undergo conformational changes with resultant effects on its ability to

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associate and/or dephosphorylate p56^{lck}. Efforts to examine substrate specificity under an *in vitro* system has also demonstrated that CD45 appeared to show no preference when presented with p56^{lck} or p60^{c-src} as substrates. Interestingly, the similar kinetic and binding results obtained using p56^{lck} or p60^{c-src}, would suggest that the details of this interaction will extend to other src-family kinases as well.

Ultimately, however, the interaction observed and presented in this body of work, provides insight into the enzymatic mechanisms that CD45 utilizes to dephosphorylate p56^{lck}. However, one must be careful in evaluating the data when using *in vitro* assays and recombinant proteins. In a physiological setting, the enzyme-substrate interaction is likely to be transient in nature, and is also likely to occur at protein concentrations far below those used in our experiments. In support of this, co-precipitation studies of CD45 and p56^{lck} have consistently demonstrated that only a small percentage of p56^{lck} is associated with CD45 (Koretzky et al., 1993; P. Johnson and M. Jabali, unpublished data). Consequently, the conditions in the *in vitro* experiments, which entail the use of inactive recombinant proteins may serve to enhance and/or stabilize an otherwise brief enzyme-substrate association. Despite this obvious caveat, this approach has enabled the initial delineation of the mechanisms involved in the binding of these two molecules towards the catalytic event. Again, the high degree of complexity is stressed. However, in light of the elaborate regulatory mechanisms of src-family kinases, it is not that surprising that CD45 must target its substrate in such a sophisticated manner.

Despite the usefulness of the data described in this thesis in providing additional information and discussion into questions of how the substrate enzyme interaction occurs and of how *in vivo* substrate preferences may arise, it is also important to assimilate these observations into an immunological context. Data presented here can therefore make the following predictions concerning the relevance of CD45 in T cell signaling and ultimately T cell function.

First, if the hypothesis of substrate preference being predominantly caused by expression levels of substrate is correct, then one would argue that the delicate control of how signaling molecules may interact (in this case, CD45 and src-family kinases) is maintained simply by expression levels as well. More importantly, it stresses the point that the putative functions of signaling molecules cannot be predicted by the gross determination of whether molecules are present or absent in a cell. Rather, one must also consider the degree to which protein expression has occurred. This notion has far reaching consequences and forces investigators, in order to ascertain the proper consequences of their observations, to include the analysis of expression levels of the proteins they are studying. This standard would apply both to work on cells directly purified from animals and also to work on manipulated cell lines that contain cDNA constructs so that protein expression can occur. One can imagine that the discrepancies of protein expression levels from cell to cell may explain many of the inconsistencies observed in the research literature.

Second, the data shown in this thesis provides further detail into the importance of CD45 during T cell activation. In particular, it stresses the complexity of the interaction between CD45 and p56^{lck}, and provides

additional evidence that CD45 would be capable of controlling the conformational state of p56^{lck} by virtue of its ability to interact with several of the discrete domains of p56^{lck} and by its ability to dephosphorylate the protein. CD45 may therefore affect both the kinase activity of p56^{lck} and the availability of its protein binding domains for interactions with other signaling molecules. The implications of this are profound as p56^{lck} is a major focal point of T cell signaling both in the periphery and in the context of T cell development (review in Weiss and Littman, 1994). Furthermore, although the interaction between p56^{lck} and CD4 has been clearly established, the demonstration of a direct and specific interaction between CD45 and p56^{lck} highlights an additional mechanism for p56^{lck} to be securely localized in areas of the cytoplasm where an activated kinase may be required to propogate a T cell activation signal.

Finally, it can be argued that continued research in CD45 function will reap many benefits. CD45 has several characteristics that make it an excellent candidate for research in the treatment of ailments that specifically affect cells of the immune system. CD45 has restricted expression on immune cells, and can therefore be used to target diseases that concern cells of the hematopoietic system. In addition, there is an even greater degree of specific tissue distribution when evaluating the expression of certain isoforms of CD45 (reviewed in Johnson et al., 1997). This would allow very precise targeting of drugs to particular cell types, and may therefore enable a drug to work at much lower dosages. Another useful trait of CD45 is that this molecule exists as a transmembrane molecule harbouring an extracellular region. Consequently, CD45 function, and more specifically, PTP activity, may be modulated with

compounds that do not even need to enter the cell cytoplasm. Exciting glimpses into this strategy have already been documented with the use of an isoform specific antibody to prevent allograft rejections (Lazarovits et al., 1992). Taken together, the aforemention traits make CD45 a very attractive molecule for therapeutic studies but will also entail the continued and substantial research in the area of the extracellular domain.

Yet without doubt, the most important characteristic of CD45, is it's central role in the antigen mediated activation signal. Consequently, the knowledge gained in CD45 research, including data presented in this thesis, will provide additional strategies upon which to base novel therapies on. This would primarily concern information on the regulation of both PTP activity and PTP substrate specificity of CD45. For example, one can envision the use of carefully designed molecules that could compete for any one of the interactions defined in this thesis. Subsequently, this may enable investigators to regulate CD45's involvement in the activation process and thus provide a means to regulate the activation signal itself. This has important implications as one could negate detrimental transforming signals that are common place in many leukemias. Moreover, it may be beneficial in many instances to upregulate activity, and thereby allow the immune cells to function in a more sensitive state. Such ideas may be useful for treatment of patients who are immunosuppressed or immunocompromised. For example, these strategies could result in enhanced immune responses in immunodeficient patients with A.I.D.S.

To conclude, there are many opportunities to be gained from the research done in this thesis. The knowledge presented here and acquired

may be added to the long list of research done on CD45 to elucidate its role in enzyme activity, substrate preferences and in T cell signalling in general. Furthermore, this same knowledge may be applied to more clinical uses in the rational design of drugs. This may one day allow physicians to essentially tailor immune responses to the particular needs and requirements of the patient in question. This thesis also highlights the strengths and weaknesses of work done under *in vitro* systems. Despite caveats created by the mere fact that experiments are not done under complete physiological settings, the use of *in vitro* techniques confers a high degree of experimental control to obtain very defined conditions, such that acquisition of information can occur with both speed and ease. These initial *in vitro* observations thus provide a good first step in elucidating functional mechanisms that may have otherwise gone undetected if investigators were restricted to *in vivo* techniques. I have no doubt that the *in vitro* methods defined and established in this thesis will have continued use in the study of CD45 function.

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