MOLECULAR ANALYSIS OF THE DOMAINS IN CD45 THAT AFFECT EXPRESSION AND FUNCTION

by

ARPITA MAITI

B. Sc.(Hon.), The University of Toronto, Trinity College, 1990

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

Department of Microbiology and Immunology

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

September, 1994

In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

(Signature)

Department of Microbiology & Immunology

The University of British Columbia
Vancouver, Canada

Date Oct 6, 1994

ABSTRACT

The aim of this thesis was to further understand the molecular function of CD45, a transmembrane glycoprotein that has intrinsic tyrosine phosphatase activity. Expression of two isoforms of CD45, CD45RABC and CD45RO, and two mutated forms of CD45, one lacking protein tyrosine phosphatase (PTP) activity, and one lacking the cytoplasmic domain, were analyzed in a fibroblast cell line, L tk^-. It was observed that only a proportion (10-30%) of CD45 expressed in all transfected cells was expressed on the surface. Deletion of the cytoplasmic tail of CD45 resulted in the expression of two CD45 proteins of 125 kDa and 160 kDa. From pulse-chase experiments, it was determined that the higher molecular weight form was derived from the lower molecular weight form.

Mutational analysis of the of the cytoplasmic domain of CD45 indicated that a conserved glutamine in the second PTP domain of CD45, expressed in *E. coli*, resulted in the loss of CD45 PTP activity. This demonstrates that a mutation in domain II can affect PTP activity thought to reside in domain I. In order to determine the effect of mutations in CD45 on T cell signalling, it was first necessary to characterize a CD45-deficient T cell line. The BW5147 CD45-negative cell line was characterized with respect to $p59^{fyn}$, a potential *in vivo* substrate for CD45. It was determined that the expression of CD45 resulted in the reduced tyrosine phosphorylation of $p59^{fyn}$, yet CD45 had no appreciable effect on the *in vitro* kinase activity of $p59^{fyn}$. However, a 120/130 kDa phosphoprotein was identified only in $p59^{fyn}$ immunoprecipitates from CD45-positive cells after an *in vitro* kinase assay and this occurred independent of T cell receptor mediated stimulation. These results implicate CD45 in regulating the associations of $p59^{fyn}$ in addition to regulating its tyrosine phosphorylation state.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deocytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DTT	Dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
Endo H	Endoglycosidase H
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorter
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
HBS	Hepes buffered saline
HPTP	Human protein tyrosine phosphatase
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
IL-2	Interleukin-2
IPTG	Isopropylthio-β-D-galactoside
LAR	Leukocyte antigen related protein
LRP	Leukocyte common antigen related protein
M	Molar
μg	Microgram

MCS	Multiple cloning site
MHC	Major histocompatibility complex
µl	Microlitre
mL	Millilitre
mM	Millimolar
µmol	Micromole
ng	Nanogram
nm	Nanometre
nmol	Nanomole
O.D.	Optical density
PBS	Phosphate buffered saline
PKC	Protein kinase C
pmol	Picomolar
PMSF	Phenylmethylsulfonyl fluoride
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
PTPase	Protein tyrosine phosphatase
PVDF	Polyvinylidene difluoride
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
TCR	T cell receptor
Tris	Tris (hydroxymethyl) amino methane
UT	Untranslated region

ACKNOWLEDGEMENT

I would like to acknowledge the technical support of Peter Borodchak and Lizabeth Kalt and I would like to thank Dr. Pauline Johnson, Dr. Julie Deans, and David Ng for stimulating scientific discussions. I especially want to thank Dr. Pauline Johnson for offering me this opportunity to do my Masters' degree in her laboratory, for her guidance, and for setting an example of academic rigour which I have tried to apply to my work. Finally, I must acknowledge the work of my supervisory committee of Dr. Wilf Jefferies, Dr. Robert McMaster, and Dr. Linda Matsuuchi. I would like to thank them for their contributions of time, new ideas and directions, good advice, and moral support.

DEDICATION

I would like to dedicate this body of work to my parents, Mr. S. K. Maiti and Mrs. J. K. Maiti, and my sister, Anita. Without their unstinting support and sacrifices too numerous to mention, I would not have been able to achieve half of what I have so far. And to John, who has put up with a lot since he met me but always listened and helped me to see things from a different perspective.

INTRODUCTION

CD45 Structure

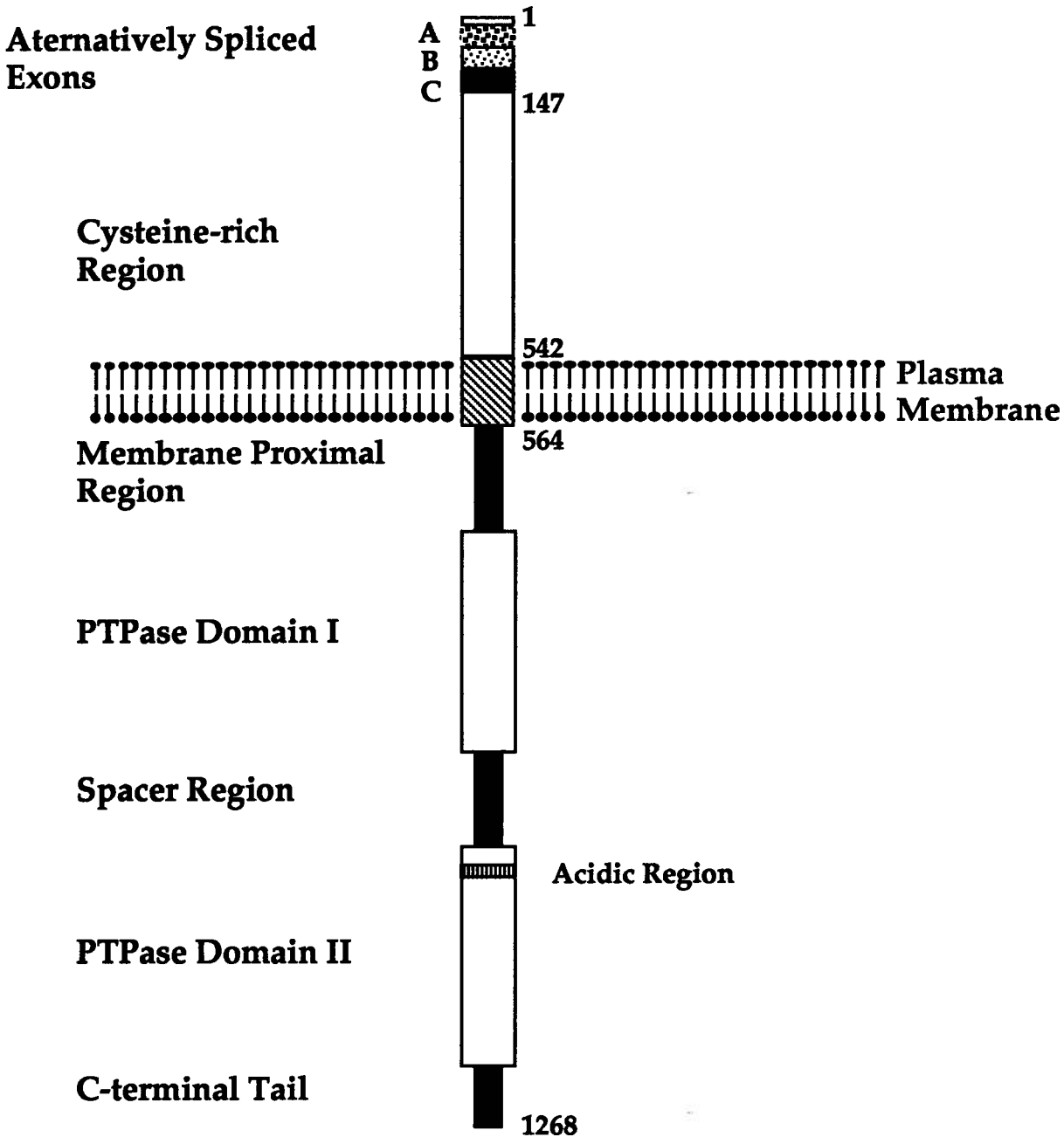
CD45 (leukocyte common antigen, T200, B220, Ly-5) is a family of glycoproteins expressed exclusively on nucleated cells of the haematopoietic lineage, reviewed in [1, 2, 3]. This family of molecules ranges in molecular weight from 180 to 220 kDa and accounts for as much as 10% of the membrane proteins expressed on the cell surface of lymphocytes. The structural heterogeneity expressed by CD45 relates to its pattern of expression in different haematopoietic cells. B cells express predominantly the higher M_r 220,000 isoform; thymocytes predominantly the lower M_r 180,000 isoform; and T cells express different isoforms that correlate with developmental stage and exposure to antigen.

The Extracellular Domain

CD45 is a transmembrane protein consisting of a heavily glycosylated variable amino terminal extracellular domain ranging from 404-543 amino acids, a single transmembrane domain, and a large, highly conserved cytoplasmic domain of 705 amino acids (Fig.1). The external domain of CD45 includes a variable region at the N-terminus and a cysteine rich region which may contain fibronectin type III repeats [4]. Alternative splicing of three exons (exons 4,5, and 6) near the amino terminus of the molecule results in the expression of different isoforms of CD45 [5]. The elucidation of the primary structure of rat, mouse, and human CD45 from the nucleotide sequence [5, 6, 7, 8, 9] demonstrated that each of the three alternatively spliced exons is encodes for about 50 amino acids each [10, 11, 12, 13]. Segments of the external domain encoded by exons 4, 5, and 6 are referred to as A, B, and C respectively. The CD45 isoform that

Figure. 1. Schematic Diagram of the Structure of CD45. Regions A, B, and C (residues 8-50, 51-99, 100-146, respectively) refer to the alternatively spliced exons 4, 5, and 6, that are present in the CD45RABC isoform. The amino acid numbering is based on the murine B cell isoform of CD45 [9]. The external domain (residues 1-541), the transmembrane domain (residues 542-563), and the cytoplasmic domain (residues 564-1268) are shown. The acidic region in the second phosphatase domain (residues 958-978) is also shown.

CD45


Aternatively Spliced Exons
A
B
C
1
147
Cysteine-rich Region
542
Plasma Membrane
564
Membrane Proximal Region
PTPase Domain I
Spacer Region
Acidic Region
PTPase Domain II
C-terminal Tail
1268

expresses all three alternatively spliced exons, for example, is named CD45RABC, while CD45RO refers to the isoform that expresses none of the three variable exons. Biochemical studies in the rat show that the regions encoded by the alternatively spliced exons are extensively modified by O-linked carbohydrates [14]. Variable usage of the exons affects both the length of the molecule as well as the amount of O-linked sugars. Many of the O-linked sugars are highly charged by the addition of sialic acid residues [15]. The visualization of purified CD45 by low-angle shadowing [16] demonstrates that the extracellular domain of the molecule is an extended rod.

The cysteine rich region of approximately 360 amino acids contains 15 potential sites for N-linked glycosylation in murine CD45. Inhibition of N-glycosylation, specifically by interfering with the transfer of dolichol phosphate-linked carbohydrate moieties onto asparagine residues by tunicamycin treatment inhibited the cell surface expression of the 180 kDa and 190 kDa isoforms of CD45 in K562 cells [17]. This suggests that the addition of N-linked sugars in the Golgi apparatus is required for cell surface expression and stability of CD45 molecules.

As a transmembrane glycoprotein and putative receptor, many groups are trying to identify CD45 ligands. It has been reported that the CD45RO isoform interacts with CD22 [18]. Recent evidence has demonstrated that a CD22-immunoglobulin fusion protein binds to multiple isoforms of CD45 on T cells [19]. Subsequent investigation has shown that CD22 is a sialic acid binding lectin with a specificity for N-linked carbohydrates containing α2-6 sialic acid moieties on all cell surface glycoproteins [20, 21], and therefore is not a CD45-specific ligand. Whether individual isoforms of CD45 interact with other ligands in a developmentally regulated or cell-type specific manner remains to be established.

The identity between species in the extracellular domain is approximately 35%, yet there are key conserved residues, cysteines, tyrosines, prolines, and tryptophans that may result in a conserved three-dimensional structure. Taken together, the protein and carbohydrate structures in the alternatively spliced region give rise to the

variability in size and charge of CD45. This diversity in CD45 isoforms may ultimately affect both intra- and intercellular interactions.

The Cytoplasmic Domain

CD45 has a receptor-like structure and a very large cytoplasmic domain which includes tandem PTPase domains [22]. The function of the cytoplasmic domain was unknown until investigators cloning a novel protein tyrosine phosphatase, PTP 1B, from human placenta, showed that it exhibited 30-40% amino acid sequence similarity to two regions of CD45 [22]. Evidence that the cytoplasmic domain of CD45 had intrinsic enzymatic activity was demonstrated by Tonks using purified CD45 [23, 24]. Subsequently, the generation of soluble recombinant cytoplasmic domain proteins in bacterial and baculovirus expression systems by others confirmed that CD45 was a protein tyrosine phosphatase [25, 26].

The cytoplasmic domain of CD45 consists of two tandem PTPase domains of ~240 amino acids separated by a 56 amino acid spacer region (Fig. 2). These two PTPase domains which shall be referred to as domain I and domain II, share ~40% sequence identity [22]. There is a unique region of 21 acidic residues within the second phosphatase domain of CD45 (domain II) that contains several potential sites for serine phosphorylation by casein kinase II [22]. Additionally, the cytoplasmic domain contains a 77 amino acid membrane proximal region, and a C-terminal tail of 78 amino acids.

It had been observed that there was a requirement for sulfhydryl compounds for the phosphatase activity of PTP 1A and PTP 1B [27] and that enzymatic activity was susceptible to inhibition by SH-modifying compounds. Thus, at least one reactive cysteine residue is essential for catalysis. Cysteine 215 of PTP 1B and cysteine 1522 of rat LAR are involved in the formation of a thiol intermediate during the

CD45 CYTOPLASMIC DOMAIN

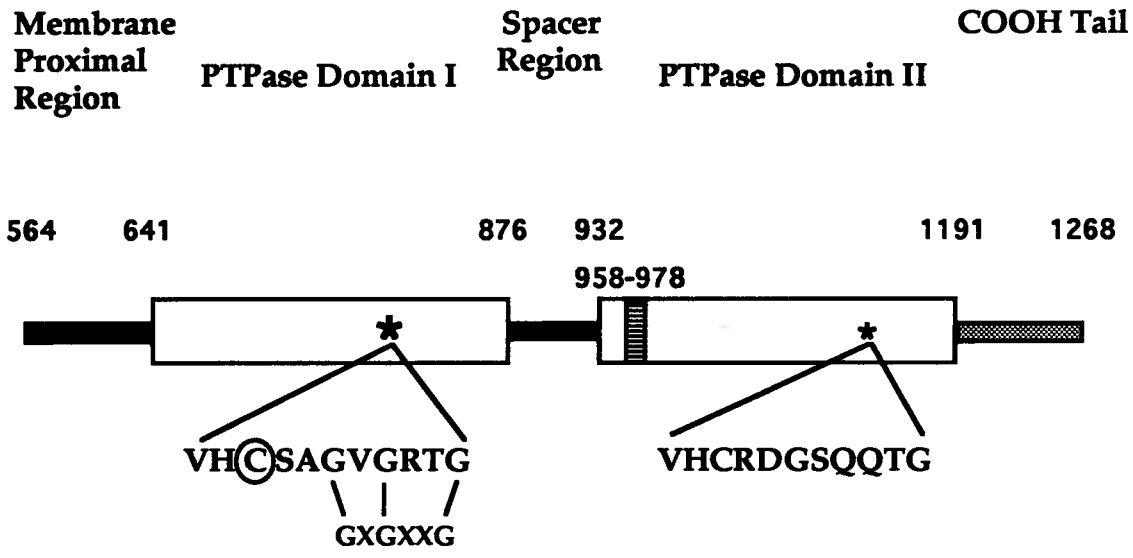


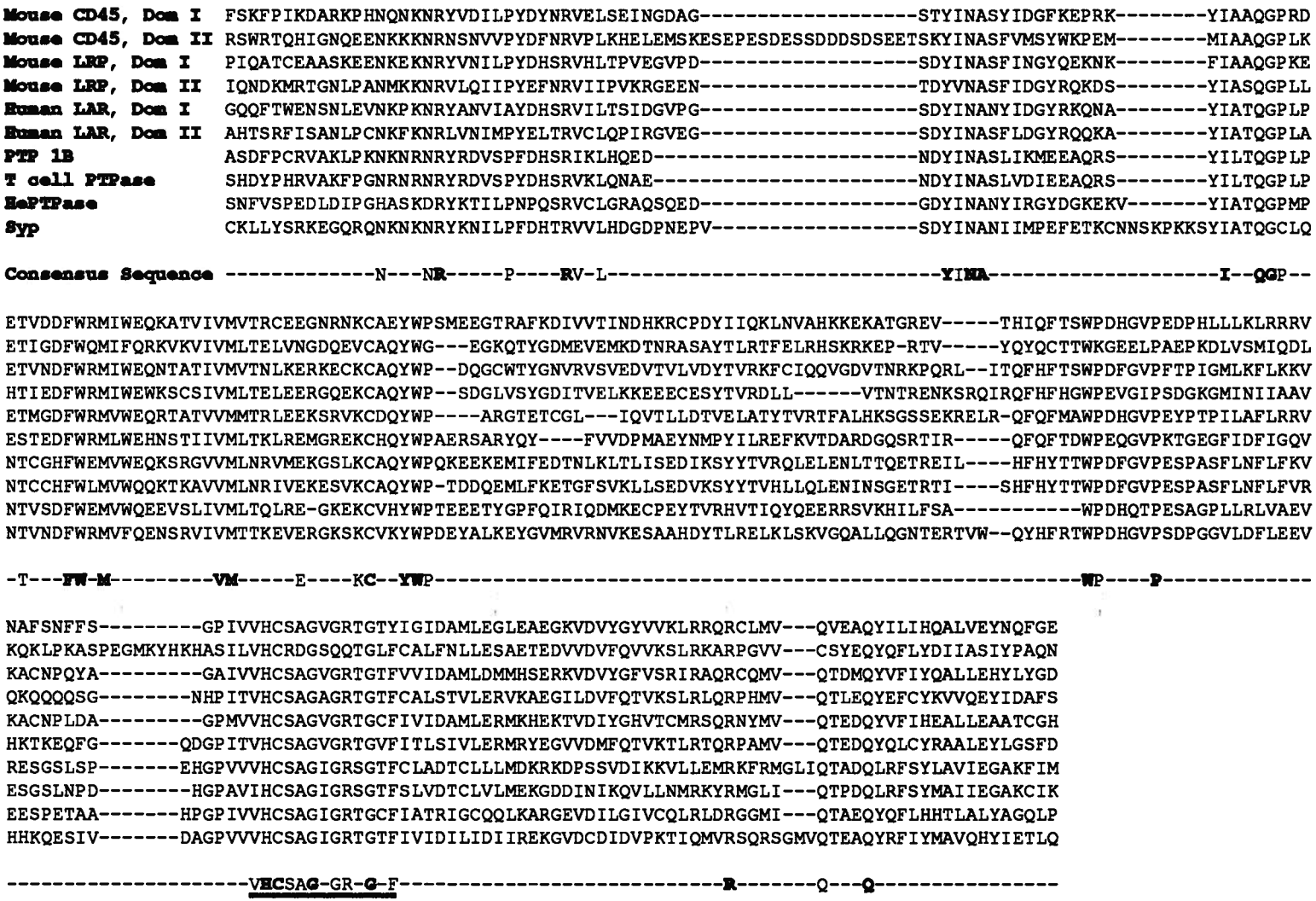
Figure. 2. Schematic Representation of the Structure of the Cytoplasmic Domain of CD45. The representation of murine CD45 cytoplasmic domain shows the tandem PTPase domains, domain I (residues 641-875), domain II (residues 932-1190), including the acidic region (residues 958-978), the membrane proximal domain (564-640), the spacer region (residues 876-931), and the C-terminal tail (1191-1268). The position and amino acid sequence of the PTPase consensus sequence is shown for both PTPase domains. The cysteine essential for activity in domain I is circled.

phosphotransfer reaction [28, 29, 30]. A point mutation of a highly conserved cysteine in the first tandemly repeated domain (domain I) of CD45 completely abolished enzymatic activity [25], indicating that domain II may be inactive against substrates used *in vitro* as it is is unable to compensate for mutations in PTPase domain I. Mutation of the analogous cysteine in domain II resulted in an active enzyme [31] or a 2-fold decrease in activity [32]. Comparison of the sequences from intracellular and transmembrane PTPases reveals a short conserved segment of amino acids surrounding the essential cysteine (Fig. 3). This PTPase consensus sequence includes a GXGXXG motif which resembles a glycine rich loop that is associated with the nucleotide binding site of G-proteins [33] and protein kinases [34].

Mutational analysis of CD45 has demonstrated that expression of bacterially expressed and *in vitro* translated PTP domain I alone does not result in an active PTPase [31, 32], suggesting that domain II is required for enzymatic function. *In vitro* translated domain II expressed alone, without domain I was also inactive [32]. Additionally, 5 non-conserved residues in the PTPase consensus sequence of domain II were altered to conform to the consensus sequence in domain I. This mutation was made in a mutant that had the essential cysteine 817 of domain I mutated in order to assess the activity of domain II only. This mutant did not possess PTPase activity. Taken together, these experiments suggest that domain II is not intriniscally active. Yet CD45 isolated from lymphoid cells and subjected to limited proteolysis to generate a 50 kDa fragment comprising part of PTPase domain I, domain II, and the spacer region was enzymatically active [35]. The same group demonstrated that CD45 isolated from fibroblasts transfected with a CD45 cDNA lacking most of domain I also had PTPase activity.

Deletion of the membrane proximal region (residues 564-640) abolished PTPase activity, but deletion of the C-terminal tail did not [32]. Interestingly, further deletion of 13 amino acids at the C-terminus of domain II was sufficient to abrogate phosphatase activity [32], again suggesting that domain II may be required for the function of

Figure. 3. Alignment of CD45 PTPase Domains with a Consensus PTPase Sequence. Amino acid alignment of the two phosphatase domains of CD45 [9] is shown. The consensus PTPase sequence was derived from the alignment of ten representative phosphatase domains. The two PTPase domains of murine CD45 [9], the two domains of LRP [37], the two domains of human LAR [38], and four cytosolic PTPases, PTP 1B [39], T cell PTPase [40], HePTPase [41], and the SH2-domain containing PTPase Syp [42] are shown. In the consensus sequence, invariant residues are indicated by boldface letters and residues that are 90% conserved by capital letters. The most conserved region and presumed active site is underlined. Phosphatase domain boundaries start before the α1 helix and end after the α6 helix as defined from the crystal structure of PTP 1B [36].


Mouse CD45, Dom I FSKFPIKDARKPHNQNKNRYVDILPYDYNRVELSEINGDAG----------STYINASYIDGFKEPRK--------YIAAQGPRD
Mouse CD45, Dom II RSWRTQHIGNQEENKKKNRNSNVVPYDFNRVPLKHELEMSKESEPESDESSDDDSDSEETSKYINASFVMSYWKPEM--------MIAAQGPLK
Mouse LRP, Dom I PIQATCEAASKEENKEKNRYVNILPYDHSRVHLTPVEGVPD----------SDYINASFINGYQEKNK--------FIAAQGPKE
Mouse LRP, Dom II IQNDKMRTGNLPANMKKNRVLQIIPYEFNRVIIPVKRGEEN----------TDYVNASFIDGYRQKDS--------YIASQGPLL
Human LAR, Dom I GQQFTWENSNLEVNKPKNRYANVIAYDHSRVILTSIDGVPG----------SDYINANYIDGYRKQNA--------YIATQGPLP
Human LAR, Dom II AHTSRFISANLPCNKFKNRLVNIMPYELTRVCLQPIRGVEG----------SDYINASFLDGYRQQKA--------YIATQGPLA
PTP 1B ASDFPCRVAKLPKNKNRNRYRDVSPFDHSRIKLHQED----------NDYINASLIKMEEAQRS--------YILTQGPLP
T cell PTPase SHDYPHRVAKFPGNRNRNRYRDVSPYDHSRVKLQNAE----------NDYINASLVDIEEAQRS--------YILTQGPLP
HePTPase SNFVSPEDLDIPGHASKDRYKTILPNPQSRVCLGRAQSQED----------GDYINANYIRGYDGKEKV-------YIATQGPMP
Syp CKLLYSRKEGQRQNKNKNRYKNILPFDHTRVVLHDGDPNEPV----------SDYINANIIMPEFETKCNNSKPKKSYIATQGCLQ
Consensus Sequence ----------N---NR-----P----RV-L----------YINA----------I--QGP--
ETVDDFWRMIWEQKATVIVMVTRCEEGNRNKCAEYWPSMEEGTRAFKDIVVTINDHKRCPDYIIQKLNVAHKKEKATGREV-----THIQFTSWPDHGVPEDPHLLLKLRRRV
ETIGDFWQMIFQRKVKVIVMLTELVNGDQEVCAQYWG---EGKQTYGDMEVEMKDTNRASAYTLRTFELRHSKRKEP-RTV-----YQYQCTTWKGEELPAEPKDLVSMIQDL
ETVNDFWRMIWEQNTATIVMVTNLKERKECKCAQYWP--DQGCWTYGNVRVSVEDVTVLVDYTVRKFCIQQVGDVTNRKPQRL--ITQFHFTSWPDFGVPFTPIGMLKFLKKV
HTIEDFWRMIWEWKSCSIVMLTELEERGQEKCAQYWP--SDGLVSYGDITVELKKEEECESYTVRDLL------VTNTRENKSRQIRQFHFHGWPEVGIPSDGKGMINIIAAV
ETMGDFWRMVWEQRTATVVMMTRLEEKSRVKCDQYWP----ARGTETCGL---IQVTLLDTVELATYTVRTFALHKSGSSEKRELR-QFQFMAWPDHGVPEYPTPILAFLRRV
ESTEDFWRMLWEHNSTIIVMLTKLREMGREKCHQYWPAERSARYQY----FVVDPMAEYNMPYILREFKVTDARDGQSRTIR-----QFQFTDWPEQGVPKTGEGFIDFIGQV
NTCGHFWEMVWEQKSRGVVMLNRVMEKGSLKCAQYWPQKEEKEMIFEDTNLKLTLISEDIKSYYTVRQLELENLTTQETREIL----HFHYTTWPDFGVPESPASFLNFLFKV
NTCCHFWLMVWQQKTKAVVMLNRIVEKESVKCAQYWP-TDDQEMLFKETGFSVKLLSEDVKSYYTVHLLQLENINSGETRTI----SHFHYTTWPDFGVPESPASFLNFLFVR
NTVSDFWEMVWQEEVSLIVMLTQLRE-GKEKCVHYWPTEEETYGPFQIRIQDMKECPEYTVRHVTIQYQEERRSVKHILFSA----------WPDHQTPESAGPLLRLVAEV
NTVNDFWRMVFQENSRVIVMTTKEVERGKSKCVKYWPDEYALKEYGVMRVRNVKESAAHDYTLRELKLSKVGQALLQGNTERTVW--QYHFRTWPDHGVPSDPGGVLDFLEEV
-T---FW-M---------VM-----E----KC--YWP----------WP----P----------
NAFSNFFS---------GPIVVHCSAGVGRTGTYIGIDAMLEGLEAEGKVDVYGYVVKLRRQRCLMV---QVEAQYILIHQALVEYNQFGE
KQKLPKASPEGMKYHKHASILVHCRDGSQQTGLFCALFNLLESAETEDVVDVFQVVKSLRKARPGVV---CSYEQYQFLYDIIASIYPAQN
KACNPQYA---------GAIVVHCSAGVGRTGTFVVIDAMLDMMHSERKVDVYGFVSRIRAQRCQMV---QTDMQYVFIYQALLEHYLYGD
QKQQQQSG--------NHPITVHCSAGAGRTGTFCALSTVLERVKAEGILDVFQTVKSLRLQRPHMV---QTLEQYEFCYKVVQEYIDAFS
KACNPLDA---------GPMVVHCSAGVGRTGCFIVIDAMLERMKHEKTVDIYGHVTCMRSQRNYMV---QTEDQYVFIHEALLEAATCGH
HKTKEQFG-------QDGPITVHCSAGVGRTGVFITLSIVLERMRYEGVVDMFQTVKTLRTQRPAMV---QTEDQYQLCYRAALEYLGSFD
RESGSLSP-------EHGPVVVHCSAGIGRSGTFCLADTCLLLMDKRKDPSSVDIKKVLLEMRKFRMGLIQTADQLRFSYLAVIEGAKFIM
ESGSLNPD--------HGPAVIHCSAGIGRSGTFSLVDTCLVLMEKGDDINIKQVLLNMRKYRMGLI---QTPDQLRFSYMAIIEGAKCIK
EESPETAA-------HPGPIVVHCSAGIGRTGCFIATRIGCQQLKARGEVDILGIVCQLRLDRGGMI---QTAEQYQFLHHTLALYAGQLP
HHKQESIV-------DAGPVVVHCSAGIGRTGTFIVIDILIDIIREKGVDCDIDVPKTIQMVRSQRSGMVQTEAQYRFIYMAVQHYIETLQ
----------VHCSAG-GR-G-F----------R-------Q---Q----------

domain I. Recently, the x-ray crystal structure of PTP 1B, a cytosolic PTPase [36] was determined. The elucidation of the crystal structure of the phosphatase domain of PTP 1B will aid in determining more accurately, which regions of the cytoplasmic domain of CD45 contribute to phosphatase activity and the regulation of that activity.

CD45 Function in the Immune System

Surface expression of CD45 has been shown to be necessary for effective activation of both B and T lymphocytes through their antigen receptors [43, 44, 45] as well as for thymic development [46, 47]. CD45 is required for the appearance of the earliest T cell receptor (TCR) mediated signalling event; the induction of protein tyrosine phosphorylation. CD45-negative clones of human Jurkat and HPB-ALL cells were unable to induce the tyrosine phosphorylation of proteins upon anti-TCR or anti-CD3 stimulation [48, 49]. Engagement of the TCR in cells lacking CD45 fails to lead to phospho-inositide hydrolysis or increases in intracellular calcium [45]. These cells are unable to proliferate or produce cytokines such as interleukin-2 in response to antigen. A CD45-negative cytotoxic T cell clone failed to proliferate and was unable to cytolyse target cells upon antigen presentation [50]. Interestingly, this cell line expressed LRP, another transmembrane PTPase, yet this PTPase was unable to compensate for a deficiency in CD45 expression [3]. Transfection of CD45-deficient cells with chimeric CD45 molecules, consisting of the cytoplasmic domain of CD45 and the external domain of either the EGF receptor, the $p60^{src}$ myristylation site, or the external domain of MHC Class I , demonstrates that the cytoplasmic domain is sufficient to restore TCR/CD3 mediated signalling events [51, 52, 53], although it remains to be determined if the extracellular domain is involved in regulating these events by differential isoform expression or ligand binding.

The induction of protein tyrosine phosphorylation events upon T cell stimulation is thought to occur as a result of the activation of protein tyrosine kinases (PTKs)

associated with the TCR/CD3 complex [54, 55, 56]. Candidate kinases include the src-family kinases such as $p56^{lck}$ and $p59^{fyn}$ as well as a member of a new family of PTK, ZAP-70 [57]. Both $p56^{lck}$ and $p59^{fyn}$ have been identified as potential substrates for CD45 [26, 58, 59, 60, 61]. CD45 can dephosphorylate the negative regulatory tyrosine (tyrosine 505 and 531, respectively) of $p56^{lck}$ and $p59^{fyn}$ and this is thought to activate the kinase [59, 62, 63]. CD45 may be required either to activate these kinases upon stimulation through the TCR/CD3 complex or to maintain these kinases in an active state ready for T cell stimulation. In addition, CD45 itself may participate in the TCR/CD3 signalling complex. The exact role of CD45 in T cell receptor mediated signalling is not yet clear.

One approach to study CD45 function is to transfect CD45 mutated genes into CD45-deficient T cells. Unfortunately, it has been difficult to reconstitute CD45-negative T cells [3, 49] and the reasons why this occurs are not understood.

Summary of Intentions

The general aim of this work was to understand further the molecular function of CD45. Although CD45 isoforms are expressed in a developmentally regulated and cell-type specific manner, the function of individual isoforms is unknown as is the identity of isoform specific ligands. To further complicate matters, lymphocytes often express multiple isoforms at any one time. By expressing individual isoforms in a fibroblast cell line, one can begin to look for potential isoform specific ligands. One aim of this thesis was to transfect two isoforms of CD45 into the L tk^- fibroblast cell line and characterize their expression. Two additional mutants of CD45 were also expressed and these cells were characterized to determine if these mutations dramatically affected cell surface expression. L cell transfectants were characterized with respect to expression of CD45 and transport of CD45 through the endoplasmic reticulum and medial golgi apparatus. These L cell transfectants may be useful for the identification CD45 ligands and in order

to determine how interactions with the external domain of CD45 influences its PTPase activity.

The role of the cytoplasmic domain in T cell activation has been shown to be crucial, but what aspects of the cytoplasmic domain are required have yet to be established. In addition, it is still unclear whether domain II of CD45 has a regulatory function or some other activity. To further examine this question, the second aim of this thesis was to determine which amino acids in domain II were important for PTPase activity.

The effect of targeted mutations on phosphatase activity can be studied *in vitro,* but the elucidation of amino acids important for regulation of CD45 activity and restoration of T cell signalling require studying cytoplasmic domain mutants *in vivo.* CD45-deficient cells provide an experimental system that can be used to study the regions of CD45 that are important for physiological functions by assessing the ability of CD45 mutants to reconstitute T cell signal transduction. In order to determine the effects of CD45 reconstitution with cytoplasmic domain mutants, it is necessary to first characterize the defects in TCR-mediated signalling in the CD45-deficient cells. The BW5147 CD45-negative (TCR^+, $CD3^+$) cells [64] were deficient in the induction of phosphoproteins upon stimulation through the T cell receptor (P. Johnson, unpublished results). In addition, hyperphosphorylation of the negative regulatory tyrosine 505 of $p56^{lck}$ was observed in the CD45-negative BW5147 parental cell line [26]. While $p59^{fyn}$ has been identified as a potential substrate of CD45 [59, 60, 61], it was not known whether $p59^{fyn}$ was expressed in these cells or if CD45 affected the phosphorylation state of *fyn.* Thus, the third aim of this thesis was to determine what the effect of CD45 expression was on the phosphorylation state and kinase activity of $p59^{fyn}$ in the CD45-negative BW5147 cells. Ultimately, the reconstitution CD45-deficient cells with CD45 mutants will help us to better understand the role of individual regions of CD45 in phosphatase activity and T cell receptor-mediated signalling.

Thus the three main aims of this thesis were 1) to characterize CD45 transfected into L tk^- cells; 2) to generate mutations in the cytoplasmic domain of CD45 and test their PTPase activity after expression in *E. coli.*; and 3) to characterize a recipient CD45-negative T cell with respect to the tyrosine phosphorylation state and kinase activity of the src-family kinase, $p59^{fyn}$.

MATERIALS AND METHODS

Cell Culture

L tk$^-$ fibroblasts (American Type Culture Collection, Rockville, Maryland) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum and 100 units/mL of penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Gibco BRL Life Technologies, Burlington, Ont.). BW5147 CD45-positive and CD45-negative T lymphoma cells (available from the ATCC, Rockville, Maryland) were transfected with CD3δ and CD3ζ which resulted in the expression of surface T cell receptor/ CD3 complexes [64] (a generous gift from Dr. B. Malissen). These cells were maintained in DMEM supplemented with 10% horse serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin B and contained 3 mM histidinol (Sigma Chemical Company, St. Louis, Missouri) to maintain the expression of transfected plasmids. All cells were incubated at 37°C in 5% CO_2. Cells were checked routinely by flow cytometry to ensure similar levels of TCR/CD3 were expressed in CD45-positive and CD45-negative BW5147 cell lines.

Antibodies

Anti CD45 antibodies used were I3/2, a rat antibody against a pan-specific CD45 determinant in mice [65] (gift of Dr. I. Trowbridge), Ly5.2, clone 104-2.1, an allele-specific antibody (gift of Dr. S. Komuro), 131, an anti-peptide blotting antiserum against amino acids 211-250 of the external domain of CD45 (gift of Dr. J. Marth), and anti-95kD, a rabbit antiserum against the cytoplasmic domain of CD45 (gift of Dr. H. Ostergaard). CD45 exon-specific antibodies used for flow cytometry were RA3-2C2 [66], a rat anti mouse IgM versus exon A, MB4B4, a rat anti mouse exon B specific antibody [67] (American Type Culture Collection, Rockville, Maryland), and DNL-1.9, an exon C specific rat anti mouse antibody [68] from Pharmingen (San Diego, Ca.).

Murine anti-fyn antiserum was a generous gift of Dr. R. Perlmutter. Anti murine CD4 antibody GK1.5, (ATCC TIB 207) was obtained fom Dr. H-S. Teh, and anti-CD44 antibody IM7.8.1 (ATCC TIB 235) was a gift from Dr. R. Hyman. Hamster anti-mouse anti-CD3ε antibodies (145-2C11) and anti-TCRβ chain antibodies (H57-597) were obtained from the ATCC (Rockville, Maryland). Goat-anti-rat and goat-anti-hamster fluorescein isothiocyanate (FITC) labeled antibodies were purchased from Pierce (Rockford, Illinois) and Southern Biotech Associates Ltd. (Birmingham, Alabama) respectively. The anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology Inc. (Lake Placid, N.Y.). Protein A-HRP and goat-anti-mouse-HRP were purchased from Bio-Rad Laboratories Ltd. (Mississauga, Ont.).

Transfected CD45 cDNA Constructs

Plasmids transfected into L cells included CD45RABC (#106), CD45RO (#36), CD45RABC C817S (#64), and CD45RABC Δcyt (#12) and were constructed by P. Johnson and L. Melito. The Cla I-Xho I fragment encompassing the complete CD45 cDNA was cloned into the Sal I site of the pHβapr-1-neo [69] mammalian expression vector. CD45 cDNAs were missing most of their 5' and 3' untranslated regions with the exception of the CD45RO construct (#36) which still contained its 5'UT and 3' UT.

Transfection of L tk^- cells with CD45 cDNAs

L cells were maintained in 10 mL DMEM, 10% fetal calf serum (FCS), and antibiotics on Nunc 100 mm^2 tissue culture dishes (Gibco BRL Life Technologies, Burlington, Ont.) until they were 20-40% confluent, at which point they were transfected by the calcium phosphate method [70]. 20 μg of DNA was prepared by ethanol precipitation and resuspended in 750 μL of sterile water and added to 250 μL of 2M $CaCl_2$ in an eppendorf tube. 1mL of 2X Hepes buffered saline (HBS; 280mM NaCl, 50 mM Hepes pH7.1, 1.5 mM NaH_2PO_4) was aliquoted into 5 mL Falcon 2058 tubes (Baxter-Canlab, Mississauga, Ont.) and the DNA/$CaCl_2$ mixture was added dropwise

while simultaneously vortexing the Falcon tube. A cloudy fine precipitated was formed which was left at room temperature for 30 minutes. 2 mL of DMEM was removed from the cells and the DNA/$CaPO_4$ precipitate was dotted onto the plate with a pasteur pipet. The plate was rocked gently and a fine precipitate was observed on the cells under a Zeiss phase-contrast microscope. The cells were left at 37°C for five hours, at which point the media and precipitate was removed and 10 mL of fresh DMEM plus 10% FCS was added to the plates. Cells were selected for stable transfectants by the addition of 1 mg/mL of G418 (Geneticin, Gibco BRL Life Technologies, Burlington, Ont.) 48 hours post-transfection.

Flow Cytometry

2×10^5 cells were incubated with 100 µL of tissue culture supernatant containing the appropriate monoclonal antibody for 20 minutes on ice. After washing the cells with phosphate buffered saline (PBS; 154 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4) containing 2% FCS, they were incubated for 20 minutes on ice with 100 µL of a 1/100 dilution of FITC labeled secondary antibody. 2 mM ethylenediamine-tetra-acetic acid (EDTA) was added to the L cells to prevent re-adherence. Cells were washed and analyzed on a FACSCAN (Becton Dickinson, Mississauga, Ont.) analyzer.

Positive Selection of $CD45^+$ L cell Transfectants with Magnetic Beads

After 14 days in G418 selection, L cells were positively selected for CD45 expression with magnetic beads. 5×10^6 cells were taken off the plates with 2 X Versene (PBS plus 0.7 mM EDTA) for 10 minutes at room temperature, washed in sterile PBS with 2% FCS and 2 mM EDTA and resuspended in 200 µL of the PBS/2% FCS/ 2 mM EDTA solution. 10 µg of purified I3/2 antibody was added to the cells and incubated for 30 minutes on ice, resuspending the cells every 10 minutes. Cells were then washed 3 times and resuspended in 300 µl of the PBS solution, to which 50 µL of goat-anti-rat magnetic beads (Collaborative Research Inc., Bedford, Massachussetts) or 15 µL of

Dynabeads M-450 sheep-anti-rat IgG (Fc) magnetic beads (Dynal Inc., Great Neck, New York) were added. The cells and beads were left on ice for 30 minutes, resuspending the cells every 10 minutes. The labeled cells were then poured into a 5 mL uncoated Falcon dish and selected on a magnetic plate. The wash solution and non-adherent cells were aspirated and the cells washed and selected on the magnetic plate two more times. This selection process was repeated at least four times on all tranfectants before the cells were cloned out and screened by flow cytometry.

CD45 Immunoprecipitation and Western Blotting

To immunoprecipitate CD45, the appropriate number of cells were lysed in ice cold lysis buffer (1% Triton-X-100, 150 mM Nacl, 20 mM Tris-HCl pH7.4, 2 mM EDTA, 5 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 mg/mL pepstatin) and left on ice for 10 minutes. Cell lysates were then centrifuged at 12 000g for 10 minutes to remove the insoluble pellet and incubated with 200 μL of Ly5.2 tissue culture supernatant for 30 minutes at 4°C rotating end-over-end, after which point 10 μL of a 50% slurry of protein G sepharose was added for an additional hour. Surface CD45 was immunoprecipitated from unlysed cells that had been washed in PBS by incubating unlysed cells for 30 minutes with 200 μL of Ly5.2 tissue culture supernatant at 4°C rotating end-over-end and then washed once in PBS. Cells were then lysed as mentioned above, and 10 μL of a 50% slurry of protein G sepharose was added to the centrifuged lysate which was then rotated end-over-end for 1 hour at 4°C. Immunoprecipitates were washed 3 times in lysis buffer and resuspended in 10 μL of 2 X SDS sample buffer (250 mM Tris-HCl pH6.8, 20% glycerol, 200 mM dithiothreitol, 0.02% bromophenol blue) and boiled for 5 minutes before loading on 10% SDS polyacrylamide gels. Gels were run by the Laemmli method with prestained standards (Bio-Rad Laboratories Ltd., Mississauga, Ont.) and tranferred to a polyvinylidene difluoride (PVDF) Immobilon-P membrane (Millipore Canada Ltd., Mississauga, Ont.) in a transblot apparatus (Bio-Rad

Laboratories Ltd., Mississauga, Ont.) according to manufacturers instructions and blotted with 131 antibody at a 1/1000 dilution in 5% BSA-TBST (0.1% Tween 20, 150 mM NaCl, 20 mM Tris-HCl pH7.5, including protease and phosphatase inhibitors) for 90 minutes after blocking for 90 minutes in 5% BSA-TBST. Membranes were washed in TBST and incubated with a 1/10 000 dilution of HRP conjugated protein A in 5% BSA-TBST for 45 minutes, washed thoroughly, and developed using the enhanced chemiluminescence assay according to manufacturers instructions (ECL kit, Amersham Canada Ltd., Oakville, Ont.).

^{35}S Pulse Chase Labelling and Endoglycosidase H Digestion of Proteins

Twelve 100 mm^2 plates of cells per clone at 50% confluency (~2.5 X 10^6 cells) were washed in 5 mL of PBS and then starved of methionine and cysteine by incubation in 4 mL warm DMEM methionine(-) and cysteine(-) media (ICN Biomedicals, Inc., St. Laurent, Que.) for 20 minutes at 37°C. The cells were then pulsed with radiolabel for 30 minutes at 37°C. The label mixture consisted of 2.5 mL of DMEM methionine(-) cysteine(-) media supplemented with 250 µL of dialyzed FCS, 25 µL of Glutamax I (Gibco BRL life Technologies, Burlington, Ont.) and 100 µCi/mL of Promix L-[^{35}S] *in vitro* cell labelling mix (specific activity >1000 Ci/mmol, 70% methionine, 30% cysteine; Amersham Canada Ltd., Mississauga, Ont.). After the pulse period, the labeling media was removed and the cells washed in 5 mL of PBS before chasing with DMEM plus 10% FCS for 0, 15, 30, 60, 120, and 180 minutes. At the end of the chase period, the media was removed, the cells washed in 5 mL of PBS and then lysed in the plates with 1 mL of lysis buffer (1% Nonidet P-40, 120 mM NaCl, 4 mM $MgCl_2$, 20 mM Tris-HCl pH7.5). The plates were left on ice for 5 minutes, then the cells were dispersed by pipetting, transferred into an eppendorf tube, vortexed, and left on ice for a further 10 minutes. Lysates were centrifuged at 12 000g for 10 minutes at 4°C and the supernatant decanted from the insoluble pellet. Labeled cell lysates were precleared twice with 20 µL of a 50% slurry of CL-4B Sepharose (Pharmacia, Baie d'Urfe, Qué.) for 1 hour at 4°C rotating end-

over-end. Ly5.2 antibody (200 µL) precoupled to protein G (Pharmacia, Baie d'Urfe, Que.) for 2 hours was then added to the precleared lysates for 1 hour at 4°C rotating end-over-end to immunoprecipitate CD45. Immunoprecipitates were washed three times in low salt buffer (1% NP-40, 10 mM Tris-HCl pH7.5, 150 mM NaCl, 2mM EDTA), twice in high salt buffer (1% NP-40, 10 mM Tris-HCl pH7.5, 500 mM NaCl, 2 mM EDTA), and once in 10mM Tris-HCl pH7.5. Cells treated with Endoglycosidase H (Boerhinger Mannheim Canada, Laval, Qué.) were digested by the addition of 12 µl of 85 mM sodium citrate pH5.5, 4 µL of Endo H (4 mU), and 2 µL of 200 mM PMSF and incubated at 37°C overnight. All immunoprecipitates were boiled for 5 minutes in 2 X SDS sample buffer and run on 16 mm^2, 7.5% SDS-polyacrylamide gels overnight at 15 mA. Gels were fixed (40% methanol, 10% acetic acid) for 1 hour, placed in En^3hance (NEN DuPont Canada, Mississauga, Ont.) for 1 hour and then placed in Nanopure water (Millipore Corporation, Bedford, Massachussetts) for 30 minutes with gentle rocking. Gels were then dried for 90 minutes at 70°C and exposed at -70°C with Kodak X-Omat AR film and an intensifying screen for three days.

Generation of Single Stranded DNA from Phagemids

Plasmid #592 [26], which encodes for the cytoplasmic domain of CD45 cloned into the Cla I-Xho I site of pBluescript SK (+/-) (Stratagene Cloning Systems, La Jolla, Ca.), was transformed into *Escherichia coli* strain CJ236 (Bio-Rad Laboratories Ltd., Mississauga, Ont.) which expresses the *dut(-) ung(-)* phenotype. Likewise, plasmid #26, which encodes for the complete CD45 protein was cloned into the Cla I-Sal I sites of pBluescript SK (+/-) and also transformed into CJ236 *E. coli*. Single stranded DNA was generated by the addition of 300 µL of helper phage R408 (Bio-Rad Laboratories Ltd., Mississauga, Ont.) to a log phase culture of plasmid #592 or plasmid #26 in CJ236 bacteria grown in Luria Broth supplemented with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol. The R408 helper phage was titrated at 10^{12} colony forming units/mL.

Single stranded DNA was isolated by established methods [71] and determined to be at a concentration of 100 ng/μL.

Oligonucleotide Purification

Oligonucleotides were ordered from the Nucleic Acid-Protein Synthesis Unit (NAPS Unit, U.B.C.) and purified either on a C_{18} Sep-Pak column (Millipore Corporation, Bedford, Massachussetts) or by the n-butanol method [72]. Lyophilized oligonucleotides were dissolved in 3 mL of 0.5 M ammonium acetate (BDH Inc., Vancouver, B. C.) and loaded onto a C_{18} column that had been reconstituted with 10 mL of 20% acetonitrile (BDH Inc., Vancouver, B. C.) and equilibrated with 10 mL of water and 10 mL of 10 mM ammonium acetate. The column was washed with 10 mL of water and 10 mL of air pushed through with a 10 mL syringe. The oligonucleotides were eluted with 3 X 1 mL of 20% acetonitrile, dried on a Savant speed-vac and resuspended on 500 μL of sterile water. N-butanol purification involved the dissolution of lyophilized oligonucleotide in 100 μL of 30% ammonium hydroxide (BDH Inc., Vancouver, B. C.) to which 1 mL of n-butanol (BDH Inc., Vancouver, B.C.) was added. The mixture was vortexed, centrifuged at 12 000g and the single aqueous phase discarded. The oligonucleotide pellet was resuspended in 100 μL of water and re-extracted with 1 mL of n-butanol once more. The pellet was dried under vacuum and resuspended in 500 μL of sterile water. The optical density at 260 nm was used to determine the concentration of the purified oligonucleotides where concentration=O.D./extinction coefficent in μmoles/mL. 10X the oligonucleotide length is the calculation used for determining the extinction coefficient. All oligonucleotides were heated to 80°C and rapidly frozen to prevent self-hybridization.

Oligonucleotide-directed Mutagenesis

Mutagenesis was performed as per the method described by Kunkel [73, 74]. The annealing reaction was carried out by heating template and oligonucleotide at 80°C and

letting the reaction cool slowly to ~30°C. Single stranded plasmid #592 and #26 were used at a concentration of 100 ng/μL and 10 ng/μL, while working amounts of oligonucleotide used were 0.1 pmol, 2 pmol, and 10 pmol. The annealing reactions were performed in 10X annealing buffer (200 mM Tris-HCl pH7.4, 20 mM $MgCl_2$, 500 mM NaCl) in a final volume of 10 μL. After annealing, the synthesis reaction commenced by the addition of 2-5 U of T4 ligase (8000U/mL; Pharmacia, Baie d'Urfe, Qué.), 1 U of T4 polymerase (6700U/mL; Pharmacia, Baie d'Urfe, Qué.), and 1 μL of 10X synthesis buffer (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP, 10 mM ATP, 100 mM Tris-HCl pH7.4, 50 mM $MgCl_2$, 20 mM DTT). The synthesis reaction was placed on ice for 5 minutes, at 25°C for 5 minutes, and then at 37°C for 90 minutes. All of the mutagenesis reactions were transformed into competent *E. coli* strain XL-1 Blue (Stratagene Cloning Systems, La Jolla, Ca.) and selected on Luria broth/1.2% agar plates supplemented with 100 μg/mL of ampicillin. Oligonucleotides used to generate mutations in the cytoplasmic domain of CD45 were UBC #9 (5'-pGGA-ACT-GGT-ACC-CCT-CAT-AGC-TGC-3') which mutated glutamine 1180 in PTPase domain II to a glycine, UBC #10 (5'-pGTT-CTT-CTT-CTT-ATT-TTC-CAC-TAA-AGC-3') which deleted the spacer region between PTPase domains I and II, and UBC #11 (5'-pGTT-CAT-CTA-AAT-TGG-CGC-CTC-TTT-TCT-TGC-3') which simultaneously mutated serine 573 and serine 574 to a glycine and alanine respectively. Both the first and last mutations incorporated a new restriction endonuclease site in the mutant, with the exception of the spacer deletion, which resulted in a loss of 168 nucleotides. Colonies were screened by restriction digest analysis for the generation of mutants. All potential mutants were sequenced by the Sanger chain termination method using double stranded template, the Sequenase Version 2.0 DNA sequencing kit (United States Biochemical Corporation, Cleveland, Ohio) and $^{35}S\alpha$-dATP (>1000 Ci/mmol; NEN DuPont Canada, Mississauga, Ont.) according to the manufacturer's instructions. Clones generated were C3.1 and C3.2 for the Q1180G mutant, H8.8, H9.1, and H9.11 for the spacer deletion mutant, and 4.18 and 18.35 for the S573GS574A mutation in the membrane proximal region.

Generation of the Shuttle Vector

The pBluescript SK (+/-) vector (Stratagene Cloning Systems, La Jolla, Ca.) was modified to remove a portion of the multiple cloning site (MCS) by cutting with Xba I and Hind III, filling in the cohesive ends and blunt-end ligating the ends. Unique sites lost from the MCS were Spe I, Bam HI, Sma I, Pst I, Eco RI, Eco RV, and Hind III. The Xba I site was regenerated by this procedure. Two independent clones of this vector were called pBS #1 and pBS #5. pBS #5 was then cleaved with Cla I-Sal I, dephosphorylated, and ligated with a Cla I-Sal I fragment encoding the full CD45 cDNA and named #264 and #268. Vectors #264 and #268 were then digested with Acc I-Xho I at the 3' end of the CD45 insert and ligated to a linker oligo with the same cohesive ends. This linker was generated by hybridizing two non-palidromic oligonucleotides (UBC #43 and UBC #44) comprising Acc I-Spe I-Bgl II-Not I-Xho I sites and the resulting plasmids were numbered #647 and #682. Plasmid #682 was digested with Cla I at the 5' end of the CD45 insert and ligated to a Cla I-Xho I-Cla I palindromic linker (UBC #29; 5'-pCGA-TAC-TCG-AGT-AT-3') to incorporate an Xho I site at the 5' end of the CD45 cDNA. This plasmid was numbered #822 and henceforth called the shuttle vector (Fig. 4). This vector was constructed to easily subclone fragments of the CD45 cytoplasmic domain encoding site-directed mutations. The resulting mutant in the shuttle vector could then be digested with Bgl II to generate a 2 kb fragment representing the cytoplasmic domain of CD45 that could be inserted into a bacterial expression vector or cut with Cla I-Sal I or Xho I-Not I to express mutants in mammalian expression vectors. The Q1180G domain II mutant on plasmid #592 was cut with Sma I-Xba I and ligated into the Sma I-Spe I sites of the shuttle vector to enable the eventual subcloning of this particular mutant into a bacterial expression vector.

Subcloning of Mutants into a Bacterial Expression Vector

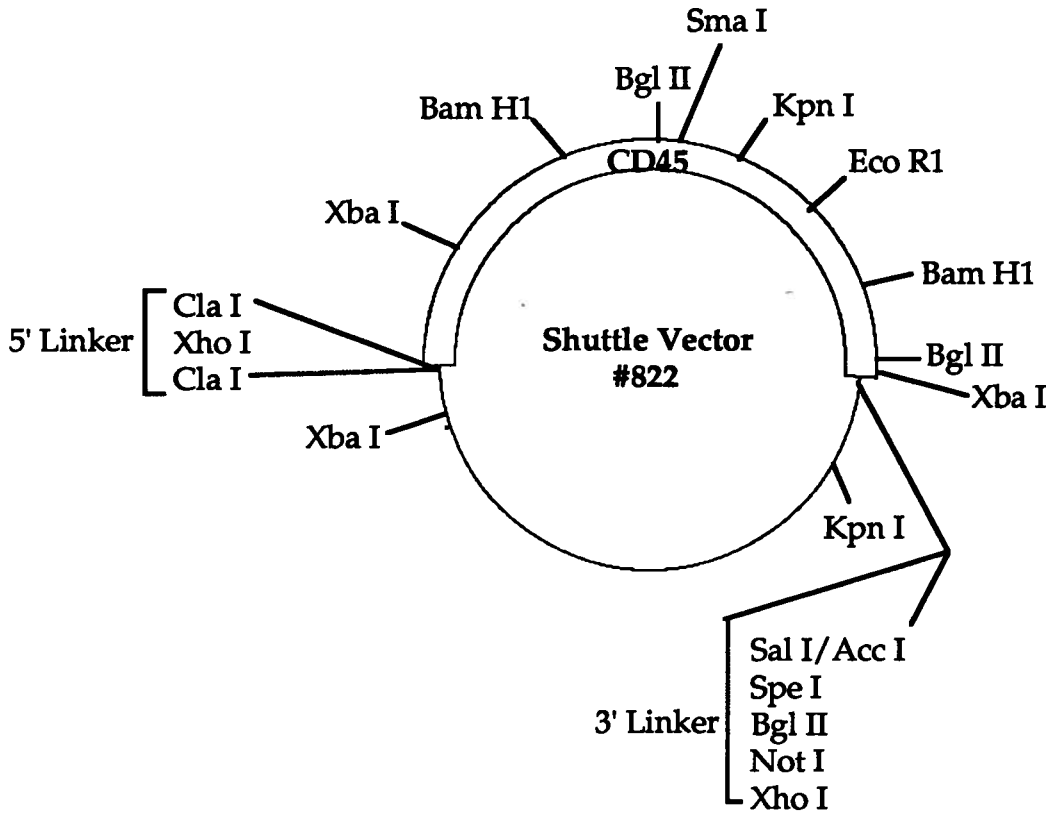


Figure. 4. Schematic Representation of the Shuttle Vector. The shuttle vector containing a CD45 insert comprising the whole molecule was constructed on the pBluescript SK +/- plasmid (see Materials and Methods). The size of the whole vector is 6.78 kb, and the CD45 insert is approximately 4 kb. Linkers at the 5' and 3' end are shown to demonstrate the extra restriction sites engineered for ease of subsequent subcloning. Selected restriction sites shown because they are unique or are useful for screening purposes. A region of the multiple cloning site 5' of the linker in pBluescript was removed. This region includes several restriction sites: (5'-3') Spe I, Bam H1, Sma I, Pst I, Eco R1, Eco RV, and Hind III.

The shuttle vector with the Q1180G mutation in CD45 was digested with Bgl II to produce a 2 kb insert comprising the cytoplasmic domain. This insert was ligated to the pET-3d-6His-IEGR bacterial expression vector (constructed by Dr. P. Johnson) that had been linearized with Bam HI and dephosphorylated and the ensuing plasmid was named C3.1px. The pET-3d [75] bacterial expression vector includes a 6 histidine tag to enable purification on a nickel column and a Factor Xa cleavage site; IEGR, to allow for purification of recombinant proteins away from the 6 histidine tag. To subclone the spacer deletion mutant into the pET-3d-6His-IEGR plasmid, the spacer deletion mutant that had been generated on the #592 plasmid (clone H8.8) was digested to produce a Kpn I-Bam HI fragment. This fragment was exchanged with a Kpn I-Bam HI fragment including the spacer region in the bacterial expression vector expressing the wild-type CD45 cytoplasmic domain (pET-3d-6His-IEGR-CD45wt) to produce H8.8px, the spacer deletion mutant in the pET-3d-6His-IEGR expression plasmid in XL-1 Blue *E. coli*. Finally, the S573GS574A mutation in the pBS-CD45 #26 vector was cut with Bgl II to isolate a 2 kb cytoplasmic domain fragment that was then ligated into the Bam HI site of pET-3d-6His-IEGR and the clones named 4.18px and 18.35px. These plasmids were subsequently transformed into BL21 (DE3) *E. coli* that contain a T7 polymerase-containing plasmid that can be induced by the addition of IPTG. The T7 polymerase then binds the T7 promoter in the pET vector system to induce the synthesis of recombinant proteins, in this case the CD45 cytoplasmic domain.

Bacterial Expression and Partial Purification of Mutant Proteins

10 mL of Luria broth supplemented with 100 μg/mL of ampicillin was inoculated with 100 μL of an overnight culture of the appropriate mutants in BL21 (DE3) *E. coli* and grown at 37°C to an $O.D._{600}$ of 0.6 (~2 hours) and then induced for 2 hours at 30°C with 40 mM IPTG. Bacteria were then lysed by spinning the bacteria in 50 mL Falcon tubes at 3000 r.p.m. in a Beckman GPR centrifuge for 15 minutes at 4°C. The bacteria were then resuspended in 2 mL of lysis buffer with 1 mM EDTA pH8.0 (50 mM

Tris-HCl pH7.5, 100 mM NaCl, 2 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, and 10 μg/mL pepstatin), re-centrifuged at 12 000g and then resuspended in lysis buffer without EDTA. 20 μL of lysozyme (10 mg/mL stock solution) was added to the lysate which was then frozen in a dry ice-ethanol bath. After allowing the lysate to thaw at room temperature, 40 μL of a 20 mg/mL solution of sodium deoxycholate was added and the solution was left at 4°C for 15 minutes, rotating end-over-end. 2 μL of DNase I (10 mg/mL) was then added to remove the nuclear material by placing the lysate at 37°C for 10 minutes. The nuclear material was removed by centrifugation at 12 000g and the resulting supernatant was immunoprecipitated with 10 μL of NTA^{2+}-agarose beads which binds the 6 histidine tag, for 90 minutes at 4°C rotating end-over-end. The immunoprecipitated recombinant proteins were eluted in 1 M imidazole pH7.2, 0.1% Triton X-100 and subsequently used in a PTPase assay.

PTPase Assays

Equal volumes of recombinant protein were assayed for tyrosine phosphatase activity against an 13-mer *fyn* peptide phosphorylated on tyrosine 531 (TATEPQpYQPGENL), synthesized by Dr. Ian-Clark Lewis (Biomedical Research Centre, U.B.C.) using a non-radioactive, colourimetric assay. This substrate was considered to be biologically relevant with respect to CD45 phosphatase activity as it had been demonstrated that CD45 was able to dephosphorylate tyrosine 531 of $p59^{fyn}$ *in vitro* [59] [59, 60, 63]. This assay measures phosphate release by the development of a green colour measured at $O.D._{650}$ [76]. Activity was normalized for equal amounts of recombinant CD45 cytoplasmic domain proteins by running equal volumes of recombinant proteins on a 7.5% SDS-polyacrylamide gel stained with Coomassie Blue (40% methanol, 10% acetic acid, 0.2% Coomassie Blue R-250). Amounts of protein were quantified by scanning densitometry of bands using Quantity One software (PDI Inc., Huntington Station, N.Y.). The recombinant protein was suspended in 10 μL of 1X PTPase buffer (50 mM Imidazole pH7.2, 1 mM EDTA, 0.1% β-mercaptoethanol, 2 mM

PMSF) and placed in a 96 well half-well plate (A/2 plates, Costar/Nuclepore Canada Inc., Toronto, Ont.) and left to equilibrate at 30°C, rotating at 140 r.p.m. The *fyn* pY531 peptide (original concentration 13.8 M) was diluted to a final concentration of 4 mM in 10X PTPase buffer (500 mM imidazole pH7.2, 10 mM EDTA, 1% β-mercaptoethanol, 20 mM PMSF), and 10 µL of this peptide was added to the 10 µL sample and rotated at 30 °C for the required time points, usually 0, 3, and 6 minutes. The reaction was stopped by the addition of 80 µL of malachite green solution (1 part 0.135% Malachite green oxalate salt, 1 part 4.2% ammonium molybdate, 0.01% Tween 20, and 2 parts sterile water), incubated at room temperature for 15 minutes to allow for the development of the green colour and read on a plate reader at $O.D._{650}$ using Softmax software (Biotechnology Lab, U.B.C.). To determine nmol of PO_4 hydrolyzed/$O.D._{650}$, 1 mM KH_2PO_4 was serially diluted twelve-fold in duplicate and assayed by the addition of the malachite green stop solution at $O.D._{650}$ and a standard curve was constructed.

CD3 Stimulation and Analysis of the src-family kinase p59fyn

9 X 10^6 BW5147 CD45-positive and CD45-negative cells were resuspended in 90 µL of pre-warmed DMEM and equilibrated at 37°C for 10 minutes. 9 µg of purified 145-2C11 was added at time zero to stimulate the cells. The cells were incubated at 37°C for the appropriate amount of time and then lysed by adding ice cold 10X lysis buffer (10% Triton X-100, 1.5 M NaCl, 200 mM Tris-HCl pH7.4, 20 mM EDTA, 5 mM sodium orthovanadate, 2 mM sodium molybdate, 2 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, and 10 µg/mL pepstatin). Cell lysates were placed on ice for 10 minutes and then centrifuged at 12 000g for 10 minutes to remove the insoluble pellet. Lysates were then added to 30 µL of protein A (Pharmacia, Baie d'Urfe, Qué.) that had been precoupled to 0.4 µL of murine fyn antiserum and 1 mL of 1X lysis buffer. These amounts had been previously determined to be sufficient to precipitate $p59^{fyn}$ from 10^7 BW5147 cells (unpublished observations). Immunoprecipitates were washed three times with 1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH7.5, 2 mM EDTA, 5 mM

sodium orthovanadate, 2 mM sodium molybdate and protease inhibitors, before being divided into three equal aliquots. 3 X 10^6 cell equivalents were run on a 7.5% SDS-polyacrylamide gels, transferred to a PVDF membrane, and blotted either with the 4G10 anti-phosphotyrosine antibody at a dilution of 1/1200 in 5% BSA-TBST or with the $p59^{fyn}$ antiserum in 5% BSA-TBST at a dilution of 1/1000. 3 X 10^6 cell equivalents were washed twice in kinase buffer (10 mM $MnCl_2$, 40 mM Pipes pH7.2) and then used in an *in vitro* kinase assay.

In Vitro Kinase Assays

Immunoprecipitated $p59^{fyn}$ was resuspended in a final volume of 10 µL kinase buffer containing 5 µCi ^{32}P-γ-ATP (specific activity ~3000 Ci/mmol, Amersham Canada, Ltd., Mississauga, Ont.) and incubated at room temperature for 10 minutes. The reaction was stopped with 25 mM EDTA in kinase buffer and the immunoprecipitate washed three times with kinase buffer prior to running on a 10% SDS polyacrylamide gel. The gel was dried and then exposed to Kodak X-Omat AR film with an intensifying screen.

RESULTS

1.0 Expression and Characterization of CD45 in L tk$^-$ cells

As expression of CD45 in lymphoid cells proved difficult, characteristics of individual isoforms were analyzed in L tk$^-$ cells. In addition, two mutant forms of CD45 were also expressed in these cells.

1.0.1 Characterization of Expression of Two CD45 Isoforms and Two CD45 Cytoplasmic Domain Mutants Transfected into L Cells

Two isoforms of CD45, CD45RABC and CD45RO, encoded by plasmid #106 and plasmid #36, were transfected into L cells (Fig. 5). Additionally, two cytoplasmic domain variants of CD45 were transfected into L cells in order to evaluate the structural and functional effects of the cytoplasmic domain on CD45 expression (Fig. 5). The mutant represented by CD45RABC C817S (plasmid #64) consists of an intact cytoplasmic domain with the tyrosine phosphatase activity rendered inactive by the mutation of cysteine 817 to a serine. This essential cysteine is required for catalysis of the phosphate hydrolysis reaction [25, 28]. The CD45RABC Δcyt construct (plasmid #12) represents a mutation in which the cytoplasmic domain has been deleted, with the exception of the first six amino acids of the membrane proximal region. The transcription of the CD45 cDNA was driven by the β-actin gene promoter in the pHβapr-1-neo mammalian expression vector [69]. For each of the isoforms transfected into L cells, approximately 25 clones were tested by flow cytometry for CD45 expression and 2 clones were found to be positive for CD45 expression (Table I). One clone for each isoform transfected was taken for further study. These clones are L106A6 (CD45RABC) and L36B3 (CD45RO). Only one CD45RABC C817S clone (L64C1) out of 64 clones tested by flow cytometry was positive for CD45 expression (Table I). In

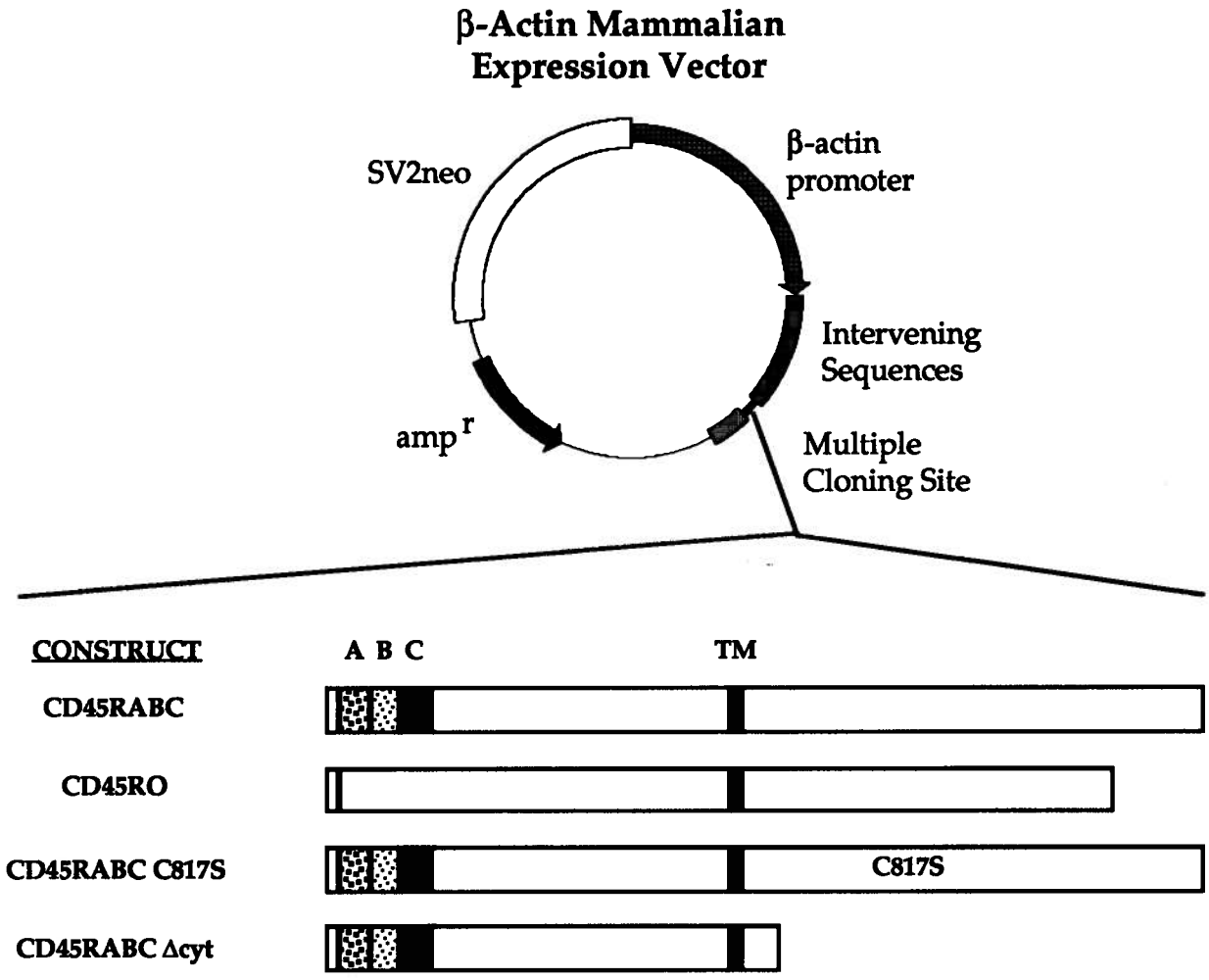


Figure 5. Schematic Diagram of the β-actin Mammalian Expression Vector and the CD45 Constructs Transfected into L cells. The β-actin mammalian expression vector and a representation of the CD45 constructs are shown. The CD45RABC, CD45RABC C817S, and CD45RABC Δcyt constructs had their 5' and 3' untranslated region shortened by the deletion of nucleotide sequences to promote mRNA stability. The CD45RABC Δcyt construct included 6 amino acids of the cytoplasmic domain to prevent secretion of the molecule. A, B, and C refer to the three alternatively spliced exons and TM refers to the transmembrane domain.

TABLE I.

CD45 Constructs	Number of Neo^r Colonies Screened	Number of CD45-positive Colonies
CD45RABC	24	2
CD45RO	25	2
CD45RABC C817S	64	1
CD45RABC Δcyt	26	7

Table. I. Results of Transfection of L tk$^-$ Cells with CD45 cDNAs. The number of noemycin resistant colonies screened and the number of CD45-positive clones obtained are shown. Colonies were screened by flow cytometry after four rounds of positive selection with anti-CD45 antibody and secondary antibody conjugated to magnetic beads (see Materials and Methods).

contrast, of the 26 CD45RABC Δcyt colonies tested, 7 were positive for CD45 expression (Table I). Clone L64C1 (CD45RABC C817S) and clones L12A5, L12B5, and L12C2 (CD45RABC Δcyt) were also taken for further study.

The total amounts of CD45 protein was determined by Western blot analysis of CD45 immunoprecipitated from transfected L cells (Fig. 6). It was determined that CD45RO transfected L cells synthesized similar amounts of CD45 protein as the CD45RABC transfected cells as determined by scanning densitometry. The apparent molecular weight of CD45 immunoprecipitated from the CD45 RABC C817S clone was determined to be 220 kDa (Fig. 6, lane 5), the same molecular weight as the wild-type CD45RABC molecule (Fig. 6, lane 3). A lower band detected at 200 kDa may be attributed to degradation of the protein during immunoprecipitation (Fig. 6, lane 5). CD45 immunoprecipitated from the CD45RABC Δcyt clones migrated as two bands of 160 kDa and 125 kDa (Fig. 6, lanes 6-8). The predicted molecular weight of this mutant was calculated to be 145 kDa, thus the presence of two CD45-specific bands, neither of which corresponded to the predicted molecular weight, was unexpected. It is possible that these two forms represent unprocessed and processed forms of the CD45 protein. No CD45 protein was detected in untransfected L tk^- cells by Western blot analysis (Fig. 6, lane 2).

Some clonal variation was observed with respect to total amounts of CD45 expressed in the three clones of the CD45RABC Δcyt mutant. The L12A5 clone expressed 3-fold to 5-fold less CD45 than the L12B5 and L12C2 clones, as determined by densitometry (Fig. 6, lanes 6-8). Because of this clonal variation, only clones L12B5 and L12C2 of the CD45RABC Δcyt mutant were used for further studies, the L12A5 clone was not characterized further. The L12C2 clone of the CD45RABC Δcyt transfectant expressed similar amounts of CD45 (~1.5-fold more) as the L12B5 clone (Fig. 6, lanes 7 and 8). The intensities of the 160 kDa band and the 125 kDa band were equivalent in individual clones of the CD45RABC Δcyt transfectant as determined by densitometry (Fig. 6, lanes 7 and 8), indicating that equivalent amounts of these species are expressed

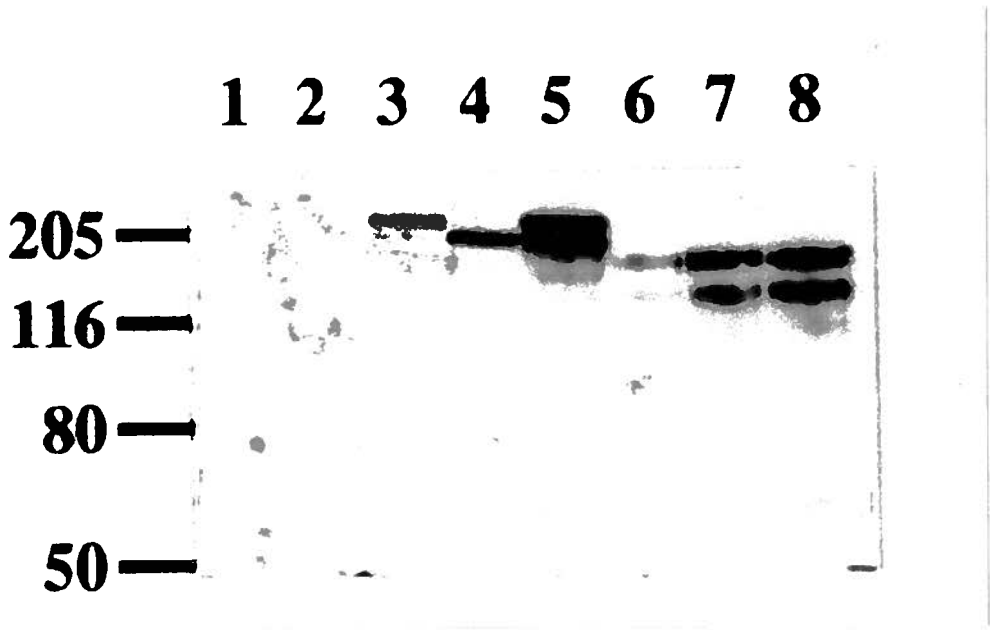


Figure. 6. Western Blot Analysis of CD45 Immunoprecipitates from Lysates of L cell Transfectants. CD45 was immunoprecipitated with the Ly5.2 antibody conjugated to Protein G from lysates of 2 X 10^6 CD45RABC *(lane 3)*, CD45RO *(lane 4)*, CD45RABC C817S *(lane 5)*, and CD45RABC Δcyt *(lanes 6-8)* transfected L cells and were blotted with an anti-CD45 antiserum (131) raised against a common peptide epitope in the CD45 extracellular domain. Three clones of the CD45RABC Δcyt mutant are shown, L12A5 *(lane 6)*, L12B5 *(lane 7)*, and L12C2 *(lane 8)*. Protein G plus the precipitating antibody *(lane 1)* and CD45 immunoprecipitates from untransfected L cells *(lane 2)* are shown for comparison. Molecular weight markers in kDa are shown on the left.

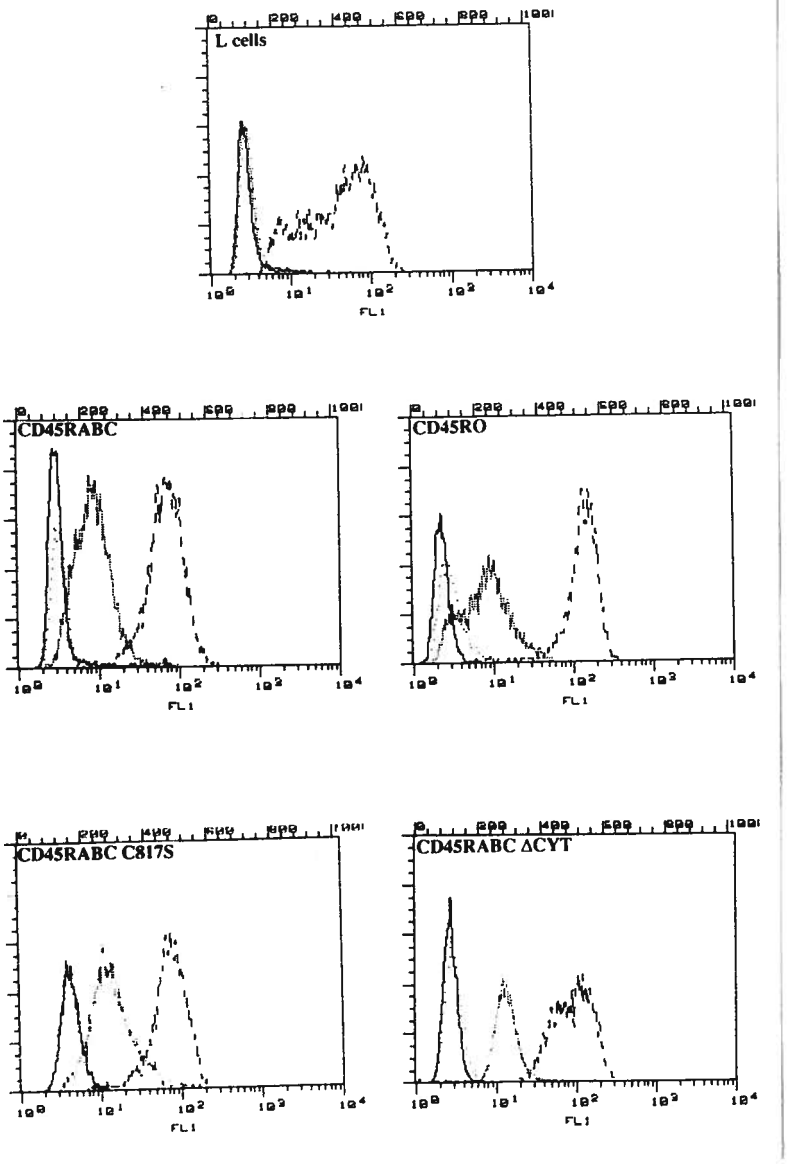


Figure. 7. Cell Surface Expression of CD45 as Determined by Flow Cytometry. L cell transfectants, CD45RABC, CD45RO, CD45RABC C817S, and CD45RABC Δcyt were screened for expression of CD45. Untransfected L cells are shown in the top panel for comparison. Cells were labeled with secondary antibody alone, FITC-labeled goat-anti-rat immunoglobulin (negative control ——), anti-CD4, GK1.5 (negative antibody control · · · · ·), anti CD45, I3/2 (·····), and anti-CD44, IM7.8.1 (positive antibody control – – – –).

in the cells. If the intensities of the two bands are added, the L12B5 and L12C2 clones of the CD45RABC Δcyt mutant express approximately the same amount of total CD45 protein as the CD45RABC C817S mutant (L64C1), which was significantly more than the CD45RABC (L106A6) and CD45RO (L36B3) clones. However, this experiment was only done once. Due to the lack of sufficient clones for each construct, it cannot be determined whether these differences are consistently seen. Indeed, subsequent data indicates that variation in the levels of expression can occur for individual clones.

CD45 cell surface expression detected by flow cytometry indicated that levels of CD45RABC (clone L106A6) were 3-fold over background (Fig. 7), that the levels of the CD45RO isoform (clone L36B3) were 4-fold over background (Fig. 7), the levels of CD45 expresion of the CD45RABC C187S transfectant (clone L64C1) were 3-fold over background (Fig. 7), and the levels of CD45RABC Δcyt (clone L12B5) were 4-fold over background (Fig. 7). Untransfected L cells were tested for CD45 expression, as a control (Fig. 7). Examination of surface expression of CD45 by flow cytometry indicated no significant differences in the levels of expression.

Surface expression of CD45 in the L cell transfectants was also characterized by immunoprecipitation of CD45 from the cell surface and subsequent Western blotting (Fig. 8). In addition, CD45 remaining in the cell lysate was also immunoprecipitated after immunoprecipitation of CD45 from the surface (Fig. 8). Immunoprecipitation of CD45 from the surface of L cell transfectants indicates that the CD45RABC Δcyt mutant is expressed at the highest level on the surface, followed by the CD45RABC C817S mutant, and then by the CD45RABC and CD45RO isoforms. Once again this experiment was only done once and without examination of multiple clones, it cannot be determined whether these differences are significant or whether they arise as a result of experimental variation or clonal variation. Immunoprecipitation of all four proteins indicate that the majority of CD45 is not present on the surface, but is found inside the cell (70-90%). Only 10% of the CD45RABC protein and 20% of the CD45RO protein is expressed on the surface compared to what is present inside the cell (Fig. 8, lanes 2S, 2C,

and 3S, 3C), as determined by densitometry. Similarly, only 30% of the CD45RABC C817S protein is expressed on the cell surface, with the rest of the protein remaining inside the cell (Fig. 8, lanes 4S and 4C). No CD45 was detected in the parental L cells (Fig. 8, lanes 1S and 1C). Bands appearing at about 200 kDa in CD45RABC immunoprecipitates (Fig.8, lane 2C) may be attributed to degradation of the protein during immunoprecipitation or alternatively, the presence of immature, unprocessed forms of CD45 inside the cell.

The observation of two distinct bands upon immunoprecipitation of total CD45 from the CD45RABC Δcyt mutant led to the characterization of these forms of CD45 on the surface. From the immunoprecipitation and Western blotting analysis of CD45 from the surface of the CD45RABC Δcyt clone L12C2, it was shown that the major species expressed on the surface of these cells was the 160 kDa form (Fig. 8, lane 5S). A lower band at about 130-140 kDa may be either a product of degradation or the lower molecular weight form (Fig. 8, lane 5S). The 160 kDa band that comprised CD45 expressed on the surface of the CD45RABC Δcyt mutant cells represented only 15% of the total amount of CD45 protein (Fig. 8, lanes 5S and 5C) expressed in these L cells, as determined by densitometry. When considering the 160 kDa band alone, 30% of the 160 kDa CD45 protein was expressed on the cell surface, and approximately 70% remained in the cell lysate. To determine the nature of these two species of CD45RABC Δcyt, pulse-chase and endoglycosidase H sensitivity experiments were performed.

1.0.2 Transport of a CD45 Protein Lacking the Cytoplasmic Domain in L cells

In order to determine the relationship between the higher and lower molecular weight forms of the CD45RABC Δcyt mutants, the transport of CD45 cytoplasmic deletion mutant through the endoplasmic reticulum (ER) and golgi apparatus was characterized by pulse-chase studies of the CD45 protein in L cells.

Deletion of the cytoplasmic domain resulted in the expression of two CD45 species of 125 kDa and 160 kDa. Pulse-chase analysis of this mutant expressed in L cells demonstrated that the first species present was the 125 kDa band (Fig. 9). Digestion of the CD45RABC Δcyt immunoprecipitates with endoglycosidase H resulted in a shift in molecular weight from 125 kDa to 80 kDa (Fig. 9), indicating that Endo H digestion reduced the molecular weight of the protein by ~40 kDa. The 125 kDa species remained Endo H sensitive (as represented by a band at ~80 kDa in the Endo H^+ lanes) to the end of the chase period of 3 hours (Fig.9). The second species, the 160 kDa form, which was the major form of CD45 expressed on the surface (Fig. 8, lanes 5S and 5C), was not observed until the 30 minute time point (Fig. 9). At 120 minutes, it was apparent that the 160 kDa species was becoming Endo H resistant, as demonstrated by the presence of a faint band at 150 kDa in the Endo H^+ lane. Additionally, the 125 kDa and 160 kDa bands were of equal intensity at 120 minutes in the Endo H^- lane but by 180 minutes, it was observed that as the intensity of the 160 kDa protein was increasing, the 125 kDa band in the Endo H^- lane was becoming less intense (Fig. 9), suggesting that the 160 kDa species was derived from the 125 kDa species. At the final time point of three hours, the 125 kDa protein was still present but the majority of the CD45 protein was represented by the upper band of 160 kDa (Fig. 9). Pulse-chase data from untransfected L cells was used as a control (Fig. 10) to determine the amount of background material that was immunoprecipitated. Background bands were detected at 55 kDa, 60 kDa, 70 kDa, and 80 kDa (Fig. 10).

DISCUSSION

The selection of CD45 expressing clones of L tk^- cells required several rounds of positive selection and the screening of 25 clones on average per contruct transfected (Table I). CD45 expression levels in L cell transfectants was low, ranging from 3-fold to 4-fold over background staining levels (Fig. 5). In comparison, others have shown that

L cells transfected with the CD45RO isoform expressed 9-fold more CD45 over background, normal lymphoid cells expressed approximately 50-100X more CD45 over background levels [77] as determined by flow cytometry.

Some inconsistencies were observed when comparing the results from Western blots of total CD45 and surface CD45. From the immunoprecipitation of total CD45 from the L cell transfectants, it appeared that clone L64C1 (CD45RABC C817S) expressed the highest levels of CD45, that the CD45RABC Δcyt mutant expressed intermediate levels, and that the full length CD45RABC and CD45RO isoforms expressed low levels of CD45 (Fig. 6). In contrast, from the examination of CD45 immunoprecipitated from the cell surface, the cytoplasmic deletion mutant (CD45RBAC Δcyt) expressed the most amounts of CD45 on the cell surface as well as inside the cell (Fig. 8). This discrepancy in expression levels may reflect the fact that these experiments were carried out at different times and that there was a certain amount of clonal variation. The CD45RABC C817S transfectant tended to be stable for CD45 expression for only a month in culture. Although these cells were periodically checked for CD45 expression by FACS analysis, the L64C1 clone may have experienced some loss of CD45 expression at the time of the surface immunoprecipitation of CD45 from the L cells. Unfortunately, this was the only clone of the CD45RABC C817S mutant that was obtained after five rounds of positive selection and the screening of 64 colonies (Table I). It is thus difficult to interpret the effect of inactivation of phosphatase activity on CD45 expression in L cells. More CD45RABC C817S clones will have to be tested. When considering the effects of the cytoplasmic deletion mutant on CD45 expression (Fig. 6 and Fig. 8), some clonal variation was observed. Clone L12A5 seemed to lose expression of CD45 on the surface over a period of six months after it had been cloned out and screened (unpublished observations). More clones of the isoforms and mutant constructs will have to be characterized with respect to CD45 expression in order to clear up the discrepancies in this data.

L cells transfected with CD45 expressed only a proportion of total CD45 protein on the surface. From the immunoprecipitation of CD45 from the surface of L cell transfectants, it was determined that between 10% and 30% of total CD45 in the cell was on the surface (Fig. 8). Whether this is also the same in lymphoid cells will be interesting to determine. Proteins destined for the plasma membrane should eventually localize to the cell surface, unless they are being degraded prior to or after localizing to the cell membrane, or if they are kept in the ER by chaperone proteins until they form dimers or multimeric complexes. It is unlikely that all the CD45 remaining in the cytosol is degraded because we observe that between 70% and 90% of total CD45 remains inside the cell (Fig. 8) and that this material is recognized by a CD45-specific blotting antibody upon immunoprecipitation. It is possible that CD45 is rapidly internalized upon expression on the cell surface and thus only 10-30% of CD45 is actually observed upon surface immunoprecipitation of CD45. Experiments to determine the rate of internalization of CD45 would address this question.

An alternative explanation of the large percentages of CD45 that remain inside the cell is that there is a requirement for some lymphoid-specific protein with which CD45 interacts and forms complexes. Since L cells may not express this other protein that CD45 interacts with, by virtue of being a fibroblast that does not express lymphoid-specific proteins, a large proportion of CD45 may be localized indefinitely inside the cell. Only a small fraction of CD45 being synthesized, between a tenth and a third, may escape this requirement for an interacting protein and localize to the cell membrane.

Deletion of the cytoplasmic domain resulted in three interesting results. The first interesting result was the observation of two bands at 160 kDa and 125 kDa of equal intensity, indicating that there were equivalent amounts of these two CD45 species in the L cells (Fig. 6). Yet when CD45 was immunoprecipitated from the surface of these transfectants, it appeared that predominantly one species, the 160 kDa protein, was being transported to the surface (Fig. 8). Even then, only a certain percentage of the CD45RABC Δcyt protein, 15%, was expressed on the cell surface, the remaining 85%

was left in the cell lysate (Fig. 8). Data from pulse-chase experiments explained some of these results. From the pulse chase experiment, it was determined that the 160 kDa form of CD45RABC Δcyt was derived from the 125 kDa form because the 160 kDa band did not appear until the 30 minute time point (Fig. 9.). In addition, between 120 and 180 minutes the higher molecular weight band gained in intensity as the intensity of the lower molecular weight form declined in the Endo H^- lanes (Fig. 9). The 125 kDa form remained Endo H sensitive for the whole chase period. In contrast, the 160 kDa form was Endo H insensitive by 3 hours (Fig. 9). These results suggest that the 125 kDa form is a precursor of the 160 kDa form. Why two forms of CD45RABC Δcyt are expressed in L cells and the carbohydrate modifications ocurring to the 125 kDa protein in order to generate the 160 kDa protein are not known.

One possible explanation for the observation of two CD45RABC Δcyt species is that CD45 transport is normally facilitated in some way, perhaps by interacting with a transporter protein. This interaction may occur through the CD45 cytoplasmic domain. Thus, when the cytoplasmic tail of CD45 is deleted, transport of the protein may be adversely affected, causing the sequestration of the protein in the ER and golgi apparatus, resulting in the differential glycoslyation of this mutant. Indeed, the time taken for 50% of this protein to become Endo H resistant was approximately 135 minutes (data not shown). Preliminary data from pulse chase studies of the full length form, CD45RABC, showed that it became Endo H resistant by 30 minutes (data not shown), suggesting that the cytoplasmic deletion mutant is transported slower than the CD45RABC isoform and that it does spend more time in the ER.

RESULTS

2.0 Mutational Analysis of the Cytoplasmic Domain of CD45

In this work, three CD45 cytoplasmic domain mutants were made and expressed in bacteria (Fig. 11). As mentioned previously, deletion of the C-terminal tail of CD45 and a further 13 amino acids at the C-terminus of domain II abrogated PTPase activity. Mutation of a conserved tyrosine in this stretch of amino acids (Y1181F), had no impact on phosphatase activity. One of the goals of the work presented here was to identify residues in the deleted area that were crucial for enzymatic activity out of the 13 amino acids in this region. Glutamine 1180 is invariantly conserved in both PTPase domains of human, mouse, and rat CD45 as well as numerous other protein tyrosine phosphatases (Fig. 3), suggesting that this residue in domain II may be crucial for the structure or function of the enzyme. Therefore a point mutation was introduced in the cytoplasmic domain of CD45 such that glutamine 1180 was changed to a glycine (Q1180G). A second mutant was made to delete the spacer region between domain I and domain II, since it had not been established in prevous work, whether or not this region was required for activity [32]. Finally, a third mutant was made to abolish a potential site of cAMP-dependent kinase or protein kinase C (PKC) phosphorylation. Ultimately, this mutant would be tested *in vivo* to determine if cAMP-dependent kinase phosphorylation occurs at this site and if so, if it affects CD45 function.

2.0.1 Generation of Three Mutations in the Cytoplasmic Domain of CD45

Mutations were generated on a single stranded template by the Kunkel method of site-directed mutagenesis [71, 72]. Oligonucleotides were engineered to include a restriction endonuclease site for screening mutant colonies. The Q1180G mutation was engineered to include an extra Kpn I site such that digestion of plasmid DNA would

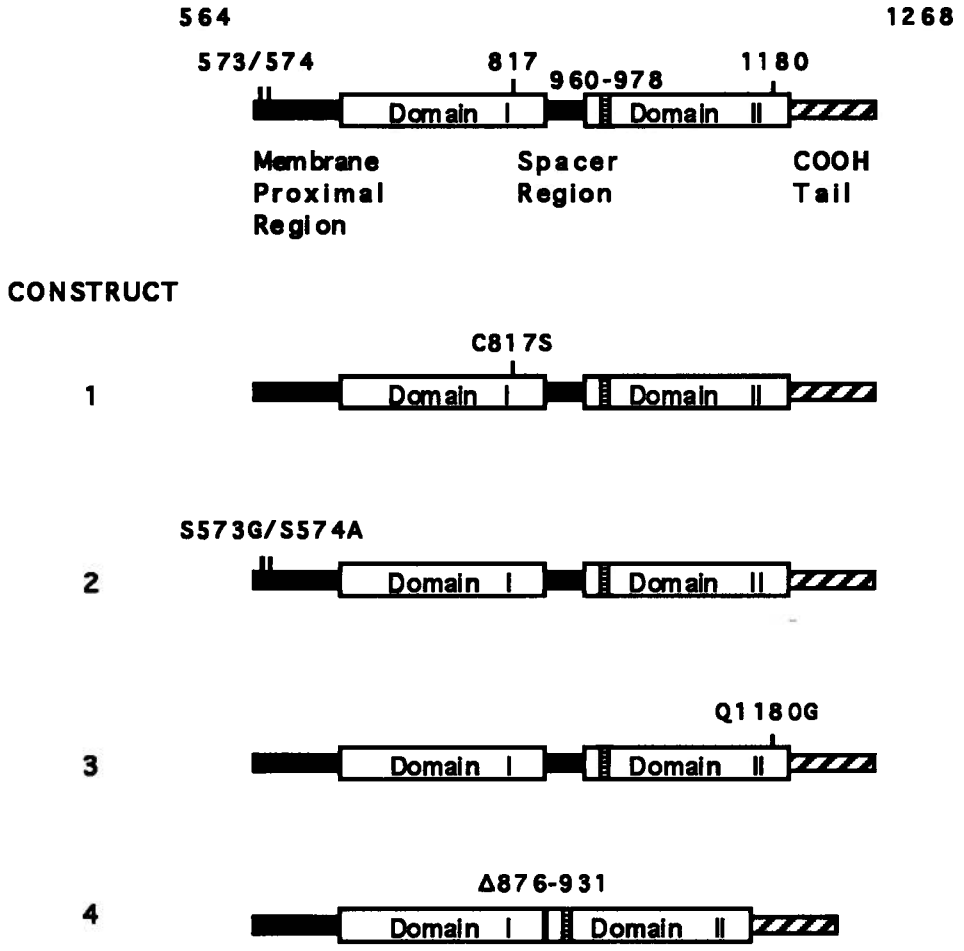


Figure. 11. Schematic Diagram of the CD45 Cytoplasmic Domain Mutants. The constructs used to generate the CD45 cytoplasmic domain proteins are illustrated. Construct 1 represents an inactivated PTPase, construct 2 represents the point mutation of two consecutive serines that may be the site of phosphorylation by cAMP-dependent kinase or PKC, construct 3 is a point mutant in PTPase domain II, and construct 4 represents a CD45 protein with the spacer region deleted. The numbering system used is from the mouse CD45RABC isoform [9].

yield bands of 3.8 kb and 2.2 kb for wild-type colonies and 3.8 kb, 1.2 kb, and 1.0 kb for colonies carrying mutant plasmids. Deletion of the spacer region between domain I and domain II resulted in the loss of 168 nucleotides which was detectable after an Eco R1 digest. Mutants with the spacer region deleted yielded bands of 4.8 kb and 1.1 kb upon digestion with Eco R1, while the wild-type colonies would have bands of 4.8 kb and 1.3 kb. The S573GS574A mutation in the membrane proximal region was engineered to include a Bsa HI site. Thus wild-type colonies would generate bands of 4.8 kb and 2.2 kb when digested with Bsa HI while colonies containing mutants would have bands of 3.2 kb, 2.2 kb, and 1.6 kb. All mutations were sequenced to verify that all mutations were accurate and in frame.

2.0.2 Bacterial Expression and Partial Purification of CD45 Mutants

The two mutants generated by site-directed mutagenesis in the pBluescript SK (+/-) vector (Stratagene Cloning Systems, La Jolla, Ca.) were then cut with Bgl II and subcloned into the Bam H1 site of the pET-3d-6His-IEGR bacterial expression vector. Unfortunately, this was not as easily accomplished for the Q1180G mutant. Therefore a shuttle vector was constructed with convenient restriction sites which allowed easy shuttling of mutant constructs out of the original pBluescript vector and into bacterial or mammalian expression vectors. The Q1180G mutant had to be subcloned into the shuttle vector (see Materials and Methods) and once in the shuttle vector, a Bgl II fragment comprising the cytoplasmic domain mutant was isolated and ligated into the Bam HI site of the pET-3d-6His-IEGR vector. The spacer deletion mutant was subcloned into pET-3d-6his-IEGR-CD45 by a Kpn I-Bam H1 fragment. Bacteria transformed with the mutant CD45 constructs were induced to express CD45 in the log phase of growth.

It was noted that levels of recombinant protein expression were equivalent for three independent clones of the Q1180G mutant (Fig. 12, lanes 7-9) and for two

independent clones of the S573GS574A mutant (Fig. 12, lanes 10-11) as demonstrated by Coomassie Blue staining of proteins run on an SDS-polyacrylamide gel. Three independent clones of the spacer deletion mutant did not express very well and so this mutant was not pursued further (Fig. 12, lanes 4-6). As controls, both wild-type CD45 (Fig. 12, lane 2) and a PTPase inactive mutant, C817S (Fig. 12, lane 3), as well as the vector without insert (Fig. 12, lane 1) were induced in BL21 (DE3) *E. coli* and soluble protein lysates were isolated and immunoprecipitated with NTA^{2+}-agarose which specifically binds to the 6 histidine tag present at the N-terminus of the recombinant protein, allowing partial purification of the recombinant protein. From Coomassie blue stained gels, a prominent band at 95 kDa was observed and verified in a previous experiment to be recombinant CD45 by western blotting with an antiserum against the cytoplasmic domain of CD45 (data not shown). The purification was considered to be partial because of the presence of contaminating bands at 110 kDa, 106 kDa, 75 kDa, 70 kDa, 60 kDa and a major species at 49 kDa which was shown to be due in part, to the degradation of the 95 kDa recombinant CD45 protein, as demonstrated by immunoblotting CD45. While the wild-type CD45 construct and the C817S mutant were expressed at relatively high and equivalent levels, the Q1180G and S573GS574A mutants expressed only a fifth and a third as much recombinant CD45 respectively (Table III). No proteins at 95 kDa were observed in the lane containing lysates from the vector control (Fig. 12, lane 1).

2.0.3 Determination of Phosphatase Activity of CD45 Mutants

Recombinant proteins generated by bacterial expression were assayed using a non-radioactive phosphatase assay [76]. The mutation of glutamine 1180 to glycine (Q1180G), contained within the 13 C-terminal residues of CD45 domain II required for PTPase activity, completely abolished the activity of the enzyme (Fig. 13). The phosphatase assays of the wild-type cytoplasmic domain of CD45 and the C817S

Table II. Quantitation of Band Density of Bacterially Expressed Proteins by Scanning Densitometry. The SDS-PAGE gel of bacterially expressed proteins (Fig. 12) was scanned using the Quantity One software (PDI Inc.) and the density of the bands determined. Bands scanned were of the vector alone (pET vector), the wild-type CD45 cytoplasmic domain (pET CD45), the mutant in PTPase domain II (Q1180G), the mutant with the spacer region deleted (Δspacer), and the mutant in the potential cAMP-dependent kinase/PKC site, (S573GS574A). Specific clones tested are noted. Lane numbers refer to lanes in Figure. 12, ND refers to densities that were not determined due to the faintness of bands.

*2/3 of the total volume of these two samples were loaded on the SDS-PAGE gel, the ratio of band intensity values were therefore normalized to represent the total volume.

TABLE II.

Lane	Construct	Band Density O.D. X mm^2	Ratio of Band Density
1	pET Vector alone	0	0
2	pET CD45	0.979*	1.0
3	C817S mutant	0.923*	0.94
4	Δspacer mutant clone H8.8	ND	ND
5	Δspacer mutant clone H9.1	ND	ND
6	Δspacer mutant clone H9.11	ND	ND
7	Q1180G mutant clone C3.1A	0.275	0.20
8	Q1180G mutant clone C3.1B	0.216	0.14
9	Q1180G mutant clone C3.1C	0.399	0.27
10	S573GS5754A mutant-clone 4.18	0.481	0.33
11	S573GS574A mutant clone 18.35	0.438	0.30

mutation in the catalytic centre of domain I as positive and negative controls respectively, are shown for comparison. It should be noted that the rates of dephosphorylation were normalized for equivalent amounts ofrecombinant CD45 assayed. The normalized activity of the S573GS574A mutant was equivalent to the wild-type recombinant protein (Fig. 13).

DISCUSSION

The fact that mutation of glutamine 1180 to glycine in domain II of CD45 abolished PTPase activity suggests that a disruption in domain II can abolish an activity that is thought to reside in domain I. Thus, a mutational event in domain II affects domain I, supporting the two domain enzyme hypothesis, which postulates that both phosphatase domains contribute to enzymatic activity by interacting with each other. Whether the glutamine at position 1180 of domain II is required for the appropriate folding of domain II or if it has a functional role remains to be determined. Recent publication of the x-ray crystal structure of PTP 1B, a single domain tyrosine phosphatase implicates glutamine 262 of PTP 1B in the interaction with the phosphotyrosine in the catalytic site [36] and suggests a role for glutamine 1180 in domain II of CD45, in the catalytic site. Since the glutamine homologous to Q1180 is invariantly conserved in all phosphatases, it may play a role in stabilizing the dephosphorylation reaction.

PTP domain II may be an important structural requirement for activity in domain I. This could occur by the involvement of residues in domain II in the catalytic site of the enzyme or in the stabilization of an enzymatically active domain I. The amide side chain of glutamine 262 of PTP 1B is involved in forming hydrogen bonds with the phosphate substrate. Mutation of glutamine 1180 in domain II to glycine may destroy this potential structural requirement for activity in domain I by making an amide group less accessible to the phosphate substrate in order to form an H-bond. The fact that

deletion of CD45 domain II or even specific mutations in domain II result in an inactive PTPase supports the idea that domain II can modulate the activity of domain I and that it is required for the optimal functioning of domain I as a phosphatase. How phosphatase domain II can regulate domain I function may be related to its unique ability to recruit substrates. There is some evidence to support this theory as it has been shown that bacterially expressed proteins encompassing domain II of HPTPα may have distinct substrate specificities from domain I [78].

A point mutation in domain II has deleterious effects on the phosphatase activity which is thought to reside in domain I of CD45. While it may be true that proteins expressed in bacteria may not fold as well as in eukaryotic cells, this result suggests that domain II has a role to play in maintaining the enzymatic activity of CD45. The Q1180G mutant will be a useful mutant that will be worth testing as a full-length form of CD45 in L cells to determine if domain II regulates the activity of domain I *in vivo*.

RESULTS

3.0 Characterization of a Recipient CD45-Negative T Lymphoma Cell Line

The CD45-deficient variant of a BW5147 cell line which expressed the TCR/CD3 complex was previously characterized with respect to induction of tyrosine phosphorylated proteins upon TCR-mediated stimulation and the phosphorylation and activity of $p56^{lck}$ (P. Johnson, personal communication). In this work, the CD45-negative and CD45-positive BW5147 cells were characterized with respect to the levels of $p59^{fyn}$ expression, phosphotyrosine levels of $p59^{fyn}$, and $p59^{fyn}$ *in vitro* kinase activity.

3.0.1 Determination of the Levels of Expression of the Src-family Kinase $p59^{fyn}$ in CD45-Negative and CD45-Positive Variants of a BW5147 T Lymphoma Cell Line

$p59^{fyn}$ was immunoprecipitated from equivalent numbers of unstimulated CD45-negative and CD45-positive cells and immunoblotted with $p59^{fyn}$ antiserum. Equivalent amounts of $p59^{fyn}$ were precipitated from the CD45-negative and CD45-positive T cells and migrated at ~59 kDa, the predicted molecular weight of *fyn* (Fig. 14). The control lane shows a band at 50 kDa that can be attributed to crossreactivity of the protein A-HRP secondary antibody to the heavy chain of the immunoprecipitating antibody (Fig. 14).

3.0.2 Evaluation of the Effect of CD45 Expression on the Phosphotyrosine Levels of $p59^{fyn}$

$p59^{fyn}$ was immunoprecipitated from equivalent numbers of CD45-negative and CD45-positive cells both prior to and after stimulation through the TCR/CD3complex with soluble anti-CD3 antibodies and Western blotted with the 4G10 anti-phosphotyrosine antibody. Like $p56^{lck}$ [26, 63], $p59^{fyn}$ was more phosphorylated in CD45-negative cells than in CD45-positive cells (Fig.15). This difference was maintained upon T cell stimulation. A transient increase in tyrosine phosphorylation of *fyn* in CD45-negative BW5147 cells was observed 90 seconds after stimulation (Fig. 15) although this subtle change in phosphorylation was not always detectable. In contrast, the tyrosine phosphorylation of $p59^{fyn}$ was observed to increase steadily over time after anti-CD3 stimulation (Fig. 15) in the CD45-positive cells.

3.0.3 Determination of the Effect of CD45 Expression on the *In Vitro* Kinase Activity of $p59^{fyn}$

$p59^{fyn}$ immunoprecipitated from 3×10^6 cells was subjected to an *in vitro* kinase assay both before and after the cells were stimulated through the TCR/CD3 complex. A slight increase in autophosphorylation of $p59^{fyn}$ was observed 90 seconds after T cell stimulation in the *in vitro* kinase assay of *fyn* immunoprecipitates from CD45-negative cells, but no such increase in activity was observed in the CD45-positive cells (Fig. 16). Instead, more significant differences were observed in other proteins phosphorylated in the *fyn* immunoprecipitates.

A prominent phosphorylated species of 120/130 kDa band was detected (Fig. 16) in an *in vitro* kinase assay on $p59^{fyn}$ immunoprecipitates from CD45-positive cells. This phosphorylated 120/130 kDa protein was observed in $p59^{fyn}$ immunoprecipitates in both unstimulated and stimulated CD45-positive cells. In the *in vitro* kinase assay of $p59^{fyn}$ isolated from CD45-negative cells, this phosphorylated species was not present. However, a faint phosphorylated band at 120/130 kDa was detected in the CD45-negative cells five minutes after T cell

stimulation. Upon stimulation with soluble anti-CD3 a 30 kDa phosphoprotein was observed (Fig.16), which was enhanced in intensity upon CD3 stimulation. This 30 kDa phosphorylated species was not observed in the kinase assay of *fyn* isolated from CD45-positive cells.

DISCUSSION

Analysis of the phosphorylation state and kinase activity of $p59^{fyn}$ led to the surprising finding that although the presence of CD45 in the cell substantially affected phosphorylation state of the kinase, it did not result in increased activity. In fact, a slight increase in $p59^{fyn}$ autophosphorylation was observed in the CD45-negative cells. This is a paradoxical result when one considers that the induction of tyrosine phosphorylated proteins upon T cell stimulation is less efficient in the CD45-negative cells (P. Johnson, personal communication). This situation was recently reported in three other CD45-negative cell lines [61]. The transient nature of the increased autophosphorylation of $p59^{fyn}$ in CD45-negative cells suggests that in the absence of CD45, other protein tyrosine phosphatases may dephosphorylate this kinase.

No correlation was found between dephosphorylation of $p59^{fyn}$ and an increased kinase activity in CD45-positive cells, counter to the current model for src-family kinase regulation which postulates that dephosphorylation of the src-family kinases leads to their activation. A correlation was observed between the presence of CD45, the phosphorylation state of the kinase, and the proteins associated with the kinase. Differences were observed in the phosphoproteins that associated with $p59^{fyn}$ in CD45-negative and CD45-positive variants of BW5147 cells. A 120/130 kDa protein was found to co-precipitate with *fyn* and be phosphorylated in an *in vitro* kinase assay by $p59^{fyn}$ or another co-precipitating kinase in CD45-positive T cells regardless of stimulation, suggesting that the association was constitutive and was not occurring as a result of TCR-mediated stimulation. This 120/130 kDa protein was not observed in *in*

vitro kinase assays from *fyn* immunoprecipitates from CD45-negative cells. The absence of the 120/130 kDa band in the *in vitro* kinase assay of *fyn* immunoprecipitates from CD45-negative cells may be due in part, to the saturation of tyrosine phosphorylation sites prior to the addition of γ-^{32}P-ATP. This would suggest that the absence of CD45 in these cells results in the hyperphosphorylation of the 120/130 kDa protein. Absence of the p120/130 from the phosphotyrosine blot of p59^{fyn} immunoprecipitates suggests that it is either not phosphorylated in the cell or that the stoichiometry of phosphorylation is below the detection level of the phosphotyrosine blot. Currently, the identity of this 120/130 kDa protein is unknown. Yet in CD45-negative cells, a faint 120/130 kDa phosphorylated band can be seen in *fyn* kinase assays 90 seconds after TCR/CD3 stimulation, indicating that this association is much weaker and much less efficient in CD45-negative cells and requires T cell stimulation for it to occur. This data indicates that CD45 is required for the efficient coupling of this 120/130 kDa *in vitro* substrate to p59^{fyn}. Currently, it is not known what mediates the association of p59^{fyn} with the 120/130 kDa protein but it has recently been demonstrated that tyrosine phosphorylated 120 kDa proteins do associate with both the SH2 [79, 80] and the SH3 domains [81]of p59^{fyn}. These proteins are rapidly phosphorylated upon T cell activation implying that they are mediators of TCR induced signalling events. The identity of these 120 kDa proteins remains to be established.

In addition, a 30 kDa phosphoprotein was observed in CD45-negative cells which was enhanced upon CD3 stimulation. As p59^{fyn} is hyperphosphorylated at its carboxy terminal tyrosine 531 in CD45-negative BW5147 cells [62, 63], it is possible that this must occur for the p30 to bind. It will be interesting to determine if this protein is involved in the regulation of TCR-mediated signalling events.

Thus, it has been determined that p59^{fyn} is hyperphosphorylated in the absence of CD45 but that this does not influence its kinase activity, assessed *in vitro*. Rather, the constitutive association of a 120/130 kDa protein to *fyn* is adversely affected by the absence of CD45. These results implicate CD45 in regulating associations of p59^{fyn} to

other proteins and may be involved in coupling the kinase to downstream TCR/CD3 induced signalling events. Both the phosphorylation of *fyn* and its association with the 120/130 kDa protein will be useful parameters with which to analyze the function of CD45 in deficient cells lines in which the expression of CD45 has been restored.

CONCLUSION

The work presented in this thesis has furthered the understanding of the molecular function of CD45. From the study of two isoforms of CD45 transfected into L tk$^-$ cells, CD45RABC and CD45RO, it was determined that expression of the three alternatively spliced exons at the N-terminus of CD45 did not significantly affect expression of the molecule. In contrast, deletion of the cytoplasmic domain of CD45 resulted in the expression of two species of 125 kDa and 160 kDa. It appears from pulse-chase experiments, that the higher molecular weight form is derived from the lower molecular weight form and that the difference in molecular weight is due additional glycosylation. The carbohydrate modifications occurring to the lower molecular weight form in order to generate the higher molecular weight form are not known.

The protein tyrosine phosphatase activity of CD45 was analyzed after targeted mutations in the cytoplasmic domain and it was concluded from this study that PTPase domain II is required for the activity of PTPase domain I. The point mutation of glutamine 1180 to a glycine in domain II abrogates phosphatase activity, suggesting that a disruption in domain II can abolish an activity which is thought to reside in domain I. This result supports the idea of a two domain enzyme in which domain II regulates the activity in domain I, potentially by interacting both with domain I and with putative substrates.

The effect of CD45 on the src-family kinase p59^{fyn} was characterized in a T cell line. p59^{fyn} is thought to be one of the kinases involved in signalling through the T cell receptor complex. It was determined that CD45 affects the tyrosine phosphorylation of p59^{fyn}, as *fyn* was hyperphosphorylated in CD45-negative BW5147 cells. Yet CD45 did not appear to affect the kinase activity of p59^{fyn} as demonstrated by its autophosphorylation. Rather, CD45 appears to affect the association between p59^{fyn} and other phosphorylated proteins, in particular, a 120/130 kDa protein. The

constitutive association of the p120/130 to $p59^{fyn}$ was not observed in CD45-negative BW5147 cells. These results implicate CD45 in regulating the associations of $p59^{fyn}$ to other proteins and CD45 may even be involved in coupling the kinase to downstream T cell receptor-mediated signalling events.

Characterization of the CD45-deficient BW5147 cell line with respect to induction of tyrosine phosphorylated proteins upon T cell stimulation, $p56^{lck}$, and in this work, $p59^{fyn}$, has now provided a suitable cell line with which to try and reconstitute deficiencies in aspects of signalling by transfecting various CD45 constructs back into these cells. In addition, further mutational analysis of the CD45 cytoplasmic domain in the work presented supports the two domain model for an active CD45 PTP enzyme and provides us with the tools to test this model further in lymphoid cell lines, such as the CD45-deficient BW5147 cells.

PUBLICATIONS

A list of publications arising from work presented in this thesis is included.

Submitted:

1. **Maiti, A., P. Borodchak, T. Brocker, M. D. Jabali, B. Malissen, and P. Johnson.** (1994) Effect of CD45 on phosphorylation and protein associations of $p56^{lck}$ and $p59^{fyn}$ during T cell receptor stimulation. *submitted to J. Biol. Chem.*

In preparation:

1. **Maiti, A., I. Haidl, W. Jefferies, and P. Johnson.** (1994) Deletion of the cytoplasmic domain of CD45 retards transport of CD45 significantly in L cells.

2. **Ng, D., A. Maiti, and P. Johnson.** (1994) Point mutation in the second phosphatase domain of CD45 abrogates phosphatase activity.

REFERENCES

1. **Trowbridge, I. S., H. Ostergaard and P. Johnson.** 1991. CD45: A leukocyte-specific member of the protein tyrosine phosphatase family. *Biochem. Biophys. Acta. 1095:46-56.*

2. **Trowbridge, I. S.** 1991. CD45: A prototype for transmembrane protein tyrosine phosphatases. *J. Biol. Chem. 266:23517-23520.*

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

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

5. **Barclay, A. N., D. I. Jackson, A. C. Willis and A. F. Williams.** 1987. Lymphocyte specific heterogeneity in the rat leukocyte common antigen (T200) is due to differences in polypeptide sequences near the NH_2-terminus. *EMBO J. 6:1259-1264.*

6. **Thomas, M. L., A. N. Barclay, J. Gagnon and A. F. Williams.** 1985. Evidence from cDNA clones that the rat leukocyte common antigen (T200) spans the lipid bilayer and contains a cytoplasmic domain of 80,000 Mr. *Cell 41:83-93.*

7. **Saga, Y., J.-S. Tung, F.-W. Shen and E. A. Boyse.** 1986. Sequences of Ly-5 cDNA: isoform related diversity of Ly-5 mRNA. *Proc. Natl. Acad. Sci. USA 83:6940-6944, and correction (1987) 84: 1991.*

8. **Ralph, S. J., M. L. Thomas, C. C. Morton and I. S. Trowbridge.** 1987. Structural variants of human T200 glycoprotein (leukocyte-common antigen). *EMBO J. 6:1251-1257.*

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

10. **Saga, Y., J.-S. Tung, F.-W. Shen and E. A. Boyse.** 1987. Alternative use of 5' exons in the specification of Ly-5 isoforms distinguishing hematopoietic cell lineages. *Proc. Natl. Acad. Sci. USA 84:5364-5368.*

11. **Streuli, M., L. R. Hall, Y. Saga, S. F. Schlossman and H. Saito.** 1987. Differential usage of three exons generates at least five different mRNAs encoding leukocyte-common antigens. *J. Exp. Med. 166:1548-1566.*

12. **Hall, L., M. Streuli, S. Schlossman and H. Saito.** 1988. Complete exon-intron organization of the human leukocyte common antigen (CD45) gene. *J. Immunology 141:2781-2787.*

13. **Johnson, N. A., C. M. Meyer, J. T. Pingel and M. L. Thomas.** 1989. Sequence conservation in potential regulatory regions of the mouse and human leukocyte common antigen gene. *J. Biol. Chem. 264:6220-6229.*

14. **Jackson, D. I. and A. N. Barclay.** 1989. The extra segments of sequence in rat leukocyte common antigen (L-CA) are derived by alternative splicing of only 3 exons and show extensive O-linked glycosylation. *Immunogenetics 29:281-287.*

15. **Pulido, R. and F. Sanchez-Madrid.** 1990. Glycosylation of CD45: Carbohydrate composition and its role in acquisition of CD45RO and CD45RB T cell maturation-related antigen specificities during biosynthesis. *Eur. J. Immunol. 20:2667-2671.*

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

17. **Pulido, R. and F. Sanchezmadrid.** 1992. Glycosylation of CD45: Carbohydrate processing through golgi apparatus is required for cell surface expression and protein stability. *Eur. J. Immunol. 22:463-468.*

18. **Stamenkovic, I., D. Sgroi, A. Aruffo, M. S. Sy and T. Anderson.** 1991. The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45R0 on T cells and α2–6 sialyltransferase, CD75, on B cells. *Cell 66:1133-1144.*

19. **Aruffo, A., S. B. Kanner, D. Sgroi, J. A. Ledbetter and I. Stamenkovic.** 1992. CD22-mediated stimulation of T cells regulates T-cell receptor/CD3-induced signaling. *Proc. Natl. Acad. Sci. USA 89:10242-10246.*

20. **Sgroi, D., A. Varki, S. Braesch-Andersen and I. Stamenkovic.** 1993. CD22, a B cell-specific immunoglobulin superfamily member, is a sialic acid-binding lectin. *J. Biol. Chem. 268:7011-7018.*

21. **Powell, L. D., D. Sgroi, E. R. Sjoberg, I. Stamenkovic and A. Varki.** 1993. Natural ligands of the B cell adhesion molecule CD22β carry N-linked oligosaccharides with a α2,6-linked sialic acids that are required for recognition. *J. Biol. Chem. 268:7019-7027.*

22. **Charbonneau, H., N. K. Tonks, K. A. Walsh and E. H. Fischer.** 1988. The leukocyte common antigen (CD45): a putative receptor-linked protein tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA 85:7182-7186.*

23. **Tonks, N. K., H. Charbonneau, C. D. Diltz, E. H. Fischer and K. A. Walsh.** 1988. Demonstration that the leukocyte common antigen CD45 is a protein tyrosine phosphatase. *Biochemistry 27:8695-8701.*

24. **Tonks, N. K., C. D. Diltz and E. H. Fischer.** 1990. CD45, an integral membrane protein tyrosine phosphatase. *J. Biol. Chem. 265:10674-10680.*

25. **Streuli, M., N. X. Krueger, A. Y. M. Tsai and H. Saito.** 1989. A family of receptor-linked protein tyrosine phosphatases in humans and Drosophila. *Proc. Natl. Acad. Sci. USA 86:8698-8702.*

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

27. **Tonks, N. K., Diltz, C. D. & Fischer, E. H.** 1988. Characterization of the major protein-tyrosine-phosphatases of human placenta. *J. Biol. Chem. 263:6731-6737.*

28. **Guan, K. L. and J. E. Dixon.** 1991. Evidence for protein-tyrosine-phosphatase catalysis proceeding via a cysteine-phosphate intermediate. *J. Biol. Chem. 266:17026-17030.*

29. **Pot, D. A., T. A. Woodford, E. Remboutsika, R. S. Haun and J. E. Dixon.** 1991. Cloning, bacterial expression, purification, and characterization of the cytoplasmic domain of rat LAR, a receptor-like protein tyrosine phosphatase. *J. Biol. Chem. 266:19688-19696.*

30. **Pot, D. A. and J. E. Dixon.** 1992. Active site labeling of a receptor-like protein tyrosine phosphatase. *J. Biol. Chem. 267:140-143.*

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

32. **Johnson, P., H. L. Ostergaard, C. Wasden and I. S. Trowbridge.** 1992. Mutational analysis of CD45, a leukocyte-specific tyrosine phosphatase. *J. Biol. Chem. 267:8035-8041.*

33. **Pai, E. F., W. Kabsch, U. Krengel, K. C. Holmes, J. John and A. Wittinghofer.** 1989. Structure of the guanine-nucleotide-binding domain of Ha-ras oncogene product p21 in the triphosphate conformation. *Nature 341:209-214.*

34. **Taylor, S. S., J. A. Buechler and W. Yonemoto.** 1990. cAMP-dependent protein kinase: framework for a diverse family of regulatory enzymes. *Annu. Rev. Biochem. 59:971-1005.*

35. **Tan, X., D. R. Stover and K. A. Walsh.** 1993. Demonstration of protein tyrosine phosphatase activity in the second of two homologous domains of CD45. *J. Biol. Chem. 268:6835-6838.*

36. **Barford, D., A. J. Flint and N. K. Tonks.** 1994. Crystal structure of human protein tyrosine phosphatase 1B. *Science 263:1397-1404.*

37. **Matthews, R. J., E. D. Cahir and M. L. Thomas.** 1990. Identification of an additional member of the protein-tyrosine-phosphatase family: Evidence for alternative splicing in the tyrosine phosphatase domain. *Proc. Natl. Acad. Sci. USA 87:4444-4448.*

38. **Streuli, M., N. X. Krueger, L. R. Hall, S. F. Schlossman and H. Saito.** 1988. A new member of the immunoglobulin superfamily that has a cytoplasmic region homologous to the leukocyte common antigen. *J. Exp. Med. 168:1553-1562.*

39. **Chernoff, J., A. R. Schievella and B. G. Neel.** 1991. Molecular cloning and expression of a major human protein tyrosine phosphatase. *Adv. in Protein Phosphatases 6:59-71.*

40. **Cool, D. E., N. K. Tonks, H. Charbonneau, K. A. Walsh, E. H. Fischer and E. G. Krebs.** 1989. cDNA isolated from a human T-cell library encodes a member of the protein-tyrosine-phosphatase family.*Proc. Natl. Acad. Sci. USA 86:5257-5261.*

41. **Zanke, B., H. Suzuki, K. Kishihara, L. Mizzen, M. Minden, A. Pawson and T. W. Mak.** 1992. Cloning and expression of an inducible lymphoid-specific, protein tyrosine phosphatase (HePTPase). *Eur. J. Immunol.22:235-239.*

42. **Feng, G-S., C-C. Hui and T. Pawson.** 1993. SH2-containing phosphotyrosine phosphatase as a target of protein-tyrosine kinases. *Science 259:1607-1611.*

43. **Justement, L. B., K. S. Campbell, N. C. Chein and J. C. Cambier.** 1991. Regulation of B cell antigen receptor signal transduction and phosphorylation by CD45. *Science 252:1839-1842.*

44. **Pingel, J. T. and M. L. Thomas.** 1989. Evidence that the leukocyte-common antigen is required for antigen-induced T lymphocyte proliferation. *Cell 58:1055-1065.*

45. **Koretzky, G. A., J. Picus, M. L. Thomas and A. Weiss.** 1990. Tyrosine phosphatase CD45 is essential for coupling T-cell antigen receptor to the phosphatidyl inositol pathway. *Nature 346:66-68.*

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

47. **Chui, D., C. J. Ong, P. Johnson, H. S. Teh and J. D. Marth.** 1994. Specific CD45 isoforms differentially regulate T cell receptor signaling. *EMBO J. 13:798-807.*

48. **Koretzky, G. A., J. Picus, T. Schultz and A. Weiss.** 1991. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc. Natl. Acad. Sci. U. S. A. 88:2037-2041.*

49. **Deans, J. P., S. B. Kanner, R. M. Torres and J. A. Ledbetter.** 1992. Interaction of CD4:lck with the T cell receptor/CD3 complex induces early signaling events in the absence of CD45 tyrosine phosphatase. *Eur. J. Immunol. 22:661-668.*

50. **Weaver, C. T., J. T. Pingel, J. O. Nelson and M. L. Thomas.** 1991. $CD8^+$ T-cell clones deficient in the expression of the CD45 protein tyrosine phosphatase have impaired responses to T cell receptor stimuli. *Mol. Cell Biol. 11:4415-4422.*

51. **Desai, D. M., J. Sap, J. Schlessinger and A. Weiss.** 1993. Ligand-mediated negative regulation of a chimeric transmembrane receptor tyrosine phosphatase. *Cell 73:541-554.*

52. **Volarevic, S., B. B. Niklinska, C. M. Burns, C. H. June, A. M. Weissman and J. D. Ashwell.** 1993. Regulation of TCR signaling by CD45 lacking transmembrane and extracellular domains. *Science 260:541-544.*

53. **Hovis, R. R., J. A. Donovan, M. A. Musci, D. G. Motto, F. D. Goldman, S. E. Ross and G. A. Koretzky.** 1993. Rescue of signaling by a chimeric protein containing the cytoplasmic domain of CD45. *Science 260:544-546.*

54. **Barber, E. K., J. D. Dasgupta, S. F. Schlossman, J. M. Trevillyan and C. E. Rudd.** 1989. The CD4 and CD8 antigens are coupled to a protein-tyrosine kinase ($p56^{lck}$) that phosphorylates the CD3 complex. *Proc. Natl. Acad. Sci. U.S.A. 6:3277-3281.*

55. **Samelson, L. E., A. F. Phillips, E. T. Luong and R. D. Klausner.** 1990. Association of the fyn protein-tyrosine kinase with the T-cell antigen receptor. *Proc. Natl. Acad. Sci. U.S.A. 87:4358-4362.*

56. **Gauen, L. K. T., A.-N. T. Kong, L. E. Samelson and A. S. Shaw.** 1992. p59fyn tyrosine kinase associates with multiple T-cell receptor subunits through its unique amino-terminal domain. *Mol. Cell Biol. 12:5438-5446.*

57. **Chan, A. C., M. Iwashima, C. W. Turck and A. Weiss.** 1992. ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCRζ chain. *Cell 71:649-662.*

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

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

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

61. **Burns, C. M., K. Sakaguchi, E. Apella and J. D. Ashwell.** 1994. CD45 regulation of tyrosine phosphorylation and enzyme activity of src family kinases. *J. Biol. Chem. 269:13594 - 13600.*

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

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

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

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

66. **Morse III, H. C., W. F. Davidson, R. A. Yetter and R. L. Coffman.** 1982. A cell-surface antigen shared by B cells and $Ly2^+$ peripheral T cells. *Cellular Immunology 70:311-320.*

67. **Birkeland, M. L., J. Metlay, V. M. Sanders, R. Fernandez-Botran, E. S. Vitetta, R. M. Steinman and E. Pure.** 1988. Epitopes on CD45R [T200] molecules define differentiation antigens on murine B and T lymphocytes. *J. Mol. Cell. Immunol. 4:71-85.*

68. **Dessner, D. S. and M. R. Loken.** 1981. DNL 1.9: A monoclonal antibody which specifically detects all murine B lineage cells. *Eur. J. Immunol. 11:282-285.*

69. **Gunning, P., J. Leavitt, G. Muscat, S.-Y. Ng and L. Kedes.** 1987. A human β-actin expression vector system directs high-level accumulation of antisense transcripts. *Proc. Natl. Acad, Sci. U.S.A. 84:4831-4835.*

70. **Chen, C. and H. Okayama.** 1988. Calcium phosphate-mediated gene transfer: A highly efficient system for stably transforming cells with plasmid DNA. *BioTechniques 6:632-638.*

71. **Sambrook, J., E. F. Fritch and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y.

72. **Sawadogo, M. and M. W. Van Dyke.** 1991. A rapid method for the purification of deprotected oligonucleotides. *Nuc. Acids. Res. 19:674.*

73. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl. Acad. Sci. U.S.A. 82:488-492.*

74. **Kunkel, T. A., J. D. Roberts and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selction. *Methods Enzymol. 154:367-382.*

75. **Studier, F. W., A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff.** 1990. Use of a T7 RNA polymerase to direct expression of cloned genes. *Meth.ods Enzymol.185:60-89.*

76. **Harder, K. W., P. Owen, L. K. H. Wong, R. Aebersold, I. Clark-Lewis and F. R. Jirik.** 1994. Characterization and kinetic analysis of the intracellular domain of human protein tyrosine phosphatase β (Hptp β) Using Synthetic Phosphopeptides. *Biochem. J. 298:395-401.*

77. **Raschke, W. C. and R. Hyman.** 1985. Stable expression of the mouse lymphocyte T200 antigen in L-cells after transfection with lymphoma DNA. *Mol. Immunol. 22:1137-43.*

78. **Wang, Y. and C. J. Pallen.** 1991. The receptor-like protein tyrosine phosphatase HPTPα has two active catalytic domains with distinct substrate specificities. *EMBO J. 10:3231-3237.*

79. **da Silva, A. J., O. Janssen and C. E. Rudd.** 1993. T cell receptor ζ/CD3-p59$^{fyn(T)}$-associated p120/130 binds to the SH2 domain of p59$^{fyn(T)}$. *J. Exp. Med. 178:2107-2113.*

80. **Tsygankov, A. Y., C. Spana, R. B. Rowley, R. C. Penhallow, A. L. Burkhardt and J. B. Bolen.** 1994. Activation-dependent tyrosine phosphorylation of fyn-associated proteins in T lymphocytes. *J. Biol. Chem. 269:7792-7800.*

81. **Reedquist, K. A., T. Fukazawa, B. Druker, G. Panchamoorthy, S. E. Shoelson and H. Band.** 1994. Rapid T cell receptor-mediated tyrosine phosphorylation of p120, an fyn/lck src homology 3 domain-binding protein. *Proc. Natl. Acad. Sci. U. S. A. 91:4135 - 4139.*

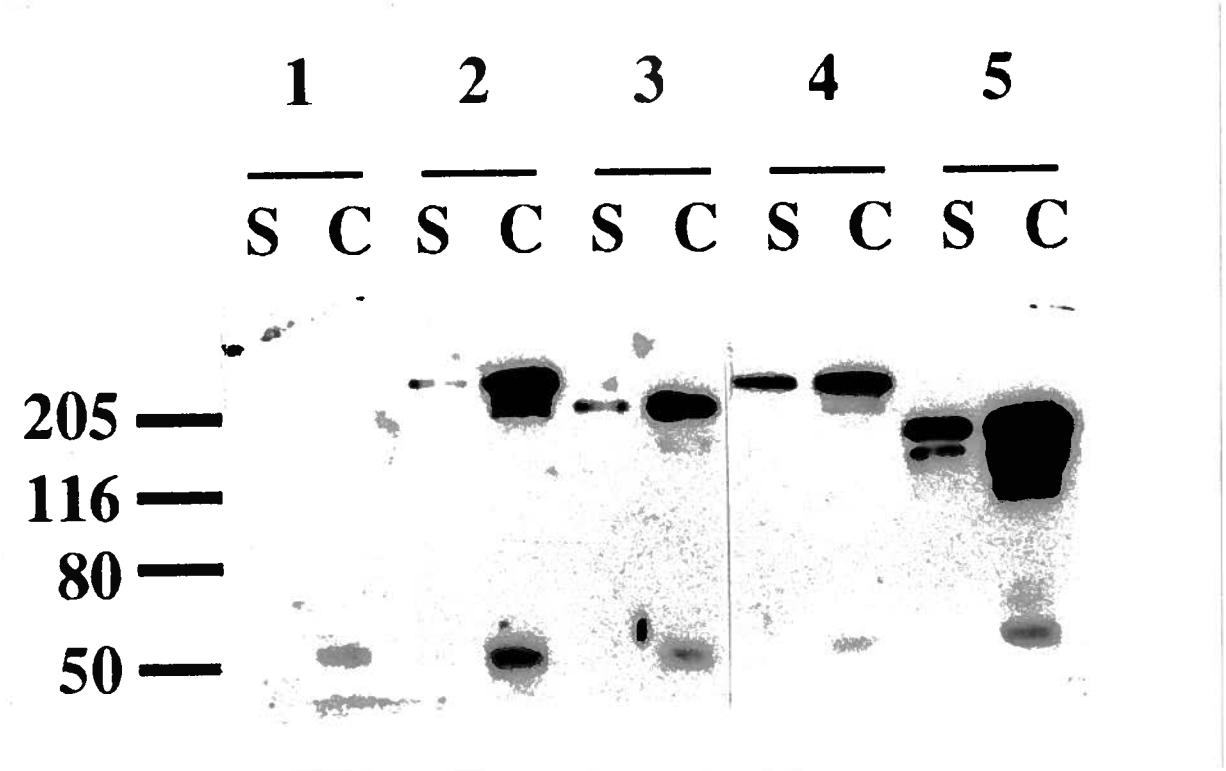


Figure. 8. Surface Expression of CD45 as Determined by Western Blot Analysis. CD45 was immunoprecipitated from the surface (S) of 5 X 10^6 untransfected L cells *(lane 1)*, CD45RABC *(lane 2)*, CD45RO *(lane 3)*, CD45RABC C817S *(lane 4)*, and CD45RABC Δcyt *(lanes 5)* transfected L cells using the Ly5.2 antibody. This was followed by reprecipitation of CD45 from the lysate remaining after surface immunoprecipitation of CD45 from the L cell transfectants (C) using the Ly5.2 antibody conjugated to Protein G. Immunoprecipitates were subsequently blotted with an anti-CD45 antiserum (131) raised against a common peptide epitope in the CD45 extracellular domain. Molecular weight markers in kDa are shown on the left.

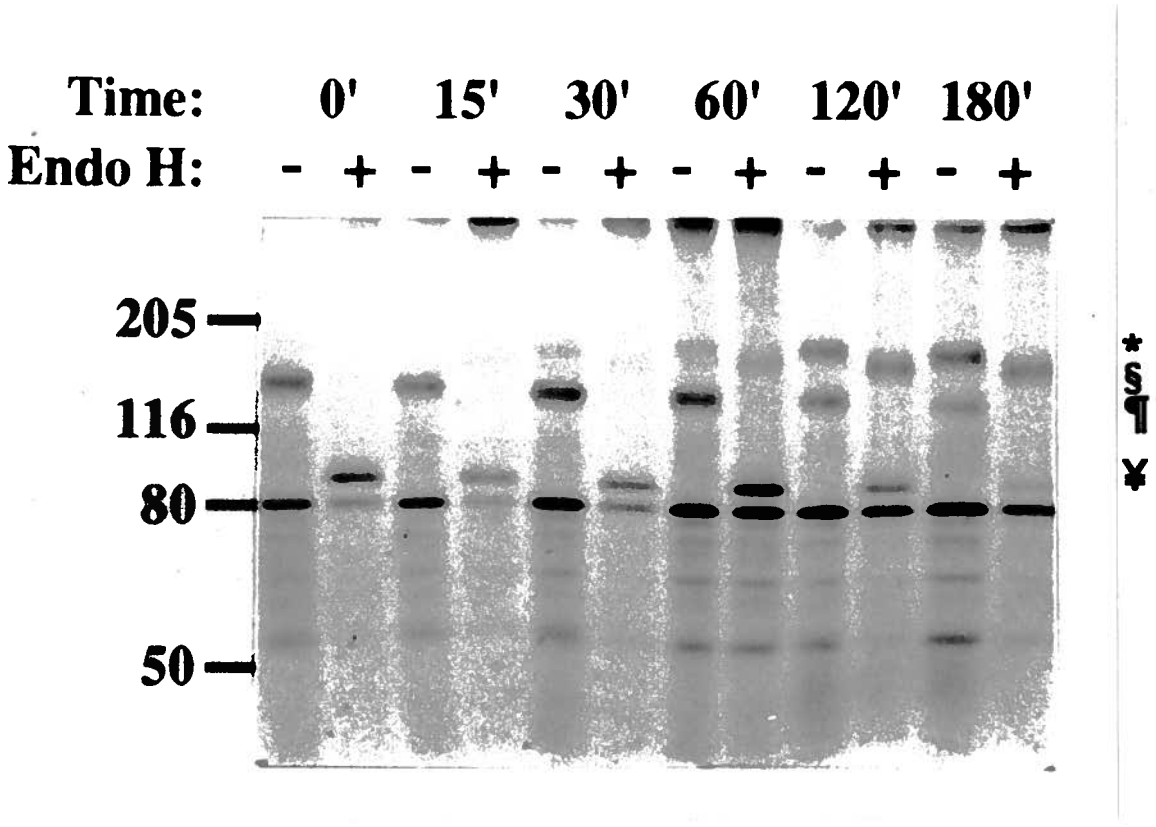


Figure. 9. Pulse-Chase and Endoglycosidase H Sensitivity of the CD45RABC Δcyt Cytoplasmic Domain Mutant Transfected into L tk⁻ cells. The transport of the CD45RABC Δcyt isoform (clone L12B5) was characterized in L cells by the pulse-chase method and endoglycosidase H treatment. Approximately 2.5 X 10^6 cells expressing the CD45RABC Δcyt isoform of CD45 were pulsed with media containing ^{35}S-methionine and ^{35}S-cysteine and chased for the indicated time with normal media. CD45 was immunoprecipitated from the cell lysates using the Ly5.2 antibody conjugated to Protein G and run on a SDS-PAGE gel (-) or digested with Endo H (+) prior to electrophoresis. Arrows indicate the high (160 kDa) and low (125 kDa) molecular weight forms of CD45RABC Δcyt. Molecular weight markers in kDa are shown on the left. CD45 proteins of 160 kDa (*), 150 kDa (§), 125 kDa (¶), and 80 kDa (¥) are shown.

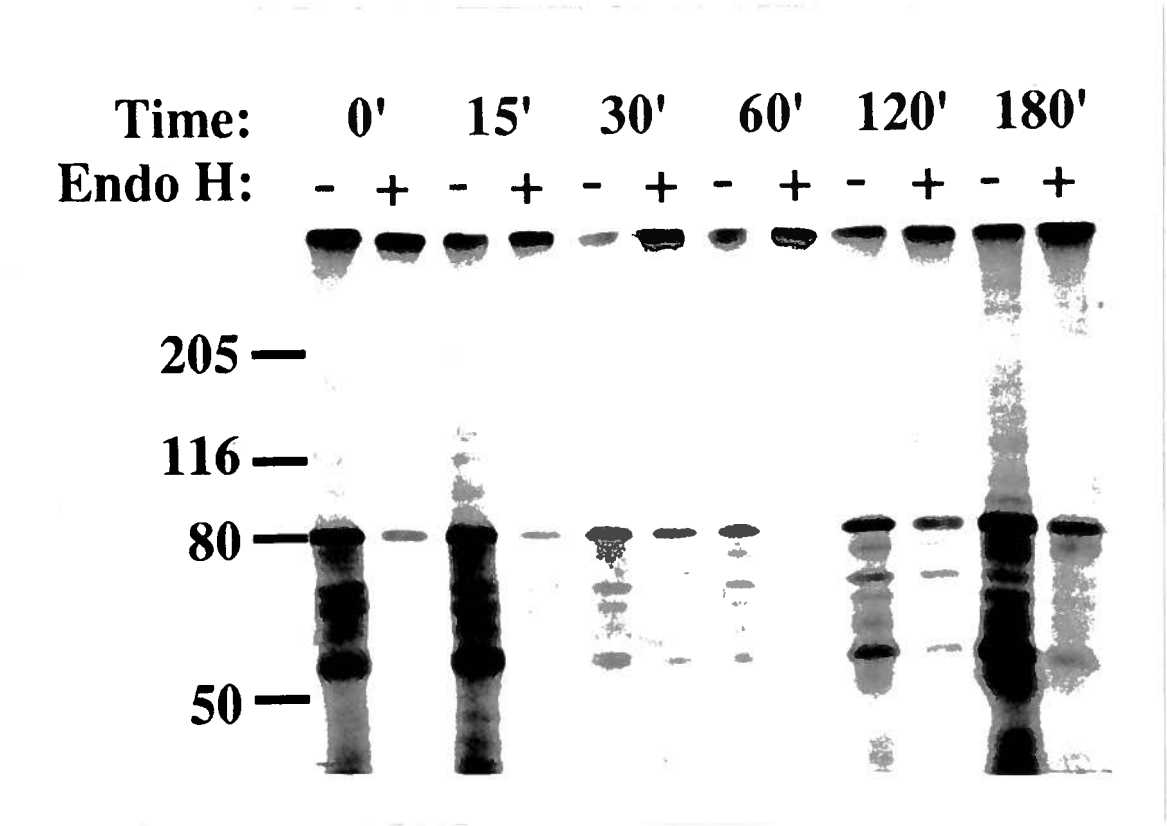


Figure. 10. Pulse-Chase and Endoglycosidase H Sensitivity of CD45 Immunoprecipitates from Untransfected L tk⁻ cells. As a control, approximately 2.5 X 10^6 L cells were pulsed with media containing ^{35}S-methionine and ^{35}S-cysteine and chased for the indicated time with normal media. CD45 was immunoprecipitated from the cell lysates using the Ly5.2 antibody conjugated to Protein G and run on a SDS-PAGE gel (-) or digested with Endo H (+) prior to electrophoresis. Molecular weight markers in kDa are shown on the left.

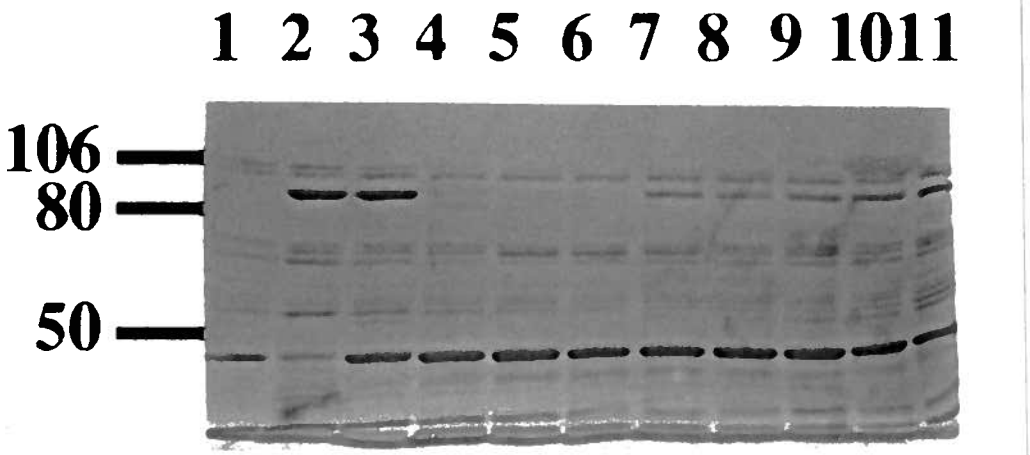


Figure. 12. Coomassie Blue Stained Gel of Recombinant CD45 Cytoplasmic Domain Proteins Generated in Bacteria. After partial purification, equal volumes of recombinant CD45 cytoplasmic domain proteins were visualized by Coomassie Blue staining of an SDS-PAGE gel. Only a 2/3 volume of the CD45 wild-type and CD45 C817S mutant were run on SDS-PAGE gels. Proteins generated by bacterial expression were pET-3d-6His-IEGR, the vector control *(lane 1)*; wild-type CD45, the positive control *(lane 2)*; the C817S mutant, the negative control whereby a point mutation inactivated the PTPase (lane 3); three clones of the spacer deletion mutant, H8.8 *(lane 4)*, H9.1 *(lane 5)*, and H9.11 *(lane 6)*; three clones of the Q1180G mutant, C3.1A *(lane 7)*, C3.1B *(lane 8)*, and C3.1C *(lane 9)*; and two clones of the S573GS574A mutant, 4.18 *(lane 10)* and 18.35 *(lane 11)*. Molecular weight markers in kDa are shown on the left of the gel.

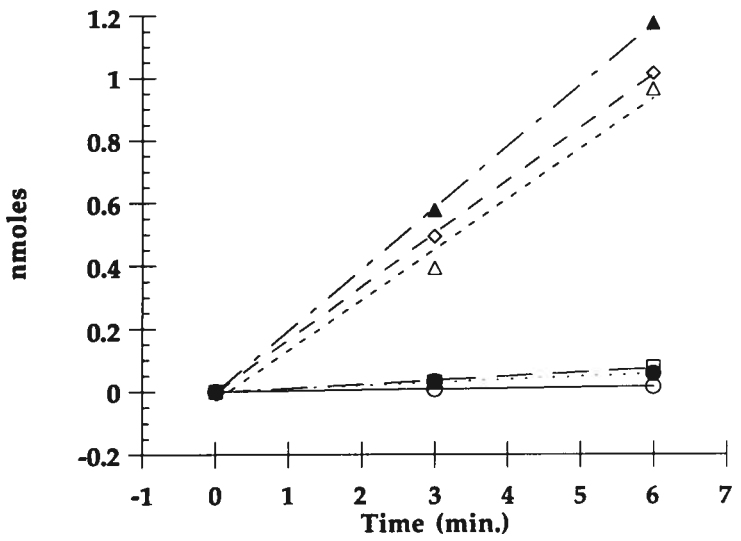


Figure. 13. PTPase Assay of Recombinant CD45 Cytoplasmic Domain Proteins Generated in Bacteria. After partial purification, equal volumes of recombinant CD45 cytoplasmic domain proteins were assayed for tyrosine phosphatase activity against a 13-mer tyrosine phosphorylated peptide comprising the negative regulatory site of $p59^{fyn}$. PTPase activity was normalized for equal amounts of protein (Table II). Recombinant proteins tested for PTPase activity were pET-3d-6His-IEGR, the vector control (--•--) wild-type CD45, the positive control (--Δ--); the C817S mutant, the negative control (—o—); the Q1180G mutant, C3.1A (···•···), two clones of the S573GS574A mutant, 4.18 (---Δ---) and 18.35 (--◊--). Only a 2/3 volume of the CD45 wild-type and CD45 C817S mutant were run on SDS-PAGE gels. Activity was calculated as nmoles of phosphate hydrolyzed by the construction of a standard curve using serial dilutions of 1 mM KH_2PO_4.

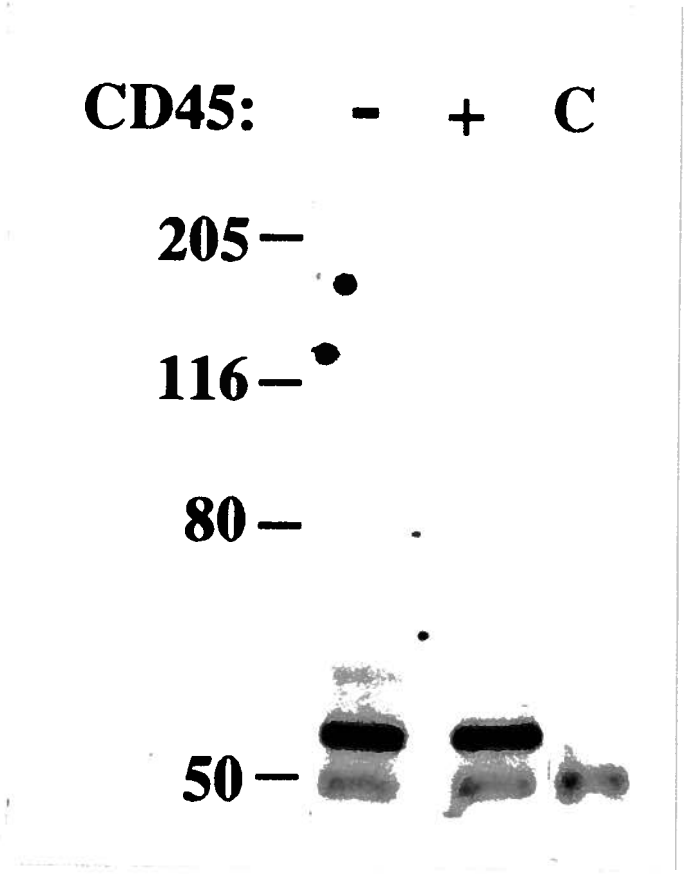


Figure. 14. Amounts of p59^{fyn} Isolated from CD45-Negative and CD45-Positive BW5147 Cells. p59^{fyn} was immunoprecipitated from lysates from 3 X10^6 unstimulated CD45-negative and CD45-positive BW5147 cells with a*fyn* antiserum conjugated to Protein A and immunoblotted with the same*fyn* antiserum. Protein A conjugated to *fyn* antiserum was run as a control (C). Molecular weight markers in kDa are shown on the left.

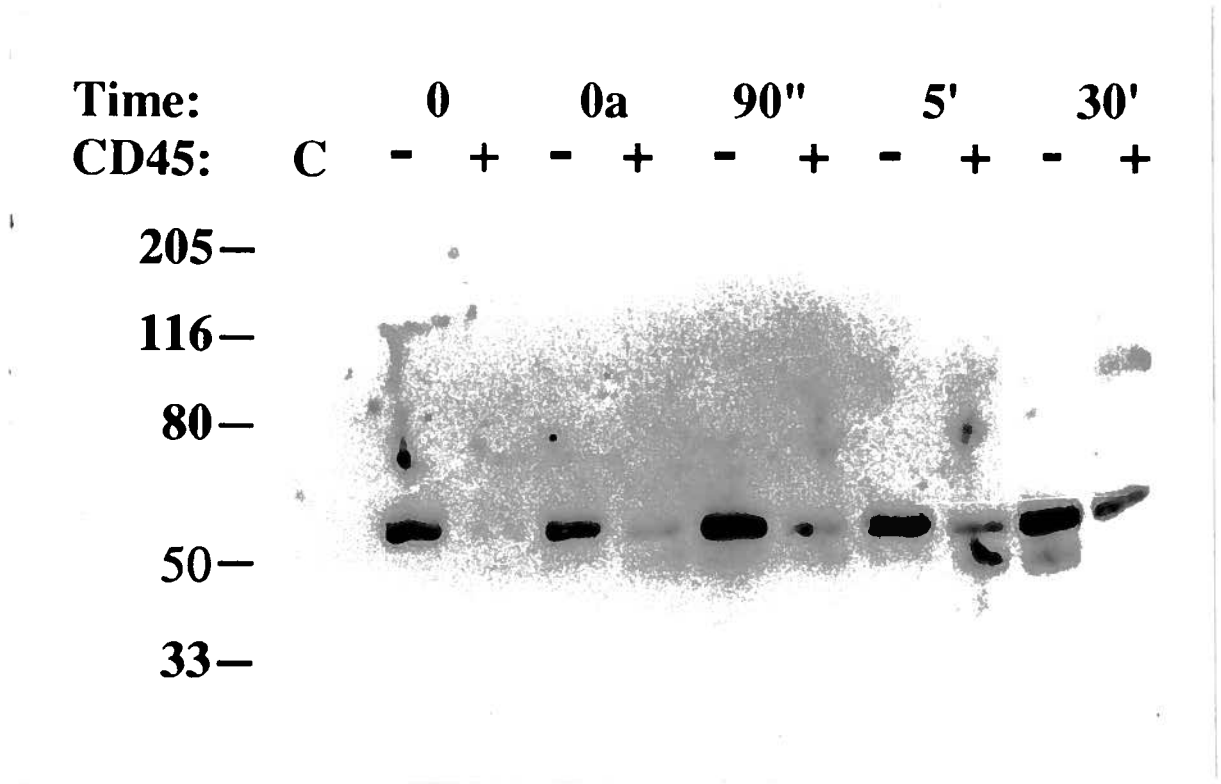


Figure. 15. Tyrosine Phosphorylation of $p59^{fyn}$ Isolated from CD45-Negative and CD45-Positive Cells Upon CD3-Mediated Stimulation. At the indicated amounts of time after stimulation with 3 μg of anti-CD3 antibody, 3 X 10^6 CD45-positive (+) and CD45-negative (-) cells were lysed in 1% Triton X-100 and $p59^{fyn}$ was immunoprecipitated from the lysates. At zero time the cells were lysed either with no CD3 antibody, (0), or lysed simultaneously with the addition of CD3 antibody (0a) and blotted with the anti-phosphotyrosine antibody (4G10). The control lane (C) contained protein A conjugated to anti-*fyn* antibody. Molecular weight markers in kDa are shown on the left.

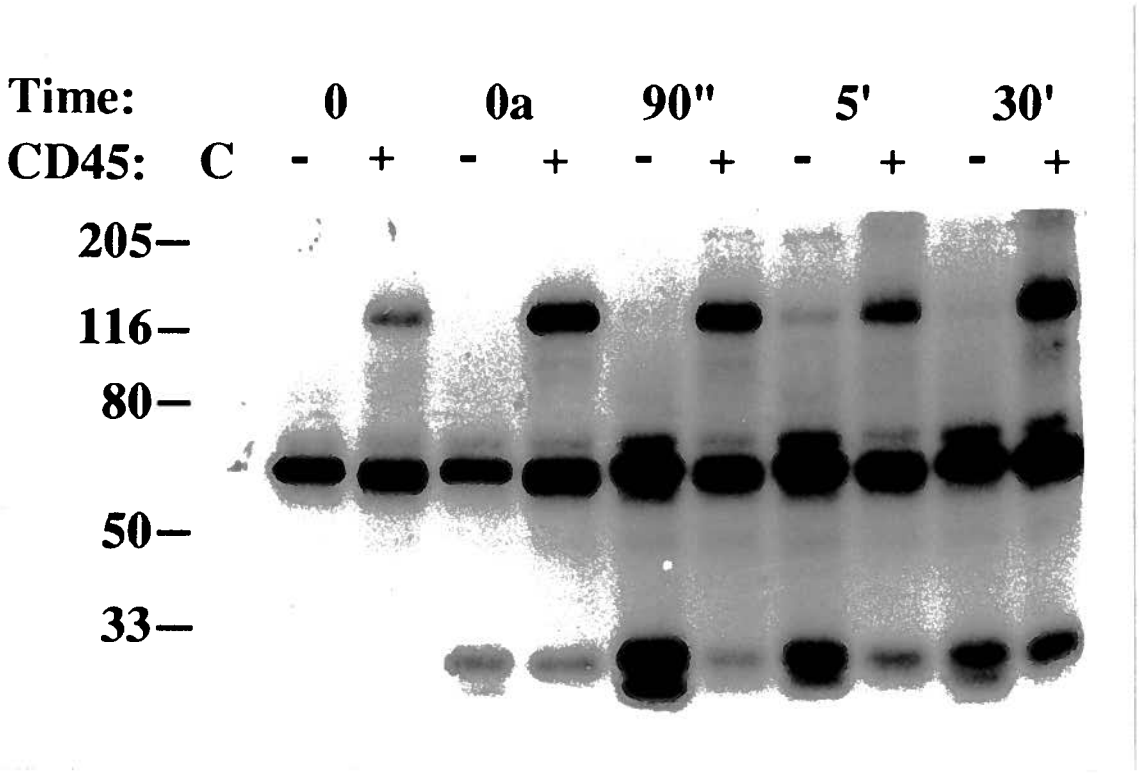


Figure. 16. ***In Vitro*** **Kinase Activity of p59^{fyn} Isolated from CD45-Negative and CD45-Positive Cells Upon CD3-Mediated Stimulation.** At the indicated amounts of time after stimulation with 3 μg of anti-CD3 antibody, 3 X 10^6 CD45-positive (+) and CD45-negative (-) cells were lysed in 1% Triton X-100 and p59^{fyn} was immunoprecipitated. At zero time the cells were lysed either with no CD3 antibody, (0), or lysed simultaneously with the addition of CD3 antibody (0a) and subjected to an *in vitro* kinase assay with γ-^{32}P-ATP prior to separation by SDS-PAGE. The control lane (C) contained protein A conjugated to anti-fyn antibody that was also subjected to an *in vitro* kinase assay. ^{32}P incorporation was measured on X-ray film. Molecular weight marker in kDa are shown on the left.