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Molecular and genetic analysis of CD45 regulation and function in T cell antigen receptor mediated-signal transduction

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Molecular and Genetic Analysis of CD45 Regulation and Function

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Dedication

To my parents, Mahendra and Manjula Desai, for their love, support and encouragement through the years.

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Figures 1-4 and 9 were kindly provided by Art Weiss.

ABSTRACT

Molecular and Genetic Analysis of CD45 Regulation and Function in T cell Antigen Receptor-Mediated Signal Transduction

Dev M. Desai

Cell surface expression of CD45, a receptor-like transmembrane protein tyrosine phosphatase (PTPase), is required for T cell antigen receptor (TCR)-mediated signal transduction. However, the regulation of CD45 function is poorly understood, chiefly resulting from the lack of information regarding CD45 ligand(s). In these studies, the regulation of a transmembrane PTPase by ligand is demonstrated, and the roles of the tandem cytoplasmic phosphatase domains in ligand-mediated regulation of PTPase function and TCR signal transduction are defined. A chimeric protein consisting of the extracellular and transmembrane domains of the epidermal growth factor receptor (EGFR) linked to the cytoplasmic domain of CD45, when introduced into a CD45-deficient cell, can restore proximal TCR signal transduction events. Ligands for the EGFR are able to negatively modulate EGFR/CD45 chimera function, in a manner dependent on dimerization of the chimeric protein. Moreover, inhibition of EGFR/CD45 function results in negative modulation of TCR signal transduction indicating the necessity for continuous CD45 function in TCR-mediated proximal signaling events. This substantiates the idea that signal transduction by tyrosine phosphorylation represents a dynamic equilibrium between PTKs and PTPases whose functions can be regulated.

CD45, like the majority of transmembrane PTPases, is composed of two tandem cytoplasmic phosphatase domains whose relative function and contribution to TCR signal transduction is not known. Analysis of EGFR/CD45

chimeric proteins containing individual and dual point mutations of the catalytic cysteine residue within the CD45 phosphatase domains has identified the function of the membrane-proximal phosphatase domain as necessary and sufficient to restore TCR signal transduction. The membrane-proximal phosphatase domain contributes to in vitro enzymatic activity, whereas the distal phosphatase domain does not contribute to in vitro enzymatic activity, nor does it function in coupling the TCR to the intracellular signaling machinery. In summary, these studies demonstrate that the function of the transmembrane PTPases can be regulated by ligands and that the phosphatase activity of the membrane-proximal phosphatase domain of CD45 is required to couple the TCR to the intracellular signal transduction machinery.

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LIST OF ABBREVIATIONS

Immunoglobulin	Ig
Major histocompatibility complex	MHC
T cell antigen receptor	TCR
Antigen presenting cell	APC
T cell receptor variable gene locus	V
T cell receptor diversity gene locus	D
T cell receptor joining gene locus	J
T cell receptor constant gene locus	C
Immunoglobulin epsilon constant region receptor I	$F_{C\varepsilon}RI$
Antigen recognition activation motif	ARAM
Mitogen activated protein kinase II	MAPK
Phospholipase C-gamma 1	PLC-γ1
Protein tyrosine kinase	PTK
Epidermal growth factor receptor	EGFR
Platelet-derived growth factor receptor	PDGFR
Diacylglycerol	DG
Inositol 1,4,5-trisphosphate	1,4,5-IP3
Intracellular free calcium concentration	$[Ca^{2+}]_i$
Endoplasmic reticulum	ER
Interleukin-2	IL-2
Cyclosporin A	CsA
Guanine triphosphate	GTP
GTPase activating protein	GAP
Protein tyrosine phosphatase	PTPase

Extracellular receptor activated kinase ..... ERK

Phosphatidylinositol ..... PI

Amino terminal ..... N-terminal

Carboxy terminal ..... C-terminal

Src homology 3 domain SH3

Src homolgy 2 domain SH2

MAPK/ERK kinase ..... MEK

Tyrosine ..... Y

Natural killer cell ..... NK cell

Severe combined immunodeficiency ..... SCID

T cell phosphatase ..... TC PTP

Fibronectin type III ..... FN III

Neural cell adhesion molecule ..... NCAM

Adenosine triphosphate ..... ATP

Polymerase chain reaction ..... PCR

Reverse-transcriptase coupled PCR ..... RT-PCR

Monoclonal anti-body ..... mAb

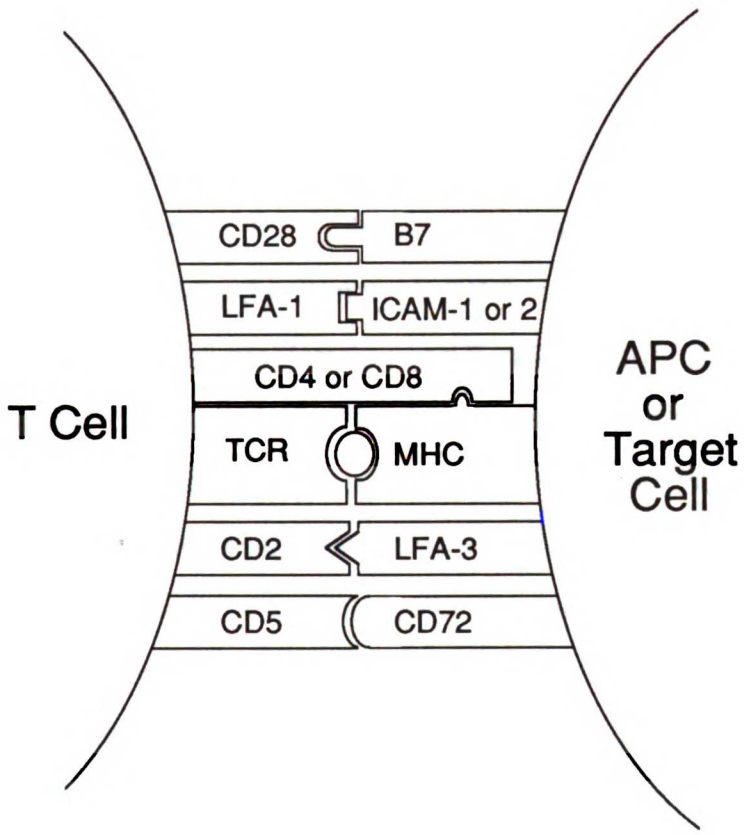
CHAPTER I: INTRODUCTION

Signal transduction in immune system function

The immune system is composed of multiple cell types which in a concerted and cooperative manner function in responding to an extraordinarily diverse number of foreign antigens, while maintaining non-responsiveness to host self-antigens. The basis for the flexible and adaptive immune response results from the highly specific interaction between antigens and antigen-specific receptors. The T and B lymphocytes comprise this antigen specific effector arm of the cellular immune response (1). In B lymphocytes, the antigen-specific surface immunoglobulin (Ig) binds to its cognate epitope located on a foreign molecule, while the antigen-specific T cell receptor recognizes peptide antigens bound to major histocompatability complex (MHC) class I and II molecules (2). In addition to T cell antigen receptor (TCR)-peptide/MHC interactions, multiple other T cell surface molecules are involved in making cognate receptor-ligand interactions with molecules expressed on the antigen presenting cell (APC) as illustrated in Figure 1. Some of these interactions increase the avidity of the T cell - APC association, whereas others such as the interaction of CD28 with its multiple ligands appears to be required for an appropriate immune response (1). The summation of these receptor-ligand interactions are translated into intracellular signal transduction events that result in the initiation of cellular responses. In addition to the antigen specific responses that occur when B and T lymphocytes encounter antigen, the other cellular components of the immune system must also be recruited and appropriately instructed in how, when and where to respond so that an orchestrated immune response to a foreign antigen can be mounted (3).

Figure 1. Ligand-receptor pairs during T cell - APC interaction

Schematic depiction of a limited number of ligand-receptor interactions at the interface of a T cell with an APC or target cell. Central to this process is the quarternary TCR, CD4/CD8 co-receptor, peptide/MHC complex.


CD28
B7
LFA-1
ICAM-1 or 2
CD4 or CD8
T Cell
TCR
MHC
APC
or
Target
Cell
CD2
LFA-3
CD5
CD72

This introduction will review the current state of knowledge on TCR structure and TCR-mediated proximal signal transduction events involved in T cell development and function. This introduction will also review the structure, function and regulation of protein tyrosine phosphatases as it pertains to TCR signal transduction to provide a rationale for the line of experimentation undertaken and described herein.

T lymphocyte development and function

T lymphocytes are the central mediators of the immune response and, thus, must be capable of distinguishing between self and foreign antigens. The recognition of antigen by the clonotypic TCR (see below) underlies the basis for T cell development, T cell proliferation and T cell effector functions, both physiological and pathological. The requirement that the immune system and, hence, T cells respond to a vast number of yet to be encountered foreign proteins is fulfilled in the equally expansive number of distinct TCRs. The generation of the diverse TCR repertoire is provided by the somatic rearrangement of the genes encoding the antigen recognition portion of the TCR (4). The stochastic nature of this process allows the production of an equivalent distribution of random TCRs required for the recognition of the myriad of foreign antigens. However, the stochastic process also results in the generation of TCRs capable of recognizing self-peptide antigens and TCRs incapable of recognizing self-MHC molecules. The immune system has evolved a mechanism of T cell development that results in the maturation of T cells that are non-autoreactive, yet recognize foreign peptide bound to self-MHC.

T cell development is a complex multistep process in which T cell precursors (thymocytes) are educated in the thymus to distinguish between foreign and self antigens. This process of thymic education, which is not

completely understood at the molecular level, involves the central processes of antigen recognition and signal transduction. Thymocytes progress through various stages of development in which changes in the cell surface expression of the CD4 and CD8 molecules can be used to demarcate the maturational stage of these cells.

Initially, thymocytes lack both CD4 and CD8 (double negative) and mature to double positive ($CD4^+8^+$) cells, a process which is thought to be triggered by the somatic rearrangement of the TCR β chain (5). At the double positive stage, a functional αβ heterodimer-containing TCR complex is expressed (see below) and it is at this stage that the immune system selects which cells will proceed to develop into mature T lymphocytes. The double positive cells undergo two selection processes: Thymocytes which express TCRs that are unable to bind self-MHC, or bind the MHC expressed on thymic epithelium with high avidity, are "negatively" selected and undergo programmed cell death (6). Thymocytes expressing a TCR which recognizes MHC plus peptide on cells of hematopoietic origin, are "positively" selected for clonal expansion (5). During this thymic selection process, only ~3-5% of the thymocytes survive to mature to either CD4 or CD8 single positive cells. The exact nature and the order of the positive and negative selection events is not clear. However, it is known that for thymocytes to be positively and negatively selected, TCR-mediated signal transduction is required.

T lymphocytes which survive the thymic selection process migrate into the circulatory system, the lymphatic vessels and the various secondary lymphoid organs, including lymph nodes, the spleen and Peyer's patches. The physiological role of the peripheral circulating T cells is to respond to foreign antigen and initiate an immune response. The CD4 and CD8 T cell specific markers are alternatively expressed on mature peripheral T lymphocytes and

functionally divide T cells into CD4 helper cells and CD8 cytotoxic cells (1). The $CD4^+$ cells secrete various cytokines and provide the necessary signals to B cells so that appropriate humoral responses are initiated. The $CD8^+$ cells are involved in cell-mediated cellular cytotoxicity. The peripheral functions of both CD4 and CD8 positive cells is mediated through signaling pathways originating from the antigen-specific TCR.

T cell antigen receptor structure and function

The TCR is a multimeric complex composed of the products of six genes, all of which are required for efficient receptor assembly and expression on the cell surface. The clonotypic Ti α and β chains form a disulfide-linked heterodimer which associates non-covalently with the CD3 complex, composed of the γ, δ, and ε chains, along with a dimer composed of members of the ζ chain family (7-9). The Ti α and β subunits, type I single transmembrane glycoproteins of 40-55 kDa in size, are responsible for peptide/MHC binding (10). The Ti chains, members of the immunoglobulin supergene family, are the products of somatic gene rearrangement between four distinct loci. These loci, termed the variable (V), diversity (D), and joining (J) regions contain multiple gene segments which are combined in a stochastic manner, contributing to the marked diversity of the TCR repertoire (4). The V/J gene segments of the α chain (11) and the V/D/J segments of the β chain (12, 13) form the antigen binding specificity of the TCR. Furthermore, during the V/D/J recombinatorial events, additional random sequences (N regions) are inserted between the V-D and D-J segments (4). Thus the N regions add another layer of diversity towards antigen recognition. The antigen binding determinants of the Ti chains are combined with constant regions (C) specifying the remainder of the extracellular domain, and the transmembrane and cytoplasmic domains.

The CD3 subunit is composed of the invariant CD3 γ, δ and ε chains. These structurally homologous molecules, also members of the immunoglobulin supergene family, contain a single immunoglobulin-like extracellular domain, a single transmembrane domain and a relatively large cytoplasmic domain. The γ and δ chains are glycoproteins of approximately 25 and 22 kDa respectively, while the 21 kDa ε chain is non-glycosylated (7). The structural similarity and tight clustering of the CD3 genes on human chromosome 11 and mouse chromosome 9, suggests that the CD3 chains arose through gene duplication (14, 15). Although the precise stoichiometry of the CD3 subunit has not been fully established, numerous biochemical and gene expression studies have suggested that each TCR complex is composed of a single Ti subunit associated with two CD3 dimers, each consisting of ε linked to either γ or δ and a ζ dimer (16, 17).

In addition to the CD3 and Ti complexes, the TCR also consists of a hetero- or homodimer containing a member of the ζ chain family (18). The ζ chain family consists of ζ; η, an alternatively sliced variant of ζ; and, the γ chain of the multisubunit IgE receptor ($F_{C\varepsilon}RI$) (19-21). The ζ family of molecules are highly related structurally, consisting of a short extracellular domain (5 - 9 amino acids), a single transmembrane domain containing an acidic residue and a relatively large conserved cytoplasmic tail. Zeta, a 16 kDa transmembrane protein exists predominantly as a disulfide-linked homodimer; however, a minority of TCRs contain a heterodimer consisting of ζ and either the 22 kDa η chain or the 7 kDa $F_{C\varepsilon}RI$ γ chain, or a homodimer of the $F_{C\varepsilon}RI$ γ chain (22).

Why is the TCR such a complex molecule, and what is the significance of the association of the Ti, CD3 and ζ chains? Consideration of the functional requirements of the TCR provides a logical basis for the evolution of a highly complex structure. The TCR must recognize a complex ligand, peptide plus

MHC, and, in turn, translate that extracellular binding event into a signal which leads to the appropriate cellular response. Numerous types of studies, including gene transfer experiments, have demonstrated that the Ti α and β chains are both necessary and sufficient for antigen recognition (7). However, the Ti chains, as indicated by their short cytoplasmic tails (5 amino acids) are unlikely to be able to directly transmit intracellular signals. On the other hand, the associated CD3 and ζ chains, with their relatively long cytoplasmic domains and short extracellular domains, are structurally suited for such a function. Furthermore, it had been known for quite some time that antibodies to the CD3 complex activate a signal transduction pathway indistinguishable from that observed through engagement of the Ti subunit with anti-Ti antibodies (23). Reconstitution studies utilizing cell lines deficient in certain TCR subunits and expression of chimeric molecules, consisting of the cytoplasmic domains of the CD3 ε or the ζ subunits fused to a large heterologous extracellular domain have demonstrated that the ζ and the CD3 ε chains can couple the Ti chains to the intracellular signaling machinery (24-27). Whether the CD3 γ and δ chains can also mediate TCR signal transduction is not known.

The ability of CD3 ε and ζ chain-containing chimeric molecules to recapitulate TCR signal transduction has been of extreme significance, as it has simplified the multimeric TCR complex into a single transmembrane protein more amenable to genetic manipulation. Furthermore, the analysis of the protein sequence of the cytoplasmic domains of the ζ chain and the CD3 chains, has revealed a common motif consisting of two YXXL sequences interspersed by six to eight amino acids (28). This motif (**A**ntigen **R**ecognition **A**ctivation **M**otif -- ARAM) sequence is found triplicated in the ζ chain and present as a single copy in the CD3 ε, γ and δ chains. Moreover, the ARAM sequence is also found in the signal transducing components of the B cell antigen receptor, a number of Fc

receptors, and in plasma membrane proteins of two viruses, known to transform lymphoid cells (29, 30). The TCR complex, composed of CD3 εγ and εδ heterodimers associated with a ζ-chain homodimer is depicted in Figure 2.

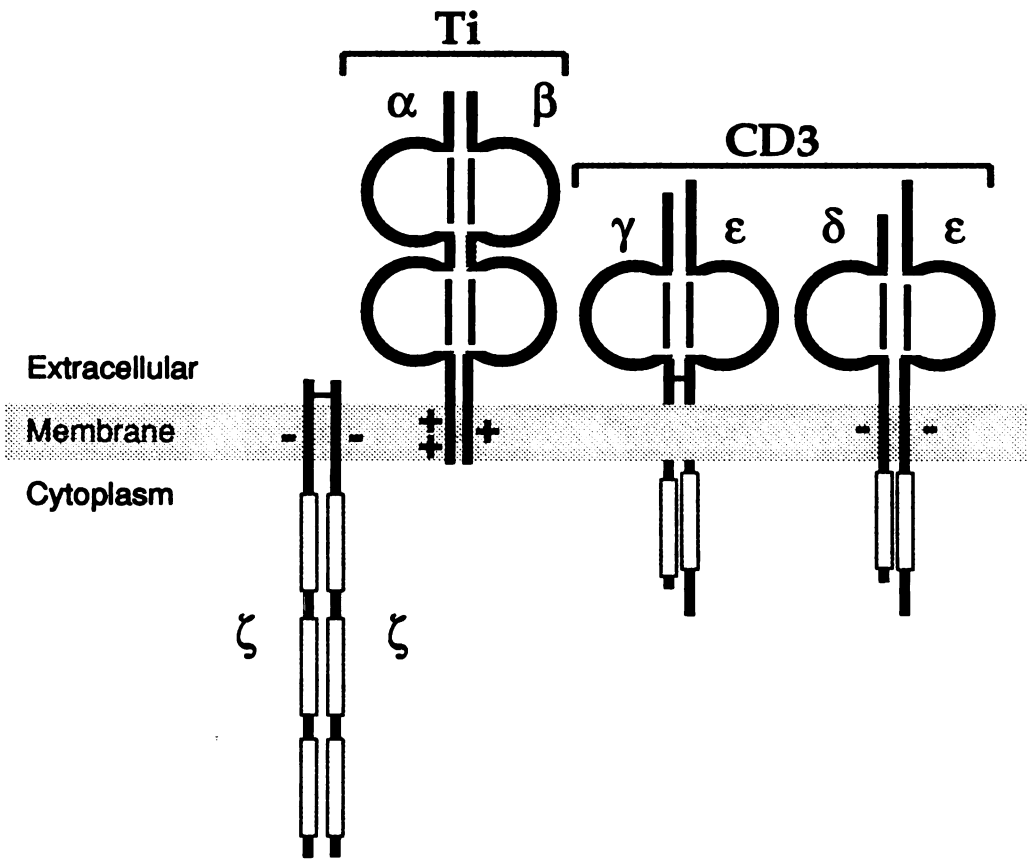
The function of the ARAMs was determined utilizing chimeric receptors containing a cytoplasmic domain consisting solely of the ARAM sequence. Induction of T cell activation events through ARAM-containing chimeric molecules established that the ARAM sequence forms the structural basis for TCR signal transduction (27, 31, 32). It is not clear why the TCR complex contains multiple ARAM sequences (at least 10), but the answer in part may be that this represents a signal amplification mechanism which is needed since a small number of TCR complexes (a few hundred) are engaged at any one time (33). Additionally, the exact sequence of the ARAM and the context in which it is located may result in the coupling of the ARAMs to different signaling pathways (27). The appearance of the ARAM sequence in other hematopoietic cell receptors indicates that a common mechanism has evolved for these receptors to couple to intracellular signal transduction molecules.

Signal transduction pathways mediated by the T cell antigen receptor

Engagement of the TCR by peptide/MHC or agonist anti-TCR monoclonal antibodies (mAbs) initiates a series of biochemical events that culminate in induction of effector functions including lymphokine secretion and cytolytic activity. The earliest detectable event following TCR engagement, occurring within seconds (34, 35), is the reversible tyrosine phosphorylation of a number of cellular proteins, including the TCR ζ (36, 37) and CD3 chains (38, 39), CD5 (40), mitogen-activated protein kinase (MAPK) (41), phospholipase $C_{\gamma 1}$ (PLCγ1) (42-44), the protein tyrosine kinase (PTK) ZAP-70 (45, 46), the structural proteins

Figure 2. The T cell antigen receptor complex

Schematic illustration of the Ti α/β heterodimeric antigen binding subunit associated with signal transducing CD3 and ζ chains. The ARAM motif, the signal transducing unit of the TCR, is depicted by the open rectangular boxes. Acidic (-) and basic (+) residues within the transmembrane segments of the TCR chains, involved in TCR assembly are also indicated.


Ti
α
β
CD3
γ
ε
δ
ε
Extracellular
Membrane
Cytoplasm
ζ
ζ

vimentin (A. Chan and D.M. Desai, unpublished observations) and ezrin (47) and the protein products of the proto-oncogenes *vav* (48) and *shc.* (49). The critical role of TCR-mediated tyrosine phosphorylation is further underscored by studies in which PTK inhibitors such as herbimycin A and genestein markedly diminish both proximal and distal T cell activation events induced through the TCR (50, 51). Unlike the tyrosine kinase growth factor receptors such as the epidermal (EGFR) and platelet-derived growth factor (PDGFR) receptors, the TCR complex does not contain intrinsic tyrosine kinase activity and, thus, is believed to couple to at least one cytoplasmic tyrosine kinase (see below).

With the exception of PLCγ1, the consequences of tyrosine phosphorylation of the majority of cellular tyrosine phosphoproteins induced following TCR engagement are not known. Phosphorylation of PLCγ1 on two critical tyrosine residues is necessary for in vivo function and increase in catalytic activity in vitro (52, 53). The mechanism by which PLCγ1 becomes tyrosine phosphorylated is unclear. The kinase responsible for PLCγ1 phosphorylation is not known; however, indirect evidence suggests that Lck and/or Fyn may function in this capacity (54). Lck has been demonstrated to co-precipitate with PLCγ1 under some circumstances and overexpression of Fyn in Cos cells expressing a ζ chain-containing chimeric receptor resulted in receptor-mediated tyrosine phosphorylation of PLCγ1 (55).

PLCγ1 activation results in the conversion of phosphatidylinositol-4,5-bisphosphate into the two second messengers diacylglycerol (DG) and inositol-1,4,5-trisphosphate (1,4,5-IP3) (56). The function of 1,4,5-IP3 in increasing intracellular calcium levels ($[Ca^{2+}]_i$) has been well established. 1,4,5-IP3 binding to its receptors located on calciosomes (intracellular Ca^{2+} stores) and the endoplasmic reticulum (ER) accounts for most of the initial increase in $[Ca^{2+}]_i$ (57). However, in T cells, the initial peak in calcium levels is followed by a

plateau phase persisting for several hours (58, 59). The plateau phase at the population level may reflect the summation of asynchronous oscillations of $[Ca^{2+}]_i$ within individual cells (60, 61). The prolonged, elevated levels of $[Ca^{2+}]_i$ appear to be critical for many cellular responses, including production of interleukin-2 (IL-2) (58, 59). The late calcium oscillations or plateau phase is mediated by the influx of extracellular calcium across the plasma membrane. Patch-clamp studies have suggested that a non-voltage-gated calcium channel may be functioning in producing the transmembrane calcium flux (62, 63). Recently, immunofluorescence and biochemical studies have identified an isoform of the intracellular 1,4,5-IP3 receptor located in the plasma membrane of T lymphocytes (64, 65). Thus 1,4,5-IP3 or its metabolites, 1,3,4,5-IP4 and 1,3,4-IP3, which have been implicated in the flux of transmembrane calcium, may be responsible for both the early and late phases of the TCR-mediated increase in $[Ca^{2+}]_i$ (66).

The other second messenger produced as a result of PLC-γ1 activation is DG. DG is a lipid moiety that has been demonstrated to regulate the pkC family of calcium/phospholipid-dependent serine/threonine kinases (67). The α, β and ε isoforms of pkC have been identified in T cells; however, the exact function of each isoform is not known (68, 69). Moreover, it appears that there may be some functional redundancy between the various isoforms (70). Engagement of the TCR or the addition of phorbol esters, potent activators of pkC (71), results in pkC activation and its translocation to the inner leaflet of the plasma membrane (72).

Although the mobilization of $[Ca^{2+}]_i$ and activation of pkC have long been documented as TCR-mediated events, the consequences of these events on subsequent cellular events remains largely unknown (73). Recent studies utilizing the immunosuppressive agents, cyclosporin A (CsA) and FK506, have

resulted in identification of one of the targets of the increase in $[Ca^{2+}]_i$ (74). Calcineurin, a calcium- and calmodulin- activated serine/threonine phosphatase is a target of the immunosuppressant/immunophilin (receptors for immunosuppressants) complex, resulting in inhibition of phosphatase activity (75, 76). Overexpression of calcineurin results in an increase in the IC_{50} for CsA and FK506 (77, 78). Moreover, expression of a constitutively active form of calcineurin results in a loss of inhibition of T cell activation by CsA or FK506, as well as permitting IL-2 gene transcription in the presence of phorbol esters only (77, 78). One direct or indirect target of calcineurin has been identified as the cytoplasmic component of the nuclear transcription factor NF-AT, which is involved in transcription of the IL-2 gene (79).

A number of cellular proteins are phosphorylated on serine and threonine residues following TCR stimulation or treatment with phorbol esters. Among these cellular substrates are the CD3 γ and δ chains (80), CD4 (81), the IL-2 receptor (82), MAP kinase (41, 83), and the Raf-1 kinase (84). Whether these proteins are direct substrates for pkC, or are substrates for pkC regulated kinases remains to be determined. Additionally, the functional consequences of phosphorylation of most of these cellular substrates is not known. However, serine phosphorylation of Raf-1, also a serine/threonine kinase, in some instances has been suggested to result in an increase in its kinase activity (84).

Recent genetic and biochemical studies have indicated that Raf-1 and the proto-oncogene, Ras, are involved in TCR-mediated IL-2 transcription (85-87). Ras, a small molecular weight guanine-nucleotide binding protein, is in an active state when bound with GTP. Ras has intrinsic GTPase activity and the hydrolysis of GTP to GDP reverts the protein to an inactive state (88). Ras function is regulated by a nucleotide exchange factor that catalyzes the exchange of GTP for GDP, and by GAP (GTPase activating protein), which accelerates the

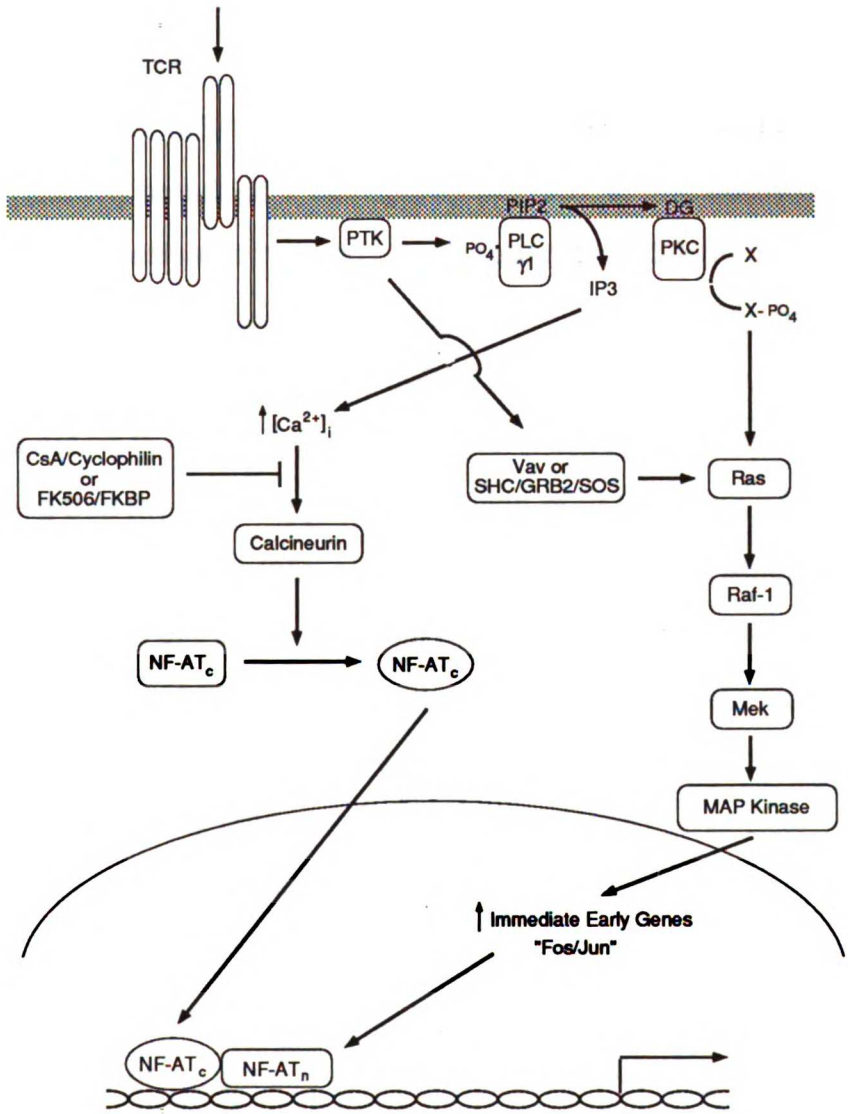
intrinsic GTPase activity of Ras (89). Expression of dominant-negative forms of Ras in T cells can prevent TCR-mediated IL-2 production. Moreover, dominant-negative Ras also prevents the activation of MAP kinase (90, 91). Numerous studies have led to the construction of a signaling pathway in which GTP-bound Ras binds to Raf-1, resulting in the initiation of a kinase cascade where Raf-1 phosphorylates MAP kinase kinase (also referred to as MEK) (92, 93), which in turn phosphorylates MAP kinase (94). MAP kinase has been demonstrated to phosphorylate the Rsk kinase, which is believed to translocate to the nucleus and phosphorylate various transcription factors (95). MAP kinase may also directly phosphorylate transcription factors, such as c-jun (95).

How the TCR couples to Ras activation is not clear. Treatment of T cells, but not fibroblasts, with phorbol esters results in Ras activation (96). This process is dependent on the function of pkC, since down-modulation of pkC expression or specific pkC inhibitors can inhibit phorbol ester-mediated Ras activation . However, inhibition of pkC function does not inhibit Ras activation by TCR stimulation, suggesting multiple TCR-mediated Ras activation pathways (97). The proto-oncogene vav, a putative guanine-nucleotide exchange factor, and a substrate for a TCR-regulated tyrosine kinase, is an attractive candidate for linking the TCR to Ras activation (48, 98). The Sos protein, also a nucleotide exchange factor, in conjunction with the SH2/SH3 containing adapter proteins Shc and Grb2/Sem5 has been implicated in Ras activation through the EGF and PDGF receptors (99). All three molecules are also expressed in T cells, thus Ras activation mediated by the TCR may occur through multiple mechanisms. A model of TCR-induced signal transduction pathways is schematized in Figure 3.

The debate over the functional consequences and importance of activation of the tyrosine kinase pathway and the phosphoinositide (PI) pathway in mediating T cell activation events has been very contentious. Compelling

Figure 3. Model of TCR-mediated signal transduction pathways

Engagement of the TCR through one or more PTKs results in the tyrosine phosphorylation of a number of cellular substrates, including PLCγ1. Tyrosine phosphorylation of PLCγ1 leads to the activation of the phosphatidylinositol pathway, which in concert with other signaling pathways culminate in lymphokine gene transcription. Arrows do not necessarily indicate direct interactions.


TCR
PIP2
DG
PTK
PO4
PLC γ1
IP3
PKC
X
X- PO4
↑[Ca2+]i
CsA/Cyclophilin or FK506/FKBP
Vav or SHC/GRB2/SOS
Ras
Calcineurin
Raf-1
NF-ATc
NF-ATc
Mek
MAP Kinase
↑ Immediate Early Genes "Fos/Jun"
NF-ATc
NF-ATn

IL-2 Gene

evidence has accumulated over the years supporting the role of the PI second messenger pathway in regulating T cell differentiation and effector functions. These include: 1) the ability of calcium ionophores and phorbol esters to mimic the distal events mediated by TCR engagement (100, 101); 2) the negative regulatory effects of pkC inhibitors and chelators of extracellular calcium on T cell activation events (83, 102); 3) the ability of heterologous receptors that activate the PI pathway to induce T cell activation (103); 4) the ability of the immunosuppressive compounds, cyclosporin or FK506 to inhibit the function of calcineurin, thereby preventing IL-2 gene transcription (74); 5) the ability of activated forms of calcineurin or pkC to substitute for calcium ionophores or phorbol esters in inducing IL-2 production (70, 77, 78). The recent findings indicating that the tyrosine phosphorylation of PLC-γ1 precedes the production of PI second messengers (35), and that the PTK inhibitors abrogate tyrosine phosphorylation of PLC-γ1 (50, 51), inhibiting production of PI metabolites (104), suggests that the PTK pathway activates the PI pathway which leads to IL-2 gene transcription. However, these observations do not exclude the possibility that activation of the PTK pathway does not contribute in other ways in regulating distal T cell effector activities.

T cell receptor signal transduction: Role of protein tyrosine kinases

As one of the earliest events detectable following TCR engagement is the induction of tyrosine phosphorylation of numerous cellular substrates, the TCR must regulate either directly, or indirectly, at least one PTK. To date, two types of cytoplasmic tyrosine kinases have been implicated in proximal TCR signal transduction: The Src-family members Lck and Fyn, and the Syk family kinases ZAP-70 and Syk. The Src-like PTKs share multiple common structural and regulatory features: (i) an amino-terminal (N-terminal) myristylation site that

permits plasma membrane localization; (ii) a unique N-terminal region which may be involved in protein-protein associations; (iii) a ~60 amino acid Src homology 3 (SH3) domain, recently demonstrated to bind proline-rich regions of other proteins (105); (iv) a ~100 amino acid Src-homology 2 (SH2) domain that recognizes and binds short linear peptide sequences containing phosphotyrosine; (v) a kinase domain containing an autophosphorylation site; (vi) and, a carboxy-terminal (C-terminal) regulatory region containing a negative regulatory site of tyrosine phosphorylation (106). Both members of the Syk family of PTKs, on the other hand, contain two tandemly arranged SH2 domains, followed by a kinase domain (107). Discrete domains involved in regulation of Syk family members have not yet been identified.

Fyn

The first PTK to be implicated in TCR signaling, the 59 kDa Fyn kinase is expressed in neuronal and lymphoid cells in two distinct forms due to tissue-specific alternative mRNA splicing (108). The Fyn and TCR coprecipitate under mild detergent solubilization conditions, suggesting a role for Fyn in TCR-mediated tyrosine phosphoprotein induction (109). However, the stoichiometry of association between Fyn and the TCR is low (< 2-4 % of TCRs are associated with Fyn) (110). Structural mapping studies in which Fyn and a chimeric form of the ζ chain were overexpressed, revealed that the first 10 amino acids of Fyn interact with a region encoding the last two ARAM sequences of ζ (111). Even in this overexpression system, the stoichiometry between Fyn and ζ remained low. These studies establish that a weak and potentially transient association occurs between Fyn and the TCR, thus suggesting that Fyn may play a role in TCR function.

Additional support for a role of Fyn in TCR signaling has come from genetic studies in which Fyn variants were overexpressed in T cell hybridomas or transgenic mice. Overexpression of the hematopoietic form of Fyn in a T cell hybridoma resulted in augmentation of both proximal and distal T cell activation events (112). A similar result was observed in thymocytes derived from transgenic mice overexpressing Fyn. Moreover, when a kinase-defective form of Fyn was overexpressed in mice, there was a diminution in TCR-mediated calcium mobilization (113). The later experiment strongly suggests that Fyn or a related kinase has a function in TCR signaling in thymocytes; however, due to the promoter used to express Fyn, overexpression was limited to thymocytes, thus the role of Fyn in peripheral TCR function was not tested. Further evidence for Fyn function in thymocyte signaling was obtained from analysis of mice in which the *fyn* locus was inactivated by homologous recombination (114, 115). Single positive thymocytes from Fyn-deficient animals demonstrated diminished intracellular calcium mobilization and proliferation; however, Fyn does not appear to be required for T cell selection as the number thymocytes and peripheral T lymphocytes are normal. Moreover, little impairment in TCR signaling was detected in either double positive thymocytes or peripheral T cells. The in vitro TCR signaling studies did not involve CD4 or CD8 co-receptor engagement, thus possibly accounting for the disparate results between normal in vivo thymic selection and diminished in vitro TCR signaling. Taken together, the evidence suggests that Fyn may have a restricted unique role in mature single positive thymocytes that can not be served by other PTKs.

Lck

In addition to Fyn, the other Src-like kinase implicated in TCR signal transduction is the 56 kDa Lck kinase (116). Lck was initially identified in the

lymphoma cell line, LSTRA, where Lck is overexpressed ~10 fold relative to normal T lymphocytes (117). Several lines of evidence indicate that Lck participates in TCR signaling, the earliest of which was the identification of the association between Lck and the cytoplasmic tails of the TCR co-receptor molecules, CD4 and CD8 (118, 119). The non-covalent stable association between Lck and CD4 and CD8α occurs through pairs of cysteine residues located in the N-terminus of Lck and in the cytoplasmic domains of CD4 and CD8 (120, 121). The interaction between the TCR and the peptide/MHC complex on the APC, is stabilized by the binding of CD4 or CD8 to the non-polymorphic regions of Class II or I respectively (122, 123), thus bringing Lck in the proximity of the TCR complex (124, 125). Furthermore, antibody cross-linking experiments demonstrated that co-engagement of CD4 and CD3 enhances both proximal and distal TCR signaling events relative to engagement of TCR alone (126-130). However, the most convincing demonstration for the importance of the CD4-Lck association comes from analysis of an antigen-specific CD4-deficient hybridoma. In the absence of CD4, this cell is unresponsive to stimulation by antigen/MHC, yet, the introduction of CD4 molecules that are capable of associating with Class II and Lck restore TCR signaling in response to antigen presentation (131). Similar results have been demonstrated with the CD8 molecule, in which expression of truncated form of the CD8α chain resulted in diminished TCR signaling (132, 133). The association of Lck with the CD4 and CD8 molecules and, thus, its proximity to ligand engaged TCR complexes makes Lck an attractive candidate for a TCR regulated PTK.

Conversely, there is also evidence suggesting Lck can function in TCR-mediated responses independently of CD4 or CD8. Expression of "activated" forms of Lck (Y505F, see below) in a CD4 and CD8 negative hybridoma

results in enhanced proximal and distal events associated with TCR stimulation (134-136). Since Lck is both myristylated and palmitylated (137), it may be targeted to the inner leaf of the plasma membrane independently of it association with CD4 or CD8.

The strongest evidence supporting a critical function for Lck in TCR-mediated signal transduction comes from genetic studies performed in cell lines and murine animals. The Jurkat, leukemic cell line, has been extensively studied as a model system for analysis of TCR signal transduction. A number of somatic cell mutants of the Jurkat line have been selected for an inability to mobilize intracellular calcium levels following TCR engagement, thus implicating a molecule involved in proximal signaling events (138, 139). One such mutant was recently identified to involve the Lck kinase, in which a splicing defect resulted in the absence of an exon encoding part of the kinase domain (140). TCR signaling is restored to this cell line by the introduction of wild-type *lck* cDNA. Interestingly, studies with the Lck-defective variant of the Jurkat cell line also suggests a function for Lck independent of its association with CD4 and CD8 as this cell lacks CD8 and has very low levels of CD4. Moreover, introduction of a myristylation mutant of Lck into this cell line is unable to restore TCR signaling (D. Straus, personal communication), suggesting that plasma membrane localization is essential for Lck function in TCR signal transduction.
In addition to the Jurkat variant, a spontaneous loss of Lck in a cytotoxic T cell clone resulted in a profound reduction in TCR-mediated cytolysis that was restored following Lck expression (141).

Further genetic evidence indicating a critical role for Lck in TCR signaling comes from mice lacking Lck as a result of specific gene targeting by homologous recombination. These mice display a severe reduction in the number of double and single positive thymocytes, while the earlier progenitor

double negative thymocytes appear at normal levels (142). In addition, there is a marked reduction in the number of peripheral T cells. Thus, unlike the Fyn-deficient mice, Lck appears to play a critical role at an early stage in thymocyte development. These Lck defective cell lines and mice, demonstrate an unique function for Lck which can not be substituted for by the endogenous Fyn and Yes kinases.

ZAP-70

A third, and most recently identified PTK implicated in proximal TCR signal transduction, is ZAP-70. Structurally dissimilar from the Src-like kinases, ZAP-70 was identified as a 70 kDa tyrosine phosphoprotein induced to associate with the TCR ζ chain following receptor engagement (45, 143). ZAP-70 is expressed exclusively in T cells and natural killer cells (NK cells) (46). The other ZAP-70-like kinase, Syk, is more widely expressed: Not only is Syk expressed in B cells, it appears to be also expressed at low levels in thymocytes, but is further reduced in peripheral T cells (144). The differential expression of Syk in T cells may have implications for the role of ZAP-70 in T cell development.

In addition to the association of ZAP-70 to the TCR ζ chain, ZAP-70 has also been demonstrated to bind the CD3 ε chain in vivo (39, 143). Furthermore, in vitro studies utilizing phosphopeptides encompassing the individual ARAM sequences from the CD3 ε, δ, and γ chains, plus the three individual ARAMs from the ζ chain demonstrates that ZAP-70 is able to bind all ARAMs in a phosphotyrosine-dependent manner (145, 146, and B. Irving, personal communication). Thus, unlike Fyn and Lck, ZAP-70 associates with the TCR following receptor engagement, and consequently may be involved in mediating events downstream of TCR phosphorylation. Similar functions may apply to Syk in thymocytes and some T cells where it is expressed at higher levels.

Molecular characterization of ZAP-70 in a heterologous cell system indicates a sequential function for the Src-like kinases and ZAP-70 (146). The interaction of ZAP-70 with the TCR ARAMs is dependent on tyrosine phosphorylation of the ARAMs by either Lck or Fyn. Optimal binding of ZAP-70 with the ARAMs requires both ZAP-70 SH2 domains and phosphorylation of both tyrosine residues of an individual ARAM. Moreover, the phosphorylation and binding of ZAP-70 to the ARAMs is not dependent on ZAP-70 kinase activity, indicating that the Src-like kinases may phosphorylate the ARAMs, recruit ZAP-70 and in turn phosphorylate ZAP-70 on yet unidentified sites. The phosphorylation of ZAP-70 following recruitment to the TCR may result in "activation" of ZAP-70 thus allowing ZAP-70 to mediate the downstream signaling cascade (147) (Figure 4). The engagement of chimeric molecules in which Syk and ZAP-70 were tethered to the extracellular and transmembrane domains of a heterologous receptor, demonstrates that Syk and ZAP-70 can mediate the tyrosine phosphorylation of cellular proteins and mobilization of $[Ca^{2+}]_i$ (148).

Recently obtained genetic evidence supports a possible role for ZAP-70 in thymic development and in TCR signal transduction. A number of non-related patients have been identified with a type of severe combined immunodeficiency (SCID) in which they have reduced numbers of peripheral $CD8^+$ T lymphocytes. Moreover, all of the $CD4^+$ T cells that are present are defective in both proximal and distal TCR-mediated signaling events (149). Biochemical characterization of these cells reveals a loss of ZAP-70 protein. In the patients examined to date, the ZAP-70 defects appear to be recessive and at the molecular level varies from deletions and missense mutations, changing critical residues or altering splicing sites. These studies suggest that ZAP-70 may be required for thymic selection of $CD8^+$ cells and demonstrate its absolute requirement in TCR signaling function in peripheral $CD4^+$ cells. As Syk is expressed in cells from these SCID patients, it

raises the intriguing question of the relative functions of Syk and ZAP-70 in thymic selection of $CD4^+$ versus $CD8^+$ cells.

The structure and function of protein tyrosine phosphatases

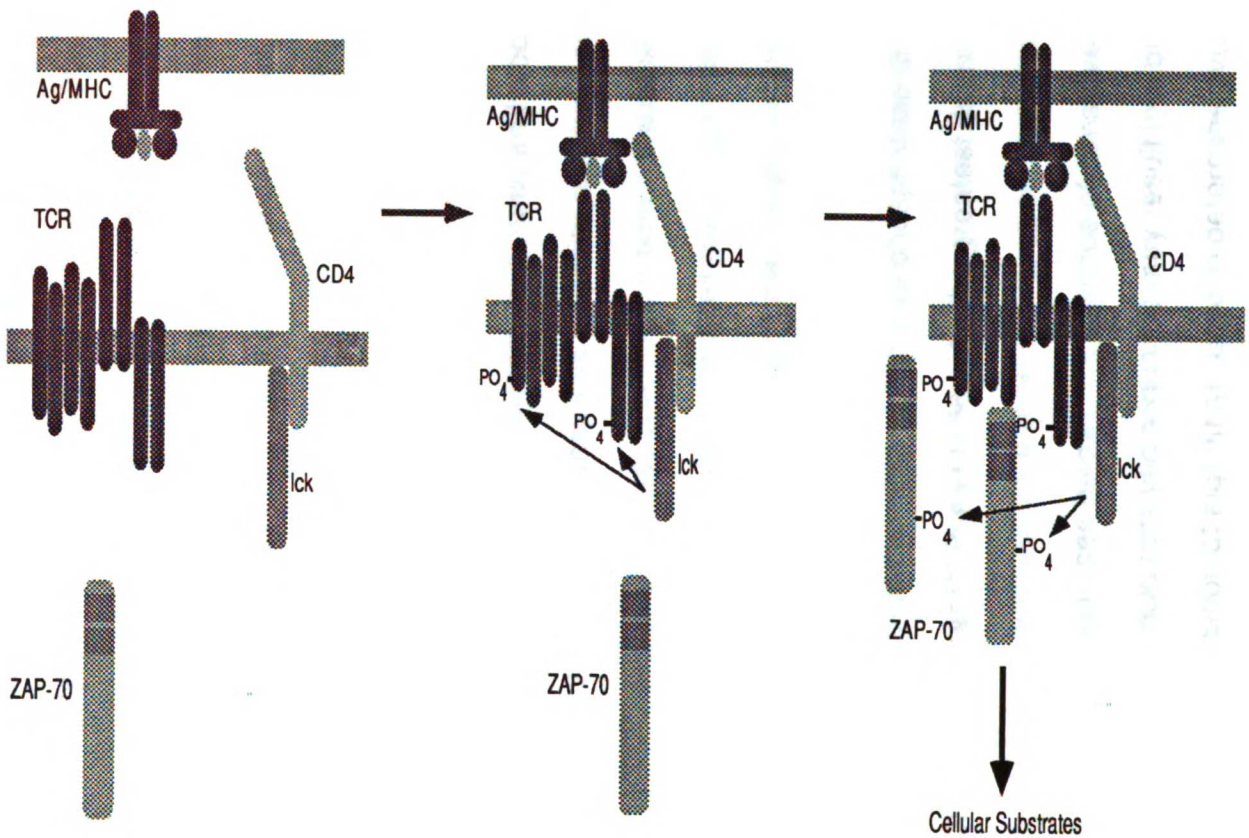
Reversible protein phosphorylation of tyrosine residues, although involving a small percentage (0.01 - 0.05%) of total cellular phosphorylation in normal cells, is a critical regulatory mechanism in controlling cellular effector functions, differentiation and proliferation (150). Since tyrosine phosphorylation of cellular proteins is an early and requisite event in TCR signal transduction, the regulatory function of protein tyrosine phosphatases (PTPases) in this process is of considerable interest (151).

The PTPases have only been recently identified relative to the counterpart PTK family of enzymes. In 1988, the first PTPase, PTP 1B, was purified to homogeneity utilizing a unique chromatography step where a modified version of lysozyme was phosphorylated *in vitro* with ATPγS (152). The resultant thiol-phosphate moiety on the lysozyme can be recognized by the PTPases; however, it is resistant to dephosphorylation, resulting in a stabilization of the PTPase-substrate complex. Amino acid sequence analysis revealed that PTP 1B bore no sequence similarity to the previously identified serine/threonine phosphatases or the broad specificity alkaline or acid phosphatases. However, the lymphocyte surface molecule, CD45, was noted to contain two cytoplasmic repeats with considerable sequence identity to PTP 1B and had phosphatase activity *in vitro* (153, 154). This will be discussed in detail below.

The initial identification of PTP 1B and CD45 as PTPases led to the cloning of numerous other family members, including molecules from such diverse species as insects, a prochordate, *Styela plicata*, *C. elegans*, bacteria, yeast and a virus, Vaccinia (155). The PTPase family of enzymes, analogous

Figure 4. A model of PTK interactions in the initiation of TCR signal transduction

Engagement of the TCR and CD4 co-receptor by the antigen/MHC complex results in the phosphorylation of the CD3 and ζ chains by Lck, recruiting ZAP-70 to the phosphorylated ARAM motif. Localization of ZAP-70 to the TCR complex allows Lck to phosphorylate and activate ZAP-70.


Ag/MHC
TCR
CD4
Ick
ZAP-70
Ag/MHC
TCR
CD4
PO4
PO4
Ick
ZAP-70
Ag/MHC
TCR
CD4
PO4
PO4
Ick
PO4
PO4
ZAP-70
Cellular Substrates

with the PTKs, can be classified into two structurally distinct groups: intracellular enzymes and transmembrane receptor-like proteins. The intracellular PTPases can be further divided into additional subfamilies. PTP 1B and approximately 20 other non-receptor like enzymes constitute the prototypes for intracellular PTPase subfamily. A second branch of the intracellular PTPases is based on the identification of a vaccina virus encoded phosphatase, Vh1 and is therefore referred to as the Vh1 subfamily (156). The Vh1 subfamily also includes the human, and insect homologs of the yeast *cdc25* gene product, the product of the immediate early gene 3CH134 known as MKP-1 (157), and the nuclear PTPase, PAC-1 (158). The Vh1 group of enzymes, unlike the other known PTPases, not only dephosphorylate phosphotyrosine substrates, but also phosphoserine and phosphothreonine proteins in vitro. Moreover, genetic and biochemical data suggests that cdc25, which demonstrates exquisite substrate specificity for the kinase $p34^{cdc2}$, dephosphorylates $p34^{cdc2}$ on both tyrosine and threonine; requisite events for $p34^{cdc2}$ activation and cell cycle progression (159, 160). The dual specificity of the Vh1-like PTPases may be accounted for by structural considerations. The 20 kDa Vh1 enzyme is coded for by 170 amino acids, considerably smaller than the ~250 amino acid phosphatase domains identified in the majority of the other PTPases (156). As the greatest degree of homology between the Vh1 subfamily and the other PTPases centers around the core consensus catalytic motif (see below), the absence of conserved amino acids flanking the 11 amino acid catalytic sequence may result in a broader substrate specificity. The application of algorithms used to determine evolutionary relationships, suggest that the Vh1 subfamily of PTPases share a common ancestry with the other PTPases and that they must have diverged long ago or at a very rapid rate.

The non-Vh1-like PTPases consist of both cytoplasmic as well as a putative nuclear protein. These enzymes are very structurally diverse except for the phosphatase domain, which can be localized at either the amino- or carboxy-termini of the molecule. The non-enzymatic regions of the intracellular PTPases are thought to be involved in subcellular localization and/or regulation of enzymatic activity (161). For example, the T cell PTPase (TC-PTP) contains a C-terminal hydrophobic region which, when deleted or cleaved by proteases, releases the TC-PTP from the particulate fraction of the cell and also results in an increase in its enzymatic activity (162). The C-terminal 35 amino acids of PTP 1B has been demonstrated to target the protein to the outer membrane of the ER (163). Moreover, the engagement of the integrin, gpIIIb/IIa, on platelets, results in calpain-mediated cleavage of PTP 1B near the C-terminus and in subcellular relocalization of PTP 1B to the cytoplasm (164). The relocalization of PTP 1B to the cytoplasm correlates with the physiological process of platelet aggregation. Lastly, the hematopoietic cell specific PTPase referred to as PTP 1C (also referred to as SH PTP1, HCP, and SHP) contains two N-terminal SH2 domains, which are probably responsible for the binding of this enzyme to activated receptors for the cytokine IL-3 (165). Analogously, the ubiquitously expressed tandem SH2 domain containing PTPase, PTP 1D (also referred to as SH PTP2 or Syp) binds to activated EGF and PDGF receptors (166, 167).

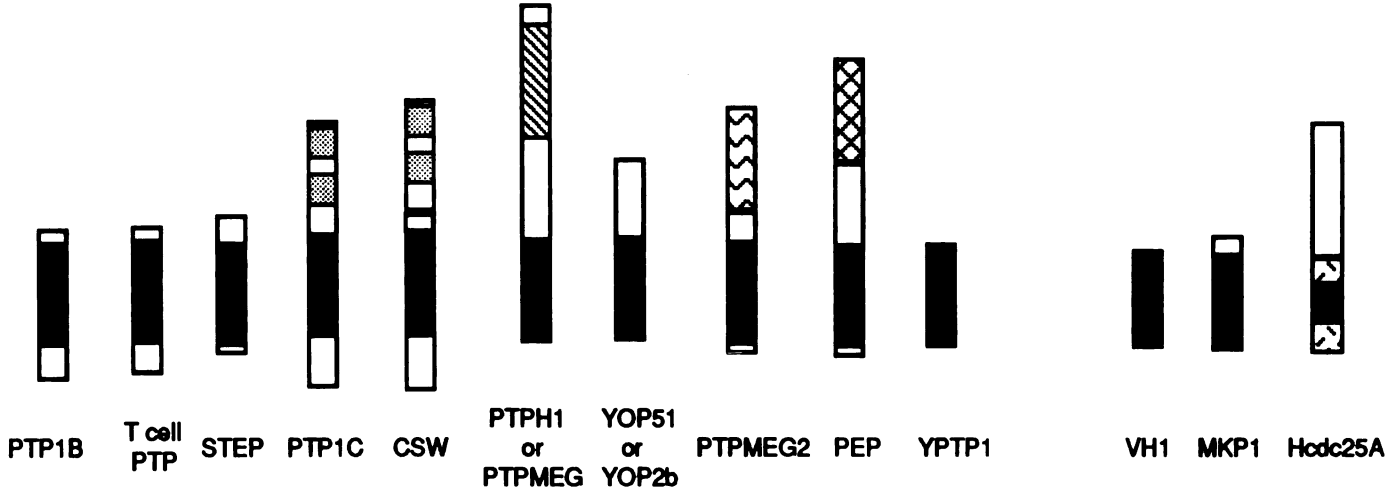
The unique domains of the other cytoplasmic PTPases have not been characterized as well and include: 1) A lipid binding domain in PTP MEG-2 (168); 2) a region rich in proline, glutamic acid, serine and threonine (PEST sequence) residues, characteristic of proteins with very short half-lives found in the PEP enzyme (169); and 3) PTP H1 and MEG-01 contain cytoskeletal Band 4.1 and ezrin-like domains (170). The homology between the unique domains of the intracellular PTPases and known proteins suggests potential functions or

means of regulation; however, the majority of the intracellular PTPases contain unique domains with no known homology or function (Figure 5).

The transmembrane receptor-like PTPases are as structurally diverse as the intracellular PTPases. The transmembrane PTPases are composed of a unique extracellular domain of variable size, a single transmembrane domain, and a cytoplasmic domain most frequently containing two phosphatase domains (171). Only HPTPβ and the drosophila protein DPTP10D contain a single phosphatase domain (171-173). While the receptor-PTPases are relatively similar through the transmembrane and cytoplasmic regions, the extracellular domains are considerably diverse. The transmembrane-PTPases can be classified based on the structural features of the extracellular domain (161). The type I transmembrane-PTPase, of which CD45 is a prototype, contains a relatively large extracellular domain with no homology to any known proteins. The type II enzymes are characterized by one to three tandem immunoglobulin-like domains followed by three to ten tandem fibronectin type III (FNIII) repeats, reminiscent of the neural cellular adhesion (NCAM) family of molecules (174). The LAR protein, a type II PTPase, sheds its extracellular domain in a cell density dependent manner, but the regulatory consequences of such an event are not known (175). Another type II PTPase, R-PTP-κ, whose expression levels are greatest in developing regions of the CNS and in areas capable of developmental plasticity also sheds its extracellular domain (176). The extracellular domains of the PTPases which comprise the type III category are distinguished by being composed solely of tandem FNIII-like repeats. Thus, the type II and III transmembrane PTPases may be involved in cellular adhesion events. Type IV molecules are characterized as consisting of relatively short extracellular domains lacking any homology to other known proteins. The extracellular domains from these molecules range from 27 to 123 amino acids

Figure 5. Schematic representation of the intracellular PTPases

The conserved catalytic domains are shown in black, while the position and relative sizes of the non-catalytic regions are indicated by rectangular boxes containing distinct symbols.


PTP1B
T cell PTP
STEP
PTP1C
CSW
PTPH1 or PTPMEG
YOP51 or YOP2b
PTPMEG2
PEP
YPTP1
VH1
MKP1
Hcdc25A

A more recently cloned molecule that does not fall into any category is RPTPγ (HPTPζ), which has a large extracellular domain with an N-terminal domain homologous to carbonic anhydrase (177, 178). While no carbonic anhydrase activity has been described, this region may have other enzymatic or regulatory functions (Figure 6).

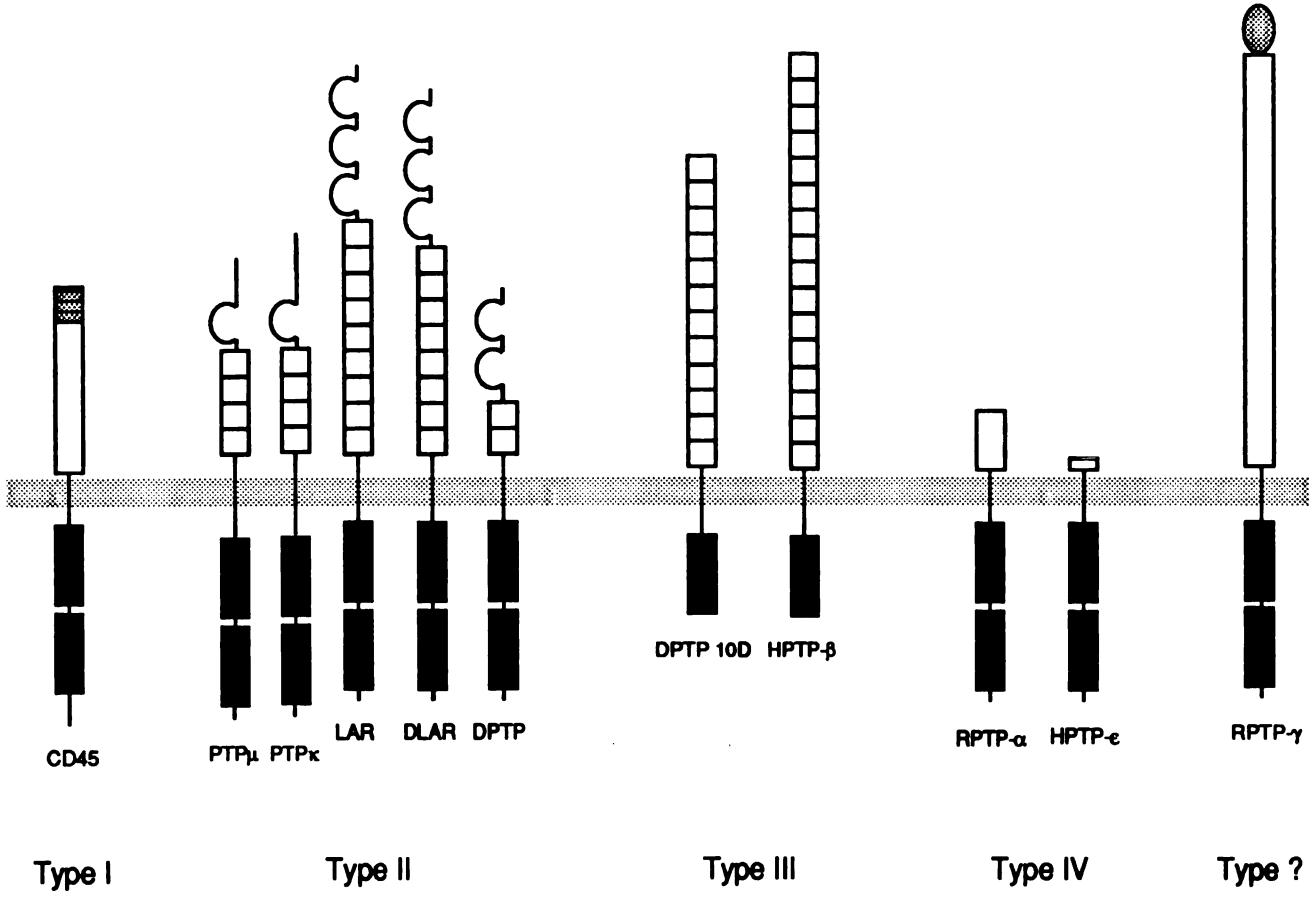
The transmembrane-PTPases were referred to as receptor-like, since no ligands had been identified. Recently a putative ligand has been identified for CD45 (see below), PTPμ and PTPκ. The ligands for the μ and κ phosphatases are the μ and κ molecules themselves (179-181). The extracellular domains of these two PTPases are capable of intercellular homophilic binding and mediating cell adhesion . The consequences of homophilic binding on phosphatase activity and cellular tyrosine phosphorylation are not known; however, reduction of cell-substrate adhesion has been documented to result in a general increase in phosphotyrosine phosphatase activity (182). Therefore, homophilic binding of μ and κ PTPases may result in inhibition of enzymatic activity. The ligands for the other transmembrane PTPases are not known. More importantly, the cellular substrates and the functional consequences of ligand binding are not understood.

Structural features of phosphatase domains

As the number of PTPases identified grows, the conserved structural features of the phosphatase domains become more readily discernable. The earliest amino acid segments to be recognized was an 11 amino acid motif present in all PTPases with demonstrated enzymatic function. The consensus sequence (I/V)HCXAGXXR(S/T)G is located at the C-terminal end of the phosphatase domain. During the purification of PTP 1B, an absolute requirement for thiol reducing agents to preserve enzymatic activity was observed, suggesting that a cysteine residue was critical for phosphatase function (152). Further

Figure 6. Schematic representation of the transmembrane receptor-like PTPases

The transmembrane PTPases are subdivided into four types based on the structure of their extracellular domain. The conserved catalytic domains are shown in black, while the non-conserved structures are depicted by rectangular boxes containing unique symbols.


CD45
PTPμ
PTPκ
LAR
DLAR
DPTP
DPTP 10D
HPTP-β
RPTP-α
HPTP-ε
RPTP-γ
Type I
Type II
Type III
Type IV
Type ?

studies with the irreversible sulfhydryl-directed inhibitors, N-ethylmaleimide, p-(hydroxymercuri)benzoate and iodoacetate resulted in absolute inhibition of phosphatase activity (183). Moreover, equivalent levels of iodoacetamide, had little effect on enzymatic activity, suggesting that the more negatively charged iodoacetate interacts with a positively charged residue at or near the active site. Consistent with the inhibitor studies, the consensus phosphatase motif contains a cysteine and an arginine residue, suggesting that this motif represents the catalytic site of PTPases. This was confirmed when ^{14}C-iodoacetate was incubated with the LAR PTPase, resulting in a stoichiometry of binding of 0.8 moles of iodoacetate per mole of LAR (184). Tryptic digestion of the LAR protein resulted in isolation of a single ^{14}C-labeled peptide, which upon amino acid sequencing analysis revealed that cysteine 1522 of the consensus phosphatase motif was the only site labeled. Although there are 11 cysteines in LAR, the labeling of cysteine 1522 indicates an unusual reactivity of this residue. Furthermore, a phospho-enzyme intermediate was isolated utilizing PTP 1B that is absent in mutants in which the cysteine from the phosphatase motif is altered, suggesting that the formation of a phospho-cysteine intermediate is critical for phosphatase activity (185). Lastly, site directed mutagenesis of the conserved cysteine, in both receptor-like and intracellular PTPases results in abrogation of phosphatase activity (159, 160, 183, 186, 187). These data, plus other biophysical studies suggest a two-step mechanism by which PTPases function: The nucleophilic cysteine attacks the phosphate group of the phosphotyrosine substrate, resulting in the formation of a thiolphosphate enzyme intermediate and the dephosphorylated substrate. The second, and rate limiting step, is the hydrolysis of the thiolphosphate intermediate by water, regenerating an active enzyme and releasing the inorganic phosphate (188). These studies indicate that only the cysteine residue in the active site is directly involved in the

phosphate transfer reaction, suggesting that the other residues of the phosphatase domain may be involved in determining the structure of the phosphatase domain, and in determining substrate specificity by potentially interacting with residues flanking the phosphotyrosine of the substrate protein.

Two other conserved motifs have been recognized within the phosphatase domains of the majority of the proteins identified to date. One motif consists of the GXGXXG sequence initially identified in the PTKs and in the dehydrogenase family of enzymes. The GXGXXG motif is located within the active site sequence, just C-terminal to the catalytic cysteine. In the PTKs, the glycine motif is thought to be involved in the coordination of the phosphate donor adenosine triphosphate (ATP) (189). The function of the glycine motif in PTPase function is not clear since, ATP is not required for catalytic activity. However, the first glycine residue appears to be critical for phosphatase activity of the LAR and CD45 PTPases, as modification of this residue abolishes activity (186, 190). The third glycine of the GXGXXG is not absolutely conserved among the PTPases, thus this motif may be functionally different in PTPases and PTKs. The third conserved motif is the FKVRES sequence found in the non-Vh1 subfamily of intracellular PTPases, that is present just N-terminal to the catalytic motif. This motif is very similar to the FLVRES sequence, the most conserved region of the SH2 domains (191). Crystallographic studies of the SH2 domains, indicates that the amino acids of the FLVRES motif directly interact with phosphotyrosine (192). Thus, the FKVRES sequence which is immediately upstream of the catalytic site in the intracellular PTPases may also be involved in coordinating phosphotyrosine. However, it is curious that the FKVRES motif is not found in the transmembrane PTPases, which unlike the intracellular PTPases contain two tandem phosphatase domains. This would suggest that the intracellular and transmembrane PTPases may be regulated differentially.

Regulatory mechanisms of protein tyrosine phosphatases

In vitro analysis of the various members of the PTPase family have indicated that the PTPases have a high specific activity. In fact, a few of the PTPases are so active that any increase in efficiency would be limited by the rate of diffusion of substrates. In comparison with the PTKs, the in vitro activity of the PTPases are one to three orders of magnitude greater, suggesting that these enzymes must be tightly regulated in vivo (161). The transmembrane PTPases, obviously, have the potential for regulation by ligand binding. The potential consequences of ligand-transmembrane PTPase interaction are discussed in Chapter 2.

A mechanism of regulation commonly used in signal transduction pathways, is that of reversible phosphorylation. Serine phosphorylation of CD45 (193), cdc25 and PTP 1B have been documented. Interestingly, PTP 1B serine phosphorylation is altered in a cell-cycle dependent manner; however, the functional consequences of phosphorylation are not known (194). There does not appear to be any measurable change in PTP 1B enzymatic activity. Cdc25, on the other hand, prior to mitosis undergoes a marked elevation in phosphatase activity that coincides with extensive serine/threonine phosphorylation at the N-terminus of the molecule (195).

Tyrosine phosphorylation is a critical means of regulating PTKs, and since the PTPase can function antagonistically, the notion that PTPases may be regulated by tyrosine phosphorylation is appealing. Many of the PTPases, both transmembrane and intracellular molecules, are tyrosine phosphorylated in vitro by a number of different kinases (155). Moreover, the SH2 domain containing PTP 1D, and PTP 1C, and the transmembrane PTPase CD45 (196, 197) have been demonstrated to be tyrosine phosphorylated in vivo. CD45 tyrosine

phosphorylation will be discussed in detail in Chapters 3 and 4. The PTP 1C molecule is reversibly phosphorylated on tyrosine residues following stimulation of the TCR (L. Tan and N. van Oers, personal communication) and the colony stimulating factor-1 receptor (198). In both cases, PTP 1C does not appear to associate with the receptors themselves, nor is the kinase responsible for PTP 1C phosphorylation known. Interestingly, PTP 1D associates with and is a substrate for the EGFR kinase (166, 167). Phosphorylation of PTP 1D results in an augmentation of enzymatic activity (166). The physiological ramifications of PTP 1D phosphorylation and the increase in its enzymatic activity are not known.

Change in subcellular localization is a mechanism postulated to be involved in c-Src regulation, and may also emerge as a general means of regulation of the intracellular PTPases. Both PTP 1B and TC-PTP contain hydrophobic C-terminal segments thought to be involved in their localization to membranous regions of the cell (162, 163). PTP 1B has been verified to be localized to the cytoplasmic face of the ER via it C-terminal 35 amino acids. Moreover, during platelet aggregation, PTP 1B is cleaved near the carboxy one-third of the molecule, liberating it from its membrane anchor (164). PTP 1B relocalization correlates with irreversible platelet aggregation, and dephosphorylation of tyrosine phosphoproteins. The TC PTP also found in the particulate fraction of the cell relocalizes to the cytosol upon removal of the C-terminal hydrophobic sequences, resulting in an increase in in vitro enzymatic activity. Furthermore, PTP 1D and PTP 1C, as a result of binding to the activated EGFR and IL-3 receptors, respectively, undergo a relocalization from the cytosol to the plasma membrane (165-167). Thus, subcellular redistribution of proteins, resulting in a significant change in the effective concentration of a protein, can be an efficient, rapid and reversible mechanism of PTPase regulation.

Some PTPases are not expressed constitutively, and rather are immediate early genes activated by a number receptor molecules, including the EGFR and the TCR. The product of the 3CH134 gene is a cytosolic dual function PTPase of the Vh1 subfamily, that appears to be involved in the dephosphorylation and inactivation of MAP kinase (199). Another Vh1-like PTPase is the mitogen-induced PAC-1 enzyme (158). PAC-1, predominantly expressed in hematopoietic tissues is localized to the nucleus and may be involved in transcriptional regulation by inactivating nuclear MAP kinase or cell cycle progression (200). Engagement of the TCR results in the induction of the hematopoietic cell specific HePTP (201). The function of HePTP is not known. Thus, it appears that the PTPase family will be regulated by multiple mechanisms, and that some individual PTPases may be regulated by numerous means.

Biology of protein tyrosine phosphatases

Although the cloning of PTPases has proceeded at a rapid rate, insights into their function has been slower to reach fruition. The biological role of CD45 in hematopoietic cell signal transduction has been intensively studied and will be discussed below. Many of the receptor-like PTPases have extracellular domains homologous with molecules involved in cell-cell or cell-extracellular matrix interactions. Indeed, PTP μ and κ have been demonstrated to mediate cell adhesion via homophilic binding (179-181). Unfortunately, the consequences of such an interaction are not known. Three drosophila transmembrane PTPases have been localized to central nervous system axons where both the timing and pattern of expression suggests a role in regulating neurite outgrowth and axonal homing (172, 173). However, the majority of the transmembrane PTPases have yet to be assigned a function.

The preponderance of intracellular PTPases, as well, do not yet have a defined function. The functions of three of the intracellular PTPases, PTP 1B, MKP1 and cdc25 have already been discussed. Another intracellular PTPase whose biological significance is known is the Yop2B PTPase produced by the *Yersinia* species of bacteria (187). The Yop protein is encoded on a virulence determining plasmid, suggesting that it may be involved in *Yersinia*-mediated host pathology. Strains of *Yersinia* which contain a Yop2B gene in which the catalytic cysteine has been altered by site-directed mutagenesis are no longer virulent (202). Moreover, the Yop2B protein has been demonstrated to result in the dephosphorylation of two macrophage tyrosine phosphoproteins following *Yersinia* infection, indicating an essential role for Yop 2B in Yersinial disease (203). These data indicate that PTPases can play key regulatory functions in mammalian cells, and that lower organisms may have subverted the function and/or expression of PTPases much in the same way as retroviruses have with PTKs.

Another arena of PTPase function may be in regulation of cellular proliferation. Approximately, one-third of the known oncogenes are encoded by PTKs (150), and as such the PTPases by virtue of their ability to dephosphorylate tyrosine phosphoproteins can be PTK antagonists and potentially function as tumor suppressors. Overexpression of PTP 1B resulted in suppression of cellular transformation by the *Neu* oncogene (204). Unfortunately, which proteins underwent an alteration in phosphorylation status due to the overexpression of the PTP 1B was not addressed. Epidemiological studies suggest a correlation between the deletion of a portion of chromosome 3, encoding the transmembrane PTPase, RPTPγ, in lung and renal carcinomas (205). More precise mapping of the genes encoding the PTPases is required for further determination of whether chromosomal abnormalities associated with tumor cells

correlates with a loss of expression of a PTPase. In addition to functioning as potential tumor suppressor genes, there is evidence that at least one PTPase may function as an oncogene. PTPases may not only oppose the action of PTKs, they may also positively regulate PTK function, such as may be the case with CD45 and RPTPα. These transmembrane PTPases have been demonstrated to dephosphorylate the C-terminal negative regulatory site of the Src-family kinases (206, 207). Moreover, overexpression of RPTPα, and not PTP 1B, in NIH 3T3 cells expressing c-Src, results in transformation of these cells (206). Therefore, PTPases may act as oncogenes or anti-oncogenes, depending on the function of the substrates they dephosphorylate.

Lastly, studies utilizing systems amenable to genetic manipulation has resulted in the identification of two PTPases involved in organismal development. First, an SH2 domain containing cytosolic PTPase, encoded by the drosophila gene *corkscrew*, is involved in a signal transduction pathway mediated by the torso receptor tyrosine kinase (208). *Corkscrew* is required for normal determination of cell fates at the termini of the embryo. Another intracellular PTPase, from *Dictyostelium*, has been identified to play a role in normal spatial and morphological development of the organism (209). Since it has been difficult to define the functions of the majority of PTPases through biochemical methods, the use of genetic systems will greatly foster the identification of the physiological roles of PTPases.

T cell receptor signal transduction: The role of protein tyrosine phosphatases

T lymphocytes express a number of PTPases whose functions are yet to be defined. Both cytoplasmic and transmembrane PTPases found in a number cell types, such as the TC-PTP and RPTPα, respectively, are also expressed in T

lymphocytes (D. M. Desai and A. Weiss, unpublished data). To date, four hematopoietic cell specific PTPases have been described HePTP, PTP 1C, PEP, and CD45. Activation of resting T cells results in the rapid induction of a 53 kilodalton PTPase, HePTP (201). Since HePTP expression is induced following TCR stimulation, it may play a function in late T cell activation events, or may function in a negative-feedback loop by dephosphorylating TCR-induced tyrosine phosphoproteins. The PEP PTPase is expressed in multiple hematopoietic cell lineages (169), as is the tandem SH2 domain containing HCP (also termed SHP, PTP-1C, and SH-PTP1) molecule (169, 210-213). While the function of PEP is not known, insight into the function of HCP has recently been obtained from genetic analysis of mice with a single gene defect resulting in stem cell dysfunction and early onset autoimmunity. Identification of HCP as the product of the motheaten and motheaten-viable locus suggests a role for this PTPase in immune cell development (214). Future reconstitution and biochemical studies should shed light on the precise role of this molecule in lymphopoiesis.

CD45 structure and expression

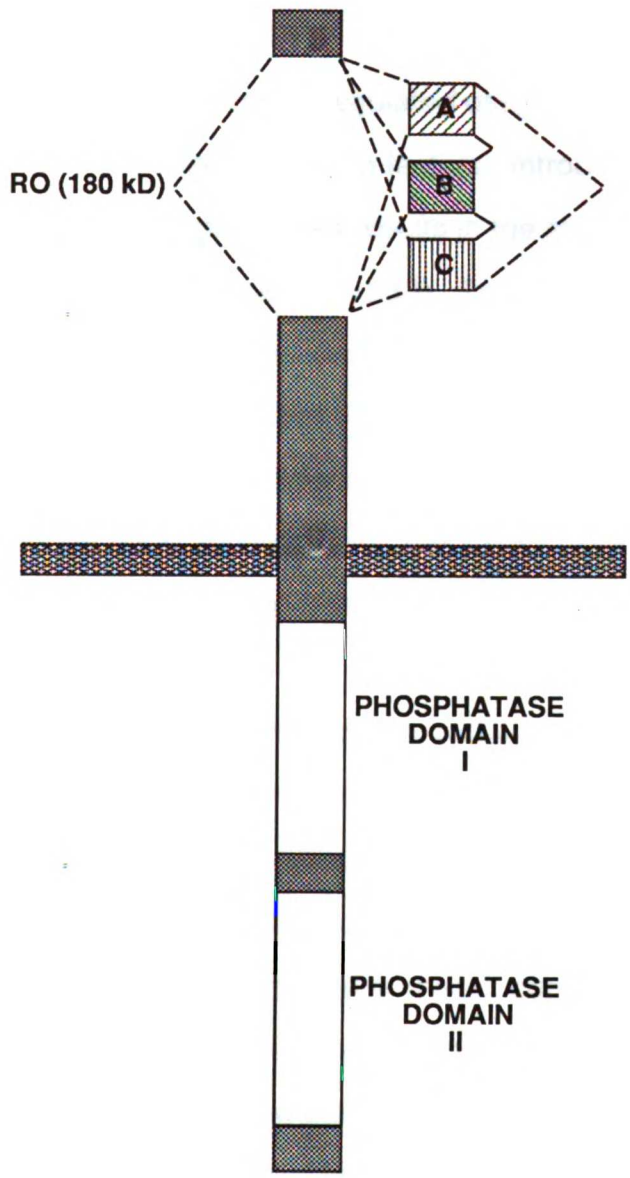
Another hematopoietic cell specific phosphatase with a defined function, is the transmembrane PTPase CD45. Long before CD45 was determined to be a member of the PTPase family, it was identified as a major cell surface polymorphic antigen (215, 216). CD45 encompasses a family of high molecular weight glycoproteins that differ in protein sequence and carbohydrate structure which are differentially expressed on various hematopoietic cell lineages (217). The basis for the different CD45 glycoproteins was elucidated following the isolation of cDNA clones and determination of the genomic organization of the CD45 gene (218-223). The CD45 gene is composed of 34 exons distributed over ~110 kB of DNA on human chromosome 1. Exons 3-33 encode for the mature

protein, of which exons 3-15 encompass the extracellular domain, exon 16 the transmembrane domain, and 17-33 the cytoplasmic domain. The CD45 isoforms are generated by regulated alternative splicing of exons 4 (A), 5 (B) and 6 (C) encoding a portion of the extracellular domain (Figure 7). The nomenclature used to describe CD45 isoforms is based on recognition of the alternative exons by specific antibodies. Isoforms containing the A (4) exon are referred to as CD45RA, whereas the B (5) exon containing isoforms are CD45RB and so on. Two isoforms that do not fit this nomenclature are those that either utilize all three alternate exons or do not utilize any and are referred to as B220 and CD45RO, respectively. Six of the eight potential isoforms have been isolated as cDNAs from different species. Moreover, recently, all eight isoforms have been isolated by reverse-transcriptase coupled polymerase chain reaction (RT-PCR) (224).

CD45 splicing appears to be regulated in both a tissue-specific and developmental manner. Most B cells express the highest molecular weight isoform of CD45, B220 (Exons A^+, B^+, C^+), whereas, mature T lymphocytes, and NK cells express multiple isoforms (217). Cells of the myeloid lineage also express one to three CD45 isoforms; however, compared to lymphoid cells, the total level of surface expression is relatively lower. Developmental regulation of CD45 isoform expression is believed to occur only in the T lymphocyte population. Mature thymocytes predominantly express the CD45RO isoform (exon A^-, B^-, C^-) whereas, immature thymocytes and peripheral blood T cells express numerous isoforms (225, 226). In addition to the developmental regulation of CD45 isoform expression, T lymphocytes alter the pattern of CD45 isoform expression following activation (227-229). T cell activation results in a significant decrease in CD45RA expression, nadiring at approximately day 7, and full reexpression by day 14 or so (228). However, as CD45RA expression is decreasing, CD45RO expression increases. The reciprocal changes in cell

Figure 7. CD45 structure and splicing pattern

Schematic representation of the structural organization of CD45. The alternatively spliced exons are indicated along with the various splicing combinations.


RO (180 kD)
A
B
C
PHOSPHATASE
DOMAIN
I
PHOSPHATASE
DOMAIN
II

surface expression of CD45RA and CD45RO results in a relatively steady state level of total CD45 surface expression. These changes in CD45 isoform expression have been used to isolate and characterize helper T cell subpopulations which secrete distinct patterns of lymphokines and have different activation requirements (230, 231).

The molecular basis for the regulated usage of CD45 exons 4-6 is due to the presence of tissue-specific splicing factors. Introduction of minigene constructs into B and T lymphocytes results in the appropriate cell type-specific splicing of the alternative exons (232, 233). Transient somatic cell fusion experiments between B cells containing a CD45 minigene and T cells results in generation of the T cell splicing pattern (234). However, the splicing pattern of T cell lines was unaltered by B cell fusion, suggesting that T lymphocytes contain negative-regulatory trans-acting splicing factors and that the B cell splicing pattern is the default pathway of exon usage. The identity of these splicing factors is not yet known.

Aside from the generation and differential expression of the various CD45 isoforms, the amino acid sequence of the extracellular domain of CD45 may yield insights into its function. The extracellular domain of CD45, composed of 391-552 (depending on isoform) amino acids, can be subdivided into three subdomains: The immediate N-terminal region, conposed of the alternatively spliced exons, contains numerous N- and O-linked glycosylation sites (217). It is estimated that CD45 is 25% by weight carbohydrate (235). Moreover, depending of the usage of exons 4-6, the glycosylation pattern of CD45 can be dramatically altered. Limited biochemical studies have demonstrated differential glycosylation of CD45 depending on the hematopoietic cell from which CD45 is isolated (236). The potential functional importance of CD45 glycosylation, is suggested by the ability of certain mAbs recognizing CD45 specific carbohydrate structures to

modulate T cell function. Furthermore, CD22, a B cell surface antigen has been demonstrated to bind to CD45 as a result of the high degree of CD45 sialylation (237).

The other two subregions of the CD45 extracellular domain, C-terminal to the carbohydrate-rich region, are characterized by the high content of cysteine residues. The cysteines are clustered in two groups of eight and are conserved between species, both numerically and spatially (238). Although the extracellular domain only exhibits 33% homology between species, the absolute conservation of the cysteine residues suggests they are of functional importance and may indicate a conserved three-dimensional structure of the extracellular domain. The implications of the cysteine-rich regions on CD45 function is discussed further in Chapter 2.

The cloning of CD45 revealed a large cytoplasmic domain (705 amino acids) containing an internal duplication of ~300 amino acids. In marked contrast to the extracellular domain, the cytoplasmic domain of CD45 is highly conserved (85-95%) between species (238). The large cytoplasmic domain of CD45 suggested a role in transmembrane signal transduction; however, it was only following the cloning of the first PTPase, PTP 1B, that the function of the CD45 cytoplasmic domain become apparent (153). The tandem 300 amino acid duplication within the cytoplasmic domain of CD45 was recognized to have considerable homology to the phosphatase domain of PTP 1B (Figure 8). CD45 possesses intrinsic phosphatase activity that is dependent on thiol-reducing agents and is inhibitable by sodium orthovanadate, a hallmark of all PTPases (154, 239). The identification of CD45 as a transmembrane PTPase suggests that CD45 may play role in regulating signal transduction pathways involving reversible tyrosine phosphorylation.

Figure 8. Alignment of the two phosphatase domains of CD45

Alignment of the amino acid sequences of the human CD45 phosphatase domains I and II. Conserved amino acids found in the enzymatic domains of nine or more transmembrane PTPases is indicated under consensus sequence. Non-conserved amino acids are indicated by a dash. Spaces are inserted into the amino acid sequences to obtain maximal alignment and are indicated by a period.


CD45 Domain I (652) NQNKNRYVDILPYDYNRVELSEINGDAG.....SNYINASYIDGF
CD45 Domain II (943) NKSKNRNSNVIPYDYNRVPLKHELEMSKESEHDSDESSDDDSDSEEPSKYINASFIMSY
Consensus N--KNRY-----YD--RV-L----------DYINA----G-
CD45 Domain I KEPRKYIAAQGPRDETVDDFWRMIWEQKATVIVMVTRCEEGNRNKCAEYWPSMEEGT
CD45 Domain II WKPEVMIAAQGPLKETIGDFWQMIFQRKVKVIVMLTELKHGDQEICAQYWG....EGK
Consensus -----YI--QGP---T--DFWRM-WEQ-----VM-T---E----KC--YWP------
CD45 Domain I RAFGDVVVKINQHKRCPDYIIQKLNIVNKKEKATGREVTHIQFTSWPDHGVPEDPHLL
CD45 Domain II QTYGDIEVDLKDTDKSSTYTLRVFELRHSKRKDSRTVYQY.QYTNWSVEQLPAEPKEL
Consensus ---G---V----------Y--R----------R-------T-WPD-GVPE-P---
CD45 Domain I L.....KL...RRRVNAFSNFSGPIVVHCSAGVGRTGTYIGIDAMLEGLEAENKV
CD45 Domain II ISMIIQVVKQKLPQKNSSEFNKHHKSTPLLIHCRDGSQQTGIPCALLNLLESAETEEVV
Consensus L----------F----------GP--VHCSAGVGRTGT----D--L--------V
CD45 domain I DVYGYVVKLRRQRCLMVQVEAQYILIHQALVEYNQFGET (893)
CD45 domain II DIFQVVKALRKARLGMVSTFEQYQFLYDVIASTYPAQNG (1209)
Consensus ---------R-QR---VQT--QY-F---AL-E------T

The earliest demonstration that CD45 may play a role in regulating antigen receptor signal transduction came from antibody cross-linking studies. Antibodies to both restricted and common epitopes of CD45 can inhibit B cell proliferation induced by anti-IgM (240). Similarly, certain anti-CD45 antibodies can inhibit NK cell mediated target cell cytolysis (241). However, antibody-mediated ligation of CD45 during TCR stimulation can result in either a potentiation or an inhibition of T cell activation depending on the anti-CD45 antibody or on the T cell line examined (242-247). In contrast, when the TCR and CD45 are aggregated together by antibodies, inhibition of T cell activation has been consistently observed (248), suggesting that CD45 could negatively regulate TCR signaling. However, this conflicts with the notion that CD45 is a positive regulator of TCR signaling function, since TCR signal transduction is abrogated in CD45-deficient cells (see below). This discrepancy may be explained by the significantly higher levels of CD45 expression, relative to the TCR. Recent studies suggest that TCR and CD45 cross-linking effectively inhibits antigen receptor oligomerization; thereby, abrogating TCR signal transduction (249). The inhibition of T cell stimulation by cross-linking the TCR with CD45 was demonstrated not to be specific for CD45 or its PTPase activity, as cross-linking the TCR with other abundant T cell surface molecules can result in a similar phenotype. The inhibition of Fcγ receptor mediated-signal transduction by cross-linking CD45 with the Fcγ receptor (250) may also be the result of preventing Fcγ receptor oligomerization. The experimental limitations of antibody cross-linking studies preclude defining a function for CD45 in TCR signal transduction.

Regulation of TCR signaling by CD45

Genetic evidence for the role of CD45 in T cell activation has come from analyses of T cell lines or clones deficient in CD45 surface expression. A murine T cell clone lacking cell surface CD45 was unable to proliferate or produce cytokines in response to antigen or anti-TCR antibodies (251). However, this cell was still able to proliferate in response to exogenous IL-2, indicating that CD45 is required for TCR-mediated cellular functions, but is not required for signaling by the IL-2 receptor. Similarly, CD45-deficient cytotoxic T lymphocytes are defective in target cell lysis as well as cytokine secretion (252). The requirement for CD45 expression in regard to signal transduction has also been demonstrated for the B cell antigen receptor (253) and for NK cell mediated cytolysis of tumor target cells (254).

In addition to the role of CD45 in T and B cell antigen receptor function, genetic evidence for the function of CD45 in development of the immune system has been recently obtained. Mice in which exon 6 (one of the alternatively spliced exons) of CD45 has been targeted by homologous recombination have a severe defect in CD45 expression on both T and B lymphocytes (255). B cell development in these mice appears normal although signaling through the B cell antigen receptor is abrogated. T lymphocyte development is arrested at the immature $CD4^+CD8^+$ double positive stage. Further studies should elucidate this differential effect of CD45 expression on T and B cell development, and on the role of B cell antigen receptor signaling and B cell ontogeny. In addition these mice can serve as a model system to analyze the effects of individual CD45 isoforms.

The biochemical basis for the defect in TCR signal transduction in CD45-deficient cells was elucidated from analysis of CD45-negative mutants derived from the Jurkat and HPB-ALL cell lines. Engagement of the TCR on these cells

failed to lead to the induction of tyrosine phosphoproteins, PI hydrolysis or increases in $[Ca^{2+}]_i$ (256, 257). As tyrosine phosphoprotein induction is one of the earliest events documented following TCR engagement, the loss of TCR-mediated tyrosine phosphoprotein induction in CD45-deficient cells indicates that the TCR is uncoupled from the intracellular signaling cascade at a very proximal step.

Since PTPases oppose the action of PTKs by dephosphorylating tyrosine phosphoproteins, it is paradoxical that the absence of the CD45 PTPase, in most T cell lines examined, does not result in any appreciable accumulation of tyrosine phosphoproteins in the resting state (257). However, a CD45-deficient cell line has been reported that appears to have elevated resting levels of tyrosine phosphoproteins compared to the parental CD45-positive cell, though the pattern observed in these CD45-negative cells differed from that seen following TCR engagement (258). As parental $CD45^+$ cell line was derived from a retroviral transformation event, integration of the viral DNA into the host genome may have resulted in the activation of a PTK. Thus, in the absence of TCR-mediated tyrosine phosphoprotein induction, the basal phosphotyrosine-containing proteins induced by viral transformation may be more prominent. Another cell with an altered phenotype is a CD45-negative variant of the Jurkat cell line, in which TCR-mediated tyrosine phosphoprotein induction is still observed (259). However, stimulation of the TCR on this CD45-deficient variant still does not result in late T cell activation events such as IL-2 production. Although the reason for this discordance in tyrosine phosphoprotein induction in two CD45-negative variants of the Jurkat leukemic line is not known; the CD45-deficient cell which can mediated early TCR signaling events may be useful in identifying molecules which may be regulated by or targets of CD45 phosphatase activity.

In most CD45-deficient cells, where a loss of TCR-mediated tyrosine phosphoprotein induction is observed, CD45 may either directly or indirectly regulate a PTK(s) which functions at a very proximal point in the TCR signaling cascade. Initial analysis of tyrosine phosphoprotein patterns in CD45-deficient cells compared to their CD45$^+$ parental cells revealed a slight increase in the tyrosine phosphorylation of a single protein of ~56,000 M_r, which was identified as the PTK Lck (260). Phosphopeptide mapping studies revealed that Lck was hyperphosphorylated on its C-terminal tyrosine residue, a site of negative-regulation for members of the Src-family PTKs (260-263). Fyn was likewise found to be hyperphosphorylated on the C-terminal tyrosine residue in CD45-deficient cells, although to a lesser degree than Lck (262, 263). Fyn and Lck appear to be specific in vivo substrates for CD45, since CD45 is unable to dephosphorylate the C-terminal tyrosine of c-Src in vivo (262). The specificity of CD45 for Lck and Fyn is further underscored by the observation that CD45-deficient T cells contain another transmembrane PTPase, RPTPα, albeit expressed at lower levels than CD45 (D. M. Desai and A. Weiss, unpublished observations). RPTPα, on the other hand, may be involved in the regulation of c-Src (206). Analyses of Lck kinase activity from CD45-positive and negative cells have revealed either no difference (260, 264) or a 2-3 fold increase (263) in the in vitro enzymatic activity of Lck isolated from CD45$^+$ cells, depending on the PTK substrate and cell line examined. Similarly, the in vitro kinase activity of Fyn was demonstrated to be approximately 2-3 fold greater in CD45-positive cells compared to CD45-negative cells (263, 264). The addition of CD45 to either Lck or Fyn in vitro also resulted in dephosphorylation at the C-terminal tyrosine and a concomitant increase in kinase activity (207, 265). These data suggest that the TCR signaling defect in CD45-deficient cells is the result of "inactivation" of Lck

and/or Fyn due to hyperphosphorylation at the negative-regulatory C-terminal tyrosine.

Another piece of evidence implicating Lck as a substrate for CD45 comes from co-immunoprecipitation studies, in which Lck and two proteins of 29,000 and 32,000 M_r co-precipitate with CD45 under mild lysis conditions (266). Utilizing these conditions, Fyn is not co-immunoprecipitated with CD45, since no kinase activity was observed in CD45 immunoprecipitates derived from the Lck-deficient J.CaM1.6 cell line (267). While it is not known if the 29 and 32 kDa proteins are different entities, or simply reflect post-translational modifications of a single protein; a cDNA clone has been isolated encoding a molecule of 19 kDa with an electrophoretic mobility of 30 kDa (268). The 30 kDa protein referred to CD45-AP shares no sequence homology with any known proteins. The expression of CD45-AP, a putative transmembrane protein, is restricted to lymphoid tissues and associates specifically with CD45. The role of CD45-AP and pp32 in CD45 and/or TCR function awaits further biochemical and molecular characterization.

Since CD45 is believed to positively regulate the functions of Fyn and Lck by dephosphorylating their C-terminal tyrosines, the PTK that phosphorylates these sites should negatively regulate Fyn and Lck functions, thereby resulting in a loss of TCR signaling function. The Csk PTK was identified as a protein that can phosphorylate c-Src at its C-terminal tyrosine residue (269). Csk, like the Src-family of PTKs contains unique, SH2, SH3 and kinase domains; however, it lacks myristylation and autophosphorylation sites and a negative-regulatory C-terminal tyrosine residue (270). In addition to phosphorylating c-Src, Csk has also been demonstrated to phosphorylate Lck and Fyn <u>in vitro</u> on the C-terminal tyrosine, resulting in a decrease in their <u>in vitro</u> enzymatic activity (271, 272). Additionally, an association between Csk and Fyn in T cell lines has been

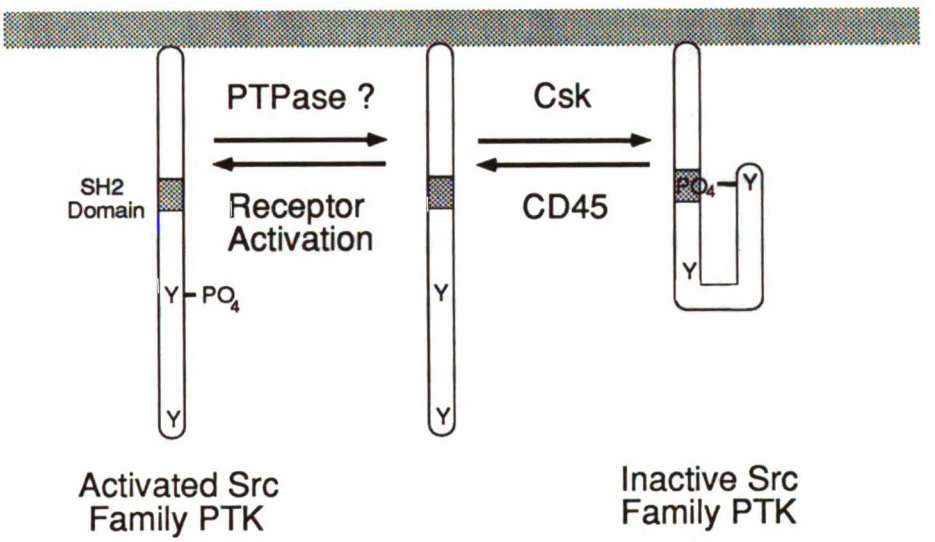
demonstrated (273). A negative regulatory role, for Csk, in TCR signaling is further suggested by the demonstration that three-fold overexpression of Csk, in CD45-positive cells, results in a decrease in TCR-mediated tyrosine phosphoprotein induction and IL-2 production (274). Surprisingly, the overexpression of Csk did not lead to demonstrable hyperphosphorylation of Lck or Fyn on the C-terminal tyrosine; however, the negative regulatory effect of Csk was overcome by overexpressing Fyn in which the C-terminal tyrosine (Y528) was changed to phenylalanine. The lack of Lck or Fyn hyperphosphorylation on the C-terminal tyrosine residue in Csk overexpressing cells may be attributed to experimental limitations in which small changes in phosphorylation status may not be readily detectable, or that the Csk-mediated inhibition of TCR signaling is effected by another molecule(s) which could regulate Lck or Fyn. Similarly, Csk overexpression dramatically decreased the in vitro kinase activity of c-Src and reversed c-Src/v-Crk-mediated cellular transformation without altering the phosphorylation status of the C-terminal tyrosine of c-Src (275). Although, it is not known if the C-terminal tyrosines of Lck or Fyn are physiologic in vivo substrates of Csk, evidence suggests that the TCR signaling defect in CD45-deficient cells (and Csk-overexpressing cells) is likely to be attributable to the hyperphosphorylation of Lck or Fyn on their C-terminal negative regulatory tyrosine residues (Figure 9).

Regulation of CD45 function

Whereas CD45 is essential for the signal transducing function of the TCR, the structural requirements and regulation of CD45 function as well as its impact on TCR function are less well understood and contradictory conclusions have been drawn from different experiments. Site-directed mutagenesis of bacterially expressed or in vitro translated CD45 cytoplasmic domain and in vitro analysis of

Figure 9. A model of Src-like PTK regulation by the CD45 PTPase and the Csk PTK

The dynamic equilibrium between the PTPase CD45 and the PTK Csk in the regulation of Src-family PTK function in lymphocytes. The depicted intramolecular conformational change in the Src-family PTK is not meant to suggest that other structural changes are not involved.


PTPase ?
Csk
SH2
Domain
Receptor
Activation
CD45
Y
PO4
Y
Y
Y
Y
Y
Activated Src
Family PTK
Inactive Src
Family PTK

PTPase activity has been used to begin defining functionally important regions within the CD45 cytoplasmic domain. Point mutation of the conserved cysteine residue involved in the phosphotransfer reaction (184, 185), reveal that only the membrane proximal phosphatase domain of CD45 has in vitro PTPase activity against a variety of artificial substrates (186, 190). Although the distal phosphatase domain does not appear to have catalytic activity, it is required for optimal function of the membrane proximal phosphatase domain, as deletion of the distal domain resulted in a loss of PTPase activity of the proximal domain (186, 190). Contrasting results have also been reported. For example, CD45 treated with limited amounts of endoproteinase Lys-C or trypsin resulted in a 50 kDa cleavage product, which contained all of the distal PTPase domain and the C-terminal one-third of the proximal PTPase domain, exhibited in vitro PTPase activity against artificial PTPase substrates (276). Moreover, when a fibroblast cell line was transfected with a CD45 molecule in which a portion of the proximal PTPase domain including the catalytic cysteine was deleted by site-directed mutagenesis, this molecule was demonstrated to have in vitro PTPase activity (276). The discrepancy between the different mutagenesis studies may reflect the distinct cellular contexts. In the studies where the distal phosphatase domain does not exhibit any PTPase activity, the cytoplasmic domain may not have been folded into the proper conformation by the bacteria or the in vitro translation system. Alternatively, by expressing the cytoplasmic domain without the extracellular and transmembrane domains, the conformation of the cytoplasmic domain may not have been the same as the wild-type molecule. A more appealing explanation for these seemingly contradictory results is that the activity of the distal phosphatase domain is regulated by the proximal phosphatase domain, and that deletion of a portion of the proximal domain unmasks the PTPase activity of the distal phosphatase domain. Thus, it is not clear whether

both phosphatase domains of CD45 are active. Moreover, the in vivo role of the two phosphatase domains of CD45 has not been determined. Thus, if both PTPase domains of CD45 are functional, it will be interesting to see how these domains function in regulating TCR signal transduction.

A function for the extracellular domain of CD45 is suggested by the complex regulated alternative RNA splicing of exons comprising a portion of the N-terminal region of the extracellular domain (221). Co-capping, immunoprecipitation and fluorescence energy-transfer experiments suggest that CD45 can associate with a number of T cell surface molecules, including the TCR (277), the CD4 and CD8 co-receptors (278), CD2 (279) and Thy-1 (277). Moreover, individual isoforms of CD45 have been reported to differentially associate with these T cell surface molecules (280). The physiological relevance of these associations require cautious interpretation due to the high density of CD45 surface expression and the use of mild detergents, coupled with the requirement of protein cross-linking reagents, to observe these associations. Instead of the extracellular domain interacting with molecules on the same cell, it could function to interact with molecules on other cells or bind soluble ligands.

The low molecular weight isoform of CD45, CD45RO, was initially demonstrated to interact with the B cell transmembrane protein, CD22 (237). More recently, a CD22-Ig fusion protein was shown to interact with a number of CD45 isoforms (281). CD22 recognizes a carbohydrate moiety on CD45, and a number of other heavily sialylated proteins; therefore, the likelihood of in vivo CD45-CD22 interactions is not certain. A potential regulatory role for CD22 was suggested by the finding that co-aggregation of the TCR and CD45, utilizing anti-TCR mAbs and the CD22-Ig fusion protein, led to an inhibition of TCR signaling (281). However, in light of the finding that CD45-TCR co-aggregation may result in a non-specific inhibition of TCR signaling, the ability of CD22 to modulate

CD45 function and TCR signaling requires further evaluation. Another potential function of the CD45-CD22 interaction may be to regulate CD22 function, which is thought to play a role in B cell activation (282, 283).

The regulation of CD45 function is not well understood. Since CD45 is a transmembrane PTPase, the regulation by ligand binding is of prime consideration; however, CD45 may also be regulated by phosphorylation. Indeed, CD45 phosphatase activity has been demonstrated to be negatively modulated by a loss of in vivo serine phosphorylation on undefined sites. The reduction in serine phosphorylation appears to be a late and indirect effect of increases in $[Ca^{2+}]_i$ levels (284). Whether changes in serine phosphorylation of CD45 influence its role in regulating TCR signaling function are not known. CD45 has also been demonstrated to become tyrosine phosphorylated following TCR stimulation (196). The effect of tyrosine phosphorylation on CD45 PTPase activity and TCR signaling have yet to be determined; however, as with the tyrosine phosphorylation of the intracellular PTPases PTP 1D, it may lead to an increase in phosphatase activity (166).

Little is known regarding how the function of CD45 or any other transmembrane PTPase is regulated owing to the absence of identified ligands. The course of experimentation described herein focuses on the in vivo regulation of CD45 and its impact on TCR signal transduction. The regulation of CD45 may serve as a paradigm for the regulation of other transmembrane PTPases.

CHAPTER II. LIGAND-MEDIATED NEGATIVE REGULATION OF A CHIMERIC TRANSMEMBRANE RECEPTOR TYROSINE PHOSPHATASE

Preface

The function and regulation of transmembrane receptor-like PTPases has been difficult to study due to the absence of identified ligands. CD45, a transmembrane PTPase, consists of a family of closely related molecules, that differ as a result of alternative splicing of exons comprising a portion of the extracellular domain. The complex manner in which CD45 splicing is regulated suggests that the extracellular domain may be critical in CD45 function. Furthermore, the potential importance of the extracellular domain is underscored by the differential association of the various CD45 isoforms with T cell surface molecules, including the TCR and the CD4 and CD8 co-receptors.

To address the importance of the extracellullar domain of CD45 in regulating TCR signal transduction, a strategy was employed where the extracellular and transmembrane domains of CD45 were replaced with those of a heterologous receptor. This approach was based on the notion that many proteins are modular in nature, and that modules from distinct, functionally unrelated molecules can be exchanged, imparting the functional characteristics of the various domains to the chimeric molecule. This idea is especially feasible with transmembrane signaling molecules and has been utilized with molecules of hematopoietic and non-hematopoietic origin (25, 285). Based on this reasoning, a chimeric protein was constructed in which the extracellular and transmembrane domains of CD45 were replaced with those of the epidermal growth factor receptor (EGFR) to determine whether the extracellular domain of CD45 influenced its function. The chimeric molecule was examined for its ability to

restore TCR signal transduction function in a CD45-deficient T cell line. The EGFR extracellular and transmembrane domains were utilized since the EGFR has been well characterized at the molecular and biophysical level (285). Moreover, by using the EGFR extracellular and transmembrane domains, the effects of ligands on the function of the chimeric transmembrane PTPase and on TCR signal transduction could be assessed.

Characterization of the EGFR/CD45 chimera in CD45-deficient cells

A chimeric molecule consisting of the EGFR extracellular and transmembrane domains linked to the cytoplasmic domain of CD45 (Figure 10) was stably transfected into the human leukemic cell lines Jurkat ($CD45^+$), HPB.ALL ($CD45^+$), and H45, an HPB.ALL variant that is deficient in CD45 mRNA transcripts and protein (256). Cell surface expression of the TCR, CD45 and the EGFR/CD45 chimera were quantitated by flow cytometry. Jurkat and HPB.ALL express high levels of CD45, whereas H45 has no detectable cell surface expression of CD45 using the GAP 8.3 mAb, which detects all CD45 isoforms (Figure 11 A, C, and E). Neither HPB.ALL, H45 nor Jurkat expresses endogenous EGFR (Figure 11 A, B, and E). However, following transfection of the EGFR/CD45 chimera, these cells express the extracellular domain of the EGFR (Figure 11 C, F, and data not shown). Numerous stable transfectants of HPB.ALL, H45 and Jurkat were obtained which displayed similar phenotypes; thus, results from three representative clones, HXL1, H45XL2, and JXL28, respectively, will be presented. H45 was also transfected with the human CD45RO isoform cDNA, resulting in the clone H45L13 (Figure 11 D). Comparison of the level of wild-type CD45, the EGFR/CD45 chimera, and CD45RO immunofluorescence indicates that the EGFR/CD45 chimera and CD45RO are expressed at comparable levels, whereas wild-type CD45 on HPB.ALL and Jurkat is expressed at higher levels (Figure 11 A, C and D). All of the cells also express high levels of TCR (Figure 11 A-F).

To characterize the structure of the EGFR/CD45 chimeric protein, cells were surface labeled, followed by immunoprecipitation with antibodies to the extracellular domains of CD45 and the EGFR (Figure 12 A). Wild-type HPB.ALL expresses three prominent CD45 isoforms which migrate

Figure 10. Schematic of the EGFR/CD45 Chimera

Schematic representation of the wild-type EGFR and CD45 molecules with the EGFR/CD45 chimera. The numbers correspond to amino acids in the mature protein and demarcate the extracellular, transmembrane, and cytoplasmic domains.

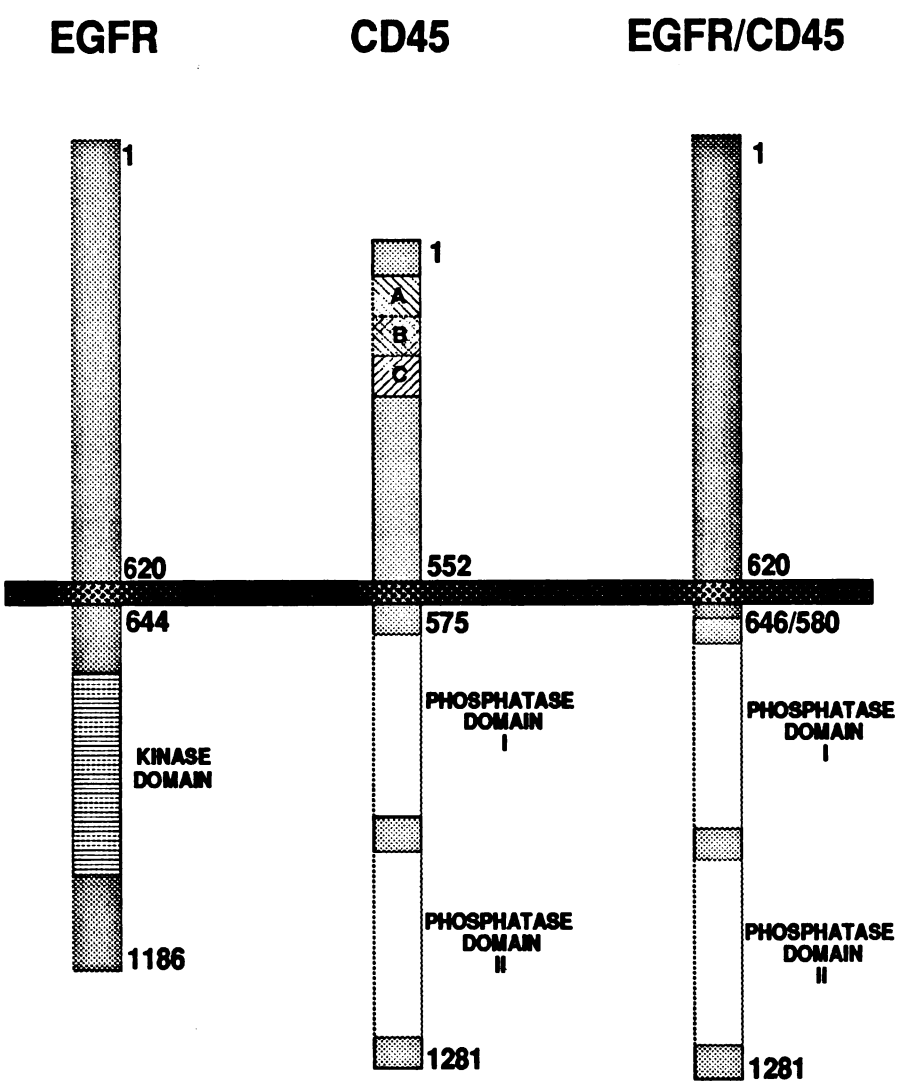
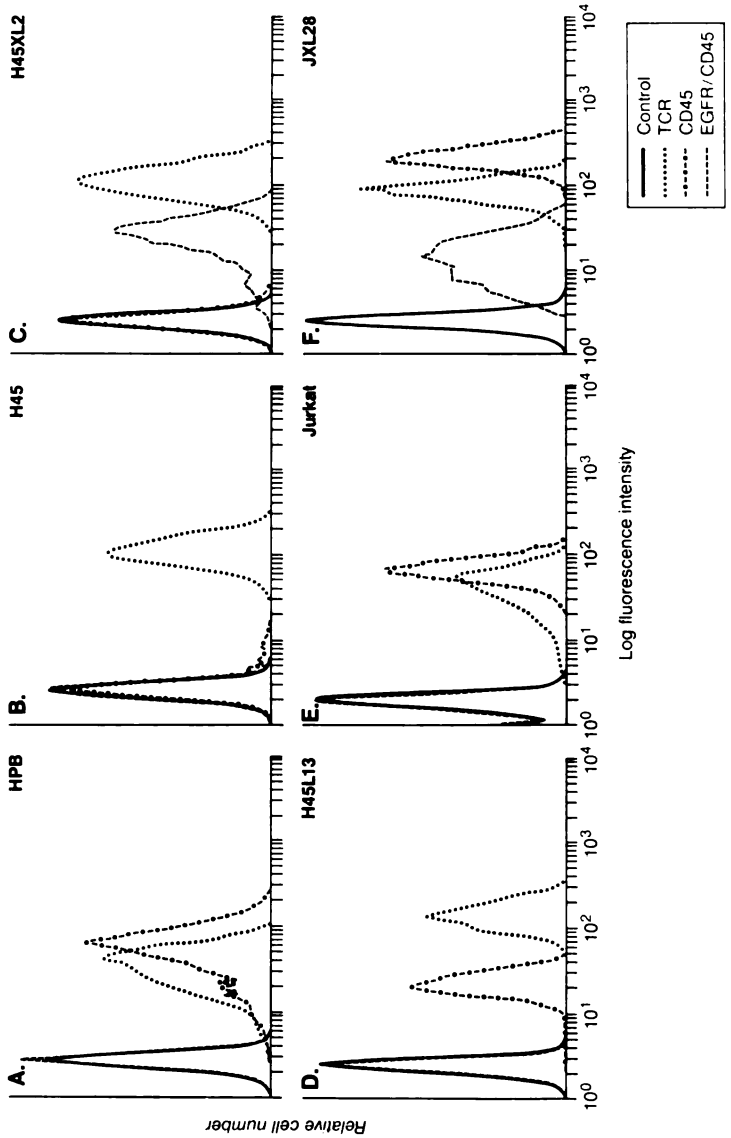

EGFR
CD45
EGFR/CD45
1
620
644
KINASE DOMAIN
1186
1
A
B
C
552
575
PHOSPHATASE DOMAIN I
PHOSPHATASE DOMAIN II
1281
1
620
646/580
PHOSPHATASE DOMAIN I
PHOSPHATASE DOMAIN II
1281

Figure 11. Cell Surface Expression of the TCR, CD45 and the EGFR/CD45 Chimera on HPB.ALL, Jurkat and Their Derivatives as Determined by Flow Cytometry

Cells were stained with a control mouse IgG2b mAb (solid line); Leu 4 (anti-CD3ε) for the TCR (dotted line); GAP 8.3 (anti-CD45, alternating dashes and dots), and LA22 (anti-EGFR, dashed line), followed by a fluoresceine-conjugated goat anti-mouse secondary mAb.


A.
HPB
B.
H45
C.
H45XL2
D.
H45L13
E.
Jurkat
F.
JXL28
Relative cell number
Log fluorescence intensity
Control
TCR
CD45
EGFR/CD45

at ~220, ~205 and ~180 kDa, whereas H45 does not express any detectable surface CD45 (Figure 12 A; lanes 2 and 5). H45L13, which has been transfected with the 180 kDa CD45RO isoform cDNA only expresses a 180 kDa cell surface protein which can be immunoprecipitated with an anti-CD45 mAb (Figure 12 A, lane 8). Neither HPB.ALL, H45, nor H45L13 express proteins recognized by an anti-EGFR antibody (Figure 12 A; lanes 3, 6, and 9); however, H45XL2, which has been transfected with the EGFR/CD45 chimera, expresses a ~190 kDa molecule recognized by the anti-EGFR mAb, 108 (Figure 12 A, lane 12). H45XL2 does not express any detectable CD45 using the mAb that detects all extracellular domain isoforms (Figure 12 A, lane 11).

The EGFR/CD45 chimera was further characterized by immunoblot analysis. Total cell lysates from HPB.ALL, H45, H45L13, and H45XL2 were subjected to immunoprecipitation with anti-CD45 or anti-EGFR antibodies which recognize epitopes on the extracellular domain of the respective receptors, followed by immunoblotting with an anti-CD45 cytoplasmic domain antiserum (Figure 12 B), or an anti-EGFR extracellular domain antiserum (Figure 12 C). As expected, CD45 immunoprecipitates from the CD45-positive cells, HPB.ALL and H45L13, are recognized by an antibody specific for the cytoplasmic domain of CD45, but no CD45 protein is detectable from the CD45-deficient H45 and H45XL2 cells (Figure 12 B, lanes 2, 5, 8, and 11). As in Figure 12 A, HPB.ALL cells contain multiple CD45 isoforms, whereas H45L13 expresses only the transfected CD45RO species. The faint lower molecular weight bands in Figure 12 B, lane 2, may represent degradation products or partially processed forms of CD45. Immunoprecipitation of H45XL2 lysates with the anti-EGFR mAb results in a single ~190 kDa protein recognized by both anti-CD45 cytoplasmic domain and anti-EGFR antibodies (Figures 12 B and C, lane 12). This suggests that the

domains comprising the EGFR/CD45 chimera are structurally similar to those of the wild-type receptors from which they are derived.

The phosphatase activity of the EGFR/CD45 chimera was characterized using o-phospho-L-tyrosine as a substrate and plasma membranes from the cells described above as the source of the PTPase. There is substantial PTPase activity associated with HPB.ALL membranes, and 80% of that activity is contributed by CD45, since very little phosphatase activity is associated with membranes from the CD45-deficient, H45 cells (Figure 13). Similar findings have been reported when comparing membrane PTPase activity from different sets of $CD45^+$ and $CD45^-$ cell lines (207, 257). Membrane associated PTPase activity was largely restored to H45 cells by the introduction of either the CD45RO isoform or the EGFR/CD45 chimera although the expression of CD45RO and the EGFR/CD45 chimera are expressed at a lower level compared wth that of CD45 on HPB.ALL cells (Figure 13). The membranes containing the EGFR/CD45 chimera and CD45RO may not have as much PTPase activity as HPB.ALL membranes because CD45 expression on HPB.ALL is higher when compared with the cells expressing the CD45RO and EGFR/CD45 chimera (Figure 11 A, C and D). Moreover, since the cell surface expression of the EGFR/CD45 chimera and CD45RO are comparable (Figure 11 C, D and 12 B), the in vitro PTPase activity of the EGFR/CD45 chimera and CD45RO are similar. Previous studies have demonstrated that of four different CD45 isoforms tested, all had equivalent in vitro PTPase activity (260). This supports the notion that the PTPase activity of CD45 may not be directly affected by the structure of the extracellular domain.

Figure 12. A. Structural Characterization of the EGFR/CD45 Chimera

Cell Surface Labeling of CD45 and the EGFR/CD45 Chimera.

HPB.ALL ($CD45^+$); H45 ($CD45^-$); H45L13 ($CD45RO^+$); and H45XL2 ($CD45^-$, $EGFR/CD45^+$) were surface biotinylated, lysed in 1% NP-40, and immunoprecipitated with control mouse IgG (lanes 1, 4, 7, and 10), anti-CD45 mAb (lanes 2, 5, 8, and 11), or anti-EGFR mAb (lanes 3, 6, 9, 12). Immune complexes were separated by SDS-PAGE, transferred to nitrocellulose and probed with ^{125}I-streptavidin, followed by autoradiography. 2×10^7 cells were used per immunoprecipitate.

Figure 12 B. Immunoblotting with an anti-CD45 cytoplasmic domain antibody

Cells, as in (A), were lysed and subject to immunoprecipitation with control mouse IgG (lanes 1, 4, 7, and 10), anti-CD45 mAb (lanes 2, 5, 8, and 11), or anti-EGFR mAb (lanes 3, 6, 9, 12). Immune complexes were separated by SDS-PAGE and western blotted with an anti-CD45 cytoplasmic domain antiserum

Figure 12 C. Immunoblotting with an anti-EGFR extracellular domain antibody

Cells, as in (A), were lysed and subject to immunoprecipitation with control mouse IgG (lanes 1, 4, 7, and 10), anti-CD45 mAb (lanes 2, 5, 8, and 11), or anti-EGFR mAb (lanes 3, 6, 9, 12). Immune complexes were separated by SDS-PAGE and western blotted with an anti-EGFR extracellular domain antiserum.

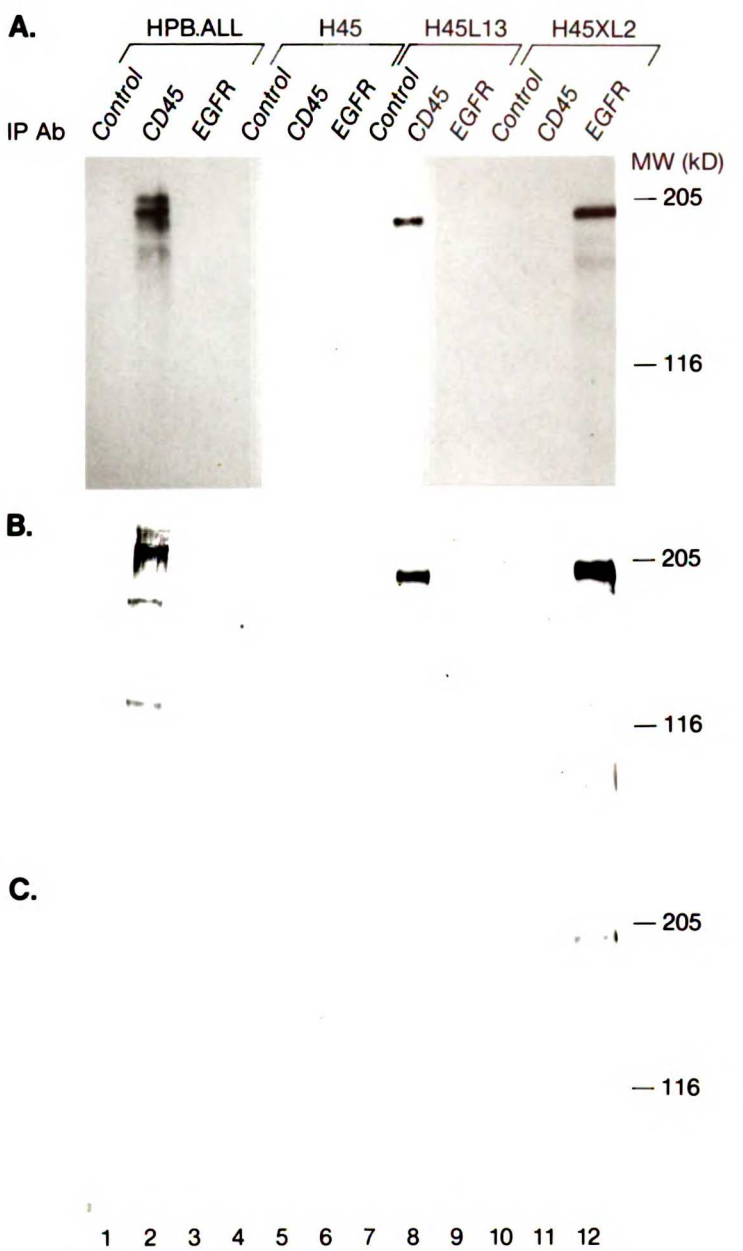
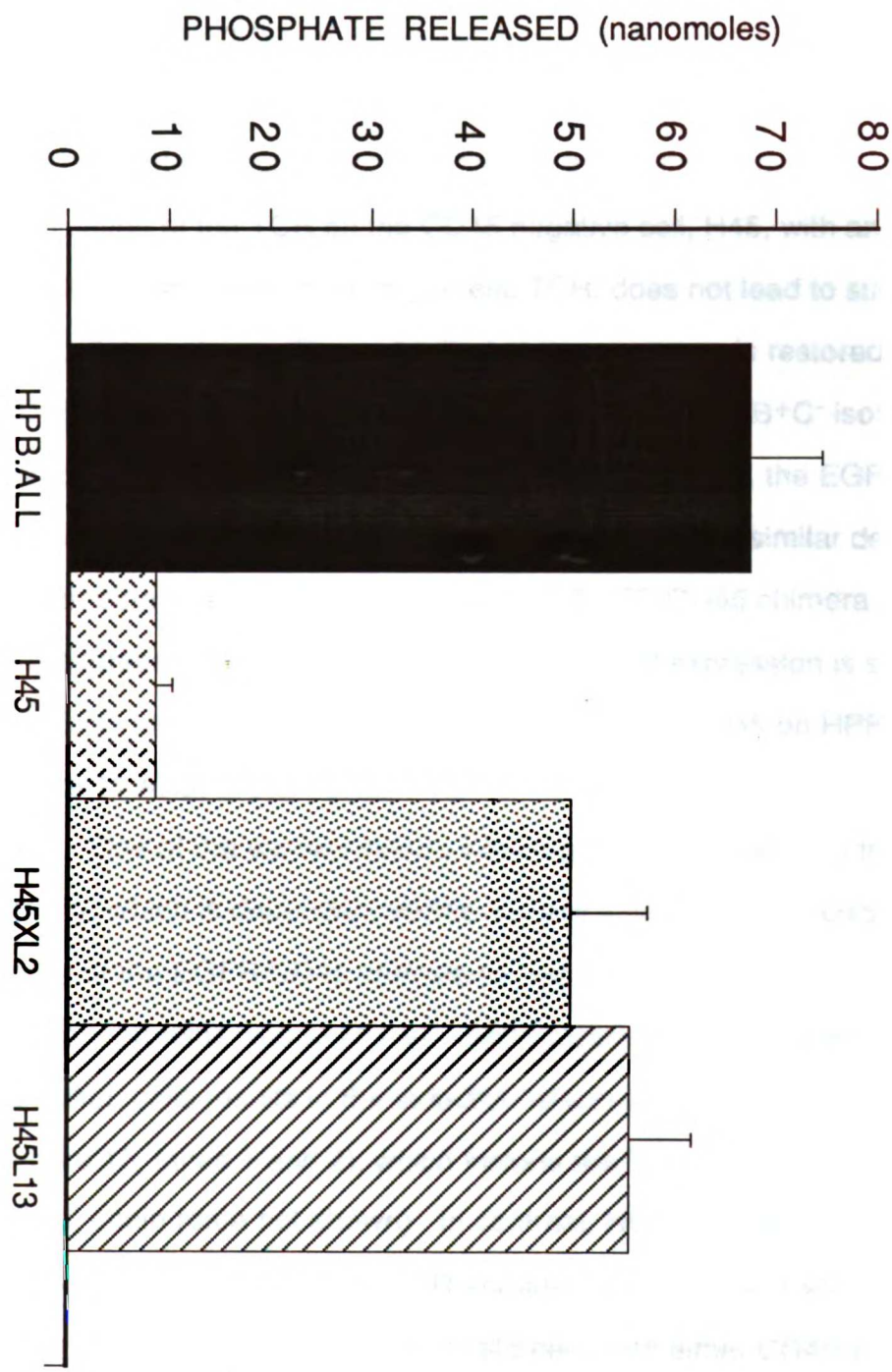

A.
HPB.ALL
H45
H45L13
H45XL2
IP Ab
Control
CD45
EGFR
Control
CD45
EGFR
Control
CD45
EGFR
Control
CD45
EGFR
MW (kD)
205
116
B.
205
116
C.
205
116
1 2 3 4 5 6 7 8 9 10 11 12

Figure 13. CD45 and the EGFR/CD45 Chimera Have Comparable Phosphatase Activity

Membranes were isolated from HPB.ALL ($CD45^+$), H45 ($CD45^-$), H45L13 ($CD45RO^+$), and H45XL2 ($CD45^-$, $EGFR/CD45^+$). 15 µg of membranes were incubated with o-phospho-L-tyrosine for 15 minutes, and the release of free phosphate was measured by a colorimetric assay.


PHOSPHATE RELEASED (nanomoles)
0
10
20
30
40
50
60
70
80
HPB.ALL
H45
H45XL2
H45L13

Restoration of T cell receptor signal transduction by the EGFR/CD45 chimera

The TCR on CD45-deficient cells is uncoupled from the intracellular signaling machinery since TCR engagement does not lead to tyrosine phosphoproteininduction or increases in intracellular free calcium (256, 257). Stimulation of the TCR on the CD45 negative cell, H45, with an antibody recognizing the CD3 component of the oligomeric TCR, does not lead to substantial increases in intracellular calcium (Figure 14 B), but this response is restored by the human CD45RO isoform (Figure 14 C), the human CD45RA^{+}B^{+}C^{-} isoform (264), as well as the 220 kDa isoform of murine CD45 (256). Strikingly, the EGFR/CD45 chimera, is also able to reconstitute TCR-mediated signaling to a similar degree (Figure 14 D). Restoration of signal transduction by the EGFR/CD45 chimera is not due to overexpression of the chimera, since its level of expression is similar to that of CD45RO in H45L13, and is less than the level of CD45 on HPB.ALL (compare Figure 11 A, C, D and 12 B).

One of the earliest events following TCR stimulation is the induction of PTK activity, which is dependent on the surface expression of CD45. Therefore, the ability of the EGFR/CD45 chimera to restore TCR-induction of tyrosine phosphoproteins was examined. Stimulation of the CD3 subunit of the TCR on HPB.ALL cells results in the induction of a number of tyrosine phosphoproteins (Figure 15, lanes 1 and 2), which include the TCR-associated PTK, ZAP-70, and the TCR ζ chain (data not shown). In contrast, levels of phosphotyrosine containing proteins do not change with TCR engagement on CD45 deficient cells (Figure 15, lanes 3 and 4). Reconstitution of H45 cells with either CD45RO or the EGFR/CD45 chimera results in comparable restoration of TCR induced tyrosine phosphoproteins (Figure 15, lanes 5-8). Moreover, the pattern of phosphoprotein induction is similar to that seen in wild-type cells. This suggests that the in vivo substrate specificity and

PTPase activity of the EGFR/CD45 chimera is similar to that of wild-type CD45, indicating that the extracellular and transmembrane regions of CD45 are not essential for initiation of signal transduction through the TCR. However, these domains may play a role in regulating CD45 function, potentially by interacting with extracellular ligands.

Regulation of TCR signal transduction by ligands to the EGFR/CD45 chimera

The mechanism by which the transmembrane PTPases are regulated is not understood. It is not known whether these PTPases are active in vivo in the basal state, or whether ligands alter PTPase activity or function. Since the EGFR/CD45 chimera is able to functionally substitute for wild-type CD45, the effect of EGFR ligands was assessed on TCR signal transduction on the CD45-negative cells which express the EGFR/CD45 chimera. As expected, EGF has no effect on TCR-mediated calcium mobilization in wild-type HPB.ALL cells (Figure 16 A) as these cells do not express the EGFR or the chimera. However, in CD45-deficient cells reconstituted with the EGFR/CD45 chimera, the addition of EGF following TCR stimulation led to an accelerated decrease in intracellular calcium to basal levels (Figure 16 B). The EGF-induced decrease in intracellular calcium was dose dependent, with maximal effectiveness at 100 ng/ml EGF (data not shown). In addition, if EGF is added prior to or simultaneously with TCR engagement, there is only a delayed and small increase in calcium levels (Figure 16 C and data not shown). Transforming growth factor α (TGFα), another ligand for the EGFR (286), has similar effects as EGF (Figure 16 D and E).

The effect of EGF on the induction of tyrosine phosphoproteins following TCR stimulation was also examined. The addition of EGF to unstimulated EGFR/CD45 chimera expressing cells has no effect on the tyrosine phosphoprotein pattern (Figure 17, lane 4). Stimulation of the TCR on cells that express the EGFR/CD45

Figure 14. Expression of the EGFR/CD45 Chimera in CD45 Negative Cells Restores TCR-Mediated Mobilization of Intracellular Free Calcium HPB.ALL ($CD45^+$) (A); H45 ($CD45^-$) (B); H45L13 ($CD45RO^+$) (C); H45XL2 ($CD45^-$, EGFR/$CD45^+$) (D) were treated, at the indicated time, with 1 µg/ml anti-TCR antibody (Leu4). Intracellular free calcium levels were measured using the calcium sensitive dye Indo-1.

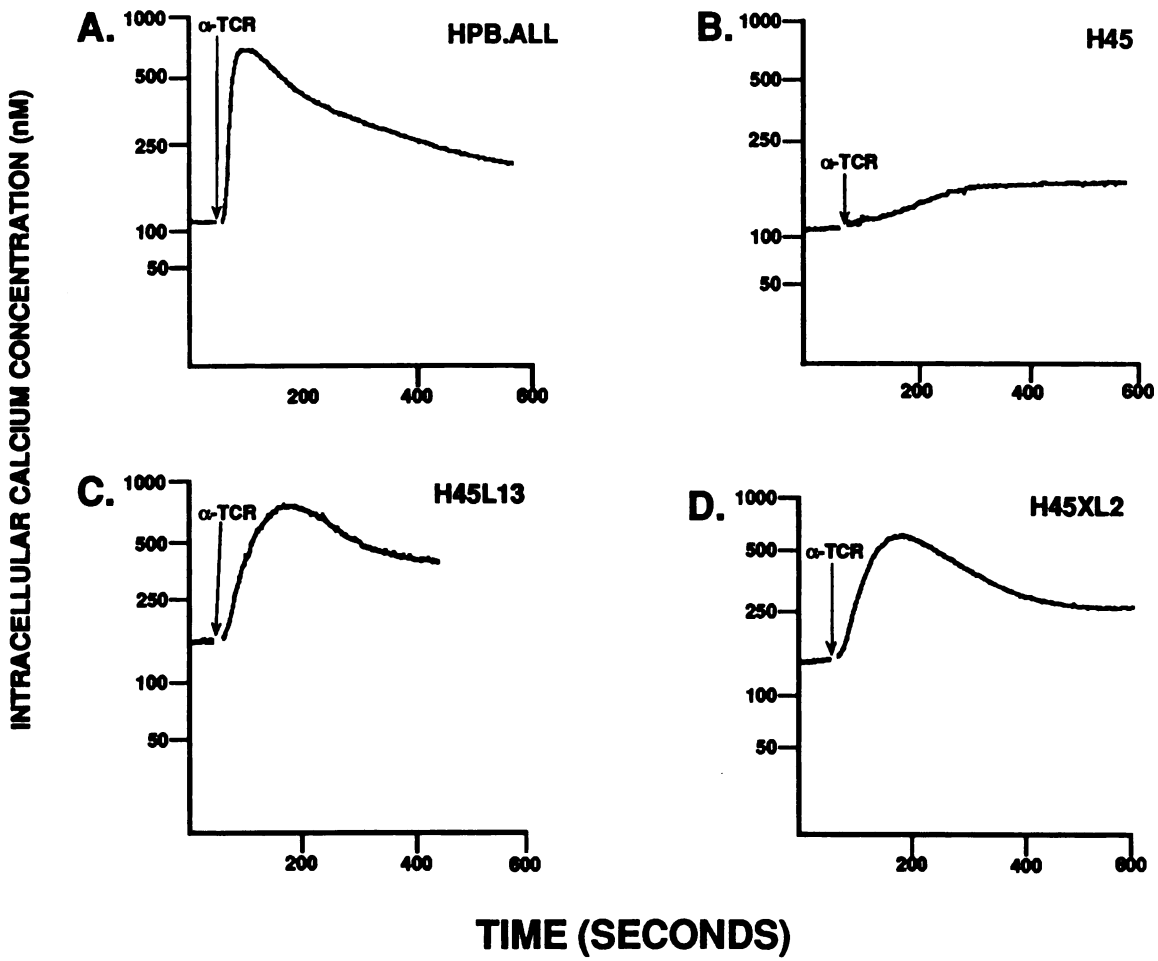
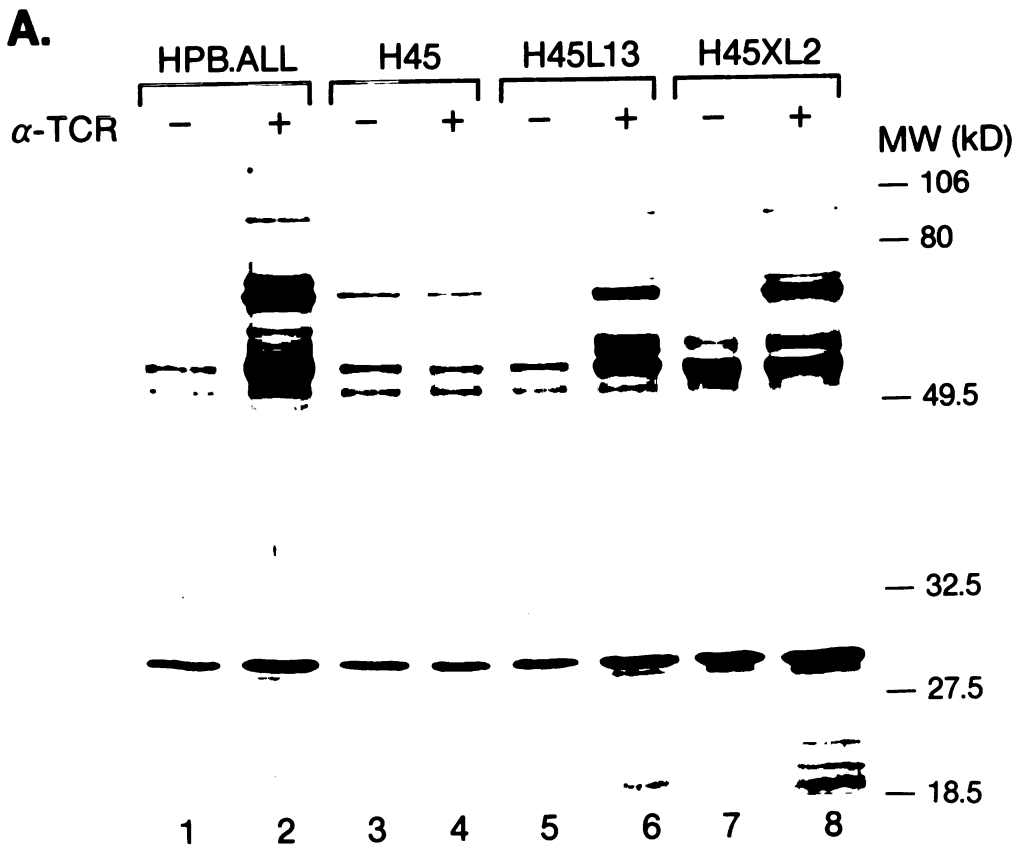

A.
HPB.ALL
α-TCR
B.
H45
α-TCR
C.
H45L13
α-TCR
D.
H45XL2
α-TCR
1000
500
250
100
50
200
400
600
INTRACELLULAR CALCIUM CONCENTRATION (nM)
TIME (SECONDS)

Figure 15. T Cell Receptor-Mediated Induction of Tyrosine-Phosphorylated Proteins is Restored in CD45 Negative Cells Upon Expression of the EGFR/CD45 Chimera

HPB.ALL ($CD45^+$), H45 ($CD45^-$), H45L13 ($CD45RO^+$), and H45XL2 ($CD45^-$; $EGFR/CD45^+$) were treated with medium (-) or with 1 μg/ml anti-TCR antibody (+) and induction of phosphotyrosine containing proteins was assessed by immunoprecipitation followed by immunoblotting with an anti-phosphotyrosine antibody.


A.
HPB.ALL
H45
H45L13
H45XL2
α-TCR
– + – + – + – +
MW (kD)
— 106
— 80
— 49.5
— 32.5
— 27.5
— 18.5
1 2 3 4 5 6 7 8

chimera leads to tyrosine phosphorylation of a number of proteins which include pp110, pp100, pp70-72, pp36 and pp21 (Figure 17, lanes 5-6). However, the addition of EGF concomitantly with anti-TCR antibody substantially inhibits tyrosine phosphorylation of cellular substrates (Figure 17; compare lanes 3, 5 and 7). Furthermore, when EGF is added subsequent to TCR stimulation, the induced tyrosine phosphoproteins are rapidly, though not completely dephosphorylated (Figure 17; compare lanes 3, 5, 6 and 8). This suggests two possible mechanisms: 1) EGF may activate the chimera, so that it can directly or indirectly dephosphorylate the tyrosine phosphoproteins. 2) Alternatively, EGF inactivates the chimera, resulting in a loss of TCR induced PTK activity, thus shifting the equilibrium between PTK and PTPase activity. This would then lead to dephosphorylation of substrates by other cellular PTPases. Regardless of the mechanism, these results demonstrate that TCR-mediated signal transduction can be inhibited or reversed by the addition of EGF, in cells expressing the EGFR/CD45 chimera.

EGFR ligands functionally inhibit EGFR/CD45 chimera activity

To address the mechanism of the effect of EGF on EGFR/CD45 chimera function and its inhibitory effect on TCR-mediated signal transduction, the chimera was expressed in the presence of wild-type CD45. If EGF functionally activates the EGFR/CD45 chimera by either increasing its PTPase activity or allowing the recognition of new substrates, then the EGF-mediated inhibition of TCR signal transduction would be predicted to function dominantly in the context of wild-type CD45. Alternatively, the addition of EGF may functionally inactivate the EGFR/CD45 chimera, so that key proteins involved in signal transduction cannot be dephosphorylated due to a reduction, inhibition or sequestration of PTPase activity. In this case, the engagement of the EGFR/CD45 chimera by EGF should have no effect on signal transduction since the loss of chimera

Figure 16. Expression of the EGFR/CD45 Chimera in CD45 Negative Cells Enables TCR Signaling to be Modulated by EGFR Ligands

HPB.ALL ($CD45^+$) (A); H45XL2 ($CD45^-$, EGFR/$CD45^+$) (B, C, D, E) were treated, at the indicated time, with either 1 μg/ml anti-TCR antibody (Leu4), 100 ng/ml EGF (Boehringer Mannheim) or 100 ng/ml TGFα (Boehringer Mannheim). Intracellular free calcium levels were measured using the calcium sensitive dye Indo-1.

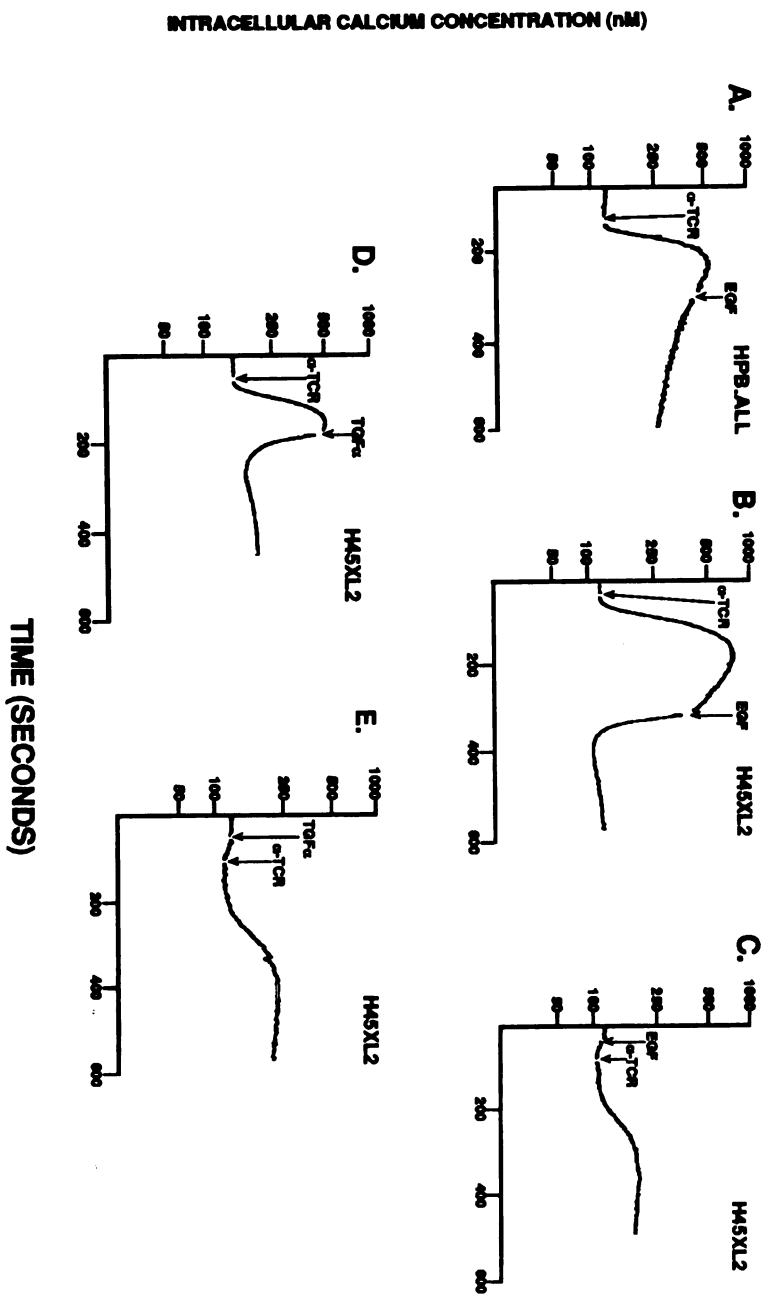
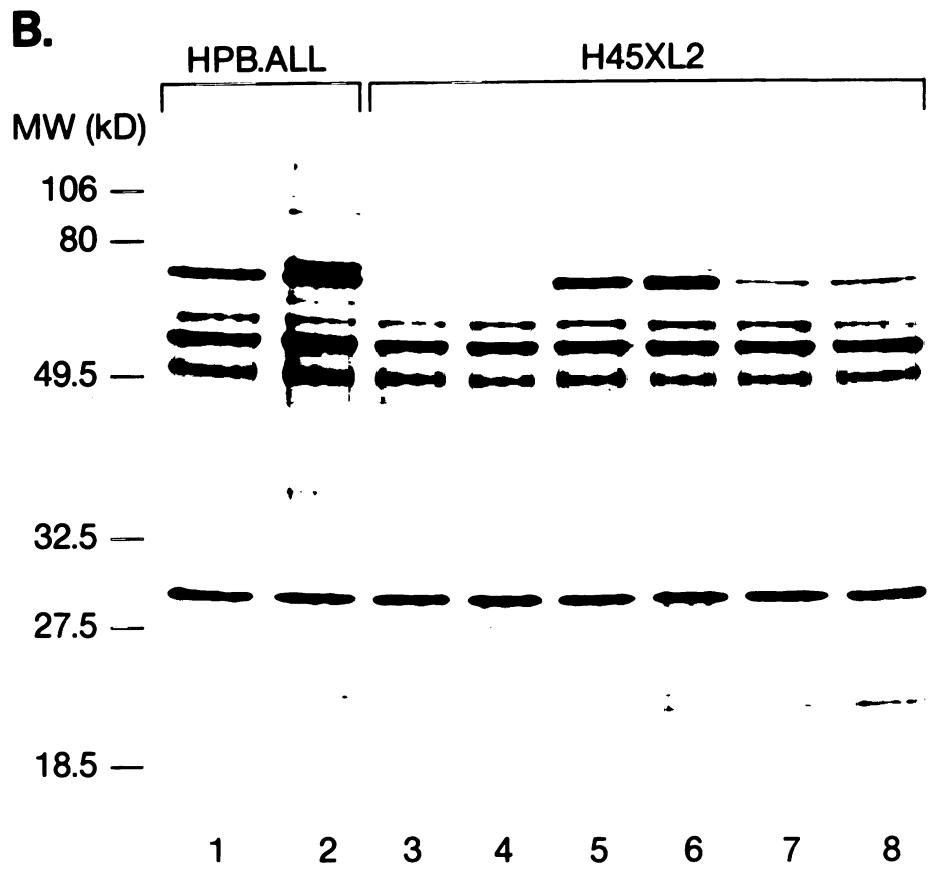

INTRACELLULAR CALCIUM CONCENTRATION (nM)
A.
B.
C.
D.
E.
HPB.ALL
H45XL2
H45XL2
H45XL2
H45XL2
α-TCR
EGF
TGFα
TIME (SECONDS)

Figure 17. T Cell Receptor-Mediated Induction of Tyrosine-Phosphorylated Proteins can be Modulated by EGFR Ligands

Tyrosine phosphoproteins were immunoprecipitated from HPB.ALL (lanes 1,2) or H45XL2 (lanes 3-8) stimulated for 2 minutes with medium (lanes 1,3); 2 minutes with anti-TCR mAb (lanes 2,5); 3 minutes with anti-TCR mAb (lane 6); 1 minute with EGF (lane 4); 2 minutes with anti-TCR mAb and EGF simultaneously (lane 7) or 2 minutes with anti-TCR mAb, followed by 1 minute with EGF (lane 8), followed by immunoblotting with an anti-phosphotyrosine mAb.


B.
HPB.ALL
H45XL2
MW (kD)
106
80
49.5
32.5
27.5
18.5
1
2
3
4
5
6
7
8

activity would be compensated for by wild-type CD45 which is not affected by the addition of EGF. Introduction of the EGFR/CD45 chimera into HPB.ALL, resulted in a clone designated HXL1. HXL1, which expresses comparable but low levels of the EGFR/CD45 chimera and CD45 (data not shown) responds normally to TCR engagement. However, unlike H45XL2, the addition of EGF, either prior to or subsequent to TCR stimulation, has no effect on intracellular calcium mobilization (Figure 18 A-D). Since HXL1 expresses low levels of both the EGFR/CD45 chimera and CD45, the chimera was also introduced into the Jurkat cell line, which expresses high levels of CD45 (Figure 11 E), and has similar signaling properties as HPB.ALL (257). A resultant clone, JXL28, expresses the EGFR/CD45 chimera at levels comparable to those of H45XL2, in which the inhibitory effects of EGF were observed (Figure 11 C and F). Stimulation of the TCR on JXL28 results in the characteristic increase in intracellular free calcium (Figure 18 E). However, this response is unaffected by the addition of EGF (Figure 18 F and G). Thus, the presence of wild-type CD45, in cells co-expressing the EGFR/CD45 chimera, is able to functionally compensate for the negative effect of EGF on chimera function. In addition, the EGF-mediated inhibition of TCR signaling is not due to internalization of the EGFR/CD45 chimera as the amount of surface bound receptor did not change following EGF treatment (data not shown). These results strongly suggest that EGF functionally inhibits the EGFR/CD45 chimera, by either directly inactivating the PTPase, changing its substrate specificity, or sequestering the PTPase activity away from critical substrates involved in TCR-mediated signal transduction. In addition, these results also indicate that CD45 function is continuously required for TCR signal transduction.

Figure 18. The EGFR/CD45 Chimera is Unable to Modulate TCR-Mediated Calcium Mobilization in Cells that Express Wild-Type CD45

HPB.ALL ($CD45^+$) (A); Jurkat ($CD45^+$)(E); HXL1 ($CD45^+$, EGFR/$CD45^+$) (B-D); and JXL28 ($CD45^+$, EGFR/$CD45^+$) (F-H) were loaded with the calcium sensitive dye Indo-1, and treated at the indicated times with anti-TCR mAb (1 μg/ml), or EGF (100 ng/ml).

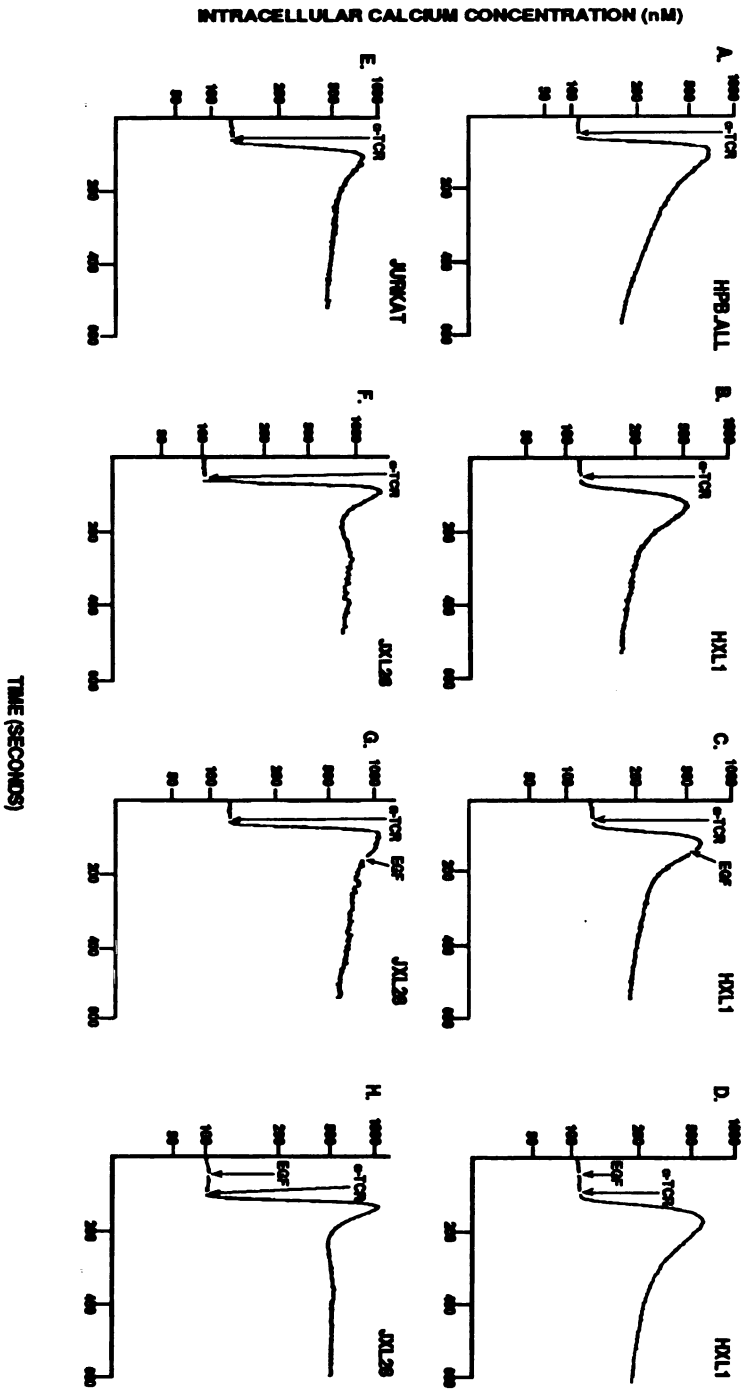
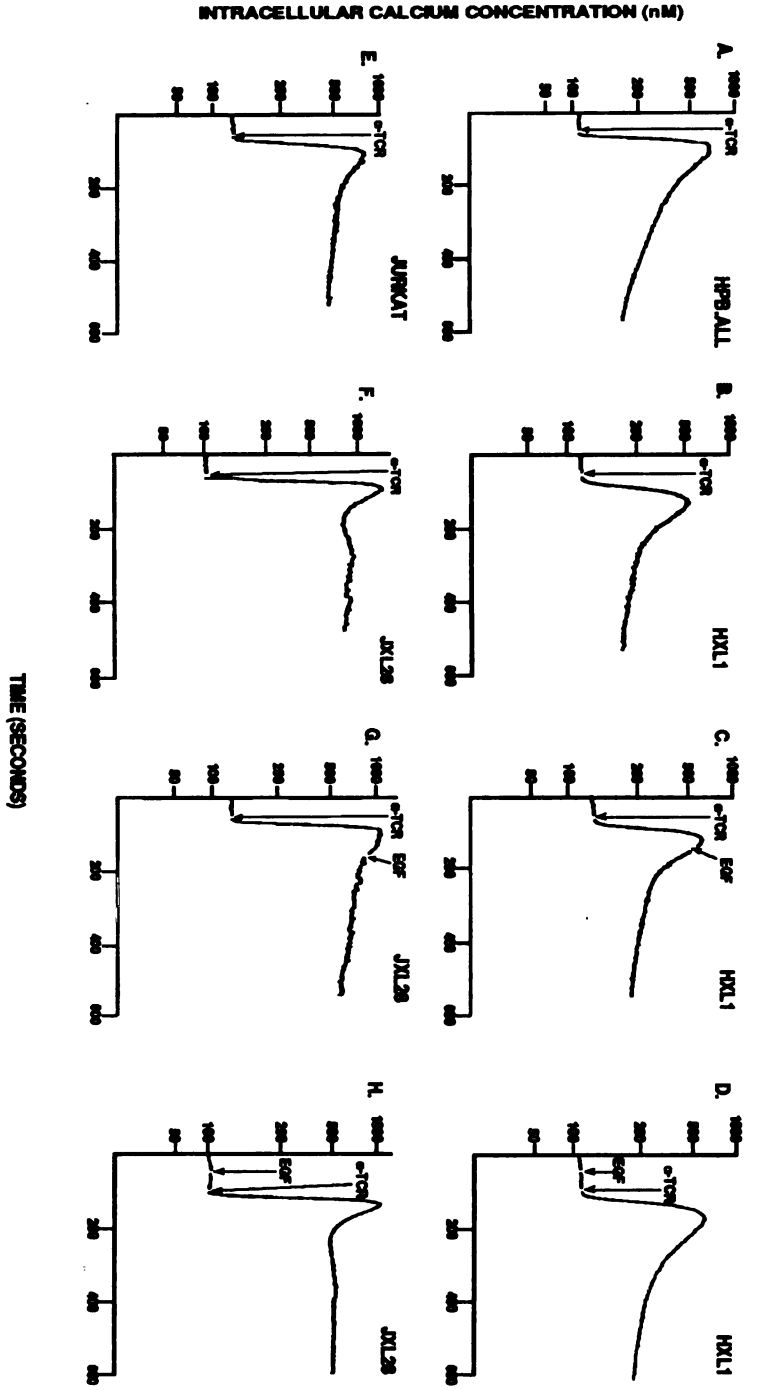

INTRACELLULAR CALCIUM CONCENTRATION (nM)
TIME (SECONDS)
A.
HPB-ALL
B.
HXL1
C.
HXL1
D.
HXL1
E.
JURKAT
F.
JXL26
G.
JXL26
H.
JXL26
e-TCR
EGF

Figure 18. The EGFR/CD45 Chimera is Unable to Modulate TCR-Mediated Calcium Mobilization in Cells that Express Wild-Type CD45

HPB.ALL ($CD45^+$) (A); Jurkat ($CD45^+$)(E); HXL1 ($CD45^+$, EGFR/$CD45^+$) (B-D); and JXL28 ($CD45^+$, EGFR/$CD45^+$) (F-H) were loaded with the calcium sensitive dye Indo-1, and treated at the indicated times with anti-TCR mAb (1 μg/ml), or EGF (100 ng/ml).


INTRACELLULAR CALCIUM CONCENTRATION (nM)
TIME (SECONDS)
A.
HPBALL
B.
HXL1
C.
HXL1
D.
HXL1
E.
JURKAT
F.
JXL26
G.
JXL26
H.
JXL26
e-TCR
EGF

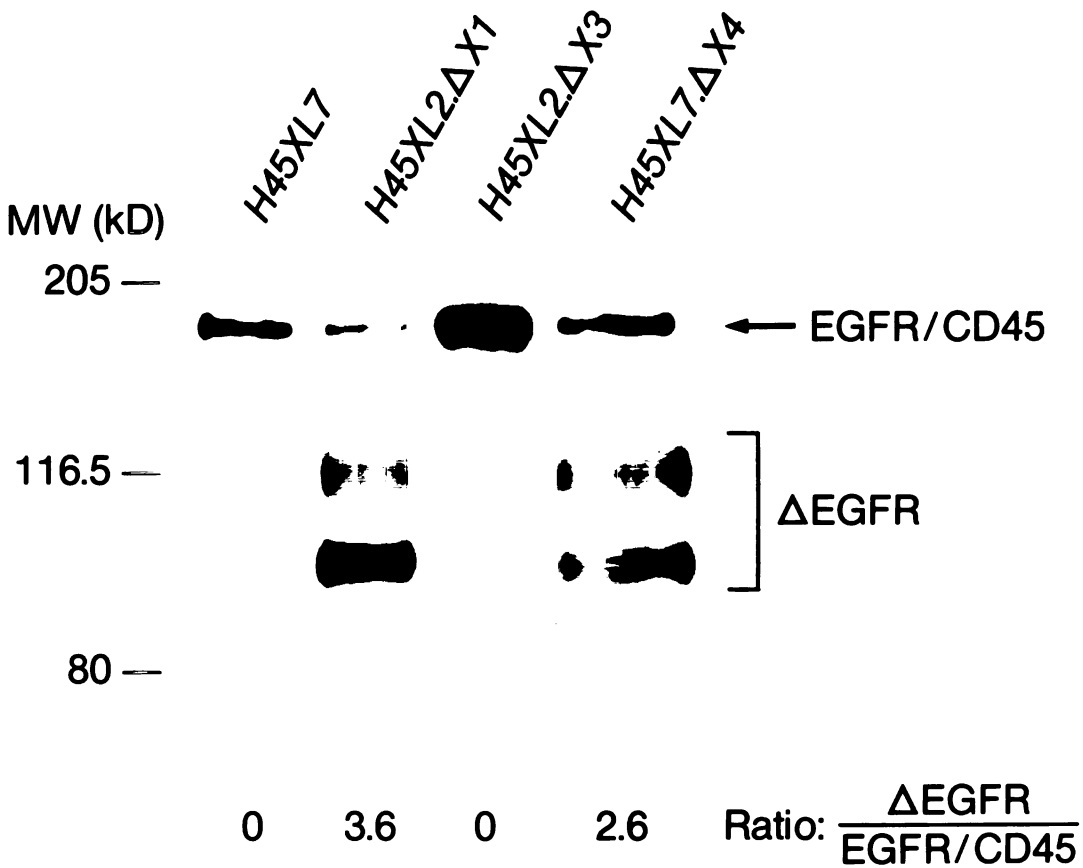
Negative regulation of EGFR/CD45 chimera function requires receptor dimerization

It is now well established that EGF binding to the EGFR causes receptor dimerization and activation of the EGFR tyrosine kinase (285). Moreover, EGF-mediated EGFR dimerization is essential for receptor activation since an EGFR in which the cytoplasmic domain has been deleted can inhibit receptor function by forming heterodimers with wild-type EGFR (287). Therefore, to determine whether dimerization of the EGFR/CD45 chimera is necessary to inhibit TCR signal transduction a truncated EGFR (ΔEGFR) was introduced into CD45-deficient cells that express the EGFR/CD45 chimera. The ΔEGFR consists of the EGFR extracellular and transmembrane domains plus four cytoplasmic amino acids. Introduction of the ΔEGFR into EGFR/CD45 chimera containing cells resulted in a number of clones with varying levels of surface expression of the ΔEGFR (Figure 19). Expression of the ΔEGFR results in two different cell surface expressed products, with the lower product migrating at ~96 kDa and the upper species at ~116 kDa. The presence of two different sized ΔEGFRs is most likely due to differential glycosylation, and not glycophosphatidyl-inositol linkage since both species were resistant to treatment with phosphatidyl-inositol specific phospholipase C (data not shown).

The effect of overexpressing the ΔEGFR relative to the EGFR/CD45 chimera in cells was determined by examining the ability of EGF to inhibit TCR-mediated mobilization of intracellular calcium. In cells where there is no detectable expression of the ΔEGFR, engagement of the EGFR/CD45 chimera results in the characteristic decrease in TCR-mediated calcium mobilization (Figure 20 A). When the ΔEGFR is expressed at 2.6 fold higher levels compared to the EGFR/CD45 chimera, the addition of saturating amounts of EGF only partially reverses the TCR-mediated increase in calcium; resulting in the

Figure 19. Immunoprecipitation of Cell Surface Labeled EGFR/CD45 Chimera and Truncated EGFR (ΔEGFR)

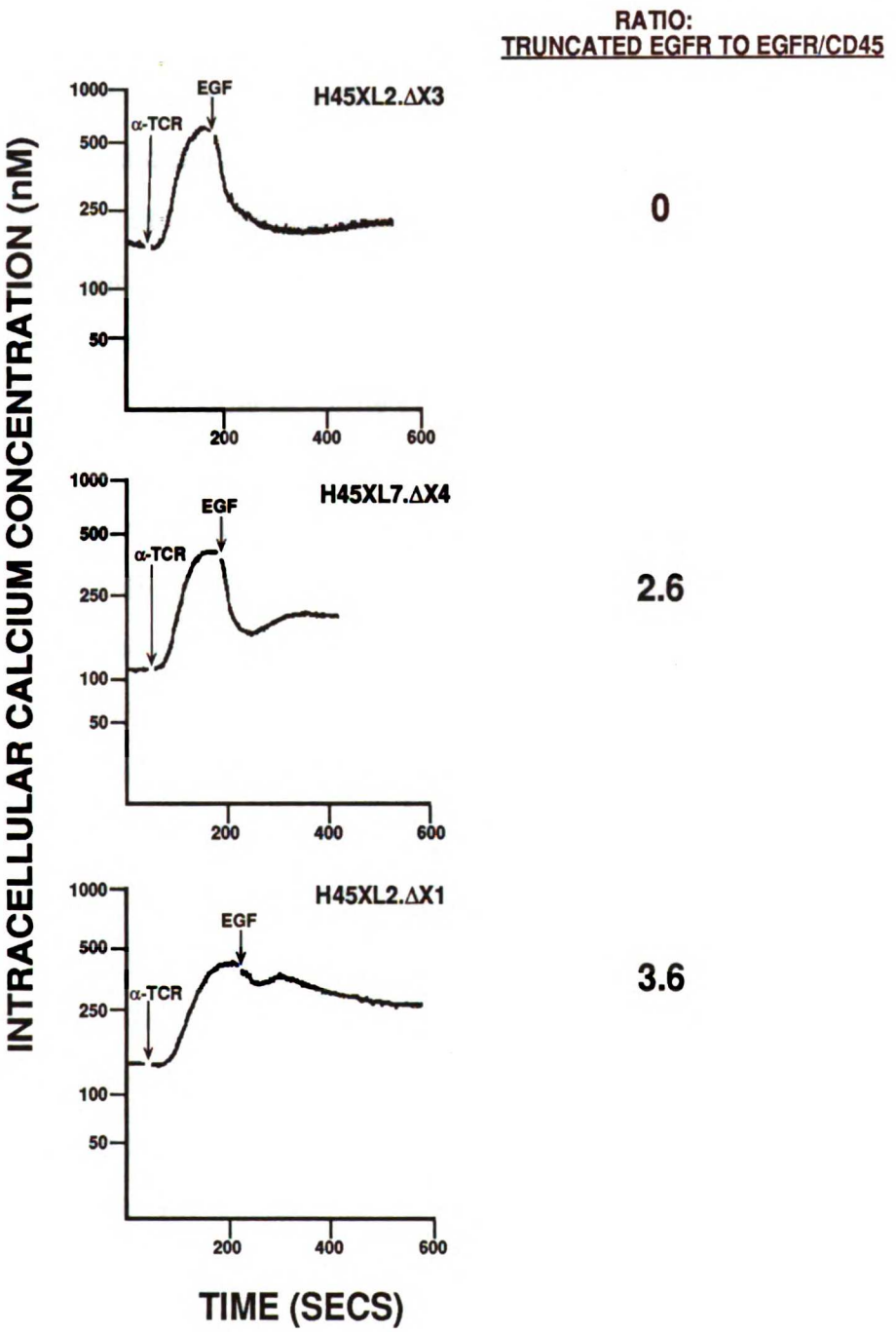
H45XL7 (EGFR/CD45$^+$) and three EGFR/CD45 chimera expressing clones which had been transfected with the ΔEGFR cDNA, H45XL2.DX1, H45XL2.DX3, and H45XL7.DX4 were surface biotinylated and subject to immunoprecipitation with anti-EGFR antibody. Immunecomplexes were resolved by SDS-PAGE, transferred to nitrocellulose and probed with ^{125}I-streptavidin followed by autoradiography. Determination of the relative ratio of the ΔEGFR to the EGFR/CD45 chimera was performed by quantitation of radioactivity using a phosphorimage scanner (Molecular Devices).


H45XL7
H45XL2.ΔX1
H45XL2.ΔX3
H45XL7.ΔX4
MW (kD)
205
116.5
80
EGFR/CD45
ΔEGFR
0
3.6
0
2.6
Ratio: ΔEGFR / EGFR/CD45

establishment of an intermediate calcium concentration (Figure 20 B). However, the expression of 3.6 times more ΔEGFR compared to the EGFR/CD45 chimera, almost completely suppresses the EGF-mediated reduction in intracellular calcium levels (Figure 20 C). This is comparable to studies in which expression of two fold more truncated EGFR relative to wild-type EGFR was sufficient to reduce by 75%, EGF-mediated receptor activation and mitogenesis (287). Thus, these data indicate that dimerization of the EGFR/CD45 chimera is required for its ability to negatively regulate TCR signal transduction, and suggest that the CD45 PTPase may only be functionally active in the monomeric state.

Figure 20. Modulation of TCR-Mediated Calcium Mobilization by the EGFR/CD45 Chimera is Inhibited by Overexpression of a Truncated EGFR.

Changes in intracellular free calcium were measured in cells expressing varying ratios of the ΔEGFR to the EGFR/CD45 chimera following treatment with anti-TCR mAb (1 μg/ml) and EGF (100 ng/ml) at the indicated times.


RATIO:
TRUNCATED EGFR TO EGFR/CD45
INTRACELLULAR CALCIUM CONCENTRATION (nM)
H45XL2.ΔX3
H45XL7.ΔX4
H45XL2.ΔX1
EGF
α-TCR
1000
500
250
100
50
200
400
600
0
2.6
3.6
TIME (SECS)

Discussion

The cytoplasmic domain of CD45 is sufficient for TCR signal transduction

CD45, a transmembrane PTPase, is essential for the signal transducing function of the T and B cell antigen receptors (251-253, 256, 257). The structural requirements and regulation of CD45 function and its impact on TCR signaling are not well understood. A function for the extracellular domain of CD45 is suggested by the regulated alternative RNA splicing of exons that encode the amino-terminal region of the extracellular domain of CD45. Moreover, the individual isoforms of CD45 have been reported to associate with distinct T cell surface molecules (280, 288). We have examined the contribution of the CD45 extracellular domain to its function by expressing the cytoplasmic domain of CD45 as a fusion protein with the EGFR extracellular and transmembrane domains. Our results demonstrate that the chimeric EGFR/CD45 protein is able to restore TCR signaling in a CD45 deficient cell line. These findings demonstrate that the extracellular and transmembrane domains are not required for CD45 function in the context of a single cell.

The EGFR/CD45 chimeric molecule structurally and functionally displays the properties of the representative domains of the wild-type proteins from which it is derived. Four mAbs directed against different epitopes of the EGFR extracellular domain, and two antisera specific for the CD45 cytoplasmic domain recognize the EGFR/CD45 chimera in native and denatured states. The integrity and appropriate conformation of the EGFR extracellular domain in the EGFR/CD45 chimera is best demonstrated by the ability of the chimeric protein to bind both known ligands for the EGFR, EGF and TGFα. The dose of EGF required for the inhibitory effects upon TCR signaling are consistent with binding to the low affinity site of the EGFR (289). The loss of high affinity EGF binding

sites is not surprising, as this has been observed with various deletions of the EGFR cytoplasmic domain (287, 290).

It is more difficult to determine if the CD45 component of the EGFR/CD45 chimera functionally mimics the cytoplasmic domain of wild-type CD45. However, since the PTPase activity associated with the EGFR/CD45 chimera is comparable with the CD45RO isoform, this suggests that replacement of the CD45 extracellular and transmembrane domains with those of the EGFR has not substantially altered the in vitro function of the PTPase domain. Moreover, since the EGFR/CD45 chimera is able to restore TCR signal transduction when expressed at levels similar to those of the transfected CD45RO, $CD45RA^{+}B^{+}C^{-}$ (264), and B220 isoforms (256), this implies that the in vivo substrate specificity of the PTPase domains of the EGFR/CD45 chimera and wild-type CD45 are the same. This is further supported by the demonstration that the TCR-induced tyrosine phosphoprotein pattern in CD45RO and EGFR/CD45 chimera expressing cells are comparable. Therefore, the ability of the EGFR/CD45 chimera to restore signal transduction by the TCR in a CD45 deficient cell line indicates that the cytoplasmic domain of CD45 is not only necessary but is also sufficient to allow the coupling of the TCR to the appropriate intracellular signal transduction machinery.

Our results suggest that the extracellular domain of CD45 is not required for signaling through the TCR. However, this does not indicate that extracellular domain of CD45 does not play a role in regulating CD45 function. Rather, our data does bring into question the functional importance of the differential association of the various CD45 isoforms with T cell surface signaling molecules including the TCR. Restoration of TCR signaling in CD45 negative cells by introduction of the CD45RO, $CD45RA^{+}B^{+}C^{-}$, and B220 isoforms as well as the EGFR/CD45 chimera indicates that if CD45 association with the TCR is

necessary for TCR function, then the CD45-TCR interaction is not isoform dependent or mediated by the extracellular and transmembrane domains of CD45. Instead, CD45 association with other cell surface molecules could potentially be mediated via interactions with cytoskeletal elements, such as fodrin (291). In light of these results, the extracellular domain of CD45 may be critical for ligand binding (see below) rather than association with signaling molecules on the same cell surface.

Ligand-mediated negative regulation of CD45 function

Numerous studies have been performed to address the role of CD45 in TCR-mediated signal transduction. Monoclonal antibodies to CD45 have been used in lieu of a known ligand but have produced varying results. CD45 cross-linking has led to inhibition or stimulation of T cell activation under different conditions (243-246). The effects observed using anti-CD45 antibodies, in many cases, have required high antibody concentrations and secondary cross-linking reagents to maximally oligomerize CD45. In most studies in which CD45 is cross-linked to the TCR, inhibition of T cell proliferation and early TCR-mediated signal transduction events have been observed (243, 248). More recently, it has been suggested that inhibition of T cell activation by CD45-TCR cross-linking is not specific for CD45 or its PTPase activity, but instead is dependent on the expression level of the surface protein to which the TCR is cross-linked (249). Thus, other cell surface proteins expressed at levels comparable to CD45 also inhibit T cell activation when cross-linked to the TCR, probably by preventing TCR oligomerization.

CD22, a B cell transmembrane protein with unknown function, can bind CD45, although this binding depends upon sialic acid residues on the CD45 molecule (237). This raises the possibility that CD22 may influence the function

of CD45. Indeed, inhibitory effects on TCR signaling by a soluble fusion protein that includes the extracellular domain CD22 have been reported; however, these inhibitory effects required the extensive crosslinking of the fusion protein bound to CD45 together with mAbs bound to the TCR (281). The extensive and saturable binding of ligand to CD45 is not likely to occur in vivo, particularly with a cell surface ligand. Further work is required to determine whether CD22 is a physiologic ligand of CD45.

Due to the uncertainty of the nature of CD45 ligands, we constructed a chimeric CD45 molecule, utilizing the extracellular and transmembrane domains of a receptor with a known ligand to study ligand-mediated PTPase regulation. The EGFR has been extensively studied, its ligand, EGF, has very defined effects on the structure and function of the EGFR (285). The ability of the EGFR/CD45 chimera to functionally restore TCR signaling in a CD45 deficient cell allowed us to study the effects of ligand binding to the EGFR/CD45 chimera on regulation of PTPase function and on TCR signal transduction. The addition of EGFR ligands prior to TCR stimulation led to an inhibition of TCR signaling. More interestingly, the addition of ligand during TCR signal transduction results in a very rapid and dramatic inactivation of TCR-mediated signals. The modulation of TCR signal transduction occurred within seconds of addition of physiological levels of EGF or TGFα and was dose dependent. This indicates that CD45 function is not only required for initiation of TCR signal transduction, but that it is also required to maintain the cascade of signaling events, since calcium mobilization and the induction of tyrosine phosphoproteins are rapidly reversed upon functional inactivation of the EGFR/CD45 chimera. This observation implies that both the calcium mobilization and the protein tyrosine phosphorylation that result from TCR stimulation requires an ongoing process. This is especially surprising, since calcium mobilization is thought to occur by an

active and rapid opening of calcium channels, followed by a slow rectification (292).

Our studies also demonstrate that modulation of TCR signaling by EGFR/CD45 chimera ligands is the result of functional inactivation of the chimeric PTPase, since EGF binding to the chimera had no effect on TCR signaling in the presence of wild-type CD45. Thus, unlike other transmembrane receptors that contain intrinsic enzymatic activity and are activated by ligand binding, the family of transmembrane PTPases may, in general, be inactivated by ligand binding. The notion that transmembrane PTPases may be negatively regulated by ligands is appealing considering that the PTPases have strikingly high intrinsic specific activities in vitro (154). Taken together, these results suggests that CD45 PTPase activity in vivo is constitutively functional and is required to couple the TCR to the intracellular signaling machinery. We demonstrate that this activity can be negatively regulated, resulting in loss of TCR function. This interpretation is supported by the signaling defect observed in CD45-deficient cell lines. Moreover, treatment of T cells with the PTPase inhibitor, phenylarsine oxide, at levels demonstrated to inhibit CD45 PTPase activity in vitro, results in a loss of TCR-mediated tyrosine phosphoprotein induction (293). These studies demonstrate that CD45 plays a critical and active role in TCR signal transduction, and suggest a very dynamic interaction of proteins involved in the signaling pathway with PTKs and PTPases whose functions can be regulated.

Potential regulation of transmembrane PTPases by dimerization

Numerous studies have shown that EGF activates the EGFR by inducing receptor dimerization and that the extracellular domain alone, in either soluble or membrane bound forms, is able to undergo ligand-dependent dimerization (287, 294). In addition, overexpression of an EGFR molecule, in which the cytoplasmic

domain has been deleted, functions to suppress wild-type EGFR activation by forming heterodimers with the wild-type receptor (287). Using the same strategy, we demonstrated that overexpression of a truncated EGFR suppresses the EGF-mediated inhibition of TCR signaling by the EGFR/CD45 chimera. This strongly suggests that dimerization of the EGFR/CD45 chimera is necessary for functional inactivation of the chimera. However, this does not indicate *a priori*, that cross-linking of wild-type CD45 with anti-CD45 antibodies should also lead to inhibition of TCR signaling. There are numerous reports of anti-EGFR antibodies that bind the EGFR with high affinity, immunoprecipitate the receptor, mediate receptor internalization, but do not activate the EGFR tyrosine kinase in vitro or in vivo (295-297). In addition these antibodies do not trigger mitogenic responses (298, 299). Therefore ligand-mediated dimerization and antibody mediated cross-linking may not be equivalent. Thus, this may explain the inconsistent findings that have been obtained with anti-CD45 antibodies.

Currently, the mechanism by which dimerization inhibits CD45 function is unclear. It is of note that recent studies suggest that a small fraction of CD45 exists as dimers on the cell surface (300). Additionally, it has been observed that CD45 is tyrosine phosphorylated following TCR stimulation (196), which could potentially lead to increased PTPase activity or function. Thus, in a manner analogous to receptor tyrosine kinases, the induced-dimerization of transmembrane PTPases by ligand may lead to trans-dephosphorylation and functional inactivation (Figure 21). Alternatively, dimerization may lead to a steric inhibition of substrate access to the phosphatase domains of CD45 (Figure 22). It can be envisaged that following T cell activation, in a para- or autocrine manner, the response may be negatively regulated by a CD45 ligand, thus keeping the immune response localized or limited in intensity.

Figure 21. A model for ligand-mediated negative regulation of EGFR/CD45 chimera function

Engagement of the TCR induces tyrosine phosphorylation of CD45, at an unidentified site, potentially augmenting CD45 function. Addition of EGFR/CD45 ligands resuts in dimerization of the chimeric molecules, leading to trans-dephosphorylation and reduction in CD45 activity.

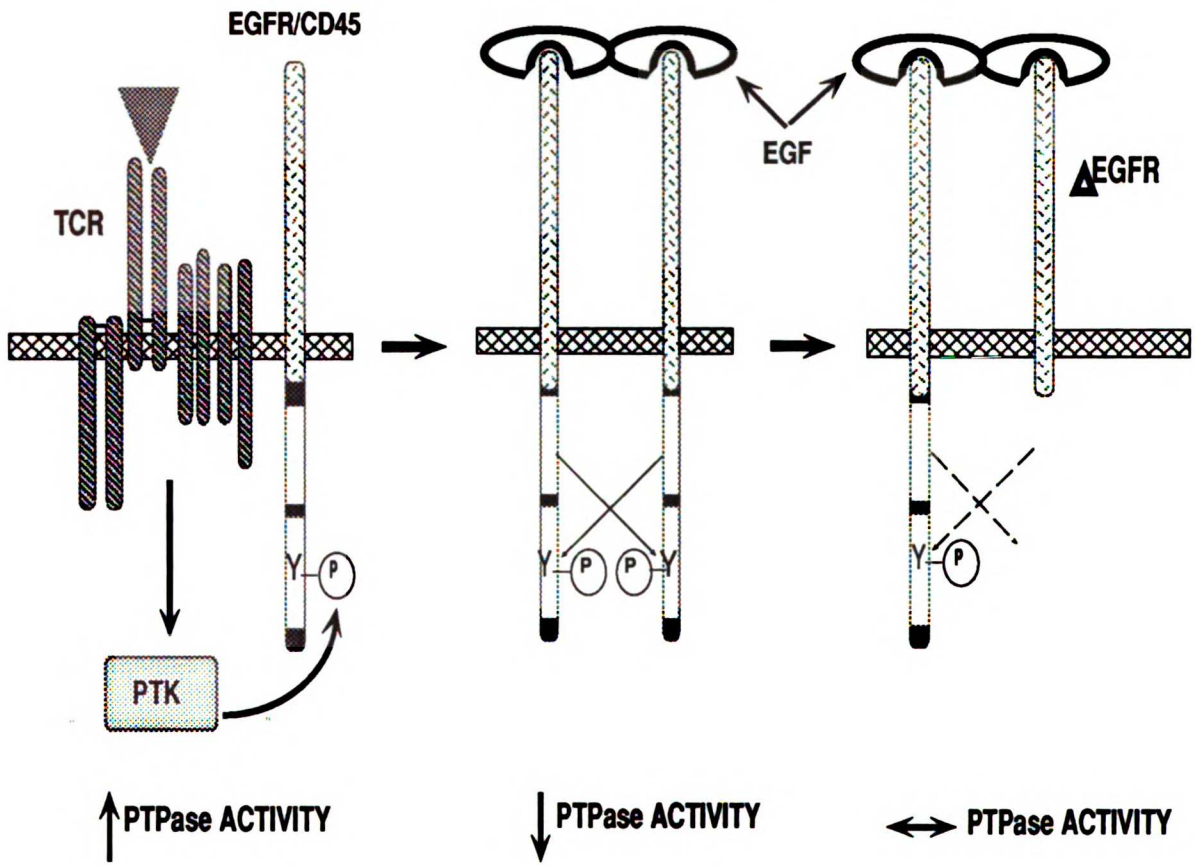

EGFR/CD45
TCR
EGF
EGFR
Y
P
PTK
PTPase ACTIVITY
PTPase ACTIVITY
PTPase ACTIVITY

Figure 22. An alternative model for ligand-mediated negative regulation of EGFR/CD45 chimera function

Dimerization of the constitutively active EGFR/CD45 chimera by EGFR ligands leads to steric inhibition of substrate access to the phosphatase domains of CD45.

EGFR/CD45
P
Y
PTK
Y
PTK
EGF
P
Y
PTK
ΔEGFR

Since EGF, a soluble peptide ligand, has such a rapid and dramatic effect on the functional activity of the EGFR/CD45 chimera, our studies suggest that there may be other ligands for CD45 besides CD22, and that such ligands may be soluble proteins. The notion that CD45 may bind a soluble ligand is further supported by structural considerations. The topographical organization of CD45 shares similarities with that of the EGFR in that it contains a large ectodomain with two cysteine-rich regions, a short, single transmembrane domain and a large intracellular domain with enzymatic activity. A number of receptors that bind soluble peptide ligands, including the EGFR, the nerve growth factor receptor, the tumor necrosis factor receptor, the insulin receptor and the insulin-like growth factor receptors all contain from one to four cysteine-rich regions that are important for ligand binding (285, 301-303). The cysteine-rich regions on these receptors contain 4-8 cysteine residues whose position and spacing are well conserved. CD45 also contains two cysteine-rich regions each containing eight cysteine residues. The second cysteine-rich region, contains pairs of cysteines that are evenly spaced, similar to the organization found in receptors that bind soluble peptide ligands (217). The potential importance of the cysteine-rich regions is evidenced by the fact that comparison of amino acid sequences of the extracellular domain of CD45 from mouse, rat and human shows only 35% homology; however, the cysteines that comprise the cysteine-rich region are absolutely conserved (238). This suggests that the cysteine-rich regions in CD45 may be functionally important and, as with other receptors, these regions may be involved directly or indirectly in ligand binding.

The function and regulation of transmembrane PTPases has been difficult to study because of the paucity of information regarding ligands and in vivo substrates. This work has demonstrated that construction of a chimeric PTPase permits the study of the function and regulation of PTPases without having

knowledge of the natural ligands. Transmembrane PTPases appear to be regulated in a manner analogous to receptor tyrosine kinases, yet are also very different since their enzymatic function may be constitutively active and negatively regulated by ligands. The precise understanding of the function and regulation of transmembrane PTPases awaits identification of their ligands. However, until such time, the use of chimeric molecules should permit a better understanding of their regulation, and may be useful in defining their in vivo function and substrates.

Summary

CD45, a transmembrane protein tyrosine phosphatase (PTPase), is required for signal transduction through the T cell antigen receptor (TCR). Multiple CD45 isoforms, differing in portions of the extracellular domain, are expressed in a tissue- and activation-specific manner suggesting an important function for this domain. A chimeric protein in which the extracellular and transmembrane domains of CD45 were replaced with those of the epidermal growth factor receptor (EGFR) is able to restore TCR signaling in a CD45 deficient cell. Thus, expression of only the cytoplasmic domain of CD45 is sufficient to restore TCR function. Moreover, EGF or TGFα functionally inactivate the EGFR/CD45 chimera in a manner which is dependent on dimerization of the chimeric protein. Inactivation of EGFR/CD45 chimera function results in the loss of TCR signaling, indicating that CD45 function is continuously required for signal transduction through the TCR. These results suggest that ligand-mediated regulation of receptor-PTPases share mechanistic similarities with those of receptor tyrosine kinases.

Experimental Procedures

Constructs and antibodies

The EGFR/CD45 chimera is comprised of the EGFR extracellular and transmembrane domains encompassing amino acids 1 to 646, fused in frame with the cytoplasmic domain of CD45 consisting of amino acids 580 to 1281. Amino acid numbering is according to Ullrich et. al. (304) and Streuli et. al. (221) for the EGFR and CD45, respectively. The EGFR/CD45 chimera construct was subcloned into the pAW-Neo3 expression vector driven by the spleen focus forming virus LTR. The truncated EGFR construct codes for amino acids 1 to 646 and was expressed utilizing the pCEP4 vector (Invitrogen). The 180 kDa CD45RO isoform cDNA was kindly provided by Drs. D. Cool and E. Fischer (Univ. of Washington), and was subcloned into the pAW-Neo3 expression vector.

Antibodies used for flow cytometry were control mouse IgG2b (Zymed laboratories); Leu 4 (anti-CD3ε, Becton Dickinson); GAP 8.3 (pan anti-CD45, ATCC); and LA22 (anti-EGFR, Upstate Biotechnology). Monoclonal antibody (mAb) 235, kindly provided by Dr. S. M. Fu (Univ. of Virginia), recognizes the CD3 subunit of the TCR (305). MAb 108.1 recognizes an epitope expressed on the extracellular domain of the EGFR (306) and was used for immunoprecipitations, while the anti-DIII rabbit antiserum, raised against bacterially expressed extracellular subdomain III of the EGFR, was utilized for Western blot analysis. Two different rabbit antisera, generously provided by Drs. Hanna Ostergaard (Univ. of Alberta) and Terry Higgins (Sterling Research), recognizing the cytoplasmic domain of CD45 were used for Western blotting. The anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology) was used for immunoprecipitation and immunoblotting phosphotyrosine containing proteins.

Cells and transfections

The human leukemic T cell lines Jurkat, HPB.ALL and its derivative H45 (CD45 negative) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Intergen), penicillin, streptomycin and glutamine (Irvine Scientific). Chimera and CD45RO transfected clones were passaged in the above medium containing 2 mg/ml Geneticin (GIBCO, Grand Island, NY). Cells transfected with the truncated EGFR were maintained by alternating passages in 2 mg/ml Geneticin and 250 μg/ml hygromycin B (Calbiochem).

Transfections were performed using the Bio-Rad Gene Pulser at a setting of 250 V and 960 μF with 20 μg of plasmid per 10^7 cells. Following electroporation, cells were maintained in RPMI medium for 2 days before limiting dilution cloning in either Geneticin or hygromycin B containing medium.

Flow cytometry

Briefly, cells were stained with saturating concentrations of primary antibody followed by fluorescein-conjugated goat anti-mouse antibody (Caltag). Cells were analyzed on a FACScan (Becton-Dickinson) as previously described (25).

Immunoprecipitations, SDS-PAGE, and western blotting

Cells were lysed at 1 x 10^8/ml in 1% NP-40, 150 mM NaCl, and 10 mM Tris (pH 7.8) supplemented with protease and phosphatase inhibitors as described (103). Lysates were incubated at 4^o C for 30 minutes, followed by centrifugation at 13,000 x g for 10 minutes, to remove insoluble material. Lysates were precleared by incubation with fixed Staphylococcus aureus (Calbiochem) for 1 hour, then centrifuged at 55,000 rpm for 20 minutes. Lysates were subject to immunoprecipitation with antibody coupled to protein A-sepharose beads.

Immune complexes were washed, resolved by SDS-PAGE (307), and transferred to nitrocellulose membranes (308). Western blotting was performed as described (140).

Stimulation of cells for assessment of tyrosine phosphoprotein induction

Cells were stimulated in serum-free medium at 1 x 10^8/ml by coating them with the anti-CD3 mAb (235) at a 1:500 dilution of ascities for 15 minutes at 4^o C. The cells were then shifted to 37^o C for the indicated time. Coating cells with antibody at 4^o C does not result in significant induction of tyrosine phosphoproteins. EGF (Boehringer Mannheim) was added at a final concentration of 500 ng/ml when the cells were placed at 37^o C, or as indicated. After stimulation, the cells were lysed as described above. Immunoprecipitates, isolated with the 4G10 anti-phosphotyrosine mAb (Upstate Biotechnology), were washed, boiled in 2X SDS sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose membranes. Phosphotyrosine containing proteins were identified by probing with the mAb 4G10, followed by alkaline phosphatase conjugated-goat anti-mouse IgG (Bio-Rad).

Cell surface labeling

5 x 10^7 cells/ml were incubated in phosphate buffered saline (PBS; pH 8.0) containing 1 mg/ml NHS-LC Biotin (Pierce) at 4^o C for 45 minutes with occasional swirling. The reaction was stopped with the addition of 50 mls PBS containing 20 mM glycine. The cells were pelleted, and washed two more times with PBS-glycine (309). Cell lysis, immunoprecipitation, SDS-PAGE, and Western blotting were performed as described earlier. The nitrocellulose membranes containing the resolved proteins were blocked with 5% non-fat dry milk (Bio-Rad) in 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20. The

blots were developed by incubating with ^{125}I-streptavidin (10 μCi, Amersham) for 30 minutes at 25^{o} C, followed by autoradiography.

Measurement of intracellular calcium

Intracellular free calcium levels were measured as previously described (310) using the calcium sensitive dye Indo-1 (Molecular Bioprobes). Cells, 3 x 10^{6}/ml, were stimulated with the mAbs Leu 4 (1μg/ml) or 235 (1:1000 dilution of ascities). EGF and TGFα (Boehringer Mannheim) were used at a final concentration of 100 ng/ml.

Tyrosine phosphatase assay

Plasma membranes were isolated by nitrogen cavitation and differential centrifugation as previously described (257). Membrane-associated tyrosine phosphatase activity was measured by incubating membranes with 10 mM O-phospho-L-tyrosine (Sigma) in assay buffer (25 mM HEPES-pH 7.5, 5 mM EDTA, and 10 mM DTT) for 15 minutes. Free phosphate released was determined by a colorimetric assay as described (207).

CHAPTER III. FUNCTIONAL ANALYSIS OF THE DUAL PHOSPHATASE DOMAINS OF THE CD45 TRANSMEMBRANE PROTEIN TYROSINE PHOSPHATASE IN T CELL RECEPTOR SIGNAL TRANSDUCTION

Preface

The demonstration that chimeric molecules consisting of the CD45 cytoplasmic domain fused to the extracellular and transmembrane regions of heterologous receptors are able to restore TCR signal transduction function in CD45-deficient cells, indicates the importance of the cytoplasmic domain of CD45 in regulating TCR signaling (311, 312). Furthermore, the extracellular and transmembrane domains of CD45 appear to be dispensable, since attachment of a myristylation site to the cytoplasmic domain of CD45 is sufficient to restore TCR signaling function in a CD45-deficient cell line (313). Although these studies indicate that the cytoplasmic domain of CD45 is both necessary and sufficient to couple the TCR to the intracellular signaling machinery, they should not be taken to indicate that the extracellular domain of CD45 is functionless.

The extracellular domain of CD45 may be involved in ligand-mediated regulation of CD45 function, as suggested by studies with the EGFR/CD45 chimera (311). The function of the EGFR/CD45 chimera is negatively regulated by ligands to the EGFR. This negative regulatory effect may represent the effect that the cognate ligand for CD45 would have. Moreover, the regulatory effect of ligand on EGFR/CD45 function requires dimerization of two full length chimeric molecules, indicating a critical regulatory role for the cytoplasmic domain of the EGFR/CD45 chimera in ligand-mediated PTPase regulation.

CD45, like the majority of transmembrane-PTPases, contains two tandem phosphatase domains; however, the in vivo functions of these domains is not

known (155). Herein, the membrane-proximal phosphatase domain will be referred to as the first phosphatase domain or domain I, while the distal phosphatase domain will be referred to as the second phosphatase domain or domain II. In vitro enzymatic studies with bacterially expressed, or with in vitro translated mRNA encoding the CD45 cytoplasmic domain suggest that only domain I has phosphatase function (186, 190). In contrast, a CD45 mutant, in which the catalytic cysteine of domain I was deleted, appeared to be enzymatically active in vitro against the substrate myelin basic protein (MBP) (276). Thus, it is not clear whether both phosphatase domains of CD45 are active. Moreover, the in vivo contribution of the two phosphatase domains of CD45 to TCR signal transduction has not been determined.

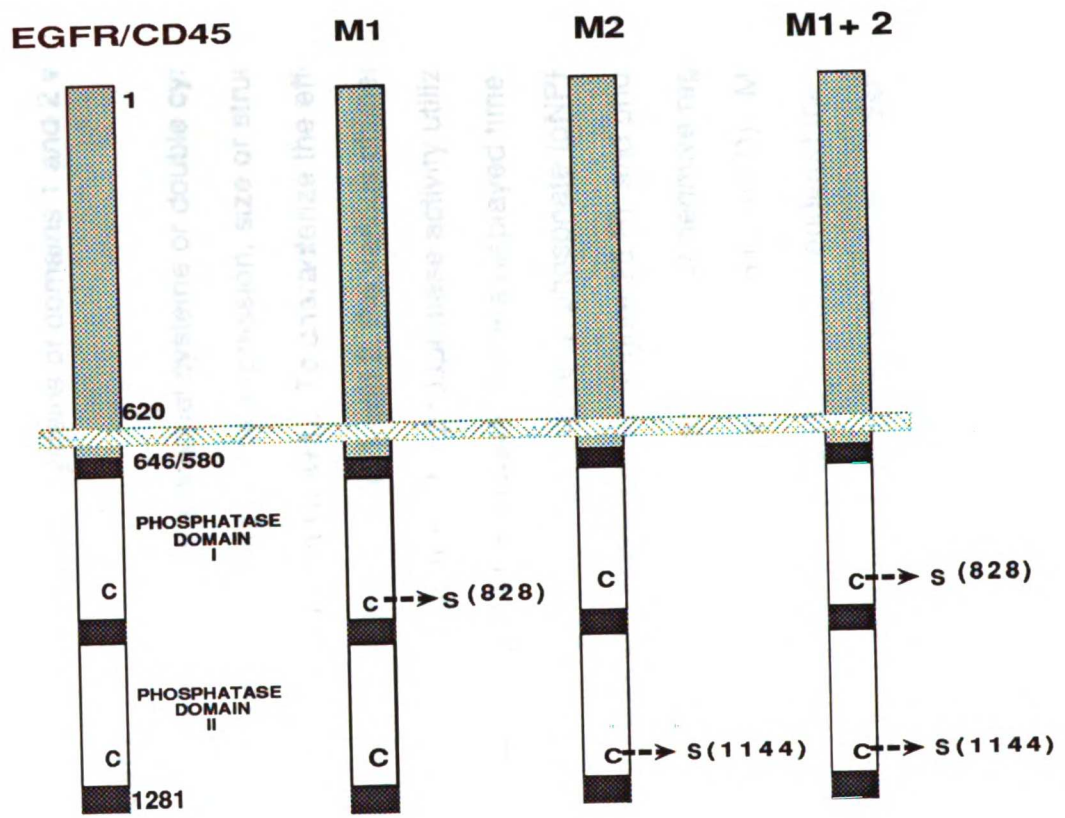
In order to determine the in vivo function of the CD45 phosphatase domains in regulating TCR signal transduction and in ligand-mediated negative regulation of CD45 function, the catalytic cysteine of the two phosphatase domains of CD45 were mutated individually and in combination, and the resulting mutated CD45 molecules were analyzed for their ability to restore TCR signaling in a CD45-deficient T cell line. Since, previous studies had demonstrated that an EGFR/CD45 chimera could functionally substitute for CD45 and that the function of the chimera could be modulated by EGFR ligands, the mutations of the CD45 phosphatase domains were performed in the context of the EGFR/CD45 chimera. Thus the relative contribution of the two phosphatase domains in ligand-mediated regulation of CD45 function could be determined. The EGFR/CD45 chimera mutants were analyzed not only for their ability to restore TCR signal transduction function in a CD45-deficient cell, but also for their contribution in ligand-mediated regulation of EGFR/CD45 function.

Construction and characterization of EGFR/CD45 chimera point mutants

Amino acid alignment of the phosphatase domains from both the cytosolic and transmembrane PTPases has revealed an 11 amino acid consensus motif located near the carboxy-terminus of the phosphatase domain [(I/V)HCXAGXXR(S/T)G] (Figure 8) (155). Site-directed mutagenesis and biochemical studies have demonstrated that the cysteine residue within this conserved motif acts as a nucleophile and forms a covalent thiol-phosphate bond with the phosphorylated-tyrosine residue of the substrate (184, 185). Mutation of the catalytic cysteine in a number of cytosolic and transmembrane PTPases results in abolition of phosphatase activity. As such, individual point mutations in the catalytic cysteine within the first (C828S) and second (C1144S) phosphatase domains of human CD45 were introduced, resulting in the generation of the M1 and M2 mutants, respectively. In addition a double point mutant was also created, referred to as M1 + 2 (Figure 23). The CD45 phosphatase domain mutations were made in the context of an EGFR/CD45 chimeric molecule, which has previously been demonstrated to function analogously to wild-type CD45 in reconstitution of TCR signal transduction function (311). All three mutant constructs were introduced into the CD45-deficient variant of the HPB-ALL leukemic cell line (H45). Multiple clones expressing each construct were isolated, analyzed and compared to H45 cells previously transfected with the wild-type EGFR/CD45 chimera. The phenotypes of representative clones expressing each construct are shown in Figure 24. Cell surface expression of the TCR, CD45 and the EGFR/CD45 chimera variants were quantitated by immunofluorescence and flow cytometry. All of the cells fail to express CD45, utilizing a pan-specific antibody which recognizes all CD45 isoforms; however, these clones express uniformly high levels of TCR.

Figure 23. Schematic representation of EGFR/CD45 chimera and phosphatase domain point mutants.

The numbers correspond to amino acids contributed by CD45 and the EGFR to the EGFR/CD45 chimera. The sites of the catalytic cysteine to serine point mutations are indicated.

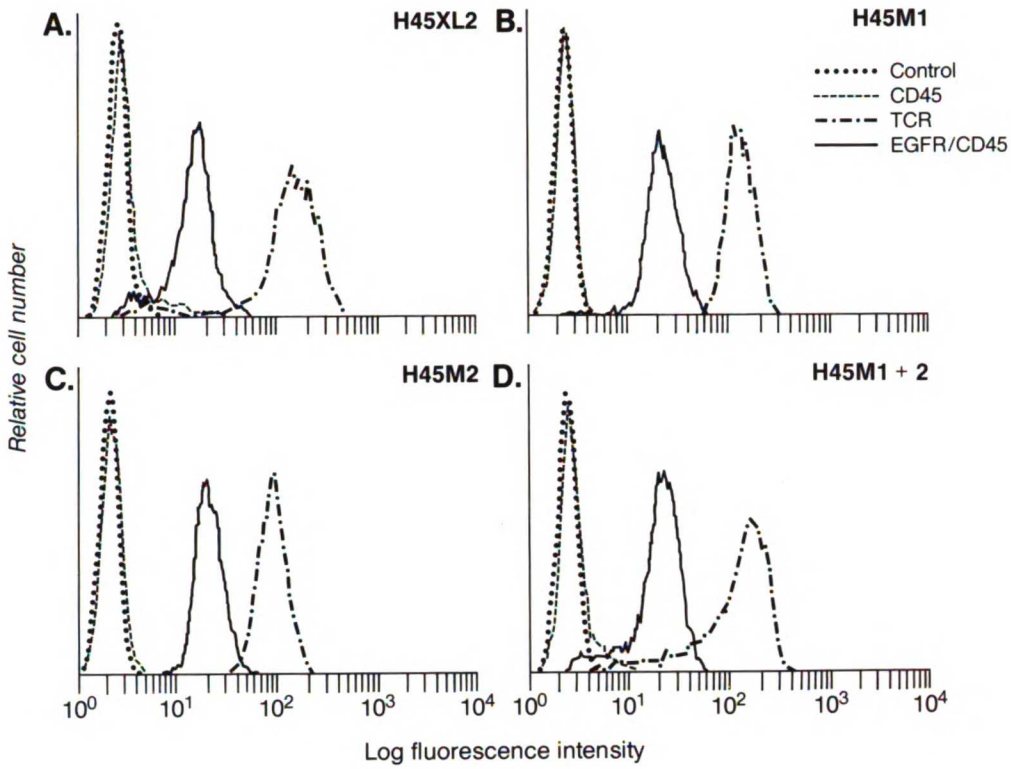

EGFR/CD45
M1
M2
M1+ 2
1
620
646/580
PHOSPHATASE DOMAIN I
PHOSPHATASE DOMAIN II
C
1281
C→S (828)
C→S(1144)

These CD45-deficient cells express similar levels of the wild-type EGFR/CD45 chimera (H45XL2; Figure 24 A), phosphatase domain 1 mutation (H45M1; Figure 24 B), domain 2 mutation (H45M2; Figure 24 C), and a double mutation (H45M1 + 2; Figure 24 D). To confirm that the various clones did indeed express the correct CD45 mutation, RNA was isolated from each clone, and RT-PCR fragments containing the catalytic cysteins of domains 1 and 2 were sequenced (data not shown).

Introduction of either the individual cysteine or double cysteine mutations did not have any apparent effect on the expression, size or structure of the EGFR/CD45 chimera (data not shown). To characterize the effect of mutating the conserved cysteines in domains 1 and 2, the various chimeras were immunoprecipitated and analyzed for phosphatase activity utilizing a number of substrates. The wild-type EGFR/CD45 chimera displayed time dependent phosphatase activity against para-nitrophenyl phosphate (pNPP) (Figure 25 A), MBP phosphorylated with purified c-Src (Figure 25 B), and phosphopeptides encompassing the autophosphorylation (Y-394) or negative regulatory (Y-505) sites of the Lck protein tyrosine kinase (Figure 25 C and D). Mutation of the catalytic site within the first phosphatase domain rendered the EGFR/CD45 chimera inactive against all substrates, and was virtually indistinguishable from the double phosphatase domain mutant. Mutation of the catalytic cysteine in the second phosphatase domain resulted in a molecule which does not appear to have catalytic activity substantially different from the wild-type molecule. Similar data were obtained utilizing v-abl phosphorylated MBP as the exogenous substrate (data not shown). These results on the in vitro enzymatic function of the wild-type and phosphatase domain point mutants of CD45 are in agreement with those determined for bacterially produced (186) or in vitro translated (190) cytoplasmic domain variants of CD45.

Figure 24. Cell surface expression of the TCR, CD45, and the EGFR/CD45 chimera mutants.

Cells were stained with a control mouse IgG2b mAb (dotted line), Leu4 (anti-CD3ε) for the TCR (alternating dashes and dots), Gap 8.3 mAb (anti-pan CD45; broken line), and LA22 (anti-EGFR, solid line). Panels A-D depict CD45-deficient (H45) clones expressing the wild-type, M1, M2 and M1 + 2 EGFR/CD45 chimeras respectively.


A.
H45XL2
B.
H45M1
Control
CD45
TCR
EGFR/CD45
C.
H45M2
D.
H45M1 + 2
Relative cell number
10^0
10^1
10^2
10^3
10^4
Log fluorescence intensity

To determine more precisely the effect of the M2 mutation on the enzymatic function of the EGFR/CD45 chimera, K_M determinations were performed using two different substrates. Surprisingly, the K_M values determined for the wild-type and M2 mutant using pNPP, and the Lck Y-505 containing phosphopeptides were similar, indicating that the putative catalytic function of the second domain does not appear to significantly influence the function of the first domain in vitro with the substrates utilized (Table I). The Lck Y-394 containing phosphopeptide was utilized equally well by both the wild-type and M2 EGFR/CD45 chimeric molecules. The K_M values obtained here, with the EGFR/CD45 chimera are slightly higher than those previously reported utilizing a molecule consisting solely of the CD45 cytoplasmic domain (188). It is not known whether this difference reflects an effect of the transmembrane and extracellular domains or whether it simply reflects experimental variation is not known.

In vivo function of the CD45 phosphatase domain mutants

In CD45-deficient cells, the TCR is uncoupled from the intracellular signaling machinery, resulting in a loss of TCR-mediated tyrosine phosphoprotein induction and mobilization of $[Ca^{2+}]_i$. TCR signal transduction can be restored in CD45-deficient cells simply by targeting the cytoplasmic domain of CD45 to the plasma membrane (313). Expression of the EGFR/CD45 chimera restores TCR signal transduction in a manner indistinguishable from wild-type CD45.

To examine the functional requirements of the two phosphatase domains of CD45 in vivo, we analyzed the ability of the various point mutants to restore TCR-mediated $[Ca^{2+}]_i$ mobilization. Engagement of the TCR on wild-type HPB-ALL cells ($CD45^+$) resulted in a rapid mobilization of intracellular calcium (Figure 26 A), but not observed in cells lacking CD45 (Figure 26 B).

Figure 25. Time-dependent dephosphorylation of different substrates by the wild-type and mutant EGFR/CD45 chimeras.

The wild-type and mutant EGFR/CD45 chimera immunoprecipitates were divided into five equivalent aliquots: one was immunoblotted with an anti-CD45 cytoplasmic domain anti-sera. The other four aliquots were incubated with phosphatase assay buffer plus: A) 10 mM pNPP; B) 50 μM MBP; C) 250 μM Lck autophosphorylation (Y-394) phosphopeptide; and D) 250 μM Lck carboxy-terminal (Y-505) phosphopeptide for the indicated times. The absorbance of the pNPP was measured at 405 nm. ^{32}P release from MBP was quantified by scintillation counting, while inorganic phosphate release from the phosphopeptides was quantitated using the ascorbic acid/ molybdenate reaction. Points shown represent values obtained following normalization for protein amount and subtraction of background.

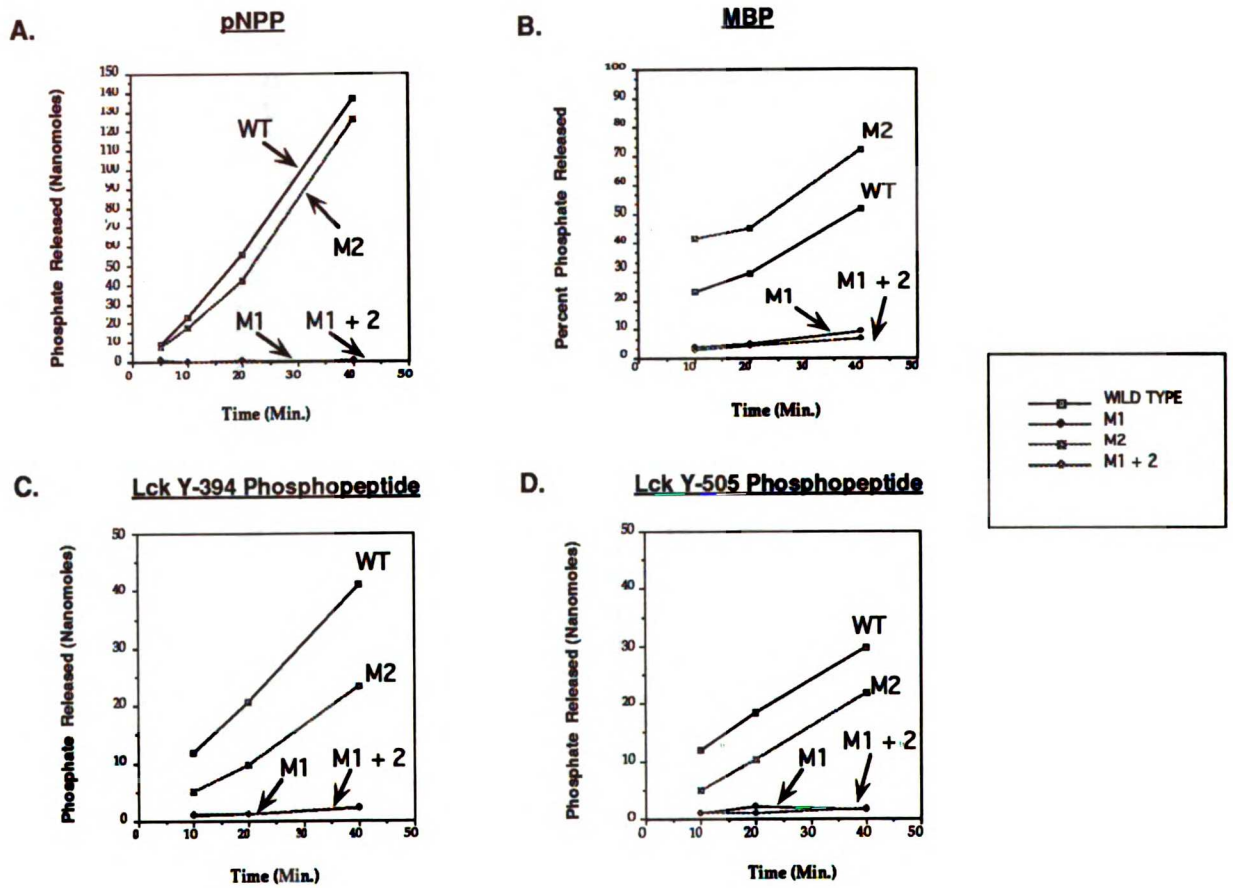

A.
pNPP
Phosphate Released (Nanomoles)
Time (Min.)
WT
M2
M1
M1 + 2
B.
MBP
Percent Phosphate Released
Time (Min.)
M2
WT
M1
M1 + 2
C.
Lck Y-394 Phosphopeptide
Phosphate Released (Nanomoles)
Time (Min.)
WT
M2
M1
M1 + 2
D.
Lck Y-505 Phosphopeptide
Phosphate Released (Nanomoles)
Time (Min.)
WT
M2
M1
M1 + 2
WILD TYPE
M1
M2
M1 + 2

TABLE I

MICHAELIS-MENTON CONSTANTS FOR WILD-TYPE EGFR/CD45 AND THE M2 EGFR/CD45 CHIMERA

ENZYME	SUBSTRATE	
	pNPP	Y505 PEPTIDE
WT - EGFR/CD45	15.53	0.392
M2 - EGFR/CD45	14.51	0.312

K_M values are expressed in millimolar. The wild-type EGFR/CD45 or the M2 EGFR/CD45 chimera was immunoprecipitated from equivalent numbers of cells expressing either the wild-type or M2 chimera. The immunoprecipitates were assayed for enzymatic activity, which was normalized for the relative amount of each protein by immunoblotting a portion of the sample.

Expression of the wild-type EGFR/CD45 chimera or the M2 mutant restored TCR signaling function (Figure 26 C and E). However, neither the M1 nor the M1 + 2 mutant was capable of restoring the TCR-regulated increase in $[Ca^{2+}]_i$ (Figure 26 D and F).

In addition to intracellular calcium mobilization, another hallmark of TCR engagement is the induction of tyrosine phosphoproteins. Examination of the ability of the various mutants to restore TCR-mediated tyrosine phosphoprotein induction revealed that engagement of the TCR, with anti-CD3 mAb, on the wild-type and M2 EGFR/CD45 chimera-expressing cells resulted qualitatively in the induction of a similar pattern of tyrosine phosphoproteins (Fig 27; lanes 1, 2, 5 and 6). The 70 kDa tyrosine phosphoprotein seen in the unstimulated H45M2 cell was not reproducibly observed in unstimulated cells. The M1 and M1 + 2 mutants were unable to restore TCR function (Figure 27; lanes 3, 4, 7, and 8). These results demonstrate for the first time that CD45 phosphatase activity was necessary for proximal signal transduction events mediate by the TCR. Moreover, the catalytic function of the first phosphatase domain of CD45 is required for this process. If the second domain has catalytic activity, which was not revealed in the in vitro assays, its activity would appear not to be required for TCR signaling function.

In vivo tyrosine phosphorylation state of Lck in cells expressing EGFR/CD45 chimera phosphatase domain mutants

Previous studies demonstrated that the Lck and Fyn PTKs are hyperphosphorylated on the carboxy-terminal tyrosine in cells deficient in CD45 expression (260-263). Fyn and Lck appear to be specific substrates since the phosphorylation state of the carboxy-terminal tyrosine residue of c-Src is not affected by the absence of CD45 (262). The phosphorylation state of the Lck

Figure 26. TCR-mediated mobilization of intracellular free calcium in wild-type and mutant EGFR/CD45 chimera expressing cells.

HPB-ALL ($CD45^+$) (A), H45 ($CD45^-$) (B), H45XL2 ($CD45^-$, EGFR/$CD45^+$) (C), H45M1 ($CD45^-$, EGFR/CD45-$M1^+$) (D), H45M2 ($CD45^-$, EGFR/CD45-$M2^+$) (E), H45M1 + 2 ($CD45^-$, EGFR/CD45-M1 + 2^+) (F) were treated at the indicated time with either 1 μg/ml anti-CD3 (Leu4) antibody or with calcium ionophore, ionomycin (Iono; 1 μm). $[Ca^{2+}]_i$ levels were measured using the calcium sensitive dye Indo-1.

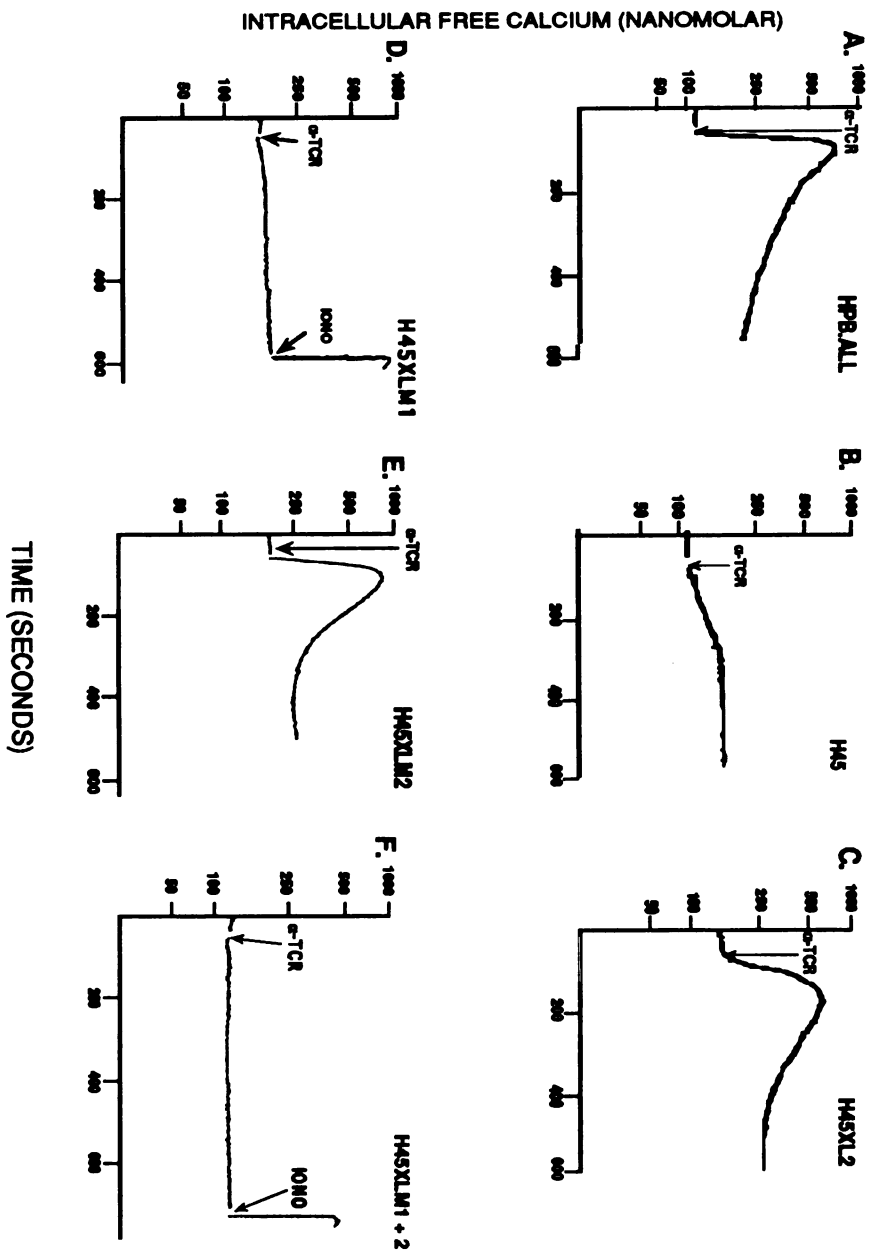
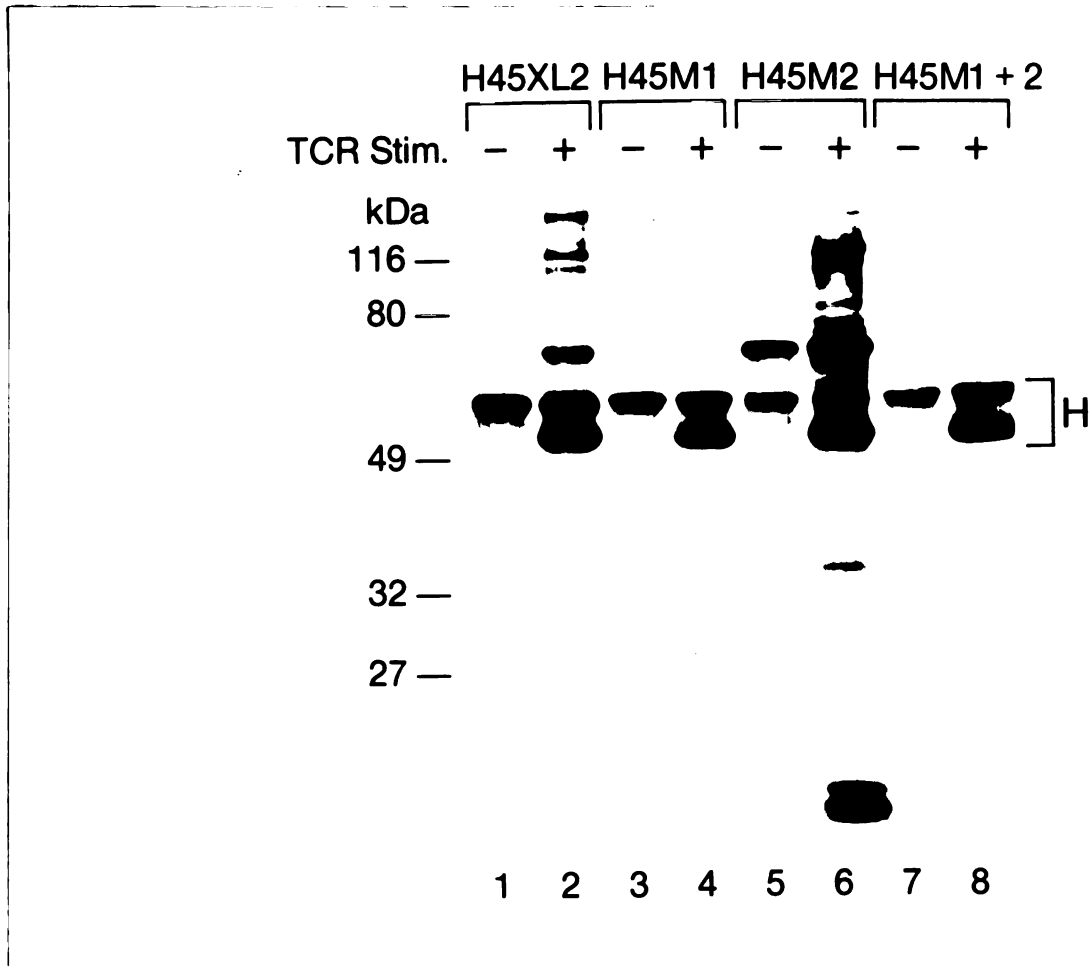

INTRACELLULAR FREE CALCIUM (NANOMOLAR)
TIME (SECONDS)
A.
HPBALL
B.
H45
C.
H45XL2
D.
H45XLM1
E.
H45XLM2
F.
H45XLM1 + 2
α-TCR
IONO

Figure 27. T cell receptor-mediated induction of tyrosine phosphoproteins in wild-type and mutant EGFR/CD45 chimera expressing cells

H45XL2 (lanes 1 and 2), H45M1 (lanes 3 and 4), H45M2 (lanes 5 and 6), and H45M1 + 2 (lanes 7 and 8) were treated with medium (-) or with a 1:1000 dilution of ascitic fluid containing anti-CD3 mAb (+) for 2 minutes. Induction of phosphotyrosine-containing proteins was assessed by immunoprecipitation followed by immunoblotting with an anti-phosphotyrosine mAb. The immunoglobulin heavy chain of the immunoprecipitating and stimulatory antibody is indicated by an H.


H45XL2
H45M1
H45M2
H45M1 + 2
TCR Stim.
– + – + – + – +
kDa
116
80
49
32
27
H
1 2 3 4 5 6 7 8

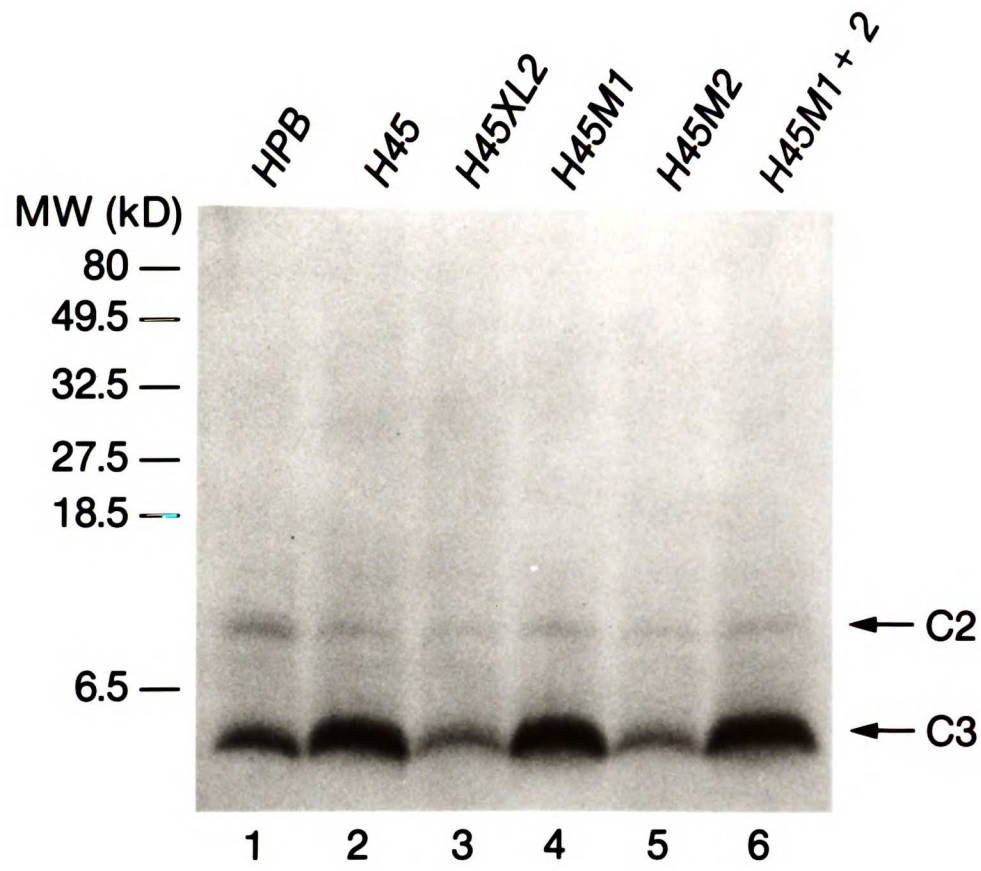
carboxy-terminal tyrosine (Y-505) in the cells expressing the various phosphatase domain mutants was examined by immunoprecipitation of Lck from cells labeled with ^{32}P-orthophosphate followed by immunoblotting, to determine the relative levels of Lck isolated from each cell line (data not shown), antecedent to treatment with CNBr. Lck contains two sets of nested CNBr cleavage sites, resulting in the generation of 3 predominant fragments: C1, a ~30 kDa peptide, encompasses the amino-terminal region of the molecule and also contains numerous serine phosphorylation sites. C2, a ~12-14 kDa peptide, contains the autophosphorylation site (Y-394). The ~4 kDa C3 fragment encompasses the carboxy-terminal negative regulatory site (Y-505). The C3 fragment was phosphorylated to a greater extent in the H45 (CD45$^-$) cells compared to the CD45$^+$, HPB-ALL cells (Figure 28; lanes 1 and 2). Introduction of the wild-type EGFR/CD45 chimera or the M2 version, both of which restore TCR signaling function, resulted in a 4-5 fold reduction in Y-505 phosphorylation (Figure 28, lanes 3 and 5). However, expression of the M1 and M1 + 2 mutants of the EGFR/CD45 chimera did not diminish the phosphorylation status of Lck Y-505 (Figure 28; lanes 4 and 6). Thus, there is a correlation between CD45 phosphatase activity in vitro, restoration of TCR signaling and dephosphorylation of Lck on tyrosine 505. The phosphorylation status of Fyn in these cells could not be satisfactorily determined due to the relatively low level of expression of Fyn.

The function of phosphatase domain one is required for ligand-mediated regulation of EGFR/CD45 function

Since previous studies demonstrated that ligands to the EGFR/CD45 chimera can negatively regulate the function of the chimera, resulting in inhibition of TCR-mediated calcium mobilization and tyrosine phosphoprotein induction,

Figure 28. In vivo phosphorylation status of the carboxy-terminal tyrosine residue (Y-505) of Lck.

Lck from HPB (lane 1), H45 (lane 2), H45XL2 (lane 3), H45M1 (lane 4), H45M2 (lane 5) and H45M1 + 2 (lane 6) cells labeled with ^{32}P-orthophosphate was immunoprecipitated, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted to ensure that relatively equal amounts of Lck where present in each immunoprecipitate. The nitrocellulose immobilized Lck was treated with CNBr and the resulting peptide fragments were separated on a 19% polyacrylamide gel. The autophosphorylation and carboxy-terminal tyrosine containing bands are indicated, as well as the positions of the molecular weight standards.


HPB
H45
H45XL2
H45M1
H45M2
H45M1 + 2
MW (kD)
80
49.5
32.5
27.5
18.5
6.5
C2
C3
1
2
3
4
5
6

assessment of the relative importance of the two phosphatase domains in this process was of importance (311). Stimulation of the TCR resulted in a mobilization of intracellular calcium, which was rapidly attenuated with the addition of ligand to the EGFR/CD45 chimera (Figure 29 A-C). As shown above, mutation of the catalytic cysteine in the second phosphatase domain resulted in an EGFR/CD45 chimera competent in restoring TCR signaling (Figure 29 D). Moreover, the M2 mutant also could still be negatively regulated by EGFR ligands, resulting in attenuation of TCR-mediated calcium mobilization (Figure 29 E). As with the wild-type chimera, engagement of the M2 chimera prior to TCR stimulation prevented the antigen receptor-mediated calcium increase (Figure 29 F).

Since tyrosine phosphoprotein induction is an early and requisite event in T cell activation, the effect of mutating the second phosphatase domain of CD45 on the pattern of protein dephosphorylation following engagement of the M2 chimera was evaluated. Stimulation of the TCR for 2 or 3 minutes on both H45XL2 and H45M2 resulted in the qualitatively similar induction of a number of tyrosine phosphoproteins (Figure 30; lanes 1-3 and 6-8). The addition of EGFR ligand concurrently with anti-TCR mAbs almost completely inhibited the induction of tyrosine phosphoproteins in cells containing the wild-type and M2 EGFR/CD45 chimeras (Figure 30; lanes 4 and 9). Moreover, subsequent to TCR stimulation for 2 minutes, the addition of EGF for one minute resulted in a rapid dephosphorylation of the majority of the induced tyrosine phosphorylation events (Figure 30; lanes 5 and 10). Thus, it appears that mutation of the catalytic site within the second phosphatase domain does not have any appreciable effect on the ability of EGFR ligands to negatively regulate the function of the EGFR/CD45 chimera and subsequent TCR signal transduction.

Figure 29. Modulation of TCR-mediated mobilization of intracellular free calcium by EGFR/CD45 ligand.

H45XL2 or H45M2 cells were treated at the indicated times with either 1 μg/ml anti-TCR mAb or 100 ng/ml EGF.

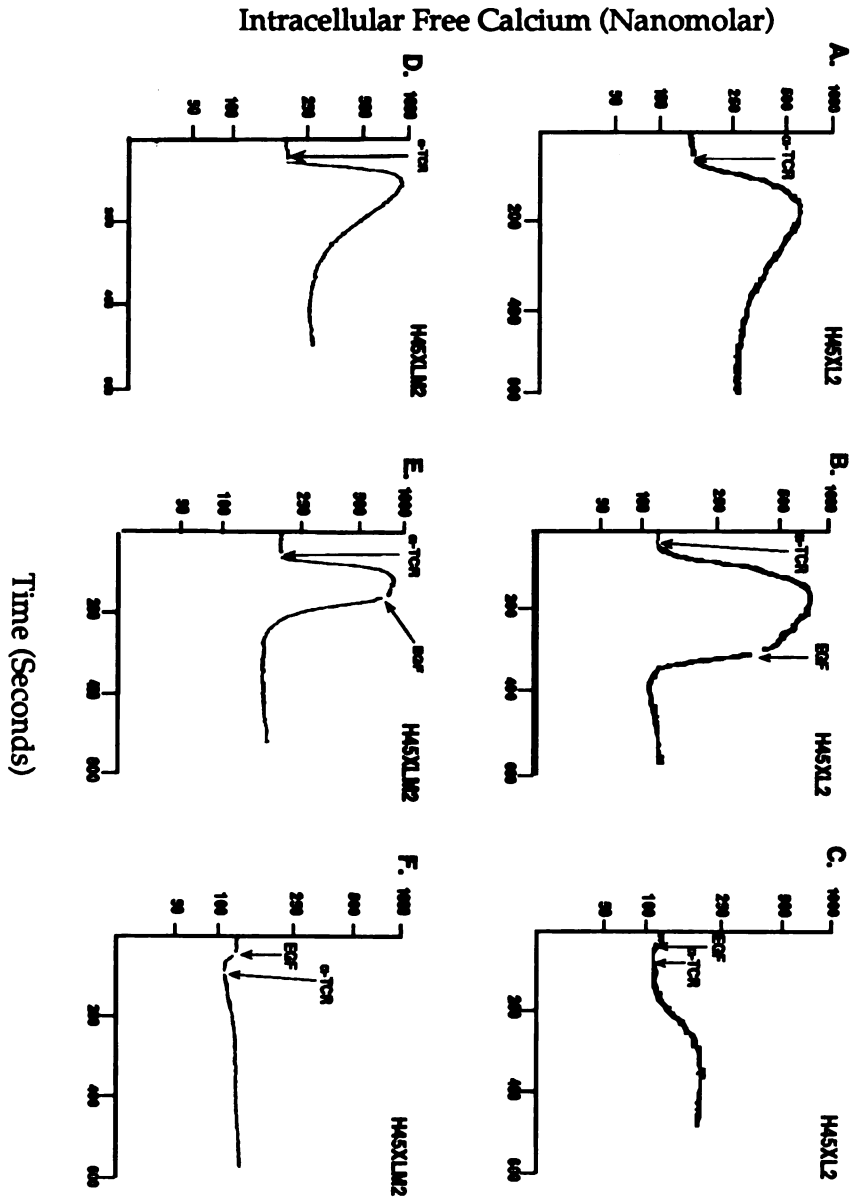
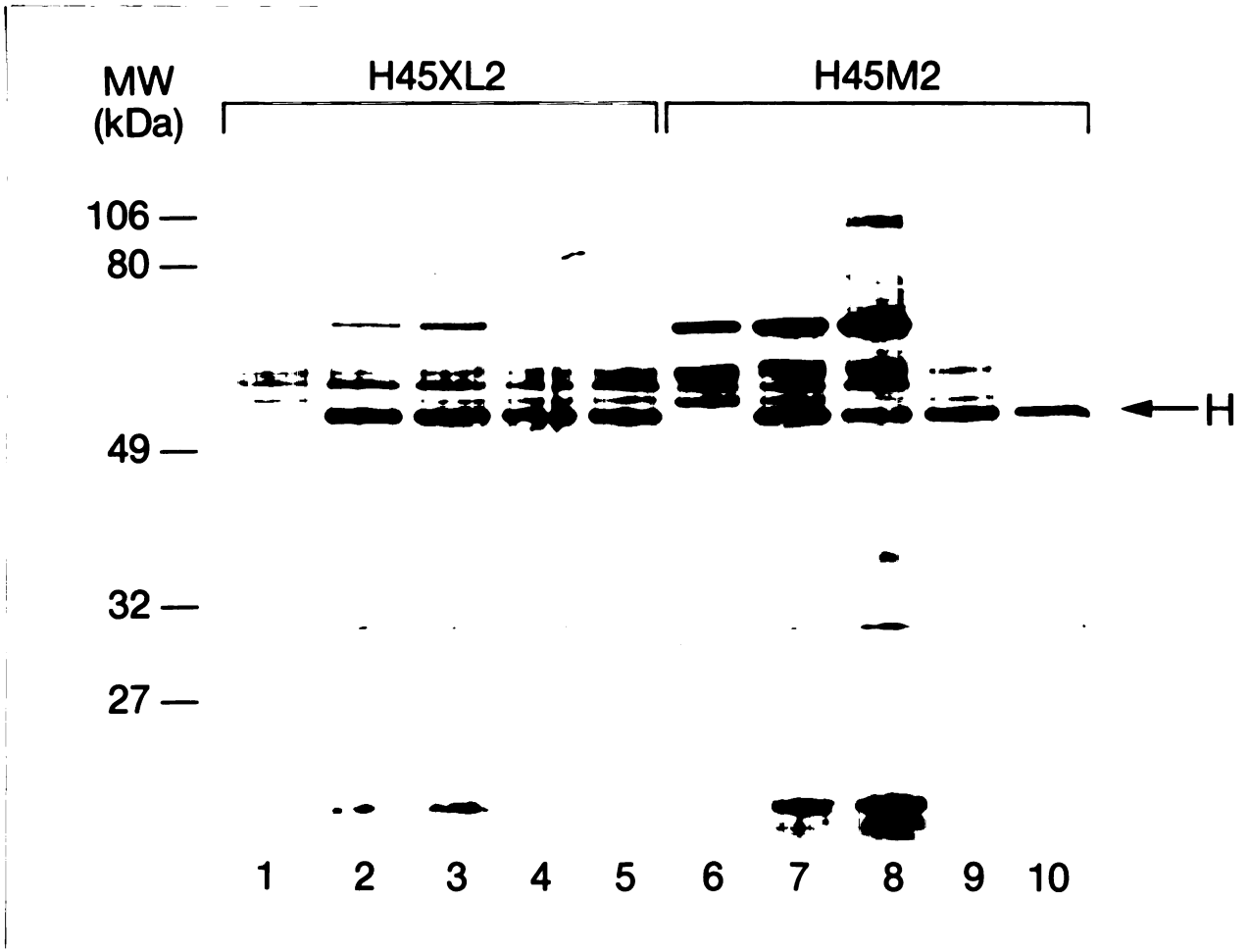

Intracellular Free Calcium (Nanomolar)
Time (Seconds)
A.
B.
C.
D.
E.
F.
H45XL2
H45XLM2
α-TCR
EGF

Figure 30. Modulation of TCR-mediated tyrosine phosphoprotein induction by EGFR/CD45 ligand.

Tyrosine phosphoproteins were immunoprecipitated from H45XL2 (lanes 1-5) or H45M2 (lane 6 -10) cells treated for 2 minutes with medium (lanes 1 and 6), 2 minutes with anti-TCR mAb (lanes 2 and 7), 3 minutes with anti-TCR mAb (lanes 3 and 8), 2 minutes with anti-TCR mAb plus EGF simultaneously (lanes 4 and 9), or 2 minutes with anti-TCR mAb, followed by 1 minute with EGF (lanes 5 and 10).


MW
(kDa)
H45XL2
H45M2
106
80
49
32
27
H
1
2
3
4
5
6
7
8
9
10

Since ligand binding to the EGFR/CD45 chimera resulted in a phenotype reminiscent of the CD45-deficient cell, internalization of the EGFR/CD45 chimera subsequent to the addition of EGF was assessed. Analysis of cell surface expression of the EGFR/CD45 chimera at various times following the addition of EGF indicates that there was no substantial change in surface expression of the EGFR/CD45 chimera (Table II). Therefore, the ability of EGF to negatively modulate TCR signaling function was not the result of reducing the cell to a state of CD45-deficiency.

TABLE II

Cell Surface Expression of the EGFR/CD45 Chimera Subsequent to EGF Treatment*

Time (Mins.)	Percent Remaining Cell Surface EGFR/CD45 Chimera
1	94.0
2	92.4
5	92.1
10	93.9

* Values are expressed as a percent of remaining cell surface EGFR/CD45 chimeric molecules compared to control treated cells. 1 X 10^6 H45XL2 cells were treated at 37° C with 100 ng/ml EGF for the indicated times. Control H45XL2 cells were treated at 4° C as above. Cell were stained for cell surface EGFR/CD45 chimera using an anti-EGFR extracellular domain mAb. Percentages reflect changes in mean fluorescence intensity.

Discussion

The catalytic function of the first phosphatase domain of CD45 is sufficient for TCR signal transduction

Numerous studies have defined the importance of CD45 expression in regulating an essential component(s) in the signal transduction pathway utilized by hematopoietic cell antigen receptors (reviewed in 107). In most CD45-deficient cells, signals emanating from the TCR, appear to be blocked at an early stage in the signaling pathway. These TCR-mediated responses can be restored by introducing any one of multiple CD45 isoforms (256, 264, 311, 314). Moreover, the extracellular and transmembrane domains of CD45 are not required to restore TCR function (311-313). Therefore, the cytoplasmic domain of CD45 is sufficient to allow TCR signaling.

CD45, like the majority of transmembrane receptor-like PTPases, contains two tandem phosphatase domains. However, the relative in vivo contribution of the two domains to CD45 function in TCR signal transduction was not known. Experiments in which individually mutating the catalytic cysteinyl residue in either or both domains and introducing the resulting mutant forms of the EGFR/CD45 cimera into CD45-deficient cells, demonstrate that the phosphatase activity of CD45 is critical for signaling through the TCR. Moreover, it appears that the catalytic function of the domain I is necessary to restore TCR signaling in the HPB-ALL leukemic cell line model system. Mutation of the cysteine residue in the catalytic site of the first phosphatase domain resulted in a molecule that was not functional in vitro against a number of structurally unique substrates, including a small molecule substrate (pNPP), a phosphoprotein (MBP) and an oligophosphopeptide encompassing Y-505 of Lck, a putative in vivo substrate. Mutation of the catalytic site in domain I also rendered the EGFR/CD45 chimera

non-functional in vivo, as it was unable to restore TCR signaling. Mutation of the catalytic site in the second phosphatase domain resulted in a molecule with in vitro and in vivo properties similar to that of the wild-type molecule. These results suggest that the catalytic activity of the first phosphatase domain of CD45 is sufficient for T cell receptor signal transduction.

Our results are in agreement with previous studies, in which no apparent phosphatase activity was present when the cytoplasmic domain of CD45 containing a mutation in the first domain catalytic site was expressed in vitro or in bacteria (186, 190). In these studies, the first domain was responsible for all observed in vitro enzymatic activity. However, in a recent study, expression of a CD45 molecule containing a partial deletion within the first phosphatase domain exhibited activity toward v-abl phosphorylated-MBP (276). However, in those experiments the residual phosphatase activity was never formally demonstrated to originate from the second phosphatase domain. In contrast, no phosphatase activity attributable to the second phosphatase domain in the M1 EGFR/CD45 chimera was detected, even when v-abl phosphorylated-MBP was utilized as the substrate. Regardless of whether the second phosphatase domain has activity against MBP, the second domain does not appear to have in vivo function with regard to restoration of TCR signaling. Moreover, the catalytic function of the second phosphatase domain, if it has any, was not required for restoration of TCR signaling, since the enzymatic function of the proximal domain was sufficient to restore TCR signal transduction.

Alteration of the catalytic cysteine residue of the second phosphatase domain did not result in any observable difference in the in vitro or in vivo function of the EGFR/CD45 chimera. The distal phosphatase domain of the receptor-like PTPase, LAR, also does not appear to be functional in vitro against the substrates tested to date (183, 186). However, the distal phosphatase

domain of R-PTPα, a ubiquitously expressed PTPase, does have enzymatic activity, albeit at a substantially reduced level compared with that of the first domain (315). In addition, the in vitro substrate specificity of the two domains of RPTPα appears to be different, which suggests that the two domains may have distinct cellular functions.

Comparison of the amino acid sequences around the catalytic site in the second phosphatase domains of CD45 and R-PTPα indicates that a number of critical and absolutely conserved amino acids in the 11 amino acid phosphatase motif are absent in CD45. However, changing the catalytic core sequence of the second phosphatase domain of CD45 to that found in the proximal domain did not generate an enzymatically functional phosphatase domain capable of dephosphorylating angiotensin or MBP (190), suggesting that other structural features are necessary to create an active phosphatase domain (Figure 8). However, the second domain may possess exquisite substrate specificity not detectable in vitro with the artificial substrates utilized. Alternatively, the activity of the second domain may be unmasked by post-translational modification such as phosphorylation, or may require the association of an activator protein, as in the case of $p80^{cdc25}$ and cyclin B (316). Regardless of the potential mechanism by which the potential enzymatic activity of the second phosphatase domain of CD45 may be unmasked, the studies presented here suggest that the potential (if existent) phosphatase function of the second domain is not required for coupling the TCR to the intracellular signaling machinery.

The fact that the majority of the receptor-like PTPases contain two phosphatase domains, in which the second domains are as well conserved as first phosphatase domains (171), suggests a function for the second domain. Mutational analysis suggests that the second phosphatase domains of CD45 and LAR rather than directly dephosphorylating tyrosine phosphoproteins, may

facilitate and regulate the activity of the first domain. Deletion of the second domain results in an enzymatically inactive proximal domain in vitro (186, 188, 190). A regulatory function for the distal phosphatase domains may be predicted for the RPTPγ (171, 317) and RPTPβ (317) enzymes, since the distal phosphatase domain from these PTPases lack the highly conserved catalytic cysteinyl residue. The in vivo function of the second phosphatase domain of CD45 remains unclear. However, as CD45 is required for T cell development and is expressed on a variety of other cell types, the second domain may play a role in other cells or during a restricted stage of T cell ontogeny.

Regulation of Lck phosphorylation by CD45

The signaling defect in CD45-deficient cells is presumed to result from hyperphosphorylation of the Lck and/or Fyn PTKs on the negative-regulatory carboxy-terminal tyrosine residue (151). In CD45-deficient cells, the action of Csk, the kinase demonstrated to phosphorylate the carboxy-terminus of Src-like kinases (271), would be unopposed, resulting in accumulation of phosphate on the carboxy-terminal tyrosine of Lck. However, introduction of a functional CD45 molecule should reverse the state of Lck phosphorylation. The results obtained with the phosphatase domain point mutants supports the notion that restoration of TCR signaling function correlates with a reduction in the level of phosphorylation of Lck on the negative regulatory site (Y-505). Moreover, only the wild-type and M2 EGFR/CD45 chimeras, which have demonstrable in vitro phosphatase activity, resulted in a reduction in phosphorylation of Lck Y-505, in addition to restoring TCR signal transduction. Additionally, overexpression of Csk has been demonstrated to result in down-modulation of TCR signaling (274). Thus, taken together, these results suggest that there is a dynamic equilibrium between Csk and CD45 in the regulation of the Fyn and Lck PTKs.

Function of the dual phosphatase domains in ligand-mediated negative regulation

Previous studies had shown that ligands to the EGFR/CD45 chimera could negatively regulate its function (311). Negative regulation required dimerization of the full length chimera, since co-expression of a truncated molecule consisting of only the EGFR extracellular and transmembrane domains prevented the ligand effect. The inhibitory effect of the truncated EGFR molecule is attributable to its ability to form heterodimers with the wild-type EGFR/CD45 chimera. Moreover, the identification of CD45 dimers (300), suggests an intermolecular mechanism of regulation of CD45 function in which either or both phosphatase domains may play a role. The finding that under some circumstances CD45 exists as a phosphoprotein (196, 197) suggests a model in which one of the phosphatase domains may regulate CD45 function by influencing its tyrosine phosphorylation status. In this model ligand would induce dephosphorylation of CD45.

The current study demonstrates that the potential phosphatase function of the distal phosphatase domain is not required for ligand-mediated negative regulation. If tyrosine phosphorylation of CD45 is a physiological occurrence and a means of regulation, then the proximal phosphatase domain may be involved in trans-dephosphorylation. However, tyrosine phosphorylation of the EGFR/CD45 chimera has not been observed utilizing a variety of stimuli (data not shown). Even the M1 + 2 EGFR/CD45 chimera, which would be incapable of autodephosphorylation, does not become tyrosine phosphorylated following stimulation of the TCR when expressed on CD45 positive cells (D. M. Desai and A. Weiss, unpublished observations). Thus, as CD45 tyrosine phosphorylation has only been observed <u>in vivo</u> following treatment of cells with tyrosine phosphatase inhibitors or by treatment of purified CD45 <u>in vitro</u> with a variety of kinases, the tyrosine phosphorylation and consequently, the phosphatase activity

of CD45 acting upon such phosphorylation may not be involved in ligand-mediated regulation (196, 197). Alternatively, dimerization of the EGFR/CD45 chimera may result in steric-inhibition of substrate access to the catalytic site, alter substrate specificity, or alter phosphatase activity of the EGFR/CD45 chimera .

CD45 is expressed in multiple isoforms and on a variety of cell types and as such it is not surprising that regulation of CD45 function is complex and may vary depending on the cellular context or developmental stage of the cell. The identification of CD45 ligands should shed valuable information on when and where CD45 function may be regulated.

Summary

Cell surface expression of CD45, a receptor-like protein tyrosine phosphatase, is required for T cell antigen receptor-mediated signal transduction. Like the majority of transmembrane-PTPases, CD45 contains two cytoplasmic phosphatase domains, whose relative in vivo function is not known. Site-directed mutagenesis of the individual cysteine residues in the catalytic sites of the two CD45 phosphatase domains indicates that the catalytic activity of the membrane-proximal domain is both necessary and sufficient for restoration of TCR signal transduction in a CD45-deficient cell. The putative catalytic activity of the distal phosphatase domain is not required for TCR-mediated proximal signaling. Moreover, in the context of a chimeric PTPase receptor, the putative catalytic activity of the distal phosphatase domain is not required for ligand-induced negative regulation of PTPase function. Additionally, the phosphorylation of the carboxy-terminal tyrosine of Lck, a site of negative regulation, is reduced only when CD45 mutants with demonstrable in vitro phosphatase activity are introduced into the CD45-deficient cells. These results demonstrate that the phosphatase activity of CD45 is critical for TCR signaling, and for preventing the accumulation of carboxy-terminal phosphorylated Lck molecules.

Experimental procedures

Constructs and antibodies

The EGFR/CD45 chimera has been described previously (311). The cysteine to serine point mutations were created in the EGFR/CD45 chimera backbone by oligonucleotide-mediated site-directed mutagenesis (Clonetech) and confirmed by nucleotide sequencing. The EGFR/CD45 chimera mutants were expressed by the pRK7 vector driven by the Cytomegalovirus long terminal repeat. The pAW-Neo3 vector has been described previously (311).

Antibodies used for flow cytometry were control mouse immunoglobulin γ2b (Zymed Lab.); Leu4 (anti-CD3ε, generously provided by R. Evans and the Memorial Sloan Kettering Institute); GAP8.3 (pan -anti-CD45, ATCC); and LA22 (anti-EGFR, Upstate Biotechnology Inc.). Monoclonal antibody (mAb) 108.1 recognizes an epitope on the extracellular domain of the EGFR and has been described elsewhere (318). The anti-Lck polyclonal antiserum and the anti-phosphotyrosine specific mAb 4G10 were purchased from UBI. The 4G10 mAb was used for immunoprecipitation and immunoblotting.

Cells and transfections

The human leukemic T cell line HPB.ALL and its derivative H45 ($CD45^-$) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (Intergen), penicillin, streptomycin, and glutamine (Irvine Scientific). EGFR/CD45 chimera expressing-cells were passaged in the above medium containing 2 mg/ml geneticin (GIBCO).

Transfections were performed as described earlier (311) with the exception that 25 μg of the expression vector containing the EGFR/CD45

chimera mutants was co-transfected with 2.5 μg of pAW-Neo vector containing the neomycin resistance gene.

Flow cytometry and measurement of intracellular calcium

Cells were stained at 4°C with saturating concentrations of primary antibody followed by fluorescein-conjugated goat anti-mouse antibody (Caltag). Cells were analyzed on a FACScan (Becton-Dickinson) as described (25). Intracellular free calcium levels were measured using the calcium sensitive dye Indo-1 (Molecular Bioprobes) as described (310). Cells (3 X 10^6/ml) were stimulated with the mAb Leu4 (1 μg/ml) or EGF (100 ng/ml) as indicated.

T cell receptor stimulation

Cell were harvested, washed twice with PBS and resuspended at 100 X 10^6/ml in PBS. Cell were incubated at 37°C for 15 minutes. Leu4 ascities (anti-CD3ε) at a 1:500 dilution was added to the cells, followed by a 2 minute incubation at 37°C. Cells were pelleted in a microfuge and lysed as described below.

Immunoprecipitations, SDS-polyacrylamide gel electrophoresis, and immunoblotting

Cells were lysed at 1 X 10^8/ml in 1% Nonidet-P40 (Boehringer Mannheim), 150 mM NaCl, and 10 mM Tris (pH 7.8) supplemented with 1 mM phenylmethylsulfonylfluoride, 2 μg/ml pepstatin A, 1 μg/ml leupeptin, 10 mM sodium pyrophosphate, 5 mM EDTA, 10 mM sodium fluoride, and 1 mM sodium orthovanadate. Lysates were incubated at 4°C for 30 minutes, followed by centrifugation at 13,000 X G for 15 minutes to remove insoluble material. Lysates were subject to immunoprecipitation with antibody coupled to protein A-

Sepharose beads (Pharmacia) for 2 hours at 4°C. Immune complexes were washed, resolved by SDS-PAGE (307) and transferred to nitrocellulose membranes (308). Immunoblotting was performed as described (140) using either alkaline phosphatase or horseradish peroxidase conjugated secondary antibodies with NBT/BCIP (Zymed Laboratories) or ECL substrates (Amersham), respectively.

Tyrosine phosphatase assay

Cells were harvested and lysed as above, except that sodium orthovanadate and sodium pyrophosphate were omitted from the lysis buffer. The wild-type and mutant EGFR/CD45 chimeras were immunoprecipitated using 1-2 μg of mAb 108 as described above. The immune complexes were washed five times with 1 ml of lysis buffer followed by 2 washes in 25 mM HEPES (pH 7.5) and 5 mM EDTA (pH 8.0). The samples are then divided into 5 equal fractions. 2X SDS-sample buffer was added to one fraction for immunoblotting, the other four fractions were resuspended in 50 μl phosphatase assay buffer (above with 10 mM DTT) plus substrate. The MBP substrate was prepared using purified c-Src (kindly provided by D. Morgan, Univ. Calif. San Fancisco) or v-abl (Oncogene Science). PNPP was purchased from Sigma and used at a final concentration of 10 mM. The phosphopeptides Lck Y-394 (IEDNEYTAREG) and Lck Y-505 (EDDFTATEGQYQPQP) were synthesized using standard FMOC chemistry by Dr. C. Turck. The reactions were placed at 37°C for the indicated times and then stopped by the addition of 50 μl 50 mg/ml BSA and 150 μl 25% trichloroacetic acid. The samples were vortexed and pelleted. The supernatant was assayed for free phosphate by either scintillation counting or by a colorimetric assay as previously described (207).

In vivo ^{32}P-orthophosphate labeling

Cells were washed twice with phosphate-free RPMI 1640 (Irvine Scientific) containing 10% dialyzed FCS. Cells were incubated at 2 X 10^6/ml in phosphate-free medium at 37°C for 1 hr. Cells were washed 2 more times with above medium and resuspended at 10 X 10^6/ml in phosphate free medium containing 1 mCi/ml ^{32}P-orthophosphate (ICN). They were then incubated for 6 hrs. at 37°C. The cells were harvested and washed once with serum-free phosphate-free medium. Cells were lysed as above. Lysates were incubated overnight with fixed Staphylococcus aureus (Calbiochem) and immunecomplexes were isolated as above. Immunecomplexes were separated by SDS-PAGE and transferred to nitrocellulose. Samples were immunoblotted to determine the relative levels of Lck protein. The bands of interest were excised and treated for 3 hrs. with 100 mg/ml cyanogen bromide in 70% formic acid. Samples were lyophylized, washed twice with deionized water and run on a 19% SDS-PAGE gel containing tricine (Sigma). The bands were visualized by autoradiography.

CHAPTER IV: CONCLUDING REMARKS

The PTPase family of enzymes is emerging as a large set of unique proteins expressed in all cell types. The structural diversity within the intracellular and transmembrane subfamilies of PTPases suggests that these enzymes will be involved in distinct cellular processes. The identification of CD45 as a hematopoietic cell-specific transmembrane PTPase involved in regulation of antigen receptor signal transduction established a function for CD45 (319). In this dissertation, the regulation of CD45 function was examined. The construction of a chimeric molecule consisting of a heterologous extracellular and transmembrane domain fused to the cytoplasmic domain of CD45 established that ligands to the extracellular domain could regulate phosphatase function. Moreover, inactivation of CD45 function dramatically modulated TCR signal transduction. Furthermore, site-directed mutational analysis of the cytoplasmic tandem phosphatase domains indicated that the enzymatic capacity of the membrane-proximal domain is required for CD45 function and consequently for TCR signal transduction. While this work, and that of others in analyzing the function and regulation of CD45 has enhanced our understanding of the role of CD45 in TCR signal transduction, a number of issues remain unanswered, and as a natural consequence of most scientific endeavors, new questions have been raised. The following discussion will address some of these issues and examine future directions for research toward a more complete understanding of CD45 function in T cell antigen receptor signal transduction.

Ligand-mediated regulation of CD45 function

The demonstration that a chimeric molecule consisting of the EGFR extracellular and transmembrane domains fused to the cytoplasmic region of

CD45 was negatively regulated by ligands to the EGFR, established that the function of the receptor-like PTPases could be regulated by ligands. The effect of ligand on the function of the EGFR/CD45 chimera and on TCR signal transduction is very dramatic and the inhibition of TCR signaling is almost complete in one minute. While the mechanism by which dimerization of the EGFR/CD45 chimera results in abrogation of TCR signal transduction has not been definitively demonstrated, some mechanisms have been ruled-out, and indirect evidence suggests that one model may be favored over another.

Although TCR signaling in EGF treated cells appears phenotypically similar to that of CD45-deficient cells, the number of EGFR/CD45 chimeric molecules expressed on the cell surface is not significantly different following the addition of EGFR ligands. Two models to account for the observation that ligand-mediated negative regulation of CD45 function requires dimerization of two wild-type molecules are depicted in figures 21 and 22. The first model is analogous to the mechanism by which transmembrane receptor PTKs are regulated, except that instead of trans-phosphorylation and activation, the EGFR/CD45 chimera would undergo trans-dephosphorylation and inactivation. The key feature of this model is the observation of CD45 tyrosine phosphorylation subsequent to TCR engagement. In light of the difficulties in detecting in vivo CD45 tyrosine phosphorylation in the absence of cellular treatment with phosphatase inhibitors, the trans-dephosphorylation model now is less appealing (see below).

The second model, although more simplistic, represents an efficient mechanism by which transmembrane PTPases could be negatively regulated. Dimerization the EGFR/CD45 chimera would result in a homodimeric complex that would be unable to interact with substrates as a result of steric obstruction of the catalytic site. Such a model does not require any post-translational modifications of the cytoplasmic domain nor does it even require that both

subunits of the homodimeric complex have phosphatase activity. This model is currently being evaluated by overexpressing the EGFR/CD45 M1 + 2 mutant in CD45-deficient cells that express the wild-type EGFR/CD45 chimera.

The negative regulatory effect of EGF on EGFR/CD45 function presumably represents the effect of the cognate CD45 ligand. However, with the exception of the transmembrane ligands with NCAM-like extracellular domains, which in a manner analogous to the neural cell adhesion molecules may bind to themselves in an intercellular interaction (179-181), the identification of ligands for the transmembrane PTPases has been difficult; especially for those PTPases which consist of unique extracellular domains lacking homology to other known proteins.

The extracellular domain of CD45 does not have any appreciable similarity with other proteins, thus not providing any clues to the nature of its ligand. A putative CD45 ligand, the B cell surface antigen CD22, has been identified (237). CD22 is able to bind to a number of CD45 isoforms, as well as another B cell molecule, in an interaction that is dependent on sialylation of the CD22 counter-receptors. The interaction of CD45 with CD22, does not appear to significantly influence CD45 function in the absence of non-physiologic manipulations (281), suggesting that CD22 may not be an important <u>in vivo</u> ligand for CD45.

The extracellular domain of CD45 contains two cysteine-rich sub-domains similar to those found in growth factor receptors, such as that for EGF and Insulin-like growth factor I (IGF-1) (285). The cysteine-rich domains of these receptors are involved in ligand binding. Although EGF and IGF-1 are soluble molecules, the EGFR and EGFR-like molecules, such as the drosophilia sevenless receptor, are able to bind membrane-bound ligands (285, 320). Thus, CD45 may bind membrane-bound and/or soluble ligands. Other confounding issues in identifying CD45 ligands relate to which isoforms bind ligands and when

and where the ligands are produced. One approach has been to isolate culture supernatants in which peripheral blood lymphocytes are stimulated with various agonists for varying periods of time, and then to examine the supernatants for TCR signal transduction inhibitory activity. Unfortunately, no activity of this nature was seen under the conditions tested (D. M. Desai and D. H. Chu; unpublished observations). The latter approach is biased toward the idea that the CD45 ligand is a soluble molecule; however, a more general line of experimentation would be to utilize an affinity reagent composed of the extracellular domain of CD45, such as a CD45-immunoglobulin fusion protein to probe tissues by in situ hybridization. The identification of a CD45 ligand(s) is absolutely critical for fully understanding CD45 function and its regulation in T cell development and during an immune response.

CD45 substrates

The identification of in vivo substrates of CD45 is essential for understanding its role in TCR-mediated cellular activation. The Lck and Fyn kinases are the only proteins that have been demonstrated to be differentially phosphorylated in CD45-positive and -negative cells, indicating that Lck and Fyn may be in vivo CD45 substrates. A number of proteins have been shown to be dephosphorylated by CD45 in vitro (52, 196). However, because PTPases have very high intrinsic enzymatic activity, it is not surprising that substrate specificity is not readily observed in vitro. Even determination of in vivo specificity has to be carefully evaluated. Overexpression of PTPases in heterologous cell systems has produced dramatic effects on protein tyrosine phosphorylation (162, 321-323).

One approach that has proven useful in identifying potential PTPase substrates utilizes the observation that mutation of the conserved catalytic

cysteine residue abrogates phosphatase activity. This mutation abolishes enzymatic function without distorting substrate recognition, thus enabling the isolation of PTPase-substrate complexes (157). This approach has been utilized with the Yob 2b and MKP1 PTPases to identify two unknown macrophage proteins and MAP kinase as putative substrates, respectively (199, 203). However, this methodology has not been effective with CD45. The EGFR/CD45-M1 + 2 mutant, in which the catalytic cysteine residue in both phosphatase domains were mutated to serine, did not bind any detectable tyrosine phosphoproteins. Similar results were obtained with the single phosphatase domain mutants (D. Desai, unpublished observations). These results suggest that the interaction between the phosphatase domains of CD45 and potential substrates is not a high affinity association. Moreover, these results suggest that Lck, which co-precipitates with wild-type CD45, may associate with CD45 through a mechanism other than one in which the phosphorylated C-terminal tyrosine residue of Lck interacts with the membrane-proximal phosphatase domain.

The Lck and Fyn kinases are hyperphosphorylated on their negative regulatory C-terminal tyrosine residues in CD45-deficient cells, indicating that Lck and Fyn may be in vivo substrates for CD45 and that their hyperphosphorylation is the basis for the TCR signaling defect in CD45-deficient cells. However, in two studies (264, 324), cross-linking of the TCR with the CD4 co-receptor molecule in CD45 deficient T cell lines paradoxically results in normal induction of proximal TCR signaling events, suggesting that Lck may be functional in some CD45-deficient cells. Since all of the CD45-deficient cell lines were derived as stable cell lines, the selection of cells in which the majority of membrane phosphatase activity is lost, probably results in a shift in the equilibrium between the PTKs and PTPases. This shift in equilibrium may result in the selection of cells in which

compensatory changes could have occurred, such as an increase in phosphorylation of Lck at the negative regulatory C-terminal tyrosine. The optimal method of analyzing the effect of CD45 loss of function would be through a conditional allele of CD45 in which CD45 function could be modulated within minutes to hours.

The EGFR/CD45 chimera is functionally a conditional allele of CD45, since the function of the EGFR/CD45 chimera can be negatively regulated by EGFR ligands. However, analysis of tyrosine phosphoproteins or the phosphorylation status of the C-terminal tyrosine of Lck has not yielded any convincing changes in the state of tyrosine phosphorylation of Lck (D. Desai, unpublished observations). The detection of changes in Lck phosphorylation on the C-terminal tyrosine may be technically limiting since a change in a small population of Lck molecules would not be discernible amidst the majority of Lck molecules which might not be affected. It is not known whether the CD4/CD8 associated or the non-CD4/CD8 associated pools of Lck are regulated by EGFR/CD45 chimera dimerization. Alternatively, an unidentified key signaling substrate may be a target for CD45. Thus, the question of CD45 substrates is still not resolved, although they are likely to include the Lck and Fyn PTKs.

The role of CD45 tyrosine phosphorylation

The concept of regulating PTPases by tyrosine phosphorylation is appealing and mechanistically an efficient way to coordinate the function of PTKs and PTPases. PTKs are both positively and negatively regulated by tyrosine phosphorylation. To date, the intracellular PTPases, PTP 1C and PTP 1D as well as the transmembrane PTPase CD45 have been demonstrated to be tyrosine phosphorylated *in vivo*. Stimulation of the TCR or the CSF-1 receptor results in PTP 1C phosphorylation (198; L. Tan and N. van Oers, personal

communication), while CD45 phosphorylation can be induced by TCR engagement (196, 197). Stimulation of the EGFR has been shown to induce PTP 1D tyrosine phosphorylation (166, 167). To date, only the enzymatic activity of PTP 1D, has been convincingly demonstrated to be modulated by in vivo tyrosine phosphorylation (166).

Unlike the tyrosine phosphorylation of PTP 1C or PTP 1D, the tyrosine phosphorylation of CD45 requires pre-treatment of cells with the phosphatase inhibitor PAO prior to TCR engagement (196). Moreover, in some instances, PAO treatment of cells alone can lead to tyrosine phosphorylation of CD45 (197). Another pharmacological agent, pervanadate, can induce tyrosine phosphorylation of numerous cellular proteins as well as induce distal T cell activation events, such as IL-2 production (325, 326). Pervanadate, like PAO, can also mediate CD45 tyrosine phosphorylation (D. M. Desai, unpublished data). One possible reason for the requirement of PTPase inhibitors to detect CD45 tyrosine phosphorylation is that CD45 is able to dephosphorylate itself in vitro (196). The use of phosphatase inhibitors to visualize CD45 tyrosine phosphorylation have precluded analysis of the effect of in vivo tyrosine phosphorylation of CD45 on its phosphatase activity. However, in vitro phosphorylation of CD45 utilizing the non-hydrolyzable phosphate donor ATPγS and either Lck or Csk results in enhancement of CD45 PTPase activity against the substrates poly-glu:tyr, Lck and Csk (197). Phosphorylation of CD45 by Csk resulted in approximately an 8-fold induction in CD45 phosphatase activity while Lck only augmented the activity by 2.5 fold. While it is reasonable to use phosphatase inhibitors and non-hydrolyzable analogs of ATP to detect CD45 tyrosine phosphorylation, it does raise the specter of concern regarding the physiological relevance of CD45 tyrosine phosphorylation, and such observations should be corroborated utilizing other methodologies.

Since the use of phosphatase inhibitors is to prevent auto-dephosphorylation by CD45, tyrosine phosphorylation should be detectable on CD45 molecules devoid of enzymatic activity. Analysis of the EGFR/CD45 chimera phosphatase domain mutants revealed no accumulation of tyrosine phosphorylation in the basal state. Surprisingly, treatment of cells with pervanadate resulted in tyrosine phosphorylation of the wild-type and single point mutants, but not the double phosphatase domain containing EGFR/CD45 chimera (D. M. Desai, unpublished data). Moreover, expression of the M1 + 2 EGFR/CD45 chimera in cells expressing endogenous CD45, does not result in tyrosine phosphorylation of the mutant chimeric molecule following TCR engagement (D. M. Desai, unpublished data). Although CD45 is capable of auto-dephosphorylation in vitro, that does not appear to be the basis for the requirement of phosphatase inhibitors to detect tyrosine phosphorylation of CD45. Interestingly, the double phosphatase domain mutant of the EGFR/CD45 chimera does not appear to be a good substrate for the PTK(s) responsible for CD45 phosphorylation.

The PTK responsible for in vivo CD45 phosphorylation is not clear. In vitro, the Csk and Lck kinase have been demonstrated to phosphorylate CD45; however, it has not been determined if the in vitro and in vivo sites of phosphorylation are the same (197). Utilizing the Lck-deficient Jurkat cell line, and pervanadate to induce CD45 tyrosine phosphorylation, indicates that in vivo CD45 phosphorylation requires Lck expression (D. M. Desai, unpublished data). Whether Lck directly phosphorylates CD45 or whether Lck regulates another PTK involved in CD45 phosphorylation is not known.

In addition to tyrosine phosphorylation, CD45 is also phosphorylated on serine residues. CD45, as well as the EGFR/CD45 chimera, are serine phosphorylated on undefined sites in resting T cells (284; D. M. Desai,

unpublised data). Treatment of cells with calcium ionophores results in a loss of serine phosphorylation and concomitant decrease in in vitro phosphatase activity (284). The effect of calcium ionophores on CD45 serine phosphorylation appears to be indirect since a 20 min. lag is required between ionophore treatment and serine dephosphorylation. How serine and tyrosine phosphorylation of CD45 influences its function and TCR signal transduction remain important unanswered questions.

The CD45 transmembrane phosphotyrosine phosphatase plays a critical role in the function of multipe cells of the immune system. A greater comprehension of the function and regulation of CD45 will allow a more indepth understanding of the regulation of immune system function, and may provide a means of therapeutic intervention in cases of autoimmunity or immunodeficiency.

CHAPTER V. BIBLIOGRAPHY

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