THE ROLE OF THE PTPH1-FAMILY OF PROTEIN TYROSINE PHOSPHATASES

IN T CELLS

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The αβ T cell receptor complex activates intracellular signaling cascades by coupling to several families of protein tyrosine kinases and protein tyrosine phosphatases. Following T cell receptor interactions with cognate peptide/major histocompatibility complexes, Src-family protein tyrosine kinases phosphorylate two tyrosine residues in a conserved amino acid motif termed the immunoreceptor tyrosine-based activation motif. The immunoreceptor tyrosine-based activation motifs are present in one or more copies in the cytoplasmic tails of the T cell receptor invariant chains, CD3 γ, δ, ε and ζ. In all T cell receptor signaling events, the immunoreceptor tyrosine-based activation motifs are transiently phosphorylated. We used a large-scale screen to identify protein tyrosine

phosphatase candidates dephosphorylating the T cell receptor ζ immunoreceptor tyrosine-based activation motifs. PTPH1 was identified in this screen, but its endogenous expression was difficult to detect in T cells. Based on sequence homology, PTPN4 was also considered as a putative regulator of phospho-ζ. T cell receptor ζ was bound and dephosphorylated by PTPN4. Overexpression of wild-type PTPN4 inhibited T cell receptor-induced AP-1 and NFκB activation in T cells. The overexpression of a substrate-trapping derivative of PTPN4 significantly augmented NFκB activation. This finding demonstrates a role for PTPN4 in the regulation of the NFκB pathway. PTPN4 knock-out mice were generated to assess the role of this PTPase in lymphocytes. This is the first description of a PTPN4-deficient animal model. No major developmental defects were observed in the PTPN4 knock-out mice. However, PTPN4-deficient animals had altered peripheral effector/memory T cell populations. While early T cell activation and T cell receptor-induced signal transduction events were normal, peripheral T cells from PTPN4-null mice secreted elevated levels of IL-4, IL-5, and IL-13 in the absence of PTPN4. This suggests that PTPN4 has a selective role in regulating effector T cell differentiation. Taken together, both PTPH1 and PTPN4 may regulate T cell receptor ζ phosphorylation, but the unique functions of these two protein tyrosine phosphatases indicate non-overlapping substrates.

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ABBREVIATIONS

Ab: antibody

AP-1: activator protein 1

APC: antigen presenting cell

APC: allophycocyanin

BCR: B cell receptor

CFSE: carboxyfluorescein succinimidyl ester

Csk: COOH-terminal Src kinase

CTLA-4: cytotoxic T lymphocyte antigen-4

DAG: diacylglycerol

DAP12: DNAX-activating protein of 12-kDa

DN: double negative

DP: double positive

ERK: extracellular signal-regulated kinase

ES: embryonic stem cell

FACS: fluorescence activated cell sorting

FcR: Fc receptor

FITC: fluorescein isothiocyanate

Gads: Grb2-related adaptor downstream of Shc

Grb2: growth factor binding protein 2

GST: glutatione *S*-transferase

HEK: human epithelial kidney cells

HRP: horseradish peroxidase

HSC: hematopoietic stem cell

IFN-γ: interferon gamma

Ig: immunoglobulin

IκBα: inhibitor of NFκB α

IL-2: interleukin-2

IP_3: inositol-1, 4, 5-triphosphate

ITAM: immunoreceptor tyrosine-based activation motif

JNK: c-Jun N-terminal kinase

kDa: kilodalton

KO: knock-out

LAT: linker for activation of T cells

LM-OVA: *Listeria monocytogenes*-OVA

mAb: monoclonal antibody

mTEP: testis-enriched phosphatase

MAPK: mitogen activated protein kinase

MFI: mean fluorescence intensity

MHC: major histocompatibility complex

NF-AT: nuclear factor of activated T cells

NFκB: nuclear factor-kappa B

NK: natural killer

NKR: natural killer receptor

OVA: ovalbumin

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PE: phycoerythrin

PEP: Pest-domain Enriched Tyrosine Phosphatase

PerCP: peridinin-chlorophyll-protein

PI3K: phosphoinositide-3-kinase

PI-4,5-P_2: phosphatidylinositol-4, 5-biphosphate

PKC: protein kinase C

PLCγ1: phospholipase C γ1

PMA: phorbol myristate acetate

PTB: protein tyrosine binding

PTK: protein tyrosine kinase

PTPase: protein tyrosine phosphatase

PTPH1: PTPH1/PTPN3

PTPN4: PTP-MEG1/mTEP

RAG: recombinase activating gene

RPTP: receptor-type PTPase

RT-PCR: reverse transcriptase polymerase chain reaction

SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis

SH2: Src homology 2

SHP-1: SH2-domain-containing protein tyrosine phosphatase-1

SLP-76: SH2-domain leukocyte protein of 76-kDa

SP: single positive

SPF: specific pathogen free

Syk: 72-kDa spleen tyrosine kinase

TACE: tumor necrosis factor α-convertase

T-bet: T-box expressed in T cells

TCR: T cell receptor

Tg: transgene

Th1/Th2: T helper cells 1/2

TNF: tumor necrosis factor

VCP: valosin-containing protein

WT: wild-type

ZAP-70: zeta-associated protein of 70-kDa

CHAPTER I

Introduction

T cells play an important role in the adaptive immune response against bacterial and viral pathogens. T cells recognize distinct foreign peptide fragments contained in the peptide-binding groove of major histocompatability complexes (MHC) on antigen presenting cells. The engagement of cognate peptide/MHC complexes by the T cell receptor (TCR) initiates intracellular signals. This signal transduction is comprised of a cascade of protein tyrosine phosphorylation events which activates multiple signaling pathways. This signaling is necessary for T cell effector functions such as cell proliferation, differentiation and cytokine production.

The T Cell Receptor Complex

The TCR is a multi-subunit complex comprised of the ligand binding TCR α and β heterodimer and the noncovalently associated invariant chains CD3 γ, δ, ε and TCR ζ (Figure 1) (1-3). The αβ TCR is capable of distinguishing foreign peptides from self-peptides bound in the groove of self-MHC molecules presented on the surface of antigen presenting cells (APC)(4). The recognition of foreign antigen by the TCR is then translated into intracellular signaling events by the invariant signaling complexes CD3 γε, δε and TCR ζζ.

The TCR α and β chains consist of a constant region and a variable region encoded by three types of genes, which are the variable (V) genes, diversity (D) genes, and joining (J) genes (3). DNA recombination of the VDJ genes is executed by the RAG-1/2 protein complex and generates diversity in the TCR repertoire, permitting the TCR to recognize a broad spectrum of antigenic peptides (3). The structure of the α and β chains includes an immunoglobulin-like domain in the extracellular region, a transmembrane span, and a short intracellular portion of approximately 9-12 amino acids (2, 3). The αβ TCR associates with the signaling subunits CD3 γε, δε, and TCR ζζ, which are also required for the surface expression of the complex (5, 6). The CD3 γ, δ, and ε invariant chains have similar structures containing an extracellular immunoglobulin-like domain, a transmembrane region, and a cytoplasmic portion containing an immunoreceptor tyrosine-base activation motif (ITAM). The cytoplasmic tail of CD3 ε also contains a basic-rich region and a proline-rich stretch. These motifs associate with a unique set of kinases and adaptor proteins (7, 8). In contrast to the CD3 subunits, each TCR ζ chain has a short nine amino acid extracellular portion, a transmembrane portion, and a cytoplasmic tail containing three ITAMs (9, 10). TCR ζ is a disulfide-linked homodimer, contributing six of the ten ITAMs in the TCR complex.

TCR surface expression results from an ordered assembly of the various TCR subunits in the endoplasmic reticulum (11, 12). The CD3 γ, δ, and ε invariant chains contain acidic residues in their transmembrane regions, and these residues interact with conserved basic residues in the transmembrane portion of TCR α and β. Their assembly is ordered such that one of the two conserved transmembrane basic residues in TCR α

interacts with acidic amino acids in the transmembrane region in CD3 δ and ε chains (12). Following formation of this complex, TCR β forms a heterotrimer with CD3 γ and ε (12). Lastly, a homodimer of the TCR ζ invariant chains is formed and interacts with a basic residue in the transmembrane of TCR α (12). The assembled TCR complex is then shuttled to the cell surface. Some reports have also demonstrated that the extracellular portions of the CD3 heterodimers interact with the extracellular regions of the αβ TCR (9). These studies indicated that specific extracellular interactions influence the organization of the TCR/CD3 complex, but the sites that contact each other are still unknown (9).

ITAMs of the T Cell Receptor Invariant Chains

The CD3 γδε and TCR ζ invariant chains form the signaling subunits of the TCR complex and are important for TCR-mediated functions. The cytoplasmic tail of each invariant chain contains one or more copies of a short conserved motif known as an ITAM. A total of ten ITAMs are present in the entire TCR complex, with CD3 γ, δ, and ε each containing a single ITAM while TCR ζ has three ITAMs per chain (Figure 1). Each ITAM contains a tyrosine-based amino acid sequence of YxxL/I x_{6-8} YxxL/I (1, 13). The ITAMs are critical in initiating the TCR signaling pathway, with the two tyrosine residues transiently phosphorylated following TCR ligation.

Signal transduction mediated by the TCR is ITAM-dependent. The ITAM motifs are also present on the signaling subunits of other immunoreceptors, such as the B cell

receptor (BCR), certain NK activating receptors (NKR), and a subset of Fc receptors (FcR)(14, 15). Maintained through evolution, the ITAMs involved in immunoreceptor signaling likely arose from a common origin (14). In addition, ITAM-containing molecules have also been found in non-immunoreceptor signaling pathways. These receptors include adhesion molecules, lectin receptors, and chemokine receptors (Table 1)(15). While these ITAM-containing molecules recruit similar protein tyrosine kinases, the genomic sequences are evolutionarily distinct from “classical” ITAMs that are associated with immunoreceptors (14). The identification of ITAM-containing proteins in different viruses has also been reported (Table 1) (16-20). These viral proteins interfere with ITAM-based signaling of the host. Therefore, the virus is capable of evading the immune response and prolonging its survival in the host.

Table 1. Distribution of ITAM-Containing Proteins

Immunoreceptors	**ITAM subunit**
TCR	CD3γδε , TCR ζ
BCR	Igα, Igβ
FcR	FcRγ
NKR	DAP12
Non-immunoreceptors	**ITAM subunit**
PSGL-1 adhesion molecule	moesin, ezrin
CXCR4 chemokine receptor	TCR ζ
C-type lectin receptors	Dectin-1, -2
Viruses	**ITAM subunit**
simian immunodeficiency virus (SIV)	Nef accessory protein
Epstein-Barr virus (EBV)	LMP2A membrane protein
Kaposi's sarcoma-associated herpesvirus (KSHV/HHV8)	K1 Type I membrane glycoprotein
murine mammary tumor virus (MMTV)	gp52 envelop protein
Bovine Leukemia Virus (BLV)	gp30 transmembrane protein
Hanta-associated Pulmonary Hanta Virus	G1 surface glycoprotein

Since the TCR ζ homodimer contains six ITAMs, it is presumed to be the predominant signaling subunit of the TCR complex (Figure 1). It is the most intensely phosphorylated subunit of all the CD3 invariant chains (21, 22). TCR ζ is a 16-kDa protein that can appear as two distinct phosphorylated forms of 21- and 23-kDa following

TCR ligation (21). The 21-kDa phosphorylated form of ζ, also known as p21, is constitutively phosphorylated in thymocytes and peripheral T lymphocytes (23, 24). Weak, nonspecific interactions between the TCR and MHC molecules maintain the p21 form of TCR ζ (24-26). Following activation of the TCR, ζ is phosphorylated on all three ITAMs, and this phosphorylated form migrates at 23-kDa, p23 (24). The tandem SH2 domains of ZAP-70 bind and stabilize the two phosphorylated tyrosine residues in each ITAM (27). The 21-kDa phospho-ζ intermediate is bound by a pool of inactive, unphosphorylated ZAP-70, while p23 is associated with active phospho-ZAP-70 (23, 24, 28). The binding of active ZAP-70 initiates downstream signaling pathways (24). Various roles in T cell biology and function have been linked to the differential levels of p21 versus p23, such as thymocyte selection, T cell activation, and T cell survival (29, 30). More recently, studies have demonstrated that the TCR ζ ITAMs and the CD3 γδε ITAMs can contribute similar functions in TCR signaling (31). The elimination of the ζ ITAMs by amino acid substitutions did not affect TCR signal transduction (32).

Proximal T Cell Receptor Signal Transduction

TCR ligation induces a cascade of protein tyrosine phosphorylation events where the transient phosphorylation of the TCR ITAMs is one of the earliest events. Phosphorylation of the ITAMs leads to the activation of multiple signaling proteins and the initiation of different signaling pathways (Figure 2). Engagement of the αβ TCR induces a conformational change in CD3 ε (7, 33). The conformational change exposes a proline-rich region of CD3 ε which is then bound by the SH3 domain of the Nck adaptor

protein (7). In addition, Lck and Fyn, two Src-family protein tyrosine kinases (PTKs), relocalize to the TCR complex (34). Following their catalytic activation, Lck and Fyn phosphorylate the two tyrosine residues in each ITAM. While the myristoylation and palmitoylation target Lck and Fyn to the cellular membrane, the location of these two kinases in the cell do not overlap (35). Abundant at the plasma membrane, Lck is capable of interacting with the cytoplasmic tails of the coreceptors CD4 and CD8 (36). Both Fyn and Lck have a unique N-terminal region followed by an SH3 domain, and SH2 domain, a kinase domain, and a C-terminal negative regulatory domain (34). In the catalytically inactive state, the SH2 domain of Lck binds to its own phosphorylated C-terminal tyrosine residue 505 (Y505) (37). Dephosphorylation of Y505 by CD45, a receptor protein tyrosine phosphatase (RPTP), induces Lck to undergo a conformational change (38, 39). Protein tyrosine phosphatases (PTPases) enzymatically remove phosphate from tyrosine phosphorylated proteins. Subsequently, Lck transphosphorylates the tyrosine residue 394 (Y394) in the activation loop and becomes catalytically active (37). Active Lck phosphorylates the two tyrosine residues in the ITAMs (40, 41). The bi-phosphorylated ITAMs serve as high-affinity binding sites for the tandem SH2 domains in ZAP-70, a Syk-family PTK (23, 27, 42, 43). Lck potentiates the enzymatic activity of ZAP-70 (44, 45). ZAP-70 is phosphorylated on tyrosine residue 493 (Y493) by Src PTKs. It then autophosphorylates on residues 292, 315, and 319 (Y292, Y315, Y319) leading to its full activation (45-47). ZAP-70 then phosphorylates multiple downstream signaling molecules, including LAT, SLP-76, and PLCγ1 (47, 48).

The tyrosine phosphorylation of linker for activation of T cells (LAT) and SH2-domain leukocyte protein of 76-kDa (SLP-76) is a critical event in the signaling pathway. These two phosphorylated proteins act as linker or adapter proteins in multi-protein complexes. LAT is a palmitoylated transmembrane protein bound to the plasma membrane (49). Nine conserved tyrosine residues are present in the cytoplasmic region of LAT, and phosphorylation of the four membrane-distal tyrosine residues is required for downstream signaling. Multiple proteins containing SH2 domains bind to the phosphorylated tyrosines in LAT. Grb2 and Gads are two adapter proteins that bind to phospho-LAT via their SH2 domains (48). The two SH3 domains of Grb2 and Gads, which flank their SH2 domain, allow these molecules to bind other proteins (48). The SH3 domain of Gads binds a proline-rich region in the SLP-76 adapter protein (50). ZAP-70 proceeds to phosphorylate the amino-terminal tyrosine residues in SLP-76. Subsequently, phosphorylated SLP-76 becomes a high-affinity ligand for multiple SH2 domain-containing proteins, such as Vav, Nck, and the Itk PTK. These proteins regulate the rearrangement of the actin cytoskeleton (48). This complex of proteins also regulates the activation of PLCγ1 (47). PLCγ1 binds phospho-LAT with its SH2 domain and becomes catalytically active following its tyrosine phosphorylation. PLCγ1 hydrolyzes phosphatidylinositol-4, 5-biphosphate (PI-4,5-P_2/PIP_2) to produce diacylglycerol (DAG) and inositol-1, 4, 5-triphosphate (IP_3)(47). Ras activation by DAG is mediated through the intermediate RasGRP and leads to the initiation of the MAPK pathway (47). PKC activity is also regulated by DAG. IP_3 mediates the release of calcium from intracellular stores (47). All these events are critical in the activation of multiple signaling pathways which eventually lead to the activation of the transcription factors NF-AT, AP-1, and

NFκB (Figure 2). Finally, transcriptional activation results in the induction of T cell activation and T cell effector functions.

T Cell Development

Hematopoietic stem cells (HSC) migrate from the bone marrow to the thymus where they develop into αβ T cells. T cell development occurs in several distinct stages that are defined by specific cell surface markers. HSCs enter the thymic cortex as double negative (DN) cells lacking the surface expression of CD3, CD4, and CD8. The DN cells transition through four double negative stages that are defined by the surface expression of CD44 and CD25 (51). At DN1, the cells only express the surface marker CD44 ($CD44^{+}CD25^{-}$). At the DN2 stage, cells upregulate CD25 on the cell surface ($CD44^{+}CD25^{+}$) in response to IL-7, which is secreted by the thymic microenvironment (51). The IL-7 cytokine signals early thymocytes to survive and proliferate. CD44 expression is reduced as the cells transition from the DN2 stage to the DN3 stage. During this transition ($CD44^{low}CD25^{+}$), the recombinase activating genes (RAG) regulate the rearrangement of the TCR β locus (3, 52). The rearranged TCR β chain is expressed on the cell surface in a complex with pre-TCR α (pTα), a TCR α-like chain (3, 52). The pre-TCR (TCRβ/pTα) associates with the invariant CD3 and TCR ζ signaling subunits, and signal transmission through this complex induces the cells to enter cell cycle and to proliferate (53-55). The successful rearrangement of TCR β triggers the cells to enter the DN4 stage, where CD25 surface expression is downregulated ($CD44^{-}CD25^{-}$) (51). At this developmental stage, the expression of RAG is reduced, halting the rearrangement of

TCR β, and the coreceptors CD8 and CD4 are expressed on the cell surface. These double positive (DP; $CD8^+CD4^+$) cells increase RAG expression and begin rearrangement of the TCR α locus (52). A successful rearrangement of the TCR α locus produces a complete αβ TCR complex on the cell surface ($CD3^+CD8^+CD4^+$)(52). In the thymus, the DP thymocyte population constitutes about 80% of all cells, but only a small proportion of these DP cells are selected and mature into functional T cells (56).

Approximately 95% of the DP population is eliminated during thymocyte selection (56). The DP thymocytes are selected based on the capacity of the TCR to recognize self-peptide/MHC class I or II during positive and negative selection. Positive selection produces single positive (SP) thymocytes from the DP population (56). DP thymocytes capable of recognizing self-peptide/MHC class I or II complexes with moderate affinity are positively selected and mature into $CD8^+$ or $CD4^+$ SP T cells, respectively. Positively selected SP T cells mature and have increased surface expression of the TCR complex. Mature CD8 or CD4 T cells then exit the thymus and migrate to the secondary lymphoid organs. The commitment to CD4 or CD8 T cells is not entirely clear. Some reports suggest that the strength of TCR signaling influences the selection of T cells (56, 57). Conversely, cells unable to recognize self-MHC molecules die by necrosis. Cells that bind self-peptide/MHC complexes with high affinity are negatively selected, and apoptosis is induced.

The contributions of the TCR signaling subunits to T cell development have been studied using different mutant animal models. Targeted-deletion of each invariant chain

revealed a role for these molecules in thymocyte development. Thymocyte numbers are significantly decreased in CD3 γ-deficient mice. In the absence of the CD3 γ chain, the pre-TCR is not expressed on the cell surface and a block in the DN3 stage ($CD44^-CD25^-$) is observed (58). In addition, a role for the CD3 γ ITAM in positive selection has also been described with the use of TCR transgenic mice (59, 60). A defect in the DN3 stage has been demonstrated in CD3 ε-deficient mice (61, 62). The association of TCR β with the pTα is incomplete in the absence of CD3 ε. This defect prevents the expression of the pre-TCR on the cell surface inhibiting the cell from transitioning to the DN4 stage. Unlike CD3 ε and γ, a deficiency in CD3 δ resulted in a partial block at the DP stage (63). CD3 δ-deficient mice had normal numbers of DP cells, but the SP population was greatly reduced. The targeted deletion of TCR ζ also led to a block at the DP stage in thymocyte development (64-67). The number of DP and peripheral T cells was significantly decreased in TCR ζ-deficient mice. In summary, the TCR invariant chains have unique functions during thymocyte development, primarily through their role in TCR assembly and surface expression.

Differentiation of Effector T cells

In the periphery, TCR stimulation induces T cell activation. Early activation is characterized by the upregulation of CD69, an early activation marker, and CD25, the high-affinity IL-2Rα chain. T cells then undergo proliferation in response to IL-2 and perform different effector functions, including cytokine production. $CD8^+$ and $CD4^+$ T cells have distinct effector functions. The activation of CD8 T cells leads to their

differentiation into cytotoxic T lymphocytes (CTLs). These cells are an important component of the adaptive immune response, as they kill virus-infected cells. The effector functions of CTLs are mediated by the expression of FasL and the secretion of the cytotoxins, perforin and granzymes, which induce cell death in the targeted cell. Furthermore, the production of IFN-γ and TNF-α cytokines by CD8 T cells further assist in the immune response by stimulating other effector cells or directly acting on the pathogen (68).

CD4 T cells differentiate into either T helper 1 or T helper 2 (Th1 or Th2) cells. Th1 and Th2 cells have different effector functions and are defined by the distinct cytokines that they produce (69). Th1 cells are important for the immune response against intracellular pathogens. CD4 commitment to Th1 depends on the cytokines IFN-γ and IL-12. Simultaneously, IL-12 prevents the differentiation of Th2 cells. The expression of the T-bet (T-box expressed in T cells), a T-box transcription factor, is specific for Th1 cells and critical for IFN-γ production (69). Following differentiation, IL-12 and IL-18 or stimulation through the TCR induces IFN-γ, TNF-α, and IL-2 production. These cytokines are capable of inhibiting viral replication and assist in the CTL response. In contrast, Th2 cells participate in the elimination of extracellular parasites, such as helminths. Production of IL-4 by bystander cells promotes the differentiation of Th2 cells. IL-4 inhibits the development of Th1 cells. While numerous transcription factors are activated during Th2 differentiation, GATA3 and c-Maf are specifically expressed in Th2 cells (69). The GATA3, a zinc-finger transcription factor, is necessary for the production of IL-4, IL-5, and IL-13 cytokines, which are characteristic of Th2 cells. On

the other hand, c-Maf is upregulated by GATA3 and transactivates IL-4. The Th2-specific cytokines generates a humoral immune response by regulating B cell functions (69).

In our studies, stimulated peripheral T cells isolated from PTPN4 knock-out mice produced increased amounts of Th2-specific cytokines compared to wild-type cells. These findings suggested a potential effect in the transcriptional activation of IL-4. TCR ligation on differentiated Th2 cells induces the transcription of IL-4. TCR-induced IL-4 production is controlled by multiple regulatory elements in the 5' promoter region (70). In response to TCR stimulation, GATA-3 is a weak transactivator of IL-4 (70). Chromatin remodeling of the IL-4 locus may be regulated by GATA-3, allowing other transcription factors to bind the promoter region. Both nuclear factor of activated T cells 1 (NF-AT1) and NF-AT2 are required for IL-4 production (70). Activator protein 1 (AP-1) also participates in complexes bound to the NF-AT-binding sites in the IL-4 promoter (70). Furthermore, the NFκB family members p65 and c-REL have been reported to enhance the activation of IL-4. During Th2 differentiation, CD28 costimulation can also induce NFκB activity, which in turn, upregulates GATA3 expression (70).

Protein Tyrosine Phosphatases

Intracellular signaling events are regulated by various protein modifications. For example, the phosphorylation of serine, threonine, tyrosine, and histidine residues is a post-translational modification that plays a key role in controlling multiple cellular

processes. While approximately 30% of intracellular proteins are phosphorylated, protein tyrosine phosphorylation makes up only a small proportion of all phosphorylation events (0.01 – 0.05% of all protein phosphorylation)(71). Yet, tyrosine phosphorylation events are essential in regulating numerous cellular functions, such as cell growth, cell cycle, gene transcription, and immune functions (72, 73). The dysregulation of tyrosine phosphorylation has been observed in different human diseases.

Protein tyrosine phosphorylation is a reversible covalent modification regulated by the opposite actions of PTKs and protein tyrosine phosphatases (PTPases). PTPases are a family of enzymes that remove the phosphate from phosphotyrosine residues (74). Approximately 107 genes encoding putative PTPases have been identified in the human genome, and 105 of these genes have a murine equivalent (75-77). The PTPase superfamily is divided into four major subfamilies that include low-molecular weight (LMW) PTPases, cdc25 phosphatases, dual specificity phosphatases (DSPs), and classical PTPases (74, 77). DSPs have a broad substrate specificity capable of targeting phosphoserine, phosphothreonine, and phosphotyrosine, whereas classical PTPases specifically dephosphorylate tyrosine residues (74, 77). Classical PTPases are further categorized as receptor-like PTPases (RPTPs) or non-transmembrane PTPases (78). Despite the low homology in the amino acid sequence between these four families of PTPases, the different PTPases share similarities in the secondary structure of the catalytic domain and loop structure of the active site, thereby utilizing parallel mechanisms of dephosphorylation (79-82).

Classical PTPases are characterized by a conserved catalytic domain of approximately 250 – 300 amino acids (78). The catalytic pocket has a depth of 9 Å, which determines specificity for phosphorylated tyrosine residues (79, 83). Sequence alignment of known PTPase domains revealed ten conserved motifs, all of which are involved in the folding of the catalytic pocket or the recognition and dephosphorylation of the substrate (84). PTPases are defined by an active site motif HCxxGxxR, which is also known as the signature motif. This highly conserved sequence forms the phosphate-binding loop at the base of the catalytic pocket (74, 84, 85). During the first step of the catalytic reaction, the active site cysteine carries out a nucleophilic attack on the phosphorylated tyrosine (85, 86). The arginine residue in the active site is important in binding the substrate and stabilizing the phospho-enzyme intermediate (87). Binding of the phosphotyrosine triggers a conformational change. A conserved WPD loop motif in the PTPase encloses the substrate in the catalytic pocket (83, 88). The invariant aspartic acid in the WPD motif participates in the second step of the catalytic reaction, functioning as a general acid and base by facilitating the protonation of the phenolic leaving group, which results in a phospho-enzyme intermediate (88, 89). Acting as a general base, the aspartic acid subsequently hydrolyzes the phospho-enzyme intermediate (89). Variations in the amino acid sequences of the catalytic domains segregate PTPases into 17 different subdivisions (90). Interestingly, PTPases that share sequence homology in the PTPase domain also contain similar flanking domains in the full-length protein (90).

The substrate specificity of classical PTPases is not only determined by the catalytic domain but also by the combination of unique flanking domains, and such domains are used to further distinguish different families of PTPases (77, 91). As modular enzymes, PTPases are similar in structure to PTKs but greatly differ from serine/threonine protein phosphatases (92). Several of the domains present in PTPases are also located in PTKs, such as SH2, SH3, and PH domains (77).The different domains localize PTPases to specific subcellular sites and facilitate protein-protein interactions with regulatory or target proteins. In addition, multiple flanking domains in one phosphatase increase substrate specificity by concurrently recognizing distinct ligands (92).

Other flanking domains that are present in PTPases include the FERM and PDZ domains. These domains are present in the PTPH1, PTPN4, and PTP-BAS PTPases. The FERM domain, approximately 300 amino acids in length, is homologous to the N-terminal region of band 4.1 superfamily members (93). This domain targets proteins to the plasma membrane (94). There are three modular subdomains formed by the FERM domain and are known as F1, F2, and F3 (94). Each of the subdomains resembles the structure of known proteins. F1, F2, and F3 are structurally similar to ubiquitin, acyl-CoA binding proteins, and PTB/PH/EVH1 domains, respectively (93, 94). PDZ domains are comprised of 80-100 amino acids and often bind other PDZ-containing proteins (95). In addition, PDZ domains normally interact with the C-terminal region of transmembrane receptors and channel proteins. Most proteins containing a PDZ domain have been found

localized and clustered at the plasma membrane (95). While PTPH1 and PTPN4 contain one PDZ domain, PTP-BAS consists of five PDZ domains.

Generation of PTPase Substrate-Trapping Mutant

The biological functions of PTPases in different signaling pathways have not been as well-characterized as PTKs. This is partially due to the difficulty in identifying PTPase substrates. The generation and use of substrate-trapping mutants has helped in the identification of some PTPase substrates. In one type of substrate-trap, the catalytic activity of the PTPase is eliminated by mutating the active site cysteine to a serine residue (74). This mutation allows the PTPase to bind the phosphotyrosine, but prevents the nucleophilic attack mediated by the catalytic cysteine residue (96-98). A second type of substrate-trapping mutant was identified with the phosphatase PTP1B by mutating different invariant amino acids involved in the catalytic reaction (87). Substitution of the invariant aspartic acid with an alanine significantly reduces the catalytic activity and protects the phosphorylated tyrosine substrate (87). This substrate-trapping mutant is capable of forming a stable substrate-enzyme complex more efficiently than the cysteine-serine mutation (87). Additional amino acid substitutions have been described that improve the substrate-trapping capabilities of different PTPases. The double mutants that have been used include a cysteine-serine, glutamine-alanine, or tyrosine-phenylalanine mutations in combination with the aspartic acid-alanine mutation (99). The ability to identify PTPase substrates with substrate-trapping mutants has improved the understanding of the physiological functions of PTPases.

Dephosphorylation of TCR ζ by Protein Tyrosine Phosphatases

A cascade of protein tyrosine phosphorylation events is initiated following TCR activation. These transient phosphorylation events are partly regulated by the catalytic activities of PTPases suggesting a role in limiting the strength and duration of signaling. T cells express approximately 68 putative PTPase genes that were identified in the human genome (100). Reports using different techniques have implicated multiple PTPases in the positive or negative regulation of T cell activation (Table 2)(101-103).

TABLE 2. PTPases that Regulate TCR Signal Transduction

PTPase	Gene Symbol	Regulatory Role in TCR Signal Transduction	Substrate
CD45	PTPRC	Positive	Inhibitory tyrosine of Src PTKs
CD148/DEP1	PTPRJ	Negative	LAT, PLC-γ1
HePTP	PTPN7	Negative	ERK
PEP/LYP	PTPN22	Negative	Positive regulatory tyrosine of Src PTKs, ZAP-70
PTPH1	PTPN3	Negative	TCR ζ ITAMs
PTP-MEG1/TEP	PTPN4	Negative	TCR ζ ITAMs
PTP-PEST	PTPN12	Negative	Shc, Cas, Pyk2, FAK
SHP-1/PTP1C	PTPN6	Negative	Positive regulatory tyrosine of Src PTKs, ZAP-70
SHP-2/PTP1D	PTPN11	Positive	Ras/ERK activation by unknown substrate
LMPTP	ACP1	Positive	ZAP-70 (Y292)

Since ζ phosphorylation is detected shortly after TCR engagement and is transient in nature, the PTPases responsible for regulating ITAM phosphorylation have been of interest. Four distinct PTPases have been described based on their ability to dephosphorylate the TCR ζ ITAMs. SHP-1 is a cytoplasmic PTPase that is specifically expressed in hematopoietic cells and contains N-terminal tandem SH2 domains followed by a C-terminal PTPase domain (101). A point mutation in the SHP-1 gene was identified in the immunodeficient *motheaten* mice. This strain of mice revealed a role for SHP-1 in immune cells (104-106). Thymocytes from these mutant mice had increased levels of tyrosine-phosphorylated proteins, including the TCR ζ ITAMs, and hyperproliferated in response to TCR stimulation and IL-2 (107, 108). Further studies demonstrated that SHP-1 dephosphorylates ZAP-70, SLP-76, and the positive regulatory tyrosine (Y394) in Src PTKs (107-110). SHP-2 is another PTPase that contains tandem SH2 domains in the N-terminus (101). SHP-2 is highly homologous to SHP-1, but these two PTPases have significantly different physiological functions. A positive role in TCR signaling has been described for SHP-2, since its catalytic activity is required for the activation of Ras/ERK following TCR stimulation (111). Facilitated by an interaction with the cytotoxic T lymphocyte antigen-4 (CTLA-4) receptor, SHP-2 has also been reported to target TCR ζ and ZAP-70 and negatively regulate TCR-mediated signaling (112, 113). However, the results demonstrating a negative regulatory role for SHP-2 are inconsistent with the other reports (111-114).

The transmembrane PTPase CD45 contains tandem catalytic domains in the cytoplasmic tail, which is characteristic of most RPTPs (91). Only the membrane-

proximal PTPase domain is catalytically active. Ligand-induced dimerization of CD45 keeps the enzyme in an inactive state (115). The catalytic domain of one CD45 molecule binds the wedge motif in another CD45 molecule inhibiting PTPase activity (115). CD45 dephosphorylates Y505, the negative regulatory tyrosine in Lck, resulting in kinase activation and subsequent reduction in ζ phosphorylation (38, 39). CD45-null thymocytes exhibited decreased levels of phospho-ζ supporting a positive regulatory role in TCR signaling (116). However, one report suggested that CD45 dephosphorylated TCR ζ (98). This negative role in TCR signaling conflicts with the former studies.

Negative regulation of TCR-mediated signal transduction has also been described for the cytoplasmic PTPase PEP, Pest-domain Enriched Tyrosine Phosphatase (101). A constitutive association between the proline-rich domain of PEP and the SH3 domain of Csk localizes PEP to Src and ZAP-70 PTKs (117, 118). PEP is then capable of dephosphorylating the positive regulatory tyrosine in Lck (Y394) and ZAP-70 (Y493) PTKs, thereby inhibiting their kinase activities (119, 120). More recently, the use of substrate-trapping derivatives of PEP identified the phosphorylated ζ ITAMs as substrates (118, 119). However, these reports are inconsistent with previous studies demonstrating that PEP does not target phospho-ζ (120). Furthermore, the catalytic domain of PEP did not associate with phospho-ζ in a substrate-trap screen (121). A targeted deletion of PEP in mice revealed a role for this PTPase in activated T cells (122). Naïve T cells exhibited normal TCR-mediated signaling and function in the absence of PEP. However, an accumulation of the effector/memory T cell subset was observed in

these mice. This subpopulation of cells demonstrated increased proliferation in response to TCR stimulation.

Due to the conflicting data in the existing reports, we attempted to identity any PTPases capable of regulating ζ phosphorylation. With two different approaches, PTPH1/PTPN3 (PTPH1) was identified as a PTPase capable of complexing with and dephosphorylating the ζ ITAMs (121). Previous studies measuring transcriptional activation had revealed a negative regulatory role for PTPH1 in TCR signal transduction, further supporting our findings (121, 123, 124). PTP-MEG1/PTPN4 (PTPN4), a PTPH1-family member, was also implicated in the negative regulation of TCR-mediated signaling in those same studies (123, 124). However, a substrate was not identified for either PTPase. PTPH1 and PTPN4 are cytoplasmic PTPases that share a high degree of homology in the amino acid sequence (125, 126). These are the only two PTPases that contain an N-terminal FERM domain followed by a central PDZ domain and a C-terminal catalytic domain (91).

PTPH1 is a PTPase of approximately 120-kDa. PTPH1 localization to the plasma membrane is facilitated by its FERM domain (124). Deletion of the FERM domain reduced the ability of PTPH1 to inhibit TCR signal transduction (123). In the course of our studies, another group reported the generation of PTPH1 knock-out mice (127). PTPH1-deficient mice exhibited normal T cell development, TCR signaling, and T cell functions (127).

PTPH1 can interact with TACE, tumor necrosis factor α-convertase (128). The PDZ domain of PTPH1 bound the C-terminal region of TACE, where the last five amino acids were necessary for the interaction (128). TACE is responsible for the cleavage of membrane-bound TNF-α into a soluble cytokine. The association of PTPH1 negatively regulated the activity of TACE and prevented the release of TNF-α from the membrane (128). However, how PTPH1 regulates TACE function was not determined. PTPH1 also interacts with 14-3-3β, a protein involved in cell cycle control (129). Phosphorylation of two serine residues on PTPH1 was required for its association with 14-3-3β (129). Phosphorylation on serine residue 359 was mediated by the serine/threonine kinase C-TAK1 (129). Currently, the function of this interaction is unknown.

The ATPase vasolin-containing protein (VCP) is also involved in cell cycle regulation and is a substrate of PTPH1 (130). The overexpression of PTPH1 in the NIH3T3 fibroblast cell line resulted in the disruption of cell growth (130). This result was due to the ability of PTPH1 to complex with VCP (130). VCP has been identified as a regulator of cell cycle. Previous reports have demonstrated that the translocation of VCP to the nucleus is required for cell cycle, and the phosphorylation of tyrosine 805 is necessary for this redistribution (131). The two C-terminal tyrosine residues 796 and 805 in VCP were dephosphorylated by PTPH1 (130). Therefore, PTPH1 inhibits cell growth by dephosphorylating VCP, preventing its translocation to the nucleus. Interestingly, TCR stimulation results in the phosphorylation of the two C-terminal tyrosine residues in VCP (132, 133).

PTPN4 is a 116-kDa PTPase that was originally cloned from a megakaryoblastic cell line (134, 135). PTPN4 has also been reported to localize to the cytoskeletal region (124, 136). Similar to PTPH1, PTPN4 also appeared to have a role in controlling cell proliferation. The overexpression of PTPN4 in COS7 cells inhibited cell growth (136). Furthermore, a catalytically inactive mutant of PTPN4 also disrupted cell growth but to a lower degree (136). PTPN4 has been implicated in potential roles in the brain and testis. One report demonstrated that the PDZ domain of PTPN4 interacted with the C-terminal tail of the glutamate receptors (GluR) δ2 and GluRε1, which are expressed in the brain (137). The phosphorylation of GluRε1 by Fyn was enhanced by PTPN4, but the mechanism of this effect was not determined (137). PTPN4 (murine TEP, testis-enriched phosphatase) was also cloned from testes (138). Two different transcripts of 3.2 and 3.7 kb were highly expressed in the testis (138). By Northern blotting, the 3.2 kb transcript was exclusive to the testis, while the 3.7 kb transcript was detected in both the testis and brain (138). Furthermore, the expression of PTPN4 was increased in adult mice compared to young mice and limited to the seminiferous tubules which contain germ cells (138).

PTPH1 and PTPN4 were previously shown to inhibit TCR-mediated TCR signaling (123, 124). In addition, we reported the identification of PTPH1 from a substrate-trap screen as the predominant PTPase in the library that could complex with phospho-ζ (121). Our findings demonstrated that the catalytic domain of PTPH1 was capable of associating with phosphorylated TCR ζ. In addition, PTPH1 directly dephosphorylated TCR ζ. These results will be discussed further in Chapter III. Since the endogenous expression of PTPH1 was difficult to detect in lymphocytes, we considered

additional PTPases to be involved in the regulation of ζ. PTPN4 was a candidate PTPase, since it shares a high degree of homology in the amino acid sequence with PTPH1. Furthermore, PTPN4-deficient mice were utilized to investigate the physiological function of PTPN4 in T cell development, TCR-mediated signal transduction, and T cell effector functions and will be presented in Chapter IV.

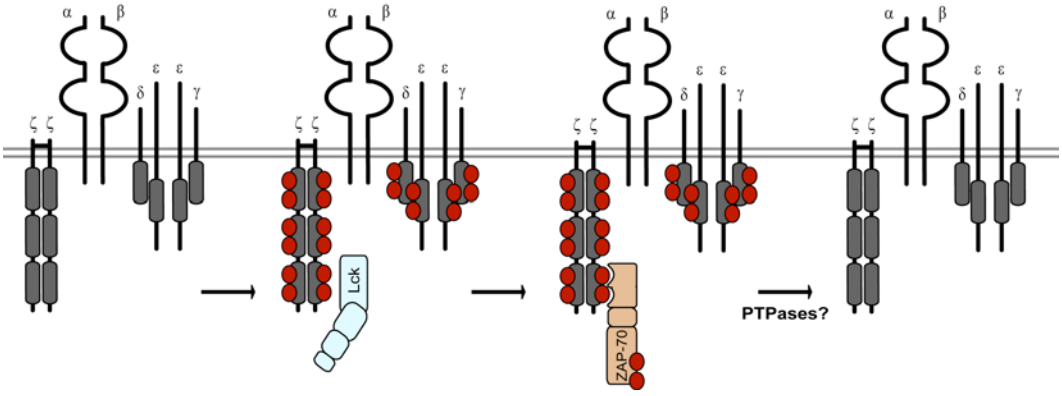

Figure 1. The T Cell Receptor Complex. The αβ TCR is a multimeric complex comprised of the αβ heterodimer and the CD3 γδε and TCR ζ invariant chains. The αβ TCR recognizes peptide/MHC ligands on the surface of APCs. Engagement of the αβ TCR with peptide-MHC complexes activates the Lck PTK. Lck phosphorylates the two tyrosine residues in each ITAM of the invariant signaling subunits (CD3 δε, γε and TCR ζζ). The tandem SH2 domains of ZAP-70 bind the bi-phosphorylated ITAMs. ZAP-70 is then activated upon tyrosine phosphorylation. Protein tyrosine phosphatases (PTPases) have been implicated in dephosphorylating the TCR ITAMs. The ITAMs are represented as gray boxes, and the red circles represent phosphorylated tyrosine residues.

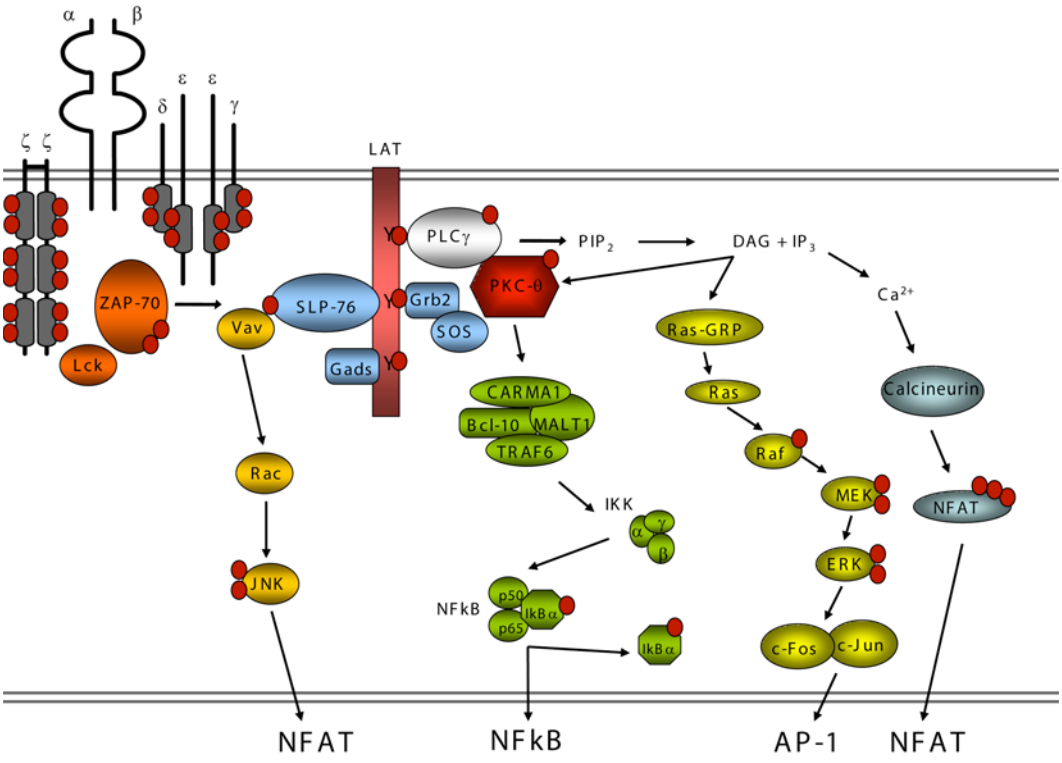

Figure 2. TCR-Induced Signaling Pathways. Following TCR cross-linking and ITAM phosphorylation, the activation of ZAP-70 leads to the phosphorylation of multiple downstream signaling molecules. These events result in the nuclear translocation of the transcription factors NF-AT, AP-1, and NFκB. The red circles represent phosphorylated residues (tyrosine, serine, or threonine).

CHAPTER II

Materials and Methods

Cell Lines

The COS7 and HEK 293T/17 cell lines used for transfection experiments were purchased from American Type Culture Collection (ATCC, Rockville, MD). The Jurkat T cell line (E6.1) was grown in RPMI 1640 supplemented with 5% FBS and 2 mM L-glutamine. The COS7 cell line (African green monkey kidney fibroblast-like cells) and HEK 293T/17 cell line (human epithelial kidney cells) were cultured in DMEM with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M β-mercaptoethanol (β-ME). All cells were maintained at 37^{o}C with 5 – 7% CO_2.

Antibodies

The 145-2C11 (anti-CD3ε) hybridoma was purchased from ATCC, and the 1F6 (anti-Lck) hybridoma was kindly provided by Dr. A. Weiss (University of California San Francisco, San Francisco, CA). The 35.71 (anti-CD28) hybridoma was a generous gift from Dr. J. Allison (Memorial Sloan-Kettering Cancer Center, New York, NY). Anti-IL-12 (TOSH) and anti-IL4 (11B11) hybridomas were obtained from Dr. J.D. Farrar (University of Texas Southwestern Medical Center, Dallas, Texas). Monoclonal antibodies (mAbs) were purified from supernatants by Protein A or Protein G affinity

chromatography (Amersham Biosciences, Piscataway, NJ). A clonotypic mAb against TCR β (C305), expressed on Jurkat T cells, was in the form of mouse ascitic fluid. Purified antibodies against human CD28 were purchased from Caltag Laboratories (Burlingame, CA). Polyclonal antibodies specific for p42/44 MAPK, β-actin, and GAPDH were purchased from Cell Signaling Technologies (Beverly, MA). Anti-FLAG and anti-phospho-p42/44 antibodies were purchased from Sigma (St. Louis, MO), anti-phosphotyrosine (4G10) from Upstate Biotechnology, Inc. (Lake Placid, NY), anti-phospho-Src (Y418) from Biosource International (Camarillo, CA), and anti-IκBα from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific for anti-TCR ζ (6B10.2) has been previously described (53). PTPH1 (4G1) mAb was generated using full-length PTPH1 as an immunogen (121). Purified rabbit polyclonal antibodies specific for PTPN4 was purchased from Orbigen (San Diego, CA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig, HRP-conjugated goat anti-rabbit Ig (Bio-Rad Laboratories, Hercules, CA), and HRP-conjugated goat anti-mouse IgG_{2b} (Zymed Laboratories, South San Francisco, CA) were used as secondary antibodies for Western blotting procedures. The following antibodies were utilized in multicolor flow cytometry (purchased from BD Pharmingen, San Jose, CA): APC-Cy7-B220, APC-CD4, PerCP 5.5-CD4, PE-Cy7-CD8, PE-Cy5-CD3, FITC-CD25, APC-CD44, PE-CD69, and APC-Cy7-CD11b. Pacific Blue-CD3 was purchased from eBiosciences (San Diego, CA), and PE-Texas Red-CD62L and –CD69 were obtained from Caltag Laboratories.

Transient Transfections

COS7 cells were transiently transfected with different amounts of plasmid DNA using the Fugene 6 transfection reagent according to the manufacturer's instructions (Roche Diagnostics Corporation, Indianapolis, IN). HEK 293T/17 cells were transfected with the calcium phosphate procedure (139). Jurkat T cells were transfected by electroporation. DNA or siRNA was mixed with the cells. The cells were transferred into 0.4 cm electroporation cuvettes (Bio-Rad Laboratories) and electroporated at 300 V, 975 μF (Bio-Rad Gene Pulser II).

Monoclonal Antibody-Mediated Stimulations

Single cell suspensions were prepared from the thymus, lymph nodes (axillary, inguinal, and mesenteric lymph nodes were pooled), or spleen. Red blood cells (RBC) were depleted from spleen cell preparations with RBC lysis buffer (0.1 mM EDTA, 155 mM ammonium chloride, 10 mM potassium bicarbonate, pH 7.2 – 7.4). After determining cell numbers, the cells were resuspended at 10^8 cells/ml in PBS and incubated at 37°C for 20 – 30 min to reduce basal levels of tyrosine phosphorylation. The cells were either left untreated or stimulated with an Ab combination of anti-CD3ε (2C11) and anti-CD28 (37.51) (10 μg/ml each) at 37°C. After various lengths of incubation, the cells were pelleted rapidly and lysed in a Triton X-100-containing lysis buffer.

Cell Lysis

Several distinct lysis buffers were used. Approximately 10^8 cells were resuspended in 1 ml of cold lysis buffer and incubated on ice for 20 min. Primary T lymphocytes and HEK 293T/17 cells were lysed in a buffer containing 0.2 – 0.5% Triton X-100, 20 mM Tris-Cl, pH 7.6, 100 mM NaCl, 10% glycerol and supplemented with protease inhibitors (10 μg/ml aprotinin, leupeptin, benzamidine, and 0.25 mM PMSF) and phosphatase inhibitors (1 mM of sodium fluoride, sodium orthovanadate, and sodium molybdate). COS7 cells were lysed in a Tris-based lysis buffer (10 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA) containing 1% Triton X-100 and 0.25% sodium deoxycholate supplemented with protease and phosphatase inhibitors. A 1% Triton X-100 lysis buffer containing 10 mM sodium phosphate, pH 7.0 and 5 mM NaF was used to examine the protein-protein interactions between PTPH1, TCR ζ, and Lck. Alternatively, cells were sonicated in a Tris-based buffer (50 mM Tris-Cl, pH 8.0) containing protease inhibitors. To detect PTPH1 and PTPN4 protein expression, cells were lysed in a buffer containing 1% NP40, 0.5% *n*-dodecyl-β-D-maltoside, 20 mM Tris-Cl pH 7.6. This buffer was supplemented with protease inhibitors. Cell lysates were centrifuged at 14,000 rpm for 15 min at 4°C to clear the insoluble fraction.

Immunoprecipitation and Western blotting

Proteins were immunoprecipitated by mixing cell lysates with 2 – 5 μg of the corresponding Ab and 25 μl of either protein A- or protein G-sepharose beads™ (Amersham Biosciences) for 2 h at 4°C. The immunoprecipitates were washed three times with chilled lysis buffer prior to boiling the samples in SDS-sample buffer for 5

min. Proteins from immunoprecipitations or total cell extracts were resolved by 8 – 12.5% SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF; Millipore Limited, Bedford, MA). For Western blotting, membranes were blocked with 4% bovine serum albumin (BSA) in a Tris-buffered saline solution (20 mM Tris-Cl, pH 7.6, 0.5 M NaCl) containing 0.05% Tween-20 (TBST) for 1 h at room temperature. Membranes were incubated for 1 h at RT with a primary Ab diluted in TBST. For immunoblotting with antibodies from Cell Signaling Technologies, the Abs were diluted in TBST containing 5% BSA, and this was added to the membranes for 12-18 h at 4^{o}C. After incubations with the primary Abs, the membranes were washed 3 – 4x with TBST followed by incubations with HRP-conjugated secondary Abs. Antibody binding was detected with the enhanced chemiluminescence reaction according to manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL) and exposure to film. For certain experiments, the antibodies were stripped off the membranes with either 0.1 M glycine, pH 3.0 for 30 min at room temperature or a Tris-buffered solution (62.5 mM Tris-Cl, pH 6.7) containing 2% SDS and 100 mM β-ME for 30 min at 50^{o}C.

GST-Fusion Proteins and Pull-Downs

The catalytic domains of wild-type or substrate-trap derivatives of PTPN4, PTPH1, SHP-1 and SHP-2 were cloned into pGEX-2TK vectors (Amersham Biosciences). The GST-fusion proteins were expressed in XL-1 blue or BL21 *Escherichia coli* (Stratagene, La Jolla, CA) following the addition of 1mM isopropyl-b-D-thiogalactoside (IPTG). After 3 – 4 h cultures, the bacteria were pelleted, resuspended

in a Tris-based lysis buffer (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 5 mM EDTA, 5 mM β-ME, 0.1% Triton X-100, and 0.1 μg/ml lysozyme with 1 mM NaF and protease inhibitors), and sonicated (3 x 30 s at 40% duty cycle and 4 output control). Bacterial lysates were cleared by centrifugation at 4^{o}C, and GST-fusion proteins were purified with glutathione sepharose beads according to manufacturer's instructions (Amersham Bioscience).

For pull-down experiments, cells were lysed in a Tris-based lysis buffer (10 mM Tris-Cl, pH 7.6, 150 mM NaCl, 1 mM EDTA) containing 0.25% Triton X-100, 5 mM iodoacetomide, 1 mM sodium fluoride, sodium orthovanadate and protease inhibitors. Following a 15 min incubation on ice, DTT was added at a final concentration of 15 mM. The lysates were then mixed with GST-fusion proteins and glutathione sepharose beads (Amersham Bioscience) for 2 h at 4^{o}C. The sepharose beads were pelleted by centrifugation and washed in lysis buffer. Proteins separated by SDS-PAGE were transferred to PVDF and immunoblotted.

Protein Tyrosine Phosphatase Assays

The ability of PTPH1 or PTPN4 to dephosphorylate TCR ζ was examined in a protein tyrosine phosphatase assay. Purified GST-ζ was tyrosine-phosphorylated with c-Src in *in vitro* kinase reactions (baculoviral stocks were a generous gift by Dr. D. Morgan, University of California, San Francisco). Recombinant PTPH1 or PTPN4 was incubated with phosphorylated ζ in a PTPase assay buffer (50 mM Tris-Cl, pH 7.6, 2 mM

EDTA, 2 mM β-ME, 0.1% Triton X-100, 2 mM NaF, and protease inhibitors) at 37^{o}C. The reaction was terminated with the addition of SDS-sample buffer. For some reactions, 0.5 mM sodium molybdate or sodium orthovanadate was added to the sample at the start of the reaction.

Transcriptional Reporter Assays

1.5 x 10^{7} Jurkat T cells were resuspended in 0.4 ml of RPMI 1640 and electroporated with 20 μg of AP-1- or NFκB-luciferase constructs in combination with 1.0, 5.0, and 10 μg of PTPH1 or PTPN4 expression vectors. Cells were cultured for 12 – 16 h and then stimulated with C305 (anti-TCR β, 1:1000 dilution) with or without anti-CD28 (1 μg/ml) for 6 – 7 h. Cells were lysed in 1% Triton X-100, 20 mM KPO_4, pH 7.8, 0.4 mM DTT. Lysates were mixed with an ATP-containing buffer (20 mM $MgCl_2$, 200 mM KPO_4, pH 7.8, 10 mM ATP) (140). Luciferase activity was measured after the addition of 1x luciferin (Pierce Biotechnologies) on a microplate reader.

Generation of PTPN4-Deficient Mice

Genomic DNA (129/Sv mice) was obtained from the University of Texas Southwestern Transgenic Technology Center (Dallas, TX). PCR reactions were used to amplify a region of PTPN4 containing exons 20-23 and another region spanning exon 24. A *loxP* site was inserted between exon 20 and 21 by PCR cloning. To generate the long targeting arm, the gene fragment including exon 20-loxP-exons 21-23 was digested and

subcloned into the NotI-BstZI sites of the pGK-neo plasmid (a generous gift from Dr. Toru Miyazaki, University of Tokyo, Japan). The PCR primers were: (intron 19-20) 5' ATC GCG GCC GCC AGA CAT GTC CAG CCT GTG GCC; (exon 21) 3' CCT TTT AAA AGG ACC CGT GTA GCA TC; (loxP) 5' AGC TAT AAC TTC GTA TAG CAT ACA TTA TAC GAA GTT AT; (loxP) 3' AGC TAT AAC TTC GTA TAA TGT ATG CTA TAC GAA GTT AT; (exon 20) 5' ATC GCG GCC GCC AAA TGT TCT TTG TAG ACT TGC; (exon 23) 3' CTC GGC CGT CTA AAT TCT CAG AGC TGG GG. The second fragment containing exon 24 was cloned into BamH1-KpnI sites downstream of the floxed Neo^r cassette. The PCR primers were: (exon 24) 5' CCA GCT CTG AGA ATT TAG ACA GAA GGA TCC CTG GG; (exon 24) 3' GAT GGT ACC AGT ATT GAT GCT GGA GCA ACG. The pGK-neo-PTPN4 targeting fragment was electroporated into SM-1 embryonic stem cells by the University of Texas Southwestern Transgenic Technology Center. Following G418 and gancyclovir selection, 576 ES cell clones were screened by Southern blotting. For Southern blot analysis, isolated genomic DNA was digested with XbaI restriction enzyme at 37^oC for 12-18 h. DNA fragments were separated by gel electrophoresis and transferred to a cation nylon membrane (Hybond-N+, Amersham Biosciences). Hybridization with radiolabelled probe 7 was performed in a sodium phosphate buffer (0.5 M $NaHPO_4$, pH 7.2, 7% SDS, 1 mM EDTA, 1% BSA) at 60^oC for 4 - 12 h. Probe 7 recognizes a region spanning exon 19 external to the targeting construct. The probe was generated with the PCR primers: (intron 18-19) 5' ACA GGA TCC CAA AGG AAT AGC AAC CCC C; (intron 19-20) 3' AGT AAG CTT GCA CAG TAG ATT AGG CCC TAA AC. The targeted recombinant ES cell clone 3F6 was identified and expanded for injections into C57BL/6 blastocysts.

The resulting chimeric male mice were mated to C57BL/6 female mice. Heterozygous mice containing the targeted allele were interbred to produce homozygous mice ($PTPN4^{fl/fl}$). These mice were crossed with Lck-Cre or MORE-Cre transgenic mice (Jackson Laboratories, Bar Harbor, ME) to obtain a targeted deletion of PTPN4 in the thymus or the entire mouse, respectively. These mice are currently back-crossed onto B6 (N4 and N5). Cag-Cre mice were generously provided by Dr. Tom Sato (University of Texas Southwestern Medical Center, Dallas, Texas).

Mice were genotyped by PCR and confirmed by Southern blotting with Probe 7. Genomic DNA was isolated either from the tail or thymus for PCR reactions. The PCR primers were: (exon 20) 5' CCT GGA ATG ACA ATG TCT TGT GCC; (intron 23-24) 3' GTC CTC GAA CTC AGA AAT CCA CCT; (intron 21-22) 3' GGT TGC AAT GTT ATT CAC CAT GGA GAG. The wild-type allele is a 645 bp product, and a 900 bp fragment identifies the knock-out allele. Messenger RNA for PTPN4 was determined by RT-PCR using RNA isolated from thymocytes with TRIZOL (Invitrogen). RNA was purified on spin columns (Qiagen, Inc., Valencia, CA) and eluted with DEPC-treated (Sigma) water. Eluted RNA was treated with DNaseI (Roche Diagnostics Corporation) at room temperature for 15-20 min. The DNaseI was inactived by incubating at 75^{o}C for 10 min. Reverse transcription reactions were performed with oligo-d(T) primers (Promega Biosciences, Inc., San Luis Obispo, CA) and AMV-Reverse Transcriptase (Promega Biosciences, Inc.) at 42^{o}C for 1 h. PTPN4 transcripts were amplified with the following primers for 32 cycles: (PTPN4 FERM) 5' CTG AAC TTG GAG ACT ACA ATC AG; (PTPN4 FERM) 3' CCT GAC ATC ACT CCA ATC AGG; (PTPN4 catalytic) 5' ATG

ATG CTA CAC GGG TCC TT; (PTPN4 catalytic) 3' GTG ACA TTT AAC TCT GCC ACG; (GAPDH) 5' TGG CAA AGT GGA GAT TGT TGC C; (GAPDH) 3' AAG ATG GTG ATG GGC TTC CCG. PCR products were separated by gel electrophoresis. The product for the FERM domain is approximately 250 bp, and the catalytic domain fragment is 324 bp. The GAPDH primers yield a 200 bp product. All mice were housed in a specific pathogen free facility. All animal care and use was approved by University of Texas Southwestern Institutional Animal Care and Use Committee.

Surface and Intracellular Staining and Flow Cytometry Analysis

All staining was performed in 96-well, round-bottom plates. $0.5 – 1 \times 10^6$ cells were washed in FACS buffer (PBS containing 1% FBS and 0.05% sodium azide) and incubated with hybridoma culture supernatant containing an anti-FcRII mAb (2.4G2, ATCC) for 10 min at 4^oC. This step prevents fluorochrome-labeled Ab from binding to Fc receptors. Cells were subsequently stained with an Ab mix for 20 – 30 min at 4^oC. The labeled cells were washed twice and resuspended in FACS buffer for analysis by flow cytometry. Samples were run on an LSRII FACS (BD Biosciences), and cell populations were analyzed with FlowJo software (Treestar, Ashland, OR).

For intracellular staining, the cells were first stained for surface proteins and then fixed with 4% paraformaldehyde at RT for 15 min. The cells were washed with cold intracellular (IC) buffer (PBS containing 0.1% FBS) and then permeabilized by adding saponin buffer (PBS containing 0.2% FBS and 0.1% saponin) for 10 min at room

temperature. The Abs were diluted in saponin buffer and mixed with the permeabilized cells. After a 30 min incubation at 4^{o}C, the cells were washed with saponin buffer and resuspended in FACS buffer for flow cytometric analysis.

T Cell Activation

Peripheral T lymphocytes were prepared from lymph nodes or spleen. T cell populations were enriched by depleting B cells with B220-specific magnetic beads (Invitrogen Dynal AS, Oslo, Norway). T cell populations were plated at 0.1 x 10^6 cells/well in 96-well, round-bottom plates. Individual wells were pre-coated with 3 μg/ml plate-bound anti-CD3ε and 1 μg/ml anti-CD28 to stimulate the cells. The production of IL-2, IL-4, IL-5, IL-13, IFN-γ and TNF-α was measured from culture supernatants using Cytometric Bead Array (BD Pharmingen) and the upregulation of activation markers, CD25 and CD69, was evaluated by flow cytometry.

To generate $CD8^+$ effector T cells, total splenocytes or lymph node cells were cultured at 10^6 cells/ml in 50 U/ml rIL-2 (NIH, Bethesda, MD) and stimulated with 10 μg/ml 145-2C11 (anti-CD3ε) for three days. On day 3, cells were re-cultured at 5 x 10^5 cells/ml in media containing 50 U/ml rIL-2. Cells were washed on day 7 and cultured in medium without rIL-2 for another 12 – 18 h. Approximately 90% of the rested cells were $CD8^+$ T cells expressing $CD62L^{lo}CD44^{hi}$. The remaining $CD4^+$ T cells were depleted with anti-CD4 magnetic beads (BD Pharmingen). $CD8^+$ effector T cells were

restimulated with 3 μg/ml 145-2C11 for 6 h or 50 ng/ml PMA and 1 μM ionomycin for 4 h. 1 μM monensin was added to the cultures for the last 2 h of the restimulation.

$CD4^+$ T cells from spleen or lymph nodes were polarized to Th1 or Th2 cells *in vitro*. $CD4^+$ T cells were purified with a CD4 negative selection kit (Invitrogen Dynal AS). The isolated cells were cultured for three days at $1 – 2 \times 10^6$ cells/ml (1 ml/well) in a 48 – well plate coated with 3 μg/ml 145-2C11 and 1 μg/ml 35.71. 2 μg/ml anti-IL-4, 50 U/ml rIL-2, and 5 ng/ml rIL-12 (R&D Systems, Minneapolis, MN) were added to the cultures to generate Th1 cells. Alternatively, cells were polarized to the Th2 subset in cultures containing 1:10 anti-IL-12 culture supernatant, 50 U/ml rIL-2, and 5 ng/ml rIL-4 (R&D Systems). After three days of stimulation, cells were diluted 1:8 in media containing 50 U/ml rIL-2 and allowed to expand for 4 days. Polarized $CD4^+$ T cells were rested and restimulated as described for CD8 T cells. Cytokine producing cells were examined by intracellular cytokine staining as described above.

T Cell Proliferation Assay

Enriched T cells were resuspended at 10^7 cells/ml in PBS and labeled with 1 μM CFSE (carboxyfluoroscein succinimidyl ester, Molecular Probes, Eugene, OR) for 10 min at 37°C. Cells were washed three times with RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 5×10^{-5} M β-ME. Labeled cells were stimulated for 72 h with plate-bound antibodies as previously described. Cell division correlated with CFSE dilution and was measured by flow

cytometry. Each round of cell division dilutes CFSE equally between daughter cells. Proliferation was determined for both $CD4^+$ and $CD8^+$ T cells.

Listeria monocytogenes Infections

Listeria monocytogenes capable of secreting soluble ovalbumin (LM-OVA) was kindly provided by Dr. J. Forman (University of Texas Southwestern Medical Center, Dallas, TX). LM-OVA bacteria were grown on Brain Heart Infusion (BHI) agar plates containing 100 μg/ml streptomycin. Bacteria were prepared for infections by growing 3 ml cultures in BHI medium containing 100 μg/ml streptomycin at RT for 12 – 18 h. The overnight bacterial cultures had densities of approximately 1.9×10^9 CFU/ml. To maintain virulence, C57BL/6 mice were injected intravenously each month, and spleens plated on BHI/streptomycin agar plates. The LD_{50} of LM-OVA in C57BL/6 mice is 2×10^5 CFU. For infections, LM-OVA were washed and resuspended at $1 – 2.5 \times 10^5$ CFU/ml in PBS prior to intravenous injections. For primary infections, PTPN4 KO mice and WT littermates were infected with 5×10^4 CFU. To measure memory responses, mice were infected with a low dose of LM-OVA (2×10^3 CFU) and re-infected with a high dose (2×10^5 CFU) 4-6 weeks after the primary infection.

Mice were sacrificed three days post-infection, and the livers and spleens were harvested. Half of the spleen was used for surface staining, and the other half was used to calculate CFU. Whole and half spleen weights were measured to calculate splenocyte cellularity. The frequency of activated ($CD62L^{lo}CD44^{hi}$) T cells was determined.

Splenocytes were stained with anti-CD3-Pacific Blue, -CD4-PerCP 5.5, -CD8-PE-Cy7, -CD62L-PE-Texas Red, -CD44-APC, -CD25-FITC, -CD69-PE, -B220-APC-Cy7 and analyzed by flow cytometry. The liver and remaining spleen were homogenized in sterile water. Serial dilutions (undiluted, 1:10, 1:100, 1:1000) of spleen and liver homogenates were plated on duplicate BHI/streptomycin agar plates. Following a 24 h incubation at 37^{o}C, the CFU was calculated for each organ.

CHAPTER III

PTPH1 Is a Predominant Protein Tyrosine Phosphatase Interacting with and Dephosphorylating the T Cell Receptor ζ Subunit

Introduction

Engagement of the T cell receptor (TCR) initiates a cascade of tyrosine phosphorylation events through a conserved motif known as the immunoreceptor tyrosine-based activation motif (ITAM) (48). Multiple ITAMs are present on the cytoplasmic domains of the TCR invariant chains (CD3 γδε and TCR ζ). TCR cross-linking leads to the activation of Src family PTKs (protein tyrosine kinases), which phosphorylate two tyrosine residues within each ITAM (141). The phosphorylated ITAMs are bound by the tandem SH2 domains of the ZAP-70 PTK (27). ZAP70 is catalytically activated following its tyrosine phosphorylation by Lck and subsequent auto- and trans-phosphorylation events (34, 142). Once activated, it phosphorylates a number of intracellular and transmembrane proteins (48). The ITAMs of TCR ζ are one of the first tyrosine-phosphorylated substrates detected after TCR engagement (21). The TCR ζ homodimer appears more heavily phosphorylated than the other invariant chains, mainly because it contains three ITAMs per chain (21, 29). Two distinct phosphorylated forms of ζ with molecular masses of 21- and 23-kDa (p21 and p23) can be generated

following TCR interactions (30). The relative ratios of p21 and p23 in T cells have been correlated with distinct biological functions (29, 30).

The transient nature of protein tyrosine phosphorylation in T cells has been partially attributed to the actions of protein tyrosine phosphatases (PTPases). Approximately 68 of the 107 putative PTPases identified from the human genome are expressed in T cells (77, 103). While several PTPases have been implicated in regulating T cell activation, the identity of those PTPases that specifically dephosphorylate the ζ ITAMs remains inconclusive. A number of studies have suggested that SHP-1, SHP-2, and CD45 can dephosphorylate phospho-ζ (98, 107, 113). SHP-1 and SHP-2 belong to a family of phosphatases distinguished by tandem SH2 domains at the amino-terminus (78). Thymocytes from SHP-1-deficient mice have numerous hyperphosphorylated proteins compared to wild-type mice (107). The T cells from these mutant mice have enhanced proliferative responses and increased IL-2 production in response to TCR stimulation (108). Since Lck, ZAP-70, and TCR ζ were hyperphosphorylated and activated in the SHP-1-mutant mice when compared to normal mice, SHP-1 was implicated in the dephosphorylation of these proteins (107-109). Yet other reports have shown that SHP-1 does not dephosphorylate ζ (110, 143). While SHP-1 is primarily expressed in hematopoietic cells, SHP-2 is ubiquitously expressed (101). SHP-2, unlike SHP-1, is a positive regulator of signal transduction. The catalytic activity of SHP-2 is required for Ras/Erk activation following TCR-mediated signaling (111, 144). One report suggested that SHP-2 could bind to the transmembrane protein CTLA-4 and localize to TCR ζ, where it could dephosphorylate the ζ ITAMs (113).

CD45 is a receptor-type PTPase (RPTP) which consists of extracellular, transmembrane, and cytoplasmic regions. Two intracellular PTPase catalytic domains are present on CD45, which is characteristic of most RPTPs (78). CD45 activates signal transduction by dephosphorylating the inhibitory tyrosine on Src family PTKs (38, 39). Dephosphorylation of this inhibitory tyrosine residue results in the activation of Src PTKs, implicating a positive regulatory function for CD45. One report demonstrated that CD45 could complex with and dephosphorylate TCR ζ, indicative of an inhibitory role (98). Yet, low levels of phospho-ζ were still observed in CD45-deficient thymocytes, suggesting that other PTPases could dephosphorylate TCR ζ (116).

In an attempt to identify the PTPases dephosphorylating TCR ζ, two different experimental approaches were utilized, biochemical purification techniques and a substrate-trap screen. The biochemical purification approach revealed that SHP-1 and PTPH1 could target phospho-ζ (121). In a separate technique, substrate-trapping mutants of PTPases were utilized in a large-scale substrate-trap screen. This screen relies on the ability of the PTPase catalytic domain to associate with its substrate, which has successfully identified physiological substrates of PTPases (87, 145). The substrate-trapping concept is based on the presence of a conserved catalytic domain containing a catalytic cysteine and an invariant aspartic acid in all PTPases (74, 85). A single amino acid mutation of the invariant aspartic acid to an alanine (DA) reduces the catalytic activity several hundred-fold resulting in a prolonged interaction between the enzyme and substrate (87). By probing 47 different substrate-trapping mutant PTPases, we identified

PTPH1 as the predominant PTPase capable of associating with phospho-ζ (121). Notably, the catalytic domains of SHP-1, SHP-2 and CD45 did not associate with phosphorylated ζ, suggesting that these PTPases do not directly target TCR ζ (121). Our biochemical and substrate-trapping approaches established PTPH1 as a candidate PTPase that dephosphorylates the ITAMs of TCR ζ.

To determine whether TCR ζ is a physiological substrate of PTPH1, its ability to complex with and dephosphorylate ζ from total cell extracts was examined. The catalytic domain of PTPH1 associated with phosphorylated ζ from cell lysates. An additional interaction was detected between the two proteins independent of the catalytic domain. Furthermore, PTPH1 dephosphorylated the ζ ITAMs when expressed in cells. These results provide evidence that TCR ζ is a substrate of PTPH1.

Results

PTPH1 Associates with the TCR ζ ITAMs

To determine whether PTPH1 could complex with phospho-ζ prepared from cell extracts, GST-pull-down assays were performed. COS7 cells were transfected with Lck and TCR ζ. Following cell lysis, TCR ζ was directly immunoprecipitated and immunoblotted with an anti-phosphotyrosine mAb. TCR ζ was heavily tyrosine phosphorylated (Figure 3A, lane 4). GST-fusion proteins consisting of substrate-trapping (DA) catalytic domains of PTPH1, SHP-1, and SHP-2 were prepared and each was mixed with the COS7 cell lysates containing phospho-ζ. The catalytic domain of PTPH1 complexed with phosphorylated ζ (Figure 3A, lane 1). In contrast, the catalytic domains of SHP-1 and SHP-2 did not associate with phospho-ζ (Figure 3A, lanes 2 and 3). A complex between GST and phospho-ζ was not detected (data not shown). The ability of PTPH1 to associate with the 21- and 23-kDa phosphorylated forms of TCR ζ, present in primary T lymphocytes, was subsequently examined. In thymocytes and peripheral lymphocytes, TCR ζ is constitutively phosphorylated and appears as a 21-kDa (p21) phosphoprotein (Figure 3B, lanes 1 and 5)(23). The 23-kDa (p23) phosphorylated form of TCR ζ is induced following stimulation (Figure 3B, lanes 2 and 6)(24, 28). Cells isolated from the thymus or lymph nodes and spleen were either left unstimulated or treated with pervanadate. TCR ζ was directly immunoprecipitated, or the GST-PTPH1 substrate-trapping mutant was mixed with cell lysates for pull-down assays (Figure 3B). The catalytic domain of PTPH1, as a substrate-trap, pulled-down p21 from cell lysates

prepared from unstimulated cells (Figure 3B, lanes 3 and 7 versus lanes 1 and 5). Following pervanadate stimulation, PTPH1 complexed with both p21 and p23 from either thymocytes or peripheral T cells (Figure 3B, lanes 4 and 8). These results demonstrated that the catalytic domain of PTPH1 associates with phosphorylated ζ.

The ability of PTPH1 to bind phosphorylated TCR ζ was further investigated by co-expressing full-length substrate-trapping mutant PTPH1 along with TCR ζ and Lck in COS7 cells. The full-length substrate-trapping mutant of PTPH1 contained an additional tyrosine to phenylalanine mutation (YFDA) to reduce tyrosine phosphorylation of PTPH1. This was previously shown to enhance the trapping capabilities of full-length PTPH1 expressed in mammalian cells (130). Transfected cells were lysed with a buffer containing different amounts of sodium orthovanadate, a PTPase-specific inhibitor. TCR ζ or PTPH1 was directly immunoprecipitated from cell lysates, and precipitates were western blotted with anti-phosphotyrosine, anti-Lck, and anti-PTPH1 antibodies (Figure 4A and B).

From ζ immunoprecipitations, an increase in phospho-ζ was observed with increasing amounts of sodium orthovanadate (Figure 4A, lanes 1-5). PTPH1 was detected in ζ immunoprecipitates, demonstrating that PTPH1 co-immunoprecipitated with TCR ζ (Figure 4A). The amounts of PTPH1 associating with phosphorylated ζ were augmented with increased concentrations of sodium orthovanadate (Figure 4A, lanes 1-5). Low levels of Lck were also observed in ζ precipitates following anti-Lck western blots (Figure 2A).

In reciprocal PTPH1 immunoprecipitations, phosphorylated ζ was not detected following anti-phosphotyrosine western blotting (Figure 4B). However, an interaction between Lck and PTPH1 was identified (Figure 4B). In PTPH1 immunoprecipitations, the levels of Lck associating with PTPH1 were not affected by the varying concentrations of sodium orthovanadate (Figure 4B). These findings suggested that PTPH1 localizes to the correct subcellular compartment to complex with both TCR ζ and Lck.

PTPH1 Regulates the Phosphorylation of the TCR ζ ITAMs and Active Lck

To determine whether PTPH1 could dephosphorylate the TCR ζ ITAMs, full-length, catalytically active PTPH1 was overexpressed in COS7 cells in combination with Lck and TCR ζ. Transfected cells were lysed, and the levels of phosphorylated ζ were assessed by western blotting. Increased amounts of PTPH1 led to diminished levels of phospho-ζ (Figure 5, lanes 1-4). The total amounts of TCR ζ and Lck were unaltered (Figure 5, lanes 1-4). The ability of PTPH1 to dephosphorylate the other CD3 ITAMs has not been determined. Overexpression of SHP-1 also resulted in reduced levels of phosphorylated ζ without any changes in the total levels of TCR ζ and Lck (Figure 5, lanes 5-8). This is likely a result of reduced catalytic activity of Lck regulated by SHP-1, which would lead to diminished ITAM phosphorylation (Figure 6, lanes 5-8)(108). The levels of phospho-ζ were not affected by the overexpression of SHP-2 (Figure 5, lanes 9-12). To determine whether PTPH1 dephosphorylated Lck, the levels of active phospho-Lck were examined with anti-phospho-Src antibodies specific for tyrosine 418.

Overexpression of both PTPH1 and SHP-1 decreased the levels of active Lck (Figure 6, lanes 1-4 and lanes 5-8). Taken together, these data demonstrated that PTPH1 can dephosphorylate the phospho-ITAMs and the tyrosine residue in the active site of phospho-Lck.

TCR ζ is Directly Dephosphorylated by Recombinant PTPH1

Purified recombinant PTPH1 was used in a phosphatase assay to assess its ability to directly dephosphorylate TCR ζ. Recombinant PTPH1 was incubated with *in vitro* phosphorylated ζ for varying lengths of time (Figure 7, lanes 1-6). A substantial decrease in ζ phosphorylation occurred by 3 min, and no phospho-ζ was detected following a 30 min incubation (Figure 7, lane 4 and lane 6). The addition of PTPase-specific inhibitors, sodium molybdate (SM) and sodium orthovanadate (SO), at the start of the incubation blocked the enzymatic activity of PTPH1 and eliminated the dephosphorylation of ζ (Figure 7, lanes 7-8). This result indicated that phospho-ζ is directly dephosphorylated by PTPH1.

Overexpression of PTPH1 in Jurkat T Cells Inhibits TCR Signaling and Alters Cell Growth

Previous reports have shown that the overexpression of WT PTPH1 inhibited the activation of the transcriptional regulators NF-AT and AP-1 (123). Our preliminary results also demonstrated that PTPH1 could inhibit AP-1 activity after TCR stimulation

(Figure 8). To examine the effects of PTPH1 in T cells, Jurkat T cell were selected for clones that overexpressed wild-type or substrate-trapping mutant PTPH1. Notably, protein expression was very difficult to detect by Western blotting regardless of the clone. Jurkat clones overexpressing WT PTPH1 exhibited visible decreased growth rates. Conversely, an effect on cell growth was not observed in cells overexpressing the substrate-trapping mutant (YFDA) PTPH1. Since the overexpression of PTPH1 Jurkat cells affected cell growth, TCR-induced protein tyrosine phosphorylation was subsequently examined. Jurkat clones were stimulated and lysed at different time-points, and total cell extracts were immunoblotted with anti-phosphotyrosine mAb. Regardless of the Jurkat clone, both wild-type and substrate-trapping mutant PTPH1 did not affect the kinetics of phospho-ζ (Figure 9). Collectively, these data suggested that PTPH1 is a potential regulator of cell cycle, which is supported by similar results in a previous report (130). In an alternative approach, Jurkat T cells were transfected with siRNA to knock-down PTPH1 protein expression. Changes in TCR-induced protein tyrosine phosphorylation could not be determined in PTPH1 siRNA-transfected cells compared to control-transfected cells. In these cells, the knock-down of PTPH1 expression could not be confirmed.

The Catalytic Domain of PTPH1 is Capable of Binding the ITAM in DAP12

Since the TCR ζ ITAMs were targeted by PTPH1, its ability to regulate other ITAM-containing proteins was examined. NK activating receptors initiate signal transduction through an associating signaling molecule, DAP12, which contains an

ITAM in its cytoplasmic tail (146). COS7 cells were transfected with DAP12 and Lck to induce tyrosine phosphorylation of DAP12. Pull-downs with GST-PTPH1 substrate-trapping mutant were performed on cell lysates. Phospho-DAP12 was detected in DAP12 immunoprecipitations (Figure 10, lane 3). The catalytic domain of PTPH1 complexed with phosphorylated DAP12 (Figure 10, lane 4). Pull-downs of phospho-ζ were used as a control for the trapping capabilities of GST-PTPH1 (Figure 10, lane 2). It is unclear whether the interaction between PTPH1 and DAP12 is physiologically relevant, since the expression of PTPH1 in NK cells has not been examined. The invariant chains of the BCR, Igα and Igβ, were also considered as substrates for PTPH1. However, an interaction could not be determined since the overexpression of Igα and Igβ could not be confirmed. These data implied that the catalytic domain of PTPH1 may associate with phospho-ITAMs present in other immunoreceptor complexes and/or other ITAM-containing proteins.

Discussion

The invariant chains of the TCR complex translate extracellular ligand binding into an intracellular cascade of transient protein tyrosine phosphorylation events. Some of the earliest tyrosine-phosphorylated substrates that appear following receptor engagement are the ITAMs of the TCR invariant chains. While the PTKs involved in tyrosine phosphorylation events have been well-characterized, the role of PTPases in dephosphorylating the ITAMs has not been extensively examined. This is partly due to the difficulty in identifying the specific substrates of PTPases. In particular, the PTPases implicated in dephosphorylating phospho-ζ have not been established. SHP-1, SHP-2 and CD45 have been linked to the regulation of ζ phosphorylation, but the data are inconsistent. Using two distinct approaches, we identified PTPH1 as a predominant PTPase capable of associating and dephosphorylating TCR ζ (121).

PTPH1 is an intracellular PTPase previously reported to negatively regulate TCR-mediated signaling (123). Its expression is detected in the Jurkat T cell line as well as bone marrow, fetal liver, and lymph nodes as determined by RT-PCR (124). PTPH1 consists of an amino-terminal FERM domain followed by a PDZ domain and the carboxyl-terminal catalytic domain (125). The FERM domain has been reported to target proteins to the cytoskeleton-membrane interface (147). In fact, localization to the plasma membrane by PTPH1 has been demonstrated in Jurkat T cells transfected with epitope-tagged PTPH1 (124). The localization of PTPH1 to the cytoskeleton corresponds with the localization of the TCR at the plasma membrane. This idea is supported by our results

that full-length substrate-trapping PTPH1 immunoprecipitated with phospho-ζ in cells. The levels of PTPH1 associating with TCR ζ were enhanced with increased amounts of sodium orthovanadate in the lysis buffer. Sodium orthovanadate interferes with the PTPase-substrate complex by mimicking phosphotyrosine and forms a covalent bond with the active site cysteine (89, 148). Consequently, vanadate ions block the interaction between the PTPase catalytic domain and the phosphorylated substrate. In our studies, sodium orthovanadate did not competitively inhibit PTPH1 binding to phospho-ζ indicating that another domain associates with ζ independently of the catalytic domain. An interaction between phospho-ζ and the FERM domain of PTPH1 may exist. The FERM domain forms three subdomains (F1, F2, and F3), where each module is similar to other known domains (94). The structures of F1 and F2 are similar to ubiquitin and acyl-CoA binding protein, respectively (94). The subdomain F3 contains a structural fold that is also present in PTB (phosphotyrosine binding), PH (pleckstrin homology), and EVH1 (Enabled/VASP Homology) domains (94). PTB domains specifically bind phosphorylated NPxY motifs (149). Previous results demonstrated that the F3 subdomain of talin recognizes a phosphorylated NPxY motif in the cytoplasmic tail of integrin (150). Therefore, the membrane-proximal ITAM of TCR ζ contains an NPxY motif that may be targeted by the FERM domain of PTPH1. In reciprocal PTPH1 immunoprecipitations, phosphorylated ζ was not detected implying that phospho-ζ did not complex with PTPH1. An alternative explanation could be that the antibodies for PTPH1 blocked the ability of PTPH1 to interact with ζ. Lck, however, co-immunoprecipitated with PTPH1 demonstrating that PTPH1 is capable of complexing with phospho-ζ and Lck. Whether

the FERM domain of PTPH1 is responsible for binding phosphorylated TCR ζ and/or Lck has yet to be determined.

Our findings demonstrated that PTPH1 can complex with phosphorylated ζ in cell extracts and directly regulate ζ phosphorylation. In addition, the phosphorylated tyrosine that positively regulates Lck activity was also found to be a substrate of PTPH1. Negative regulation of active Lck results in decreased levels of phospho-ζ. The effects of PTPH1 in T cells were examined in Jurkat T cells transfected with WT or substrate-trapping mutant (YFDA) PTPH1. Stable clones were stimulated through the TCR, and total protein tyrosine phosphorylation was examined. No differences in tyrosine phosphorylation of TCR ζ or other proximal signaling molecules were detected, although PTPH1 protein expression in these clones could not be confirmed by western blotting. The inability to detect PTPH1 protein may suggest that it is not overexpressed in these clones, thereby accounting for the similarities in proximal signaling following TCR stimulation. However, even though PTPH1 expression could not be detected, Jurkat clones expressing WT PTPH1 had decreased growth rates compared to control cells or clones expressing YFDA PTPH1. The rate of cell division was different between the various stable cell lines suggesting that the PTPH1 constructs were expressed in the clones. Endogenous PTPH1 has been difficult to detect in most cell populations with the current reagents. This may be due to improper extraction methods for PTPH1, which is cytoskeleton-associated. Alternatively, the levels of PTPH1 may be out of the range of detection. Recent studies have demonstrated that PTPH1 expression is upregulated in

peripheral T lymphocytes following TCR stimulation (127). Therefore, activated T cells are most likely the relevant cell population to examine PTPH1 expression.

PTPH1 was previously reported to associate with 14-3-3β and vasolin-containing protein (VCP) (129, 130). Both proteins have been linked to cell cycle regulation. Interestingly, VCP is tyrosine phosphorylated following TCR engagement (132). Dephosphorylation of VCP by PTPH1 results in cell growth inhibition (130). It is possible that the regulation of phospho-VCP and not phospho-ζ could account for the decreased proliferation observed in the Jurkat cells. PTPH1 likely targets the ζ ITAMs but may not be the primary PTPase involved in regulating ζ phosphorylation. Recently, Bauler *et al.* demonstrated that PTPH1 knock-out mice had normal TCR signal transduction (127). Additionally, T cell development and T cell activation were unaltered in these mice suggesting that PTPH1 was not essential in negatively regulating TCR signaling (127). This may be due to another PTPH1-family member and/or other PTPases that may also dephosphorylate TCR ζ. One PTPH1-family member is PTPN4 (PTP-MEG1)(78, 126). The catalytic domains of PTPH1 and PTPN4 share 64% identity in the amino acid sequence (126, 151). RT-PCR techniques have confirmed PTPN4 expression in multiple lymphoid organs, including the bone marrow, lymph nodes, spleen and thymus (124). PTPN4 can also negatively regulate TCR-induced activation of NFAT/AP-1 in transcriptional reporter assays (123, 124).

In summary, PTPH1 complexes with and directly dephosphorylates the TCR ζ ITAMs and other ITAM-containing proteins, such as DAP12. In addition to its catalytic

domain, a separate region of PTPH1 was capable of associating with phospho-ζ. There is the possibility that PTPH1 is capable of regulating additional signaling pathways that utilize ITAMs. Recent studies have demonstrated the presence of ITAM-containing proteins in non-immunoreceptor signaling. Adhesion molecules, lectin receptors, and chemokine receptors have all been reported to utilize ITAM-like signaling pathways (15). An ITAM-like motif is present in the amino-terminus of ERM proteins (152). These proteins, such as ezrin and moesin, are associated with the plasma membrane (153). PTPH1 would then have a potential role in multiple immune cell functions.

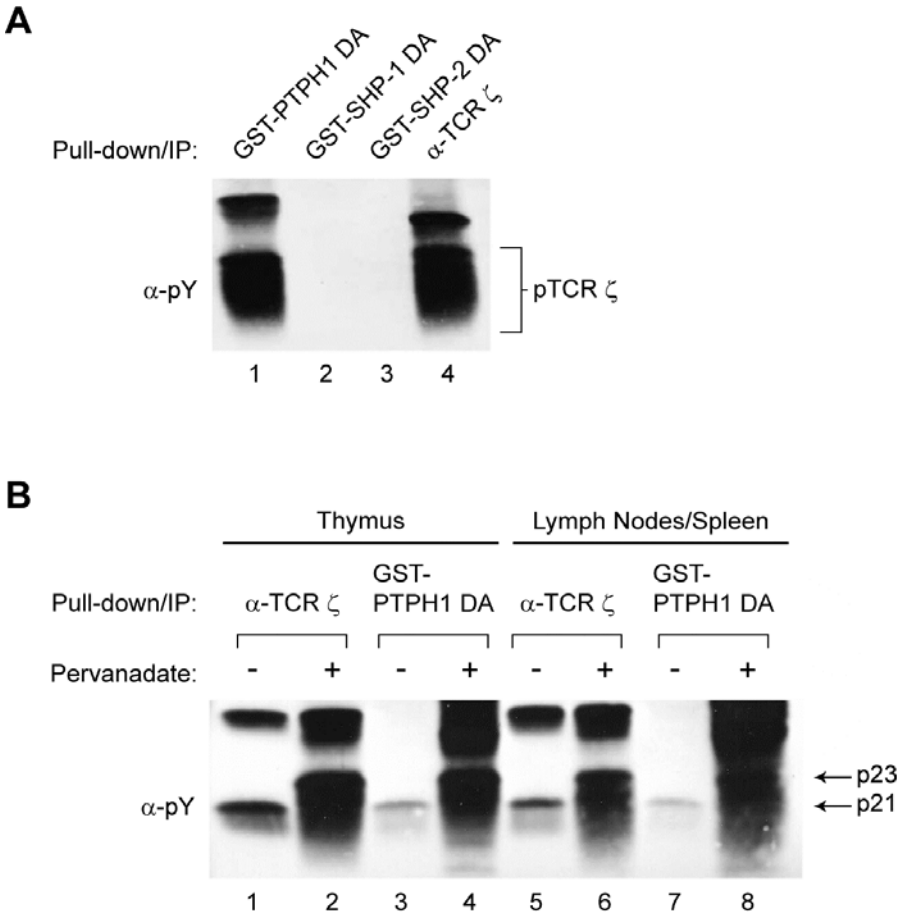

Figure 3. The Catalytic Domain of PTPH1 Associates with the Phosphorylated ζ ITAMs. (A) COS7 cells were transfected with Lck and TCR ζ. TCR ζ was directly immunoprecipitated (IP, *lane 4*) or equal amounts of the indicated GST-fusion proteins were mixed with cell lysates (*lanes 1-3*). (B) Thymocytes and peripheral T cells were left untreated or stimulated with pervanadate for 10 min at 37^{o}C. Cells were lysed, and total cell lysates were mixed with substrate-trap mutant GST-PTPH1 or anti-TCR ζ mAb. Proteins from the IPs or pull-downs were resolved on SDS-PAGE and Western blotted with anti-phosphotyrosine (pY) mAb. The data are representative of three independent experiments.

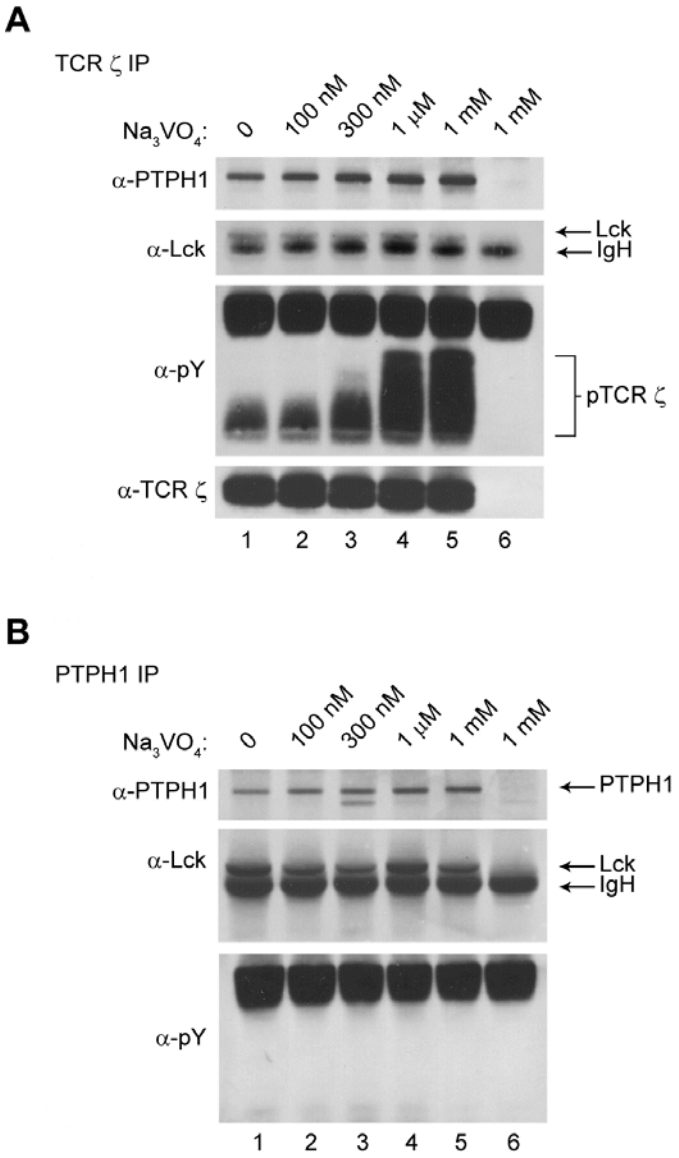

Figure 4. PTPH1 Co-Immunoprecipitates with TCR ζ and Associates with Lck. COS7 cells were co-transfected with substrate-trap PTPH1 (YFDA), Lck, and TCR ζ. Cell lysates were generated in lysis buffer with varying concentrations of sodium orthovanadate (Na_3VO_4). (A) TCR ζ or (B) PTPH1 was immunoprecipitated from cell extracts. Membranes were Western blotted with anti-TCR ζ (A), anti-PTPH1, anti-Lck, and anti-phosphotyrosine. Lysates from untransfected cells were included (A and B, *lane 6*).The data are representative of three independent experiments.

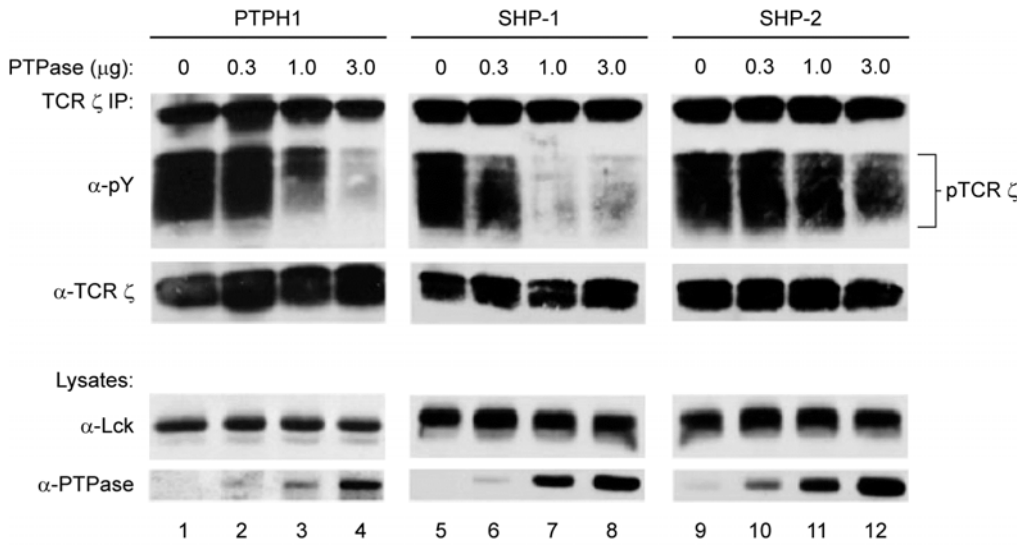

Figure 5. Overexpression of PTPH1 Decreases the Levels of Phospho-ζ in Mammalian Cells. COS7 cells were transfected with TCR ζ, Lck and increasing amounts (0, 0.3, 1.0, 3.0 μg) of PTPH1 (*lanes 1-4*), SHP-1 (*lanes 5-8*), or SHP-2 (*lanes 9-12*). TCR ζ was directly immunoprecipitated from cell lysates and resolved by SDS-PAGE. Immunoprecipitates were Western blotted with anti-phosphotyrosine and re-probed with anti-TCR ζ mAbs. Total cell extracts were immunoblotted with anti-Lck mAbs and anti-PTPH1 (*lanes 1-4*), anti-SHP-1 (*lanes 5-8*), or anti-SHP-2 (*lanes 9-12*) mAbs. The data are representative of seven independent experiments.

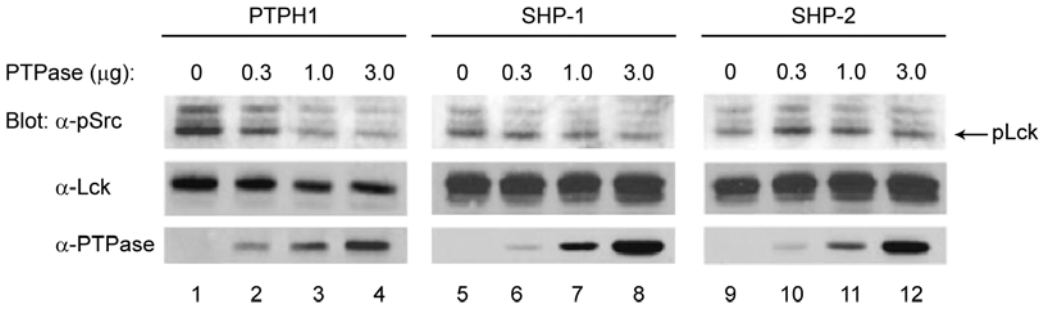

Figure 6. PTPH1 Diminishes Active Phospho-Lck. COS7 cells were co-transfected with Lck and increasing amounts (0, 0.3, 1.0, 3.0 μg) of PTPH1 (*lanes 1-4*), SHP-1 (*lanes 5-8*), or SHP-2 (*lanes 9-12*). Total cell lysates were Western blotted with anti-phospho-Src (pY418) and re-blotted with anti-Lck. Lysates were also immunoblotted with anti-PTPH1 (*lanes 1-4*), anti-SHP-1 (*lanes 5-8*), or anti-SHP-2 (*lanes 9-12*) mAbs. The data is representative of two independent experiments.

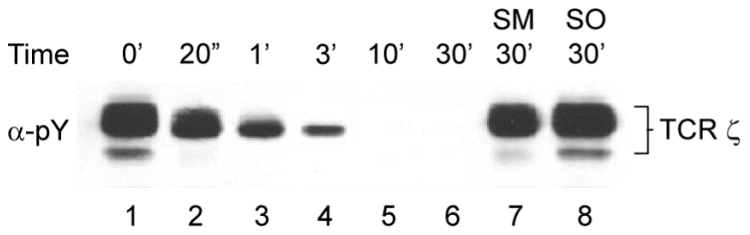

Figure 7. Recombinant PTPH1 Directly Dephosphorylates Phospho-TCR ζ. *In vitro* phosphorylated GST-ζ was incubated with recombinant PTPH1, purified from Sf9 insect cells. The reaction was stopped at various time points (0, 20 s, 1, 3, 10, or 30 min). Proteins were resolved by SDS-PAGE and anti-phosphotyrosine Western blotted. 0.5 mM sodium molybdate (*lane 7*) or sodium orthovanadate (*lane 8*) was added at the start of the reaction which was later terminated after 30 min. The data are representative of three independent experiments.

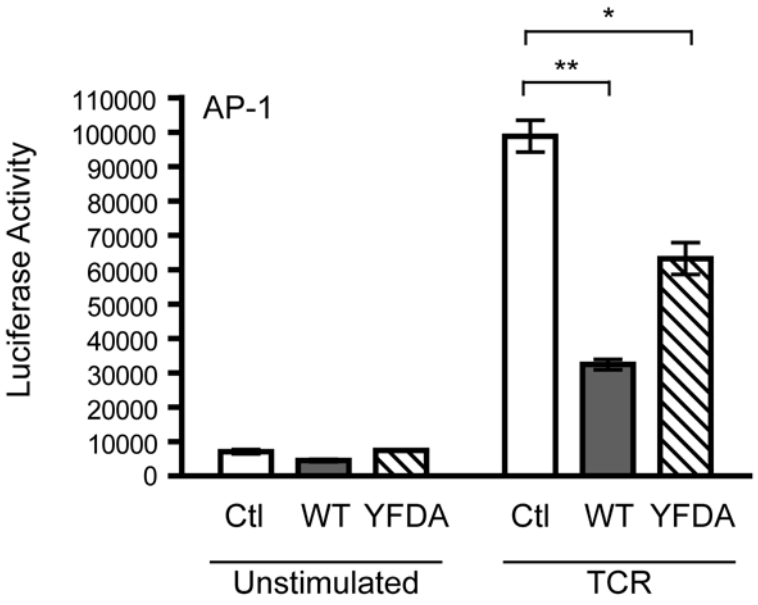

Figure 8. PTPH1 Inhibits TCR-Induced AP-1 Activation. Jurkat T cells were co-transfected with 20 μg of AP-1 reporter construct 3.0 μg of wild-type (WT) or substrate-trapping mutant (YFDA) PTPH1 or an empty control vector. Luciferase activity was measured in unstimulated and TCR -stimulated cells. Data are shown as mean ± SD (n = 3; Student's t-test *p = 0.005, **p = 0.002).

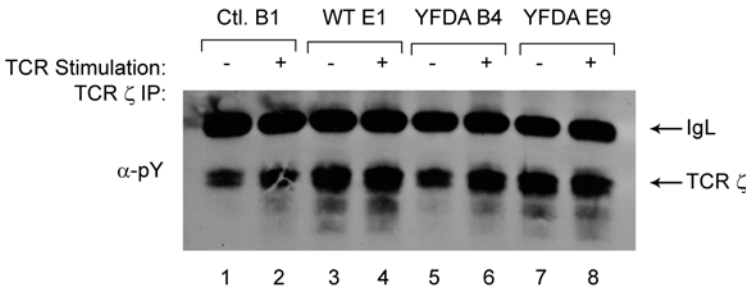

Figure 9. The Levels of Phospho-ζ are Similar Between Jurkat T Cell Clones Overexpressing Wild-Type and Substrate-Trapping Mutant PTPH1. Jurkat T cell clones stably expressing a control vector (clone B1, *lanes 1-2*), wild-type PTPH1 (clone E1, *lanes 3-4*), or substrate-trapping mutant PTPH1 (clones B4 and E9, *lanes 5-8*) were stimulated with C305.2 (α-TCR β) at 37°C for 10 min. TCR ζ was directly immunoprecipitated from cell lysates and resolved by SDS-PAGE. Immunoprecipitates were Western blotted with anti-phosphotyrosine mAb.

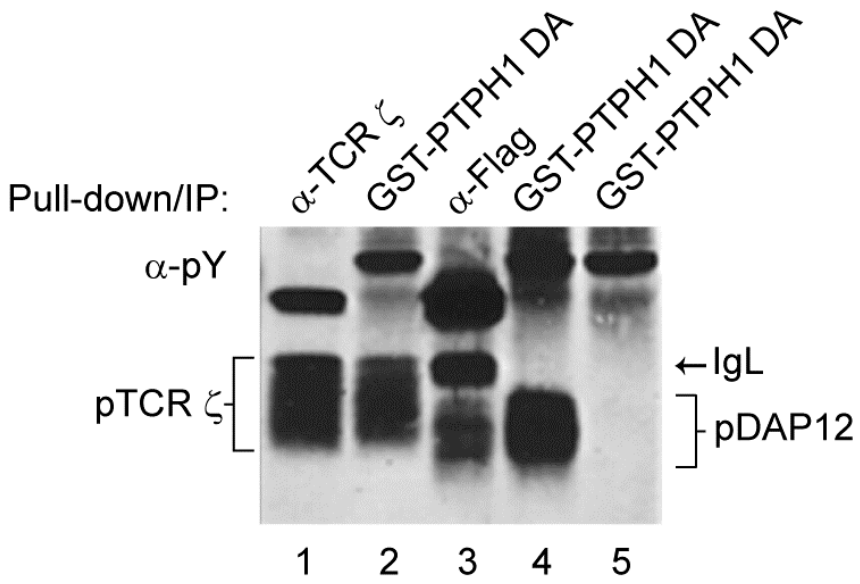

Figure 10. PTPH1 is Capable of Associating with the ITAM of DAP12. COS7 cells were co-transfected with Lck and TCR ζ (*lanes 1 and 2*) or the cytoplasmic portion of DAP12 (*lanes 3 and 4*). The DAP12 construct was Flag-tagged on the N-terminus. Substrate-trap GST-PTPH1 was mixed with cell lysates. Anti-phosphotyrosine Western blots were performed to examine the levels of phospho-ζ (*lane 1 and 2*) or phospho-DAP12 (*lanes 3 and 4*). Lysates from untransfected cells were mixed with GST-PTPH1 DA and run in lane 5 as a negative control. The data are representative of four independent experiments.

CHAPTER IV

The Protein Tyrosine Phosphatase PTPN4/PTP-MEG1 Regulates Peripheral Effector/Memory T Cell Subsets and Cytokine Production

Introduction

The T cell receptor (TCR) is a multimeric complex consisting of the ligand binding chains (αβ or γδ TCR) non-covalently associated with the signal transducing dimers, CD3 γε, δε, and TCR ζζ. TCR recognition of cognate peptide/MHC complexes on antigen presenting cells results in a cascade of intracellular signaling processes. These intracellular signals are critical for T cell development, cytokine production, and/or proliferation (34, 56, 154). Seconds after TCR cross-linking, a conformational change in the cytoplasmic tail of CD3 ε can be detected (7, 33). This conformational change along with simultaneous or subsequent activation of protein tyrosine kinases (PTKs) result in increased protein phosphorylations (155). Two Src-family PTKs, Lck and Fyn, are re-localized and activated following TCR engagement (34). In a catalytically active state, Lck and Fyn phosphorylate the two tyrosine residues in the ITAMs, a conserved signaling motif present in one or more copies in the cytoplasmic tails of the CD3 γ, δ, ε and TCR ζ subunits (31). The bi-phosphorylated ITAMs are bound by the tandem SH2 domains of ZAP-70, a Syk-family PTK (27). ZAP-70 is catalytically activated upon tyrosine phosphorylation by Src PTKs (45, 46). The

signaling cascade continues with activated ZAP-70 phosphorylating several adaptor proteins, kinases, and effector proteins. Downstream signaling events lead to the activation of multiple transcription factors, including NF-AT, AP-1 and NFκB, which results in the production of different cytokines (47, 156, 157). IL-2, in particular, is the primary cytokine produced following receptor ligation and promotes T cell proliferation and other distinct effector functions (48, 142, 157, 158).

TCR-induced intracellular signals are transient in nature with most of the tyrosine phosphorylation increases eventually diminishing back to basal levels. The magnitude and duration of TCR signaling is partly controlled by the catalytic activities of protein tyrosine phosphatases (PTPases), a family of enzymes that dephosphorylate tyrosine residues. T cells express 68 of the 107 putative PTPases identified in the human genome (75-77, 84, 100). A role for many of these PTPases in TCR-signaling pathways has been elucidated by the analysis of mice containing mutations or lacking particular PTPases (101, 159). SHP-1 and SHP-2 are homologous cytoplasmic PTPases containing tandem SH2 domains followed by a catalytic domain (78). SHP-1 is specifically expressed in hematopoietic cells and is mutated in *motheaten* mice. Thymocytes from these mutant mice have elevated levels of phosphoproteins compared to those of normal mice (104, 107, 108). Some potential substrates of SHP-1 include the positive regulatory tyrosine phosphorylation sites present in Src PTKs and ZAP-70 (108-110). SHP-2 is ubiquitously expressed and is thought of as a positive regulator of the Ras/ERK pathway during TCR-mediated signaling (111, 144). However, SHP-2 has also been reported to negatively regulate TCR signaling by dephosphorylating the TCR ζ ITAMs (113). PEP,

Pest-domain Enriched Tyrosine Phosphatase (PEP) is an additional PTPase that negatively regulates TCR signaling. The proline-rich domain of PEP constitutively associates with the SH3 domain of the PTK Csk (117). The PEP-Csk interaction permits PEP to localize near and to dephosphorylate the positive regulatory sites of Src and ZAP-70 PTKs, which effectively regulates kinase activities (101, 119, 120). PEP knock-out mice demonstrated a selective role for PEP in the regulation of effector/memory T cell subsets (122). TCR-stimulated effector cells exhibited increased proliferation and cytokine production in the absence of PEP.

We identified previously PTPH1 (PTPN3) as a PTPase that interacts with and dephosphorylates TCR ζ (121). Assays measuring transcriptional activation in T cells also supported the finding that PTPH1 negatively regulates the TCR signaling pathway (123, 124). Yet, mice containing a targeted deletion of PTPH1 maintained normal T cell development, TCR signaling events, and T cell effector functions (127). These results suggested that additional PTPases could target the TCR ITAMs and potentially negatively regulate TCR-mediated signaling. One candidate PTPase is PTPN4/PTP-MEG1 (PTPN4), a PTPH1-family member, that also contains an N-terminal FERM domain, a central PDZ domain, and a C-terminal catalytic domain (77, 126). Transcriptional reporter assays in T cells revealed that the overexpression of PTPN4 suppresses TCR-mediated signaling (123).

To determine whether PTPN4 negatively regulates TCR signaling events, we analyzed the biochemical interactions between PTPN4 and phospho-ζ. We also generated

PTPN4-deficient mice to examine the physiological function of PTPN4. We report here that PTPN4 can complex with and dephosphorylate TCR ζ. Transcriptional reporter assays using a wild-type and substrate-trapping mutant of PTPN4 revealed an important role for this PTPase in the NFκB pathway. PTPN4 knock-out mice had normal T cell development in the thymus and periphery. In contrast, knock-out mice contained increased numbers of peripheral $CD8^+$ effector/memory T cells compared to wild-type littermates. The absence of PTPN4 resulted in the production of T cells with a polarized Th2 response. These cells produced elevated amounts of IL-4, IL-5, and IL-13 cytokines, indicating an important role for PTPN4 in peripheral T cell expansion and differentiation.

Results

PTPN4 Dephosphorylates Tyrosine Phosphorylated TCR ζ

We previously reported that PTPH1 associates with and dephosphorylates tyrosine phosphorylated TCR-ζ (phospho-ζ)(121). To determine if phospho-ζ was also a substrate for PTPN4, we examined first whether a substrate-trapping derivative of PTPN4 could complex phospho-ζ. The catalytic domain of PTPN4 was engineered as a substrate-trapping mutant and expressed as a GST-fusion protein. This GST-fusion protein was used in pull-down assays with cell lysates containing phospho-ζ. The PTPN4 substrate-trapping mutant complexed phospho-ζ, the amounts of which were comparable to that pulled-down with PTPH1 (Figure 11, lanes 1-2). Consistent with previous data, SHP-1 and SHP-2 substrate-trapping mutants failed to associate with phospho-ζ (Figure 11, lanes 3-4) (121). While both the phosphorylated and non-phosphorylated pools of TCR ζ were immunoprecipitated with ζ-specific mAbs, only phospho-ζ was complexed by the PTPN4 and PTPH1 substrate-trapping mutants (Figure 11, lane 5 versus 1-2). These results indicated that both PTPN4 and PTPH1 are capable of complexing the phosphorylated ITAMs of TCR ζ.

We next examined whether full-length PTPN4 could dephosphorylate TCR ζ, using two distinct assays. First, recombinant PTPN4, purified from insect cells, was incubated with phospho-ζ for different lengths of time. A reduction in phospho-ζ occurred within 1 min of the incubation, with TCR ζ completely dephosphorylated by 30

min (Figure 12, lanes 2-6 versus 1). The addition of sodium molybdate or sodium orthovanadate, two PTPase-specific inhibitors, completely blocked the phosphatase activity of PTPN4 when added for the entire 30 min incubation (Figure 12, lanes 7-8). The enzymatic activity was not measured in these assays. In a separate approach, wild-type or substrate-trapping derivatives of PTPN4 were transfected into HEK 293T cells in combination with TCR ζ and the Lck PTK. Phospho-ζ was detected as a smear of phosphorylated intermediates in the absence of PTPN4 (Figure 13, lane 1). Overexpression of wild-type (WT) PTPN4 reduced the amount of phospho-ζ 10-20 fold (Figure 13, lane 2 versus 1). The PTPN4 substrate-trapping mutant (DA) did not affect the levels of phospho-ζ (Figure 13, lane 3). Our previous report revealed that PTPH1 could dephosphorylate Lck at tyrosine residue 394 (121). PTPN4 did not dephosphorylate Lck (Figure 14). Taken together, these data demonstrated that PTPN4 dephosphorylates TCR ζ but not phospho-Lck (Y394), suggesting it has a more selective substrate specificity compared to PTPH1.

Overexpression of PTPN4 in Jurkat T cells modestly inhibits NFAT/AP-1-controlled luciferase expression following TCR stimulation (123). To determine the role of PTPN4 in multiple TCR signaling pathways, we assessed the effects of its overexpression on NFκB- and AP-1-driven luciferase reporter constructs. Jurkat T cells were co-transfected with either an NFκB- or an AP-1 reporter construct and increasing amounts of WT or DA PTPN4. The cells were then left untreated or stimulated with anti-CD3 and anti-CD28 mAb, lysed, and luciferase activities were measured. WT PTPN4 significantly inhibited TCR-induced activation of NFκB (Figure 15A, $p < 0.05$, $p < 0.02$,

and $p < 0.006$). These cells also appeared to have decreased cell growth compared to control transfected cells (visual observation). The overexpression of the substrate-trapping derivative (DA PTPN4) increased the levels of NFκB activation both before and after stimulation (Figure 15A, $p < 0.02$ and $p < 0.006$). This finding suggested that the substrate-trapping mutant potentiated the NFκB signaling pathway, possibly by protecting a tyrosine-phosphorylated protein involved in TCR-mediated NFκB activation. We also examined the effects of PTPN4 on the transcriptional activity of AP-1. Increasing amounts of WT PTPN4 suppressed both basal and TCR-induced AP-1 activity (Figure 15B, $p < 0.05$ and $p < 0.005$). The overexpression of the substrate-trapping mutant PTPN4 (DA) was less efficient at inhibiting AP-1 activity following TCR stimulation (Figure 15B, $p < 0.05$ and $p < 0.01$). Taken together, these results indicated that PTPN4 negatively regulates the TCR-mediated signaling pathway leading to both NFκB and AP-1 activation.

Generation of PTPN4-Deficient Mice

To investigate the role of PTPN4 in T cells, we generated a PTPN4 conditional knock-out mouse. PTPN4 is predominantly expressed in testis as 3.2, 3.7, and 7.0 kb transcripts, with the 7.0 kb transcript present in thymic tissue (Figure 16) (138). These various transcripts are presumably alternatively spliced transcripts, but truncated PTPN4 proteins have not been identified. A targeting vector was designed with *loxP* sites preceding exon 21, and two others embedding a neomycin cassette following exon 23 (Figure 17). This construct was designed to disrupt the catalytic domain of PTPN4. One

ES cell clone (3F6) was identified based on the presence of a floxed allele and this was used to generate chimeric mice (Figure 18, left panel). Two independent chimeric lines were established. The wild type (WT, 13.5 kb) and targeted (flox, 7.0 kb) alleles were identified by Southern blotting (Figure 17 and 18, right panel). To delete PTPN4 in T cells, homozygous mice containing the targeted allele ($PTPN4^{flox/flox}$) were backcrossed onto Lck-Cre transgenic mice. Despite multiple backcrosses, the targeted allele never deleted in these mice. Inaccessibility of the locus may prevent recombination of the targeted allele by Lck-Cre. Consequently, the PTPN4-targeted mice were backcrossed onto the MORE-Cre transgenic line. In this line, the Cre recombinase is controlled by the Mox2 promoter which is ubiquitously expressed as early as E5 of embryonic development (160). Complete recombination of the PTPN4-targeted allele was detected by analyzing DNA, isolated from both the tail and thymus, with Southern blotting and PCR techniques (Figures 19A and 18). PTPN4-deficient mice were born at normal Mendelian ratios with no apparent developmental or phenotypic defects. The complete deletion of the catalytic domain of PTPN4 was confirmed by RT-PCR (Figure 19B). Message for the FERM domain of PTPN4 was detected in PTPN4 knock-out mice, but the mRNA levels were significantly decreased compared to wild-type littermates (Figure 19B). Therefore, the production of a truncated protein was unlikely due to the instability of the transcript. If a truncated protein was produced, it would likely act as a dominant negative molecule. We were unable to detect endogenous PTPN4 by Western blotting, most likely due to its low expression levels and tight association with the actin cytoskeleton (93, 124, 147).

PTPN4 is Not Essential for Normal T Cell Development

To determine whether PTPN4 was required for T cell development, thymocytes and peripheral T cell populations were compared between PTPN4 knock-out mice and WT littermates. The cellularity in the thymus, lymph nodes, and spleen were similar between WT and PTPN4-deficient mice. Thymocytes from WT and PTPN4-deficient littermates were stained with a panel of antibodies to characterize the various thymic subsets. The percentages of the double negative (DN), double positive (DP), and $CD8^+$ or $CD4^+$ single positive (SP) populations in PTPN4-null mice were similar to those detected in WT littermates (Figure 20A). The DN population is comprised of four developmental stages that are defined by CD44 and CD25 expression. Deletion of PTPN4 did not appear to impair the transition of thymocytes through the four DN stages (Figure 20A). The intensities of CD3 and CD5 surface expression were similar between WT and PTPN4-deficient DP and SP thymocyte subpopulations (Figure 20B). In summary, we conclude that PTPN4 is not required for early T cell development in the thymus.

We characterized the peripheral lymphocyte populations from the lymph nodes and spleen in both WT and PTPN4-null mice. WT and PTPN4-deficient mice contained equivalent percentages of B and T cells in the lymph nodes (Figure 21A). The knock-out mice had normal proportions of mature $CD4^+$ and $CD8^+$ T cells when compared to WT littermates (Figure 21A). Similar findings were observed for the spleen (Figure 22). One major phenotypic difference was noted when comparing CD3 surface levels on the peripheral T cell populations. Both peripheral $CD4^+$ and $CD8^+$ T cell populations had

significantly reduced cell surface expression of CD3 in the PTPN4-deficient mice (Figure 21B, $p < 0.03$). Intracellular staining for CD3 was performed to determine if the surface expression of CD3 was specifically modulated in the knock-out mice. The intracellular amounts of CD3 were also decreased in the PTPN4 knock-out mice. Furthermore, the reduced expression of CD3 was maintained even when peripheral T cells were cultured for 12-18 hours (Figure 23). These experiments suggested that the absence of PTPN4 reduces the expression and/or assembly of the TCR complex in peripheral T cells.

A closer examination of the naïve and effector/memory cells in the peripheral CD4 and CD8 populations revealed an increase in $CD62L^{lo}CD44^{hi}$ effector/memory subset in PTPN4 knock-out mice when compared to WT littermates (Figure 24). This increase was significant for the $CD8^{+}$ T cell population (Figure 24, $p = 0.003$). A similar aberration in the effector/memory subset was also observed in PTPN4-null mice generated from Cag-Cre backcrosses (Figure 25). Therefore, these findings were not a consequence of the Cre transgene and suggested that PTPN4 regulates the differentiation of the CD8 effector/memory subset and TCR expression.

PTPN4 is Not Required for Normal TCR Signal Transduction

The results from the substrate-trapping experiments suggested that the phosphorylated ITAMs were a likely PTPN4 substrate. To characterize the effects of PTPN4-deficiency on TCR signaling events, the phosphorylation state of various intracellular signaling proteins were analyzed prior to and following anti-CD3/CD28

stimulation. The 21-kDa phosphorylated form of TCR ζ (p21) is constitutively phosphorylated in thymocytes and peripheral lymphocytes, while the 23-kDa phosphorylated form of ζ (p23) is induced following TCR cross-linking (23, 24, 28). The magnitude and duration of p21 and p23 phosphorylation were similar in peripheral T cells from wild-type and PTPN4 knock-out cells (Figure 26). These findings suggested that either the TCR ζ ITAMs are not the primary target of PTPN4, or that a functional redundancy exists among several PTPases. Additionally, the phosphorylation of Lck, Zap70, Erk1/2, and total IκBα were comparable in intensity and duration in WT and PTPN4 knock-out peripheral T cells (Figure 26). Finally, whole cell lysates were immunoblotted for the presence of tyrosine-phosphorylated proteins. The absence of PTPN4 had no effect on overall protein tyrosine phosphorylation patterns (Figure 26). These findings implied that PTPN4 does not regulate the tyrosine phosphorylation of TCR ζ or other common signaling molecules.

PTPN4-Deficient T Cells Have Enhanced Th2 Cytokine Production

Since peripheral T cells had reduced CD3 expression in the absence of PTPN4, we wanted to determine whether the effector functions of these cells were altered. Peripheral T cells were isolated from PTPN4 knock-out mice and WT littermates, stimulated with anti-CD3/CD28 mAb, and assayed for the upregulation of selected activation markers, including CD69 and CD25. The induction of CD69 on $CD4^+$ or $CD8^+$ T cells appeared to be similar between WT and knock-out mice (Figure 27A). Unlike CD69, the levels of CD25 were increased in PTPN4-deficient cells relative to WT

controls (Figure 27A). However, it is important to note that we detected variable levels of CD25 after TCR stimulation in each mouse. These findings suggested that the absence of PTPN4 has differential effects on the cell surface expression of CD3 (lower) and CD25 (higher). To examine how such differences affected T cell proliferation, purified T cells from WT and knock-out mice were labeled with CFSE (carboxyfluoroscein succinimidyl ester) and stimulated with plate-bound anti-CD3/anti-CD28 mAbs. Following three days of stimulation, the extent of cell division was compared by examining CFSE dilution. Cell division was similar in $CD4^+$ or $CD8^+$ T cells when comparing WT and PTPN4 knock-out mice (Figure 27B).

To determine whether the types and levels of distinct cytokines were regulated by PTPN4, different Th1- and Th2-specific cytokines were measured from the culture supernatant of CD3/CD28-stimulated T cells from WT and knock-out mice. The production of IL-2, IFN-γ, and TNF cytokines was similar between PTPN4 knock-out and WT cells (Figure 28A). A statistically significant increase in cytokines that are characteristic of Th2-polarized cells (IL-4, IL-5, IL-13) was detected in PTPN4-deficient T cells when compared to WT littermates (Figure 28B, $p < 0.02$). These results suggested that PTPN4 controls TCR-mediated cytokine production and/or T cell polarization.

Discussion

In this report, we describe the first characterization of PTPN4-deficient mice. PTPN4-null animals were born at normal Mendelian ratios, no developmental defects were detected. The targeted deletion of PTPN4 did not impair T cell development in the thymus or periphery. Instead, PTPN4 deficiency resulted in an increase in the number of effector/memory T cells compared to WT mice. T cells lacking PTPN4 upregulated cell surface CD25 expression, the high affinity IL-2Rα chain, and produced elevated levels of Th2 cytokines after T cell stimulation. These results suggested that PTPN4 has a specialized role in the differentiation of effector/memory T cells and the polarization of the Th2 T cell subsets.

We initially characterized PTPN4 based on its ability to bind and dephosphorylate the ITAMs in TCR ζ. Additional transient transfection and overexpression studies suggested that WT PTPN4 negatively regulated TCR signaling. Moreover, a substrate-trapping derivative of PTPN4 potentiated the NFκB pathway. This suggests that PTPN4, in physiological systems, dephosphorylates a key TCR-induced signaling intermediate that regulates NFκB activation. The findings from our biochemical experiments and transfection assays implied a critical functional contribution of PTPN4 to the T cell receptor signaling pathway. However, our analyses of PTPN4-deficient mice revealed normal T cell development and TCR signaling profiles. In fact, all of the tyrosine phosphorylated proteins induced following TCR signaling, including the ITAMs of TCR ζ, appeared unaffected by the absence of the intact PTPN4 catalytic domain. We

considered several alternate explanations to account for the minimal effects of a PTPN4-deficiency on TCR signaling. First, the phosphorylated ITAMs of TCR ζ and the other TCR subunits might not be the primary targets of PTPN4. PTPN4 contains both FERM and PDZ domains, with the FERM domain responsible for localizing PTPN4 to the detergent-insoluble cytoskeleton. That restricted localization may prevent PTPN4 from localizing to the TCR substrates. More recent reports have revealed the presence of ITAM-like molecules in signaling pathways mediated by adhesion molecules, lectin receptors, and chemokine receptors (15). Ezrin and moesin are two such proteins containing ITAM-like sequences that associate with the plasma membrane and the adhesion molecule PSGL-1 (153, 161). The mutation of the ITAM sequence in moesin eliminates Syk association and subsequent signal transduction mediated by PSGL-1 (162). PTPN4, through its localization to the actin cytoskeleton, may participate in the regulation of multiple signaling pathways which utilize ITAM-containing proteins that are localized in the cytoskeleton. Current experiments are addressing this.

A second explanation is the ability of related family members to target the same substrate. A probable candidate is PTPH1, which is a homologous-PTPN4 family member that can complex and dephosphorylate TCR ζ. However, PTPH1-deficient mice have normal T cell development and TCR signaling functions (121, 127). It is possible that PTPN4 and PTPH1 coordinately regulate the TCR signaling pathways. Therefore, both PTPN4 and PTPH1 could regulate ζ phosphorylation, thereby accounting for the normal TCR signaling observed in the PTPN4-deficient cells. The combined disruption of PTPN4 and PTPH1 will be necessary to resolve this issue. Multiple PTPases targeting

a single phospho-substrate has been observed in other studies of PTPase-deficient mice. The absence of Hematopoietic Protein Tyrosine Phosphatase (HePTP) in lymphocytes led to an increase in ERK activation following stimulation (163). Yet, these mice maintain normal T cell development and lymphocyte activation, suggesting that a HePTP-deficiency is compensated (163). Similarly, the targeted deletion of PEP resulted in the expansion of effector/memory T cell subsets, but had no affect on T cell development or naïve T cell functions (122). These data suggest that several PTPases are capable of regulating the same phosphoprotein.

A third explanation is that PTPN4 may have a non-redundant role in activated rather than naïve cells, analogous to PEP and PTPH1, with PTPH1 upregulated in activated T cells (122, 127). While we could not detect endogenous protein in T cells or testes, where PTPN4 is also expressed, the increase in the effector/memory subset suggests that PTPN4 levels might be induced in a subpopulation of T cells during TCR/co-stimulatory molecule signaling. This would be consistent with the enhanced production of IL-4, IL-5, and IL-13 cytokines in PTPN4-null cells. Such results suggested that PTPN4 is capable of controlling TCR-induced cytokine production, influencing the extent of T cell polarization. Mechanistically, the increased levels of Th2 cytokines in PTPN4 knock-out mice might arise from increased NFκB activation. Two NFκB binding sites have been identified in the IL-4 promoter region (164). In fact, the transcriptional activation of IL-4 by NF-AT is enhanced by p65 and c-Rel (165). Therefore, the regulation of NFκB activation by PTPN4 may control IL-4 production, thereby influencing Th2 polarization and cytokine production. In addition, c-Rel and p50

bind to the NFκB site in the promoter region of CD25 regulating its expression (166). As a result, the variable levels of CD25 may also be a consequence of the dysregulation of NFκB activation and cytokine production. These interpretations are consistent with the augmented NFκB activation revealed in transcriptional reporter assays using a substrate-trapping derivative of PTPN4. Those findings implicate a key tyrosine phosphorylated substrate in the NFκB pathway as a critical substrate of PTPN4, with increases in IL-4 cytokine production as a consequence of increased NFκB activity.

In summary, the development of PTPN4 knock-out mice revealed a specific functional contribution of PTPN4 in peripheral Th cell polarization and effector/memory generation. Further experiments will be necessary to elucidate the exact biochemical mechanism whereby this PTPase exerts its selective effect at a very late developmental point for T lymphocytes.

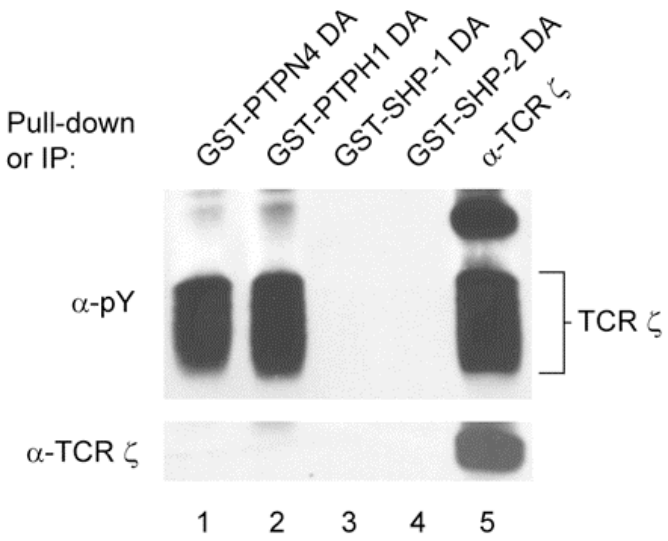

Figure 11. The Substrate-Trapping Derivative of PTPN4 Complexes with Phospho-ζ. COS7 cells were co-transfected with Lck and TCR ζ. Cell lysates were subsequently prepared and mixed with GST-fusion proteins containing the substrate-trapping catalytic domains of the indicated PTPases. Alternatively, TCR ζ was directly immunoprecipitated. The pull-downs and immunoprecipitates were resolved by SDS-PAGE and Western blotted with anti-phosphotyrosine (pY) followed by anti-TCR ζ. Data are representative of three independent experiments.

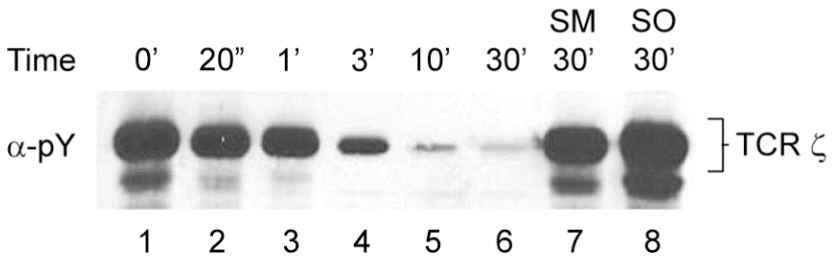

Figure 12. Recombinant PTPN4 Directly Dephosphorylates Phosphorylated TCR ζ. Recombinant PTPN4, purified from Sf9 insect cells, was incubated with purified, *in vitro*-phosphorylated ζ at 37°C for the indicated time points. Two samples were incubated in the presence of 0.5 mM sodium molybdate (SM, *lane 7*) or sodium orthovanadate (SO, *lane 8*) for 30 min. The proteins from each sample were immunoblotted with phosphotyrosine-specific mAb. Data are representative of three independent experiments.

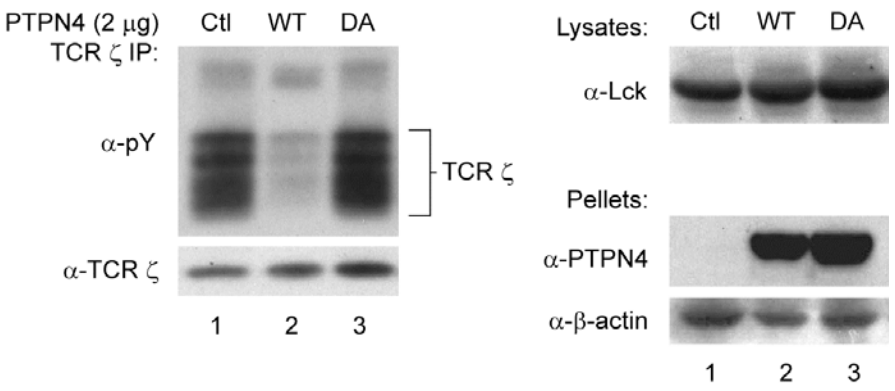

Figure 13. Overexpression of Wild-Type PTPN4 Decreases the Levels of Phospho-ζ. Lck and TCR ζ were co-transfected with a control vector (ctl, *lane 1*), full-length wild-type (WT, *lane 2*) PTPN4, or full-length substrate-trapping mutant (DA, *lane 3*) PTPN4 into HEK 293T cells. After 48 h, the cells were lysed, and TCR ζ was directly immunoprecipitated from the cell lysates. The precipitates were Western blotted with anti-pY and anti-TCR ζ mAbs. Total cell extracts were also prepared and immunoblotted with anti-Lck mAbs. The insoluble fraction was immunoblotted with anti-PTPN4 and anti-β-actin mAbs. Data are representative of five independent experiments.

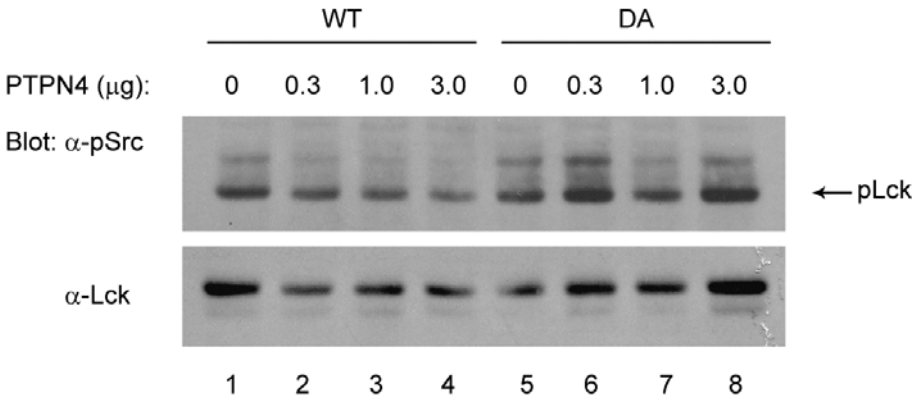

Figure 14. The Overexpression of PTPN4 Does Not Decrease Phospho-Lck in Mammalian Cells. COS7 cells were transfected with TCR ζ, Lck and increasing amounts (0, 0.3, 1.0, 3.0 μg) of wild-type (*lanes 1-4*) or substrate-trapping PTPN4 (*lanes 5-8*). Total cell extracts were Western blotted with anti-phospho-Src (pY418) and re-blotted with anti-Lck.

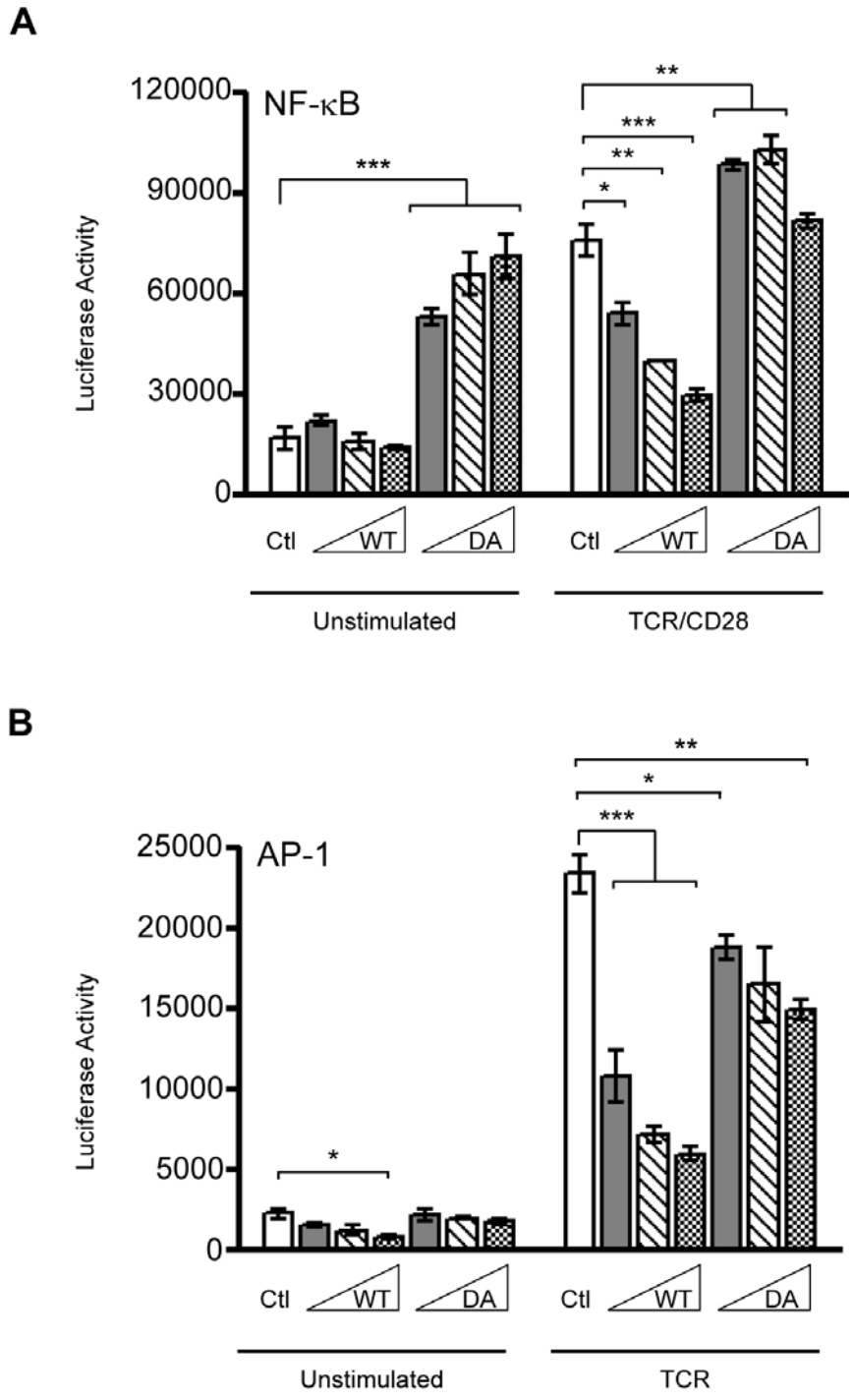
A
NF-κB
120000
90000
60000
30000
0
Luciferase Activity
***
**
***
**
*
Ctl
WT
DA
Ctl
WT
DA
Unstimulated
TCR/CD28
B
AP-1
25000
20000
15000
10000
5000
0
Luciferase Activity
*
**
*
***
Ctl
WT
DA
Ctl
WT
DA
Unstimulated
TCR

Figure 15. PTPN4 Negatively Regulates NFκB and AP-1 Activation. (A) Jurkat T cells were co-transfected with 20 μg of NFκB reporter construct and 2.5, 5.0, and 10 μg of WT or DA PTPN4. Total DNA was equalized with empty vector. Luciferase activity was measured in unstimulated and TCR/CD28-stimulated cells. Data are shown as mean ± SD (n = 3; Student's t-test *$p < 0.05$, **$p < 0.02$, ***$p < 0.006$, compared to control-transfected cells). (B) AP-1 transcriptional activity was measured as described in (A). Luciferase activity was measured in unstimulated and TCR-stimulated cells. Data are shown as mean ± SD (n = 3; Student's t-test *$p < 0.05$; **$p < 0.01$, ***$p < 0.005$, compared to control-transfected cells). Results are representative of three (A) and two (B) independent experiments.

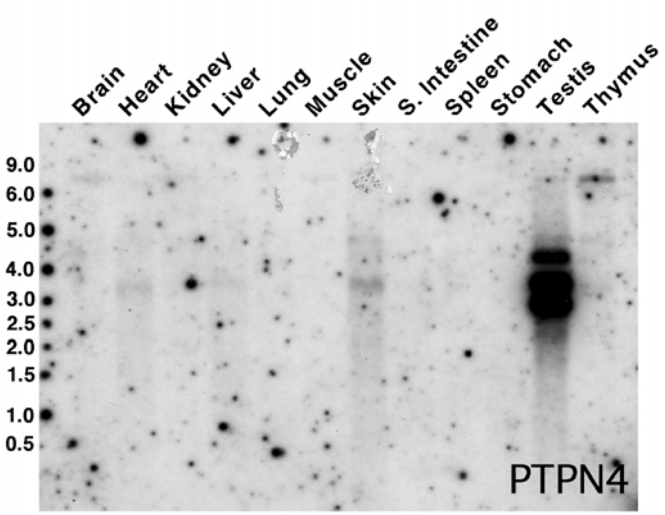

Figure 16. PTPN4 Expression in Murine Tissues. PTPN4 expression was examined in various murine tissues by Northern blotting. Three transcripts were highly expressed in the testis. A 7.0 kb transcript was detected in both the testis and thymus. The probe corresponded to the catalytic domain of PTPN4.

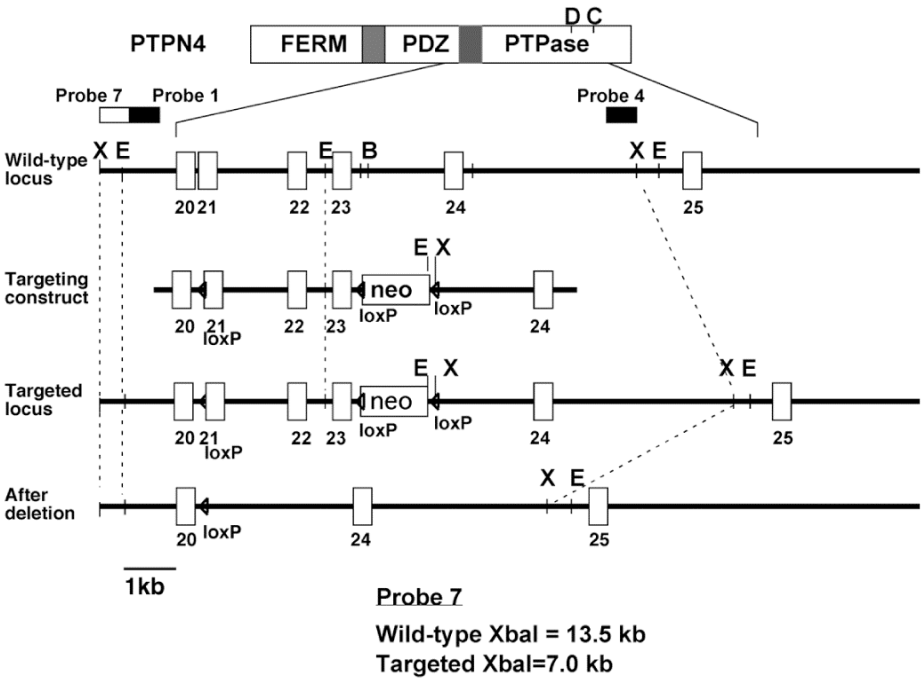

Figure 17. A Schematic Diagram of the FERM, PDZ, and Catalytic Domain (PTPase) of PTPN4. The targeting construct was designed with a loxP site between exon 20 and 21 followed by loxP sites flanking the neomycin cassette. Probe 7 was used in Southern blot analysis to detect the targeted locus and the recombined locus. B, BamHI; E, EcoRI; X, XbaI; neo, neomycin cassette.

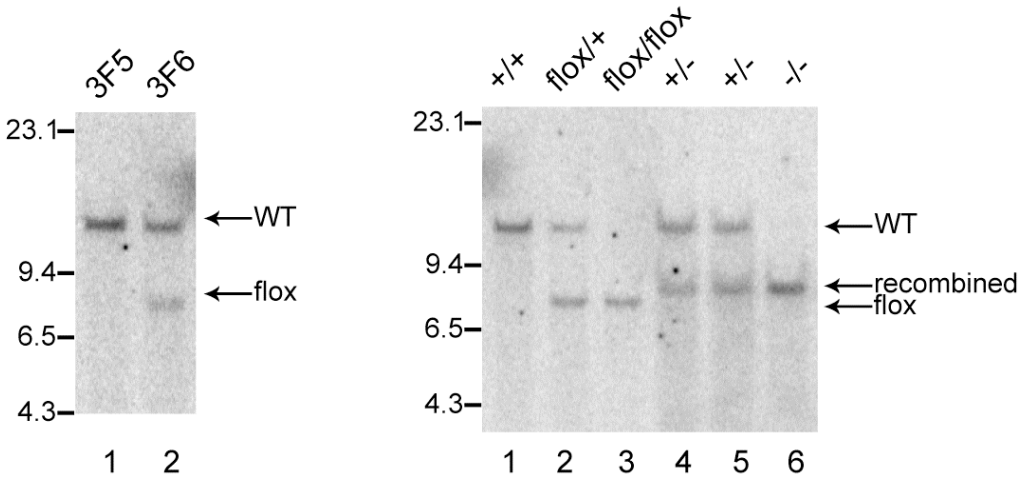

Figure 18. Detection of the Targeted and Recombined Alleles by Southern Blotting. Two ES cell clones (left panel: control, 3F5; positive for the targeted allele, 3F6) and F1 progeny from B6 crosses (right panel: *lanes 1-3*) were analyzed by Southern blotting to confirm the presence of the flanking loxP (floxed) targeted allele. Progeny from Meox-Cre crosses were examined for recombination of the floxed allele by Southern blotting (right panel: *lanes 4-6*; wild-type mice, +/+; heterozygotes, +/-; knock-out mice, -/-). Genomic DNA was digested with XbaI and hybridized with radiolabelled probe 7. Wild-type (WT), targeted (flox), and recombined alleles are indicated.

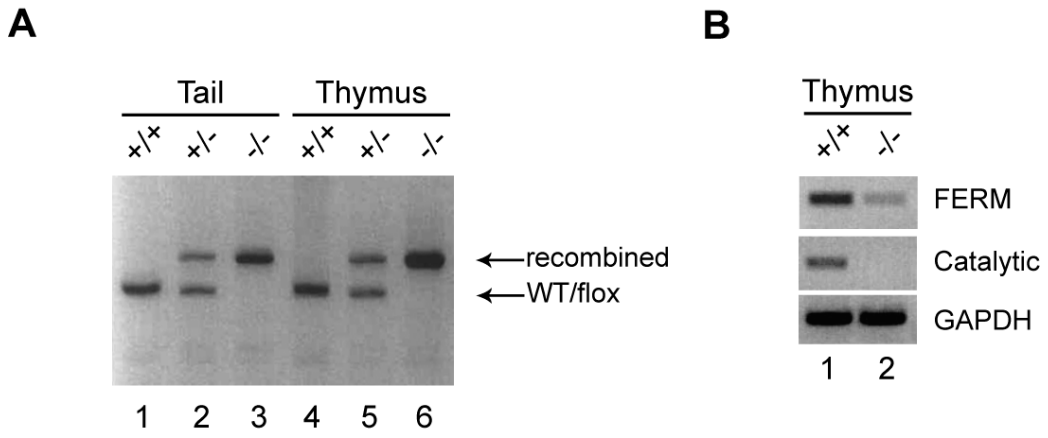

Figure 19. Recombination of the Targeted Allele Results in Complete Deletion of PTPN4. (A) To detect recombination of the targeted allele in mice, DNA was purified from either the tail or thymus of WT and PTPN4-deficient mice and amplified with PCR primers described in the Experimental Procedures. (B) PTPN4 mRNA from the thymus of WT and PTPN4 knock-out mice was amplified using RT-PCR. The primer pairs for the FERM and catalytic domains of PTPN4 and GAPDH are described in the Material and Methods.

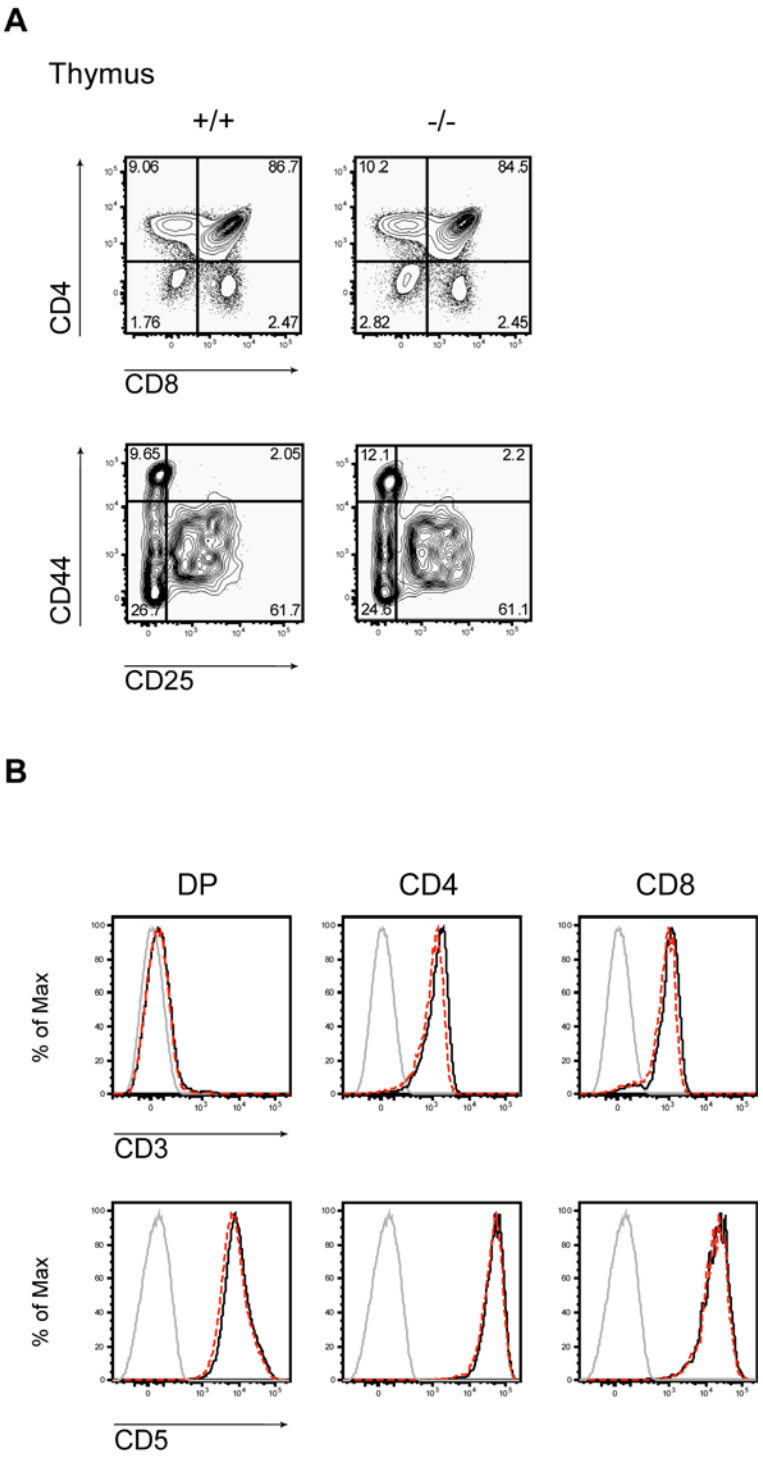
A
Thymus
+/+
-/-
9.06
86.7
1.76
2.47
10.2
84.5
2.82
2.45
CD4
CD8
9.65
2.05
26.7
61.7
12.1
2.2
24.6
61.1
CD44
CD25
B
DP
CD4
CD8
% of Max
CD3
% of Max
CD5

Figure 20. Normal Thymocyte Development in the Absence of PTPN4. (A) Thymocytes isolated from WT (+/+) and knock-out (-/-) mice were stained with anti-CD4-PerCP 5.5 and anti-CD8-PE-Cy7 Abs. The double negative populations ($CD4^-CD8^-B220^-CD11b^-$) were further distinguished by staining cells with anti-CD44-APC and anti-CD25-FITC Ab. (B) CD3 and CD5 Surface Expression on Thymocytes is Similar Between Wild-Type and Knock-out Mice. Gated double positive (DP) and $CD4^+$ and $CD8^+$ single positive populations from a representative WT mouse (black solid line) or knock-out mouse (red dashed line) were examined for CD3 and CD5 surface expression. Thymocytes were stained with anti-CD4-PerCP 5.5, anti-CD8-PE-Cy7, anti-CD3-Pacific Blue, and anti-CD5-PE Abs. An isotype control for CD3 or CD5 was used (light gray line). All cells were analyzed by flow cytometry. Data are represented as overlaid histograms and comparable to six independent experiments.

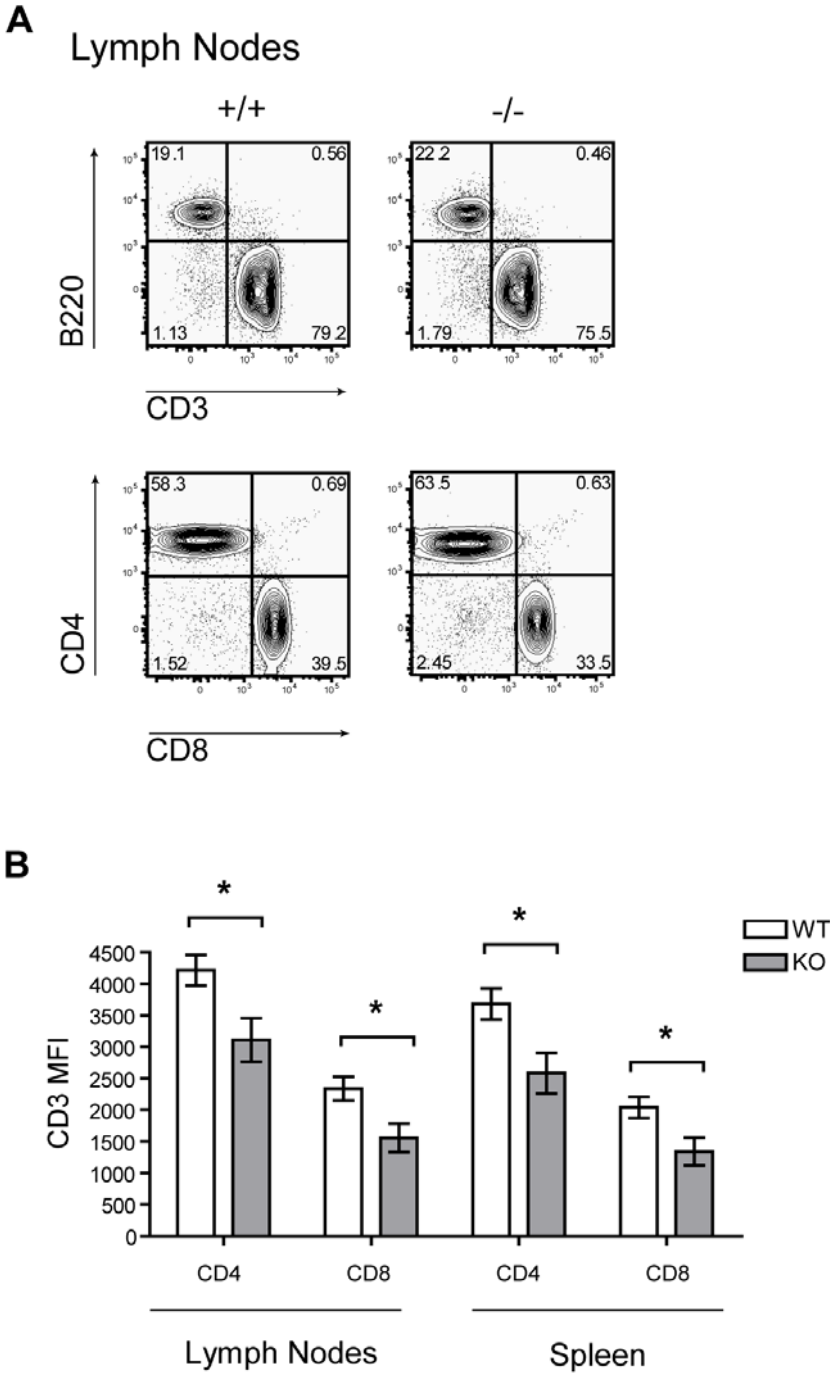
A
Lymph Nodes
+/+
-/-
19.1
0.56
1.13
79.2
22.2
0.46
1.79
75.5
B220
CD3
58.3
0.69
1.52
39.5
63.5
0.63
2.45
33.5
CD4
CD8
B
CD3 MFI
4500
4000
3500
3000
2500
2000
1500
1000
500
0
*
WT
KO
CD4
CD8
CD4
CD8
Lymph Nodes
Spleen

Figure 21. **PTPN4 Regulates CD3 Expression on Peripheral T cells.** (A) Lymph node cells of the indicated mice were isolated and stained with anti-CD3-Pacific Blue, anti-B220-APC-Cy7, anti-CD4-PerCP 5.5, and anti-CD8-PE-Cy7 Abs to examine peripheral lymphocytes populations by flow cytometry. (B) Cells, isolated from lymph nodes and spleen, were gated on $CD4^+$ and $CD8^+$ T cells. CD3 surface expression was examined by measuring CD3-Pacific Blue mean fluorescence intensity (MFI). Data are shown as mean ± SD ($n = 6$; $*p < 0.03$). Results are representative of six (A) and three (B) independent experiments.

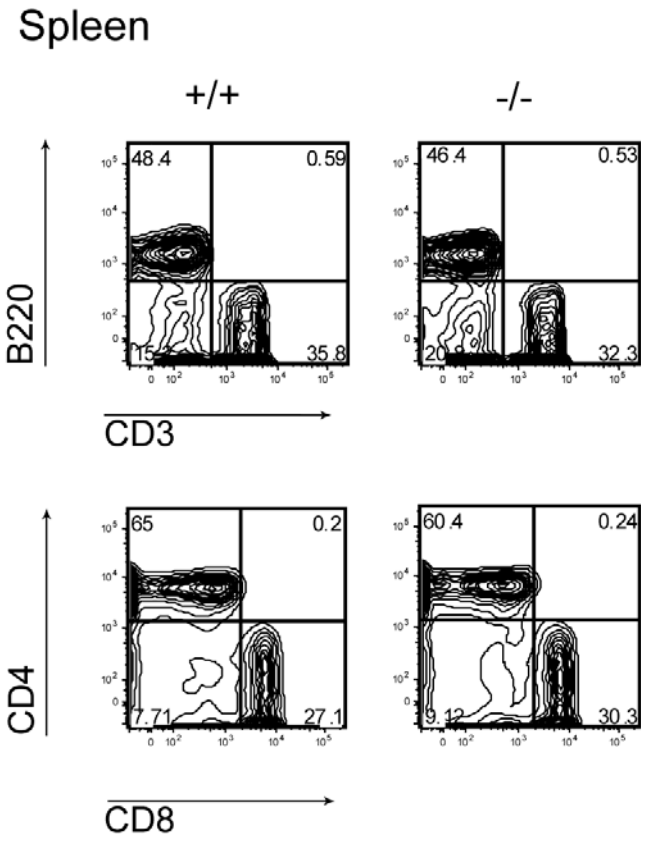

Figure 22. **Normal Lymphocyte Populations in the Spleen.** Splenocytes of the indicated mice were isolated and stained with anti-CD3-Pacific Blue, anti-B220-APC-Cy7, anti-CD4-PerCP 5.5, and anti-CD8-PE-Cy7 Abs to examine peripheral lymphocytes populations by flow cytometry.

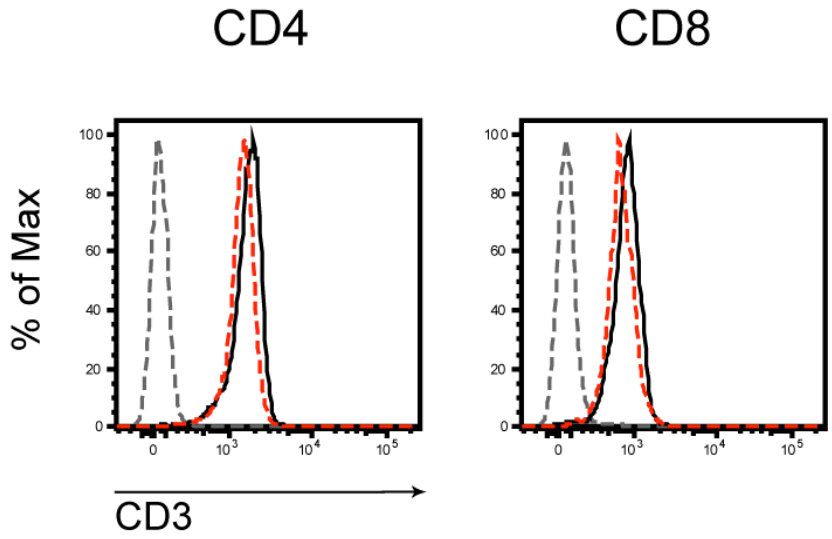

Figure 23. Peripheral T Cells Maintain Decreased CD3 Levels Following an Overnight Incubation. Lymph node cells were cultured in complete RPMI media for 12-18 h. The cells were then stained with anti-CD4-APC, anti-CD8-PE-Cy7, and anti-CD3 Pacific Blue. The levels of CD3 were assessed on CD4 and CD8 T cells by flow cytometry. Data are shown as overlaid histograms (light gray lines, isotype control; black solid line, WT cells; red dashed line, PTPN4$^{-/-}$ cells).

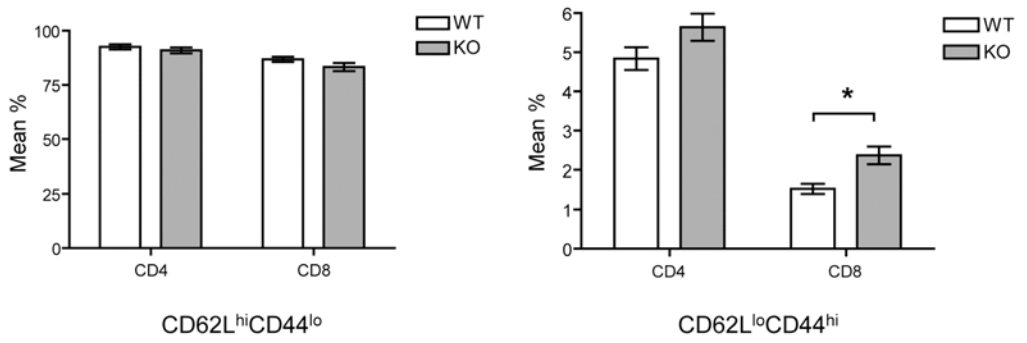

Figure 24. Increased Numbers of Effector/Memory T Cells in the Absence of PTPN4. Peripheral $CD8^+$ and $CD4^+$ T cells were stained with anti-CD62L-PE-Texas Red and anti-CD44-APC Abs to examine naïve and effector/memory subsets by flow cytometry. Data are shown as mean % ± SD (n = 4; *p = 0.003) and representative of three independent experiments.

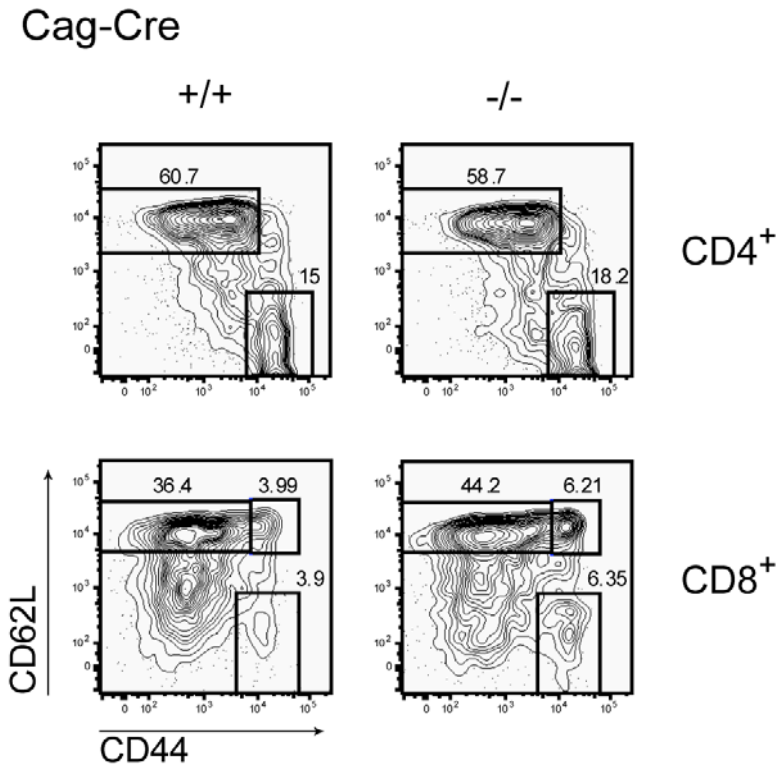

Figure 25. **Cag-Cre Knock-out Mice Have Increased Effector/Memory CD8 T Cells.** Splenocytes were isolated from Cag-Cre knock-out mice and wild-type littermates. Peripheral $CD4^+$ and $CD8^+$ T cells were gated, and the effector/memory population was examined. Cells were stained with anti-CD62L-PE-Texas Red and anti-CD44-APC Abs and examined by flow cytometry.

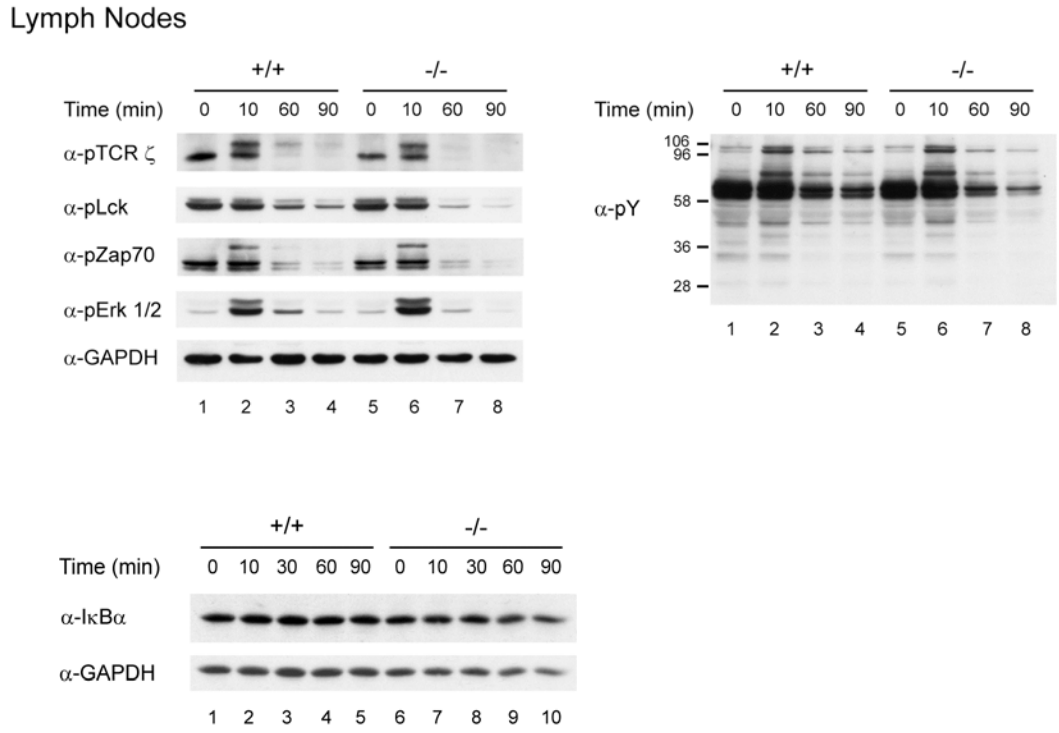

Figure 26. PTPN4 is Not Required for Normal TCR Signal Transduction. Cells from lymph nodes of PTPN4 WT (+/+) and knock-out (-/-) were stimulated and lysed as described in the Material and Methods. Phospho-ζ, phospho-Lck, phospho-ZAP70, phospho-Erk1/2, IκBα and GAPDH were detected by immunoblotting total cell extracts (left panel). Total protein tyrosine phosphorylation was detected from whole cell lysates by western blotting with anti-phosphotyrosine mAb (pY; right panel). Results are representative of three independent experiments.

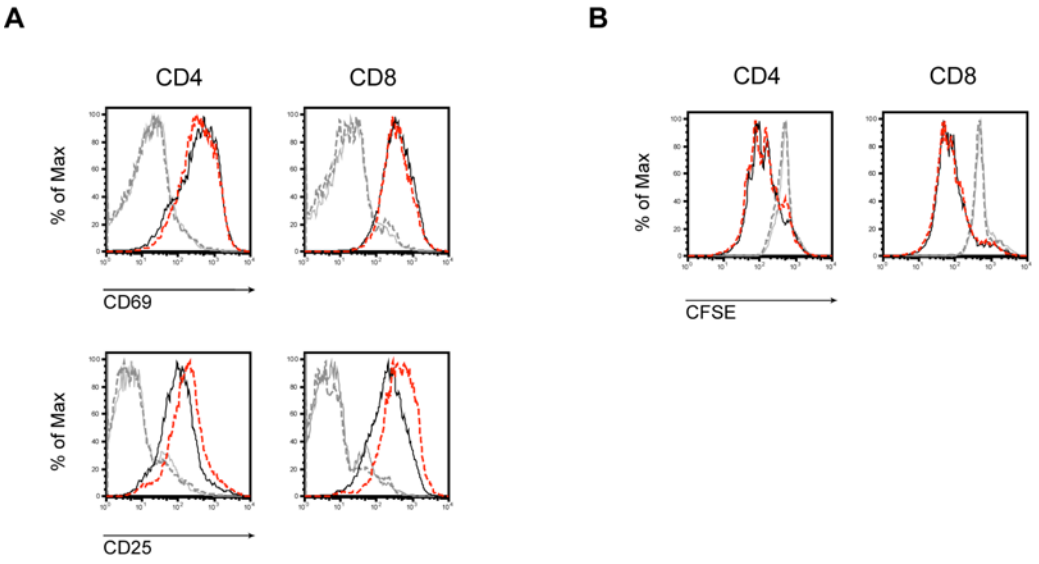

Figure 27. PTPN4-Null Cells Have Increased CD25 Levels but Normal Proliferation in Response to Stimulation. Purified T cells from a representative WT mouse (solid lines) or PTPN4 knock-out mouse (dashed lines) were stimulated (A) or labeled with CFSE and stimulated (B) as described in the Material and Methods. (A) Cells were harvested on Day 3 and stained with anti-CD4-APC, anti-CD8-PE-Cy7, anti-CD69 PE, and anti-CD25-FITC Abs to analyze upregulation of activation markers. (B) Labeled cells were analyzed by flow cytometry, and cell division was measured by CFSE dilution on Day 3. Data are shown as overlaid histograms (light gray lines, unstimulated cells; black solid line, stimulated WT cells; red dashed line, stimulated PTPN4$^{-/-}$ cells). Data are representative of three independent experiments.

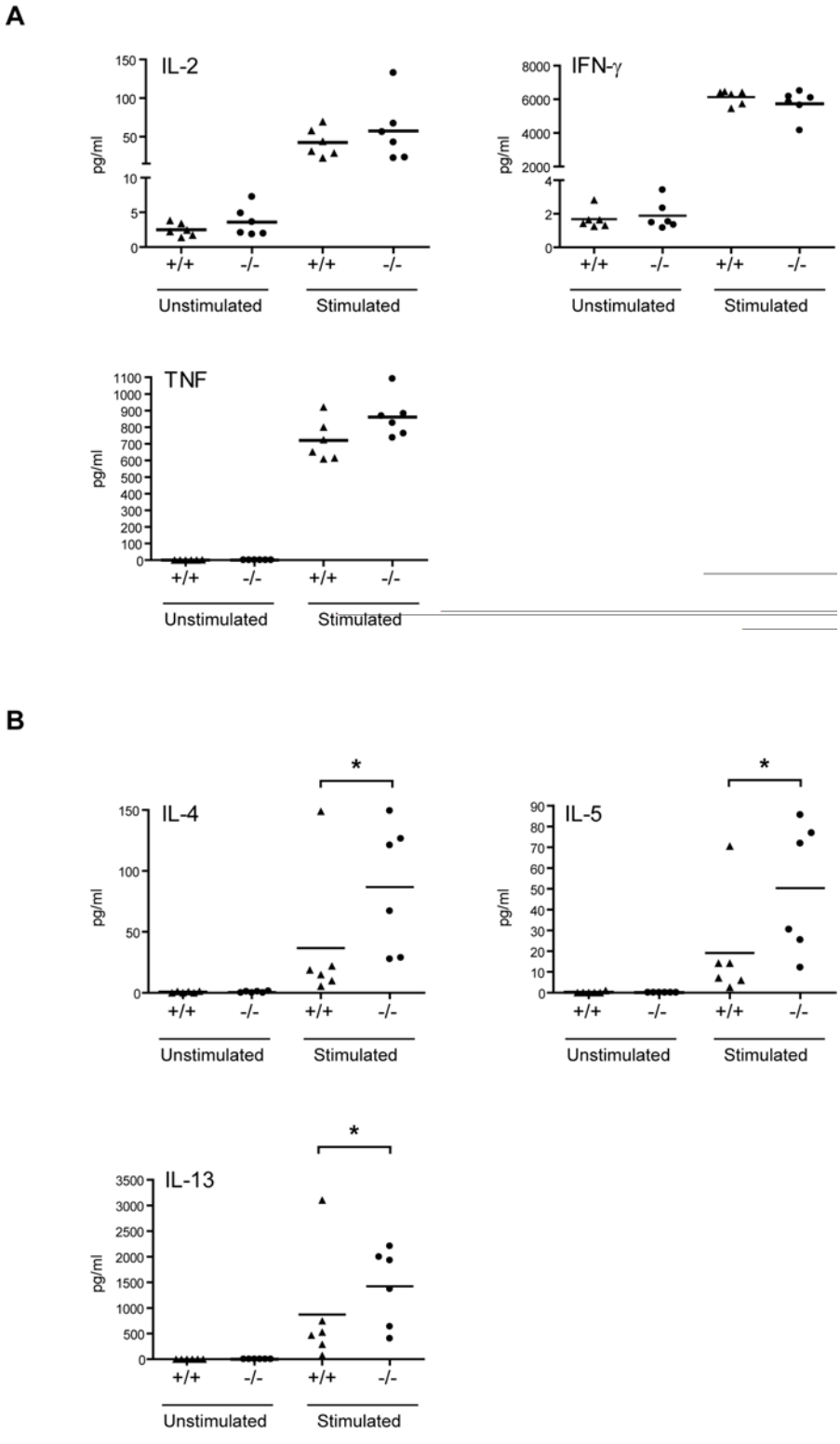
A
IL-2
pg/ml
150
100
50
10
5
0
+/+
-/-
+/+
-/-
Unstimulated
Stimulated
IFN-γ
pg/ml
8000
6000
4000
2000
4
2
0
+/+
-/-
+/+
-/-
Unstimulated
Stimulated
TNF
pg/ml
1100
1000
900
800
700
600
500
400
300
200
100
0
+/+
-/-
+/+
-/-
Unstimulated
Stimulated
B
IL-4
pg/ml
150
100
50
0
*
+/+
-/-
+/+
-/-
Unstimulated
Stimulated
IL-5
pg/ml
90
80
70
60
50
40
30
20
10
0
*
+/+
-/-
+/+
-/-
Unstimulated
Stimulated
IL-13
pg/ml
3500
3000
2500
2000
1500
1000
500
0
*
+/+
-/-
+/+
-/-
Unstimulated
Stimulated

Figure 28. PTPN4-Deficient T Cells Have Enhanced Th2 Cytokine Production. Culture supernatants were collected from unstimulated and stimulated T cells from WT (+/+) or PTPN4 knock-out (-/-) mice (n = 6). (A) IL-2, IFN-γ, and TNF were measured as described in the Material and Methods. (B) IL-4, IL-5, and IL-13 were measured from culture supernatants as previously described (n = 6; *$p < 0.02$).

CHAPTER V

Discussion

TCR signal transduction is controlled by the integrated and opposing actions of PTKs and PTPases. TCR signaling studies have focused mainly on the PTKs responsible for the activation of the pathway, since PTKs are easier to work with than PTPases. Most studies have had difficulty in identifying PTPase targets and correlating these substrates to PTPase function. Consequently, understanding the functions of PTPases during TCR signaling has progressed at a slower pace. Engagement of the TCR triggers transient protein tyrosine phosphorylation events, which activate the signals responsible for T cell effector functions. One of the earliest events following TCR stimulation is the tyrosine phosphorylation of two to three of the TCR ζ ITAMs (21, 22). Since TCR ζ contributes six ITAMs to the TCR complex, it has been presumed to be the predominant signaling subunit in the TCR. The phosphorylation of the ITAMs activates multiple downstream signaling molecules, and this ultimately results in T cell activation. We hypothesized that PTPases would play a critical role in controlling the magnitude of ζ phosphorylation during TCR signal transduction.

To identify any PTPases capable of regulating TCR ζ phosphorylation, two separate techniques were used. Biochemical purifications yielded PTPH1 and SHP-1 as two PTPases capable of dephosphorylating phospho-ζ (121). Furthermore, a screen

utilizing substrate-trapping derivatives of 47 distinct PTPases revealed that only PTPH1 complexed with phosphorylated ζ (121). Additional PTPases were considered in the dephosphorylation of TCR ζ since endogenous PTPH1 was difficult to detect in T lymphocytes. Based on the sequence homology, PTPN4, a PTPH1-family member, was also considered as a candidate PTPase capable of targeting TCR ζ. Both PTPH1 and PTPN4 were previously demonstrated to regulate T cell activation in transcriptional activation assays, but the substrates for either PTPase were not identified (123, 124). In order to determine whether TCR ζ is a substrate of PTPH1 and PTPN4, several experimental approaches were utilized in our studies. GST-fusion protein pull-downs, as described in Chapters III and IV demonstrated that the catalytic domains of PTPH1 and PTPN4 were capable of complexing with phosphorylated ζ. Both PTPH1 and PTPN4 dephosphorylated phospho-ζ when overexpressed in mammalian cell lines. Additional studies included the generation of PTPN4-deficient mice to investigate the role of PTPN4 in T cell development and function. The PTPN4 knock-out mice provided the most direct method to study the physiological function of PTPN4 in T cells. This is the first description of mice deficient in PTPN4 expression.

Based on our findings, it is possible that both PTPH1 and PTPN4 are capable of dephosphorylating the phosphorylated TCR ζ ITAMs. However, PTPN4 and PTPH1 knock-out mice demonstrate normal T cell development and T cell signal transduction in the absence of either PTPase. These data suggest PTPN4 and PTPH1 likely target unique substrates other than the ITAMs. Furthermore, PTPN4 may have a role in other biological processes in addition to the immune response.

Dephosphorylation of TCR ζ by PTPH1 and PTPN4

Both PTPH1 and PTPN4 may dephosphorylate the TCR ζ ITAMs. The results from the overexpression studies demonstrated that phospho-ζ is a substrate of PTPH1 and PTPN4, but normal TCR signaling is observed in cells isolated from PTPH1- and PTPN4-deficient mice (Figure 19 and (127)). We have demonstrated that substrate-trapping derivatives of PTPH1 and PTPN4 were capable of complexing with phosphorylated ζ in a heterologous cell lines. In addition, PTPH1 and PTPN4 directly dephosphorylated phospho-ζ, while the reduction in ζ phosphorylation was not detected with overexpression of the substrate-trapping mutants. In previous reports, the overexpression of either PTPH1 or PTPN4 in transcriptional reporter assays inhibited TCR-mediated signaling (123, 124). These molecular approaches provide plausible evidence that the phosphorylated ζ ITAMs are substrates for PTPH1 and PTPN4. In contrast, T cells isolated from PTPN4-deficient mice exhibited normal signaling following TCR stimulations. Likewise, TCR signaling was unaffected in PTPH1 knock-out mice (127). Based on the *in vitro* data, it would be expected that TCR ζ would be hyperphosphorylated in either of the knock-out mice compared to wild-type littermates. Therefore, one explanation is that both PTPH1 and PTPN4 regulate phospho-ζ levels. This would account for the normal levels of ζ phosphorylation in the absence of either PTPase.

PTPH1 and PTPN4 are related PTPases shown to be expressed in multiple lymphoid organs (124-126). These two PTPases contain an N-terminal FERM domain, followed by a PDZ domain, and a C-terminal catalytic domain (125, 126). PTPH1 and PTPN4 share high homology in the amino acid sequence (125, 126, 151). Furthermore, PTPH1 and PTPN4 were 64% identical in the catalytic domain and had the exact signature motifs in the catalytic pocket (151). Both PTPH1 and PTPN4 accumulate at the plasma membrane, and this subcellular localization is facilitated by the FERM domain (124, 136). Our studies also support this finding. In our observations, PTPH1 and PTPN4 are enriched in the membrane insoluble fraction following cell lysis (Figure 29). However, PTPN4 was redistributed to the soluble fraction when the FERM domain was deleted (Figure 29). This data demonstrates the FERM domain is necessary for PTPN4 to localize to the plasma membrane. Similar results were demonstrated with PTPH1 (124). Localization of PTPH1 and PTPN4 to the cytoskeleton would then bring these two PTPases into the vicinity of phospho-ζ. Due to the homology in the catalytic domain and the active site, it is possible that both PTPases dephosphorylate the TCR ζ ITAMs.

Overlapping substrates between PTPases has been observed in other PTPase-deficient mice. For example, HePTP is a PTPase that is expressed in lymphocytes and has been reported to specifically regulate ERK2 phosphorylation (167, 168). Consistent with the *in vitro* results, T lymphocytes from HePTP-deficient mice had increased ERK phosphorylation following stimulation (163). However, no defects were observed in the development of thymocytes or in the activation of peripheral lymphocytes (163). This is surprising considering ERK2 regulates different stages of thymocyte development (169).

Specifically, ERK2 is necessary for the expansion of DN3 cells and for the positive selection and differentiation of CD4 and CD8 SP cells (169). The mild phenotype in the HePTP-null mice suggests that other phosphatases may compensate for the absence of HePTP. In fact, multiple phosphatases have been reported to regulate ERK2 phosphorylation. The dual-specific phosphatase MKP-3 and the serine/threonine phosphatase PP2A can both target ERK2 (168, 170, 171). Therefore PTPH1 and PTPN4 may function in a similar manner.

A second explanation is that an unrelated PTPase dephosphorylates phospho-ζ, and PTPH1 and PTPN4 do not target ζ. While the overexpression of either wild-type PTPH1 or wild-type PTPN4 led to a reduction in ζ phosphorylation, the overexpression of the substrate-trapping mutants did not result in the hyperphosphorylation of ζ (Figures 5 and 13). The substrate-trapping mutant PTPase should protect the phospho-substrate from endogenous PTPases leading to hyperphosphorylation of the phosphorylated protein. A recent study reported that the low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) was capable of dephosphorylating FcγRIIA, a transmembrane receptor containing an ITAM motif (172). It is possible that LMW-PTP may also dephosphorylate the ITAMs of the TCR complex. Alternatively, a specific PTPase may not be involved in regulating the levels of phospho-ζ. PTPases were originally categorized as “housekeeping” proteins with non-restrictive specificities. Therefore, the regulation of phosphorylation in the TCR signaling pathway may be a result of non-specific dephosphorylation events by promiscuous PTPases in the vicinity of phosphoproteins.

Regulation of ITAM-Like Molecules in Non-Immunoreceptor Pathways

It is also possible that PTPH1 and PTPN4 do not regulate TCR ζ phosphorylation. For example, the induction of TCR-mediated signals was unaffected by the absence of either PTPH1 or PTPN4. These data demonstrated that PTPH1 and PTPN4 are not required for TCR signaling. One explanation is that neither PTPH1 nor PTPN4 regulate ζ phosphorylation *in vivo*. Rather, these two PTPases may target molecules present in other signaling pathways. Since the substrate-trapping catalytic domains of PTPH1 and PTPN4 exhibited a relatively high affinity for the phosphorylated ζ ITAMs, both PTPases probably interact with other ITAM-containing proteins. In fact, we showed that PTPH1 was capable of associating with the ITAM of DAP12 (Figure 8). The importance of ITAM-like molecules has been described for various non-immunoreceptor signaling pathways, including adhesion molecules, lectin receptors, and chemokine receptors (15). ITAM-like sequences have been identified in several cytoplasmic proteins. These include the cytoskeleton-associated proteins ezrin and moesin, which have been shown to interact with the adhesion molecule PSGL-1 and the PTK Syk (153, 161, 162). The association between moesin and Syk allows the kinase to participate in PSGL-1 signal transduction. The deletion of the ITAM sequence in moesin abrogates PSGL-1 signaling mediated through Syk activation (162). Therefore, PTPH1 and PTPN4 may regulate other signaling pathways that utilize ITAM-containing subunits. Another possibility is that PTPN4 and PTPH1 target a distinct set of substrates completely unrelated to ITAMs. This will be discussed further.

The Regulation of NFκB by PTPN4 Influences Th2-Specific Cytokine Production

Our findings for the PTPN4-deficient mice suggest that PTPN4 may have a more important role in T cells than PTPH1. As mentioned above, the PTPN4 knock-out mice had an increase in the effector/memory T cell population compared to wild-type mice (Figure 19). TCR stimulation induced PTPN4-null cells to produce higher amounts of IL-4, IL-5, and IL-13 versus wild-type cells. In the absence of PTPH1, restimulation of activated cells did not produce elevated amounts of IL-4 (127). Therefore, this effect is likely specific to PTPN4. The data indicate that PTPN4 may limit Th2-specific cytokine production by regulating NFκB activation. Thus, the increased magnitude NFκB activation in the absence of PTPN4 may result in the transcriptional activation of IL-4 and the subsequent production of IL-4, IL-5, and IL-13.

The cytokines IL-4, IL-5 and IL-13 are characteristic of a Th2 immune response. Th2 cells have critical roles in antibody-dependent responses and the clearance of extracellular parasites. The polarization of CD4 T cells to the Th2 lineage is largely influenced by the initial exposure of the cells to IL-4 during TCR activation (Figure 30). Signaling through the IL-4 receptor on CD4 cells induces the activation of Th2-specific transcription factors (GATA3 and c-Maf)(69, 173). GATA3 and c-Maf bind the IL-4 3' enhancer and IL-4 promoter region, respectively, inducing the production of IL-4, which provides a positive feedback loop to promote the differentiation to the Th2 lineage (173). TCR restimulation and GATA3 activation result in the production of IL-4, IL-5 and IL-

13 (69, 173). It is the initial production of IL-4 that triggers the differentiation of Th2 cells. IL-4 is produced by many different cells (dendritic cells (DC), mast cells, basophils, and Natural Killer T cells (NKT cells) but primarily secreted by CD4 T cells. The elevated production of Th2-specific cytokines in PTPN4-deficient mice may be a consequence of increased NFκB activity. The NFκB subunits, p65 and p50, have been shown to bind multiple sites of the IL-4 promoter and enhance the transcription of IL-4 (Figure 30)(165). Therefore, the transcriptional activation of IL-4 may be increased in the PTPN4-null mice compared to the wild-type mice. The data from our transient transfection assays support this hypothesis. The overexpression of the substrate-trapping derivative of PTPN4 resulted in the potentiation of NFκB activation. These data suggested that PTPN4 targets a tyrosine-phosphorylated substrate in the NFκB pathway. Specifically, interaction with the substrate-trapping mutant likely prevented the dephosphorylation of the substrate. In this case, the inability to dephosphorylate this substrate promotes the activation of NFκB.

Potential Dephosphorylation of IκBα by PTPN4

A potential PTPN4 substrate in the NFκB pathways is the inhibitor of NFκB α (IκBα). In T cells, the activation of PKCθ leads to the activation of NFκB. NFκB is bound by IκBα in the cytoplasm. Following the activation of this pathway, serine phosphorylation of IκBα results in its degradation, releasing NFκB. NFκB then translocates to the nucleus. The phosphorylation of IκBα on serine residues 32 and 36 has been extensively studied. However, reports have demonstrated that IκBα can be

phosphorylated on tyrosine residue 42 following pervanadate treatment, and this phosphorylation leads to the activation of NFκB (174, 175). The tyrosine phosphorylation of IκBα was also dependent on Lck (174). Therefore, the ability of PTPN4 to regulate IκBα tyrosine phosphorylation may be a mechanism for the regulation of NFκB activation. In the absence of PTPN4, or during the overexpression of the substrate-trapping mutant, IκBα remains tyrosine phosphorylated, and NFκB activation occurs. An increase in IκBα degradation after TCR stimulation was not detected in PTPN4-deficient cell when compared to wild-type cells (Figure 26). It is possible that the difference in protein degradation is below the threshold of detection. The relevant cell population, such as differentiated T cells, exhibiting enhanced IκBα degradation may only be a small percentage of the bulk peripheral T cell population. Therefore, the effect would not be detected unless that population was identified and purified.

A Role for PTPN4 in Other Cell Types to Regulate Cytokine Production

In the PTPN4-deficient mice, the initial production of IL-4 may come from cells, such as mast cells or dendritic cells, initiating the differentiation of Th2 cells. More specifically, PTPN4 may regulate IL-4 production in these other cellular sources rather than in the CD4 cells. The backcross with Meox-Cre transgenic mice deletes PTPN4 expression in the entire mouse. Therefore, the deficiency in PTPN4 could lead to increased production of IL-4 by these other cell types, which would then promote the differentiation of Th2 cells. PTPN4 may have a role in the signaling pathway leading the transcriptional activation of IL-4 by these other cells. As a consequence, Th2

differentiation is elicited and Th2-specific cytokines are subsequently produced. Mixed bone marrow chimeras would identify whether other cell types are involved in regulating cytokine production. Briefly, bone marrow from wild-type and PTPN4-null mice would be mixed and injected into irradiated wild-type or PTPN4-deficient mice. The production of cytokines would be examined from isolated T cells from these mice. Wild-type and knock-out T cells can be differentiated by the unique expression of Thy1.1 and Thy1.2. This would determine whether the elevated Th2 cytokine production by PTPN4-deficient cell is T cell intrinsic or influenced by other cell types.

A Role for PTPN4 in Other Biological Processes

The distribution of PTPN4 expression suggests that its main function may be in the testis. It was reported previously that two PTPN4 transcripts were highly expressed in the testis (138). By Northern blotting, we also detected high levels of PTPN4 message in the testis (Figure 13). Previous findings demonstrated the upregulation of PTPN4 expression in the testis of 9-week old mice versus 3-week old mice (138). More specifically, the PTPN4 was detected in the spermatocytes of the seminiferous tubule (138). These studies suggest a potential role in spermatogenesis. The activities of several families of PTKs have been reported to function during spermatogenesis (176). PTPN4 could function to regulate the tyrosine phosphorylation events during spermatogenesis. The role of PTPN4 in testis could then be studied with the PTPN4 knock-out mice. In our initial observations, the knock-out mice were fertile and produced normal litter numbers. Whether PTPN4 has a regulatory role in spermatogenesis remains to be determined.

Interestingly, we observed enlarged gall bladders in several PTPN4-deficient mice compared to wild-type mice. This effect was more pronounced in older mice but has not been quantitated at the present time. One explanation for this is that PTPN4 may play a role in the regulation of bile metabolism. Farsenoid X Receptor (FXR) is known as the bile receptor and shown to be involved in the feedback regulation of bile acid synthesis (177). FXR participates in the negative regulation of bile acid synthesis by transcriptionally activating other factors that inhibit bile acid synthesis (177). Furthermore, bile acids can trigger the transcription of inhibitory molecules through the c-Jun N-terminal kinase (JNK)-pathway (177). It is unclear at this time whether PTPN4 has a role in the regulation of bile metabolism or directly in the gall bladder.

Conclusions

While the overexpression studies presented in Chapters III and IV demonstrated that PTPH1 and PTPN4 were capable of dephosphorylating the TCR ζ ITAMs, the PTPH1 and PTPN4 knock-out mice indicated that TCR signaling pathway is not regulated by PTPH1 or PTPN4. Instead, these PTPases likely participate in different signaling pathways with different biological outcomes.

Additional studies are necessary to further examine the regulation of TCR signaling by PTPN4 and PTPH1. First, to address whether both PTPH1 and PTPN4 dephosphorylate the ζ ITAMs, the generation of double knock-out mice would be

required. This model would reveal the coordinate function of PTPH1 and PTPN4 in the regulation of ζ phosphorylation and the TCR signaling pathway. Alternatively, the actions of other PTPases on phospho-ζ cannot be excluded. Some of these PTPases may include PEP and LMW-PTP, which can target phospho-ζ or phospho-ITAMs, respectively (118, 172). Second, the mechanism regulating Th2-specific cytokine production in PTPN4-deficient mice will need to be elucidated. Does PTPN4 influence the increased development of Th2 cells or does PTPN4 regulate TCR-induced IL-4 activation? These studies will determine whether PTPN4 is functioning in the TCR or cytokine receptor signaling pathway to regulate the IL-4 locus (Figure 30). Finally, a disease model would implicate a functional effect of PTPN4. Infection of PTPN4 knock-out mice with *Leishmania major* would uncover defects in the ability to mount a proper immune response. A Th1 response is required for the clearance of this intracellular pathogen, while a Th2 response mounts a non-healing response. Even though PTPase substrates are identified with *in vitro* approaches, the molecular mechanism does not always explain the physiological functions of PTPases.

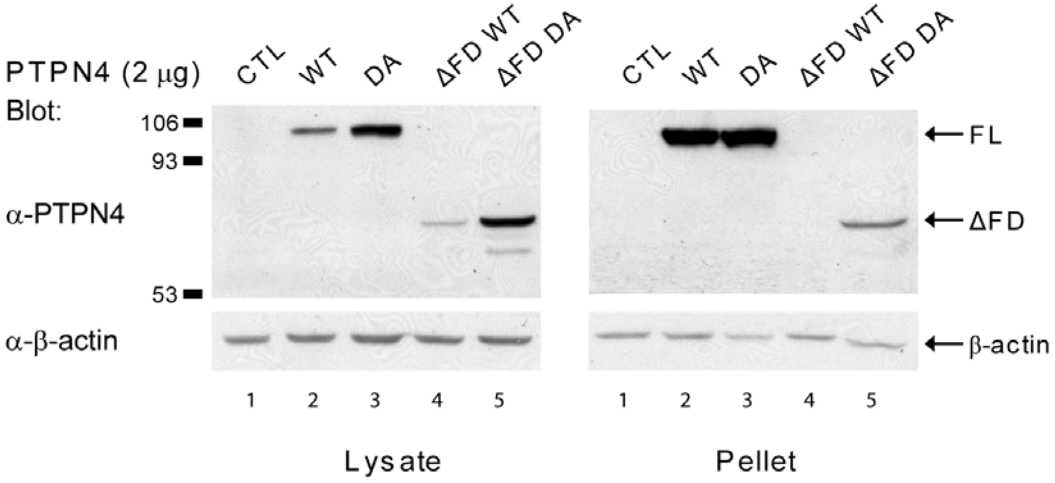

Figure 29. Localization of PTPN4 to the Membrane-Insoluble Fraction is Mediated by the FERM Domain. HEK 293T cells were transfected with a control vector (CTL, *lane 1*), full-length wild-type (WT, *lane 2*) PTPN4, or substrate-trapping mutant (DA, *lane 3*) PTPN4. Cells were also transfected with wild-type and substrate-trapping mutant PTPN4 constructs that lacked the FERM domain (ΔFD WT and ΔFD DA, *lanes 4-5*). After 48 h, cells were lysed, and both the soluble (Lysate) and insoluble (Pellet) fractions were Western blotted with anti-PTPN4 and anti-β-actin antibodies. Data are representative of 3 independent experiments.

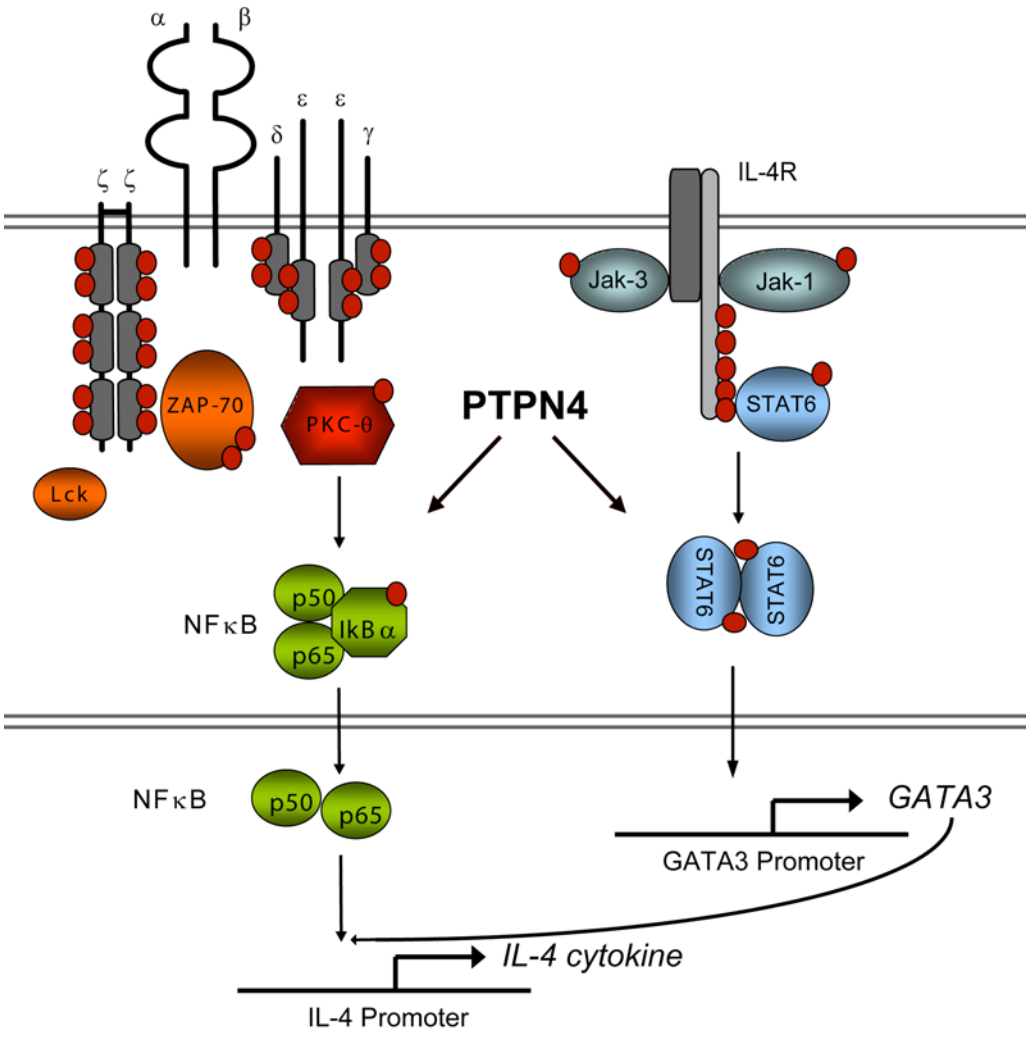

Figure 30. Model of PTPN4 Function in Th2 Cytokine Production. TCR-induced NFκB activation leads to acute IL-4 production. STAT6 is phosphorylated and activated after stimulation of the IL-4 receptor (IL-4R). Signaling through STAT6 induces the expression of the transcription factor GATA3. Subsequently, GATA3 upregulates the Th2 cytokines and promotes Th2 commitment. PTPN4 may function to inhibit IL-4 production by regulating the NFκB pathway. Alternatively, PTPN4 may regulate the cytokine signaling pathway. The red circles represent phosphorylated residues (tyrosine, serine, or theronine).

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VITAE

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