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The protein tyrosine phosphatase PTPN4/PTP-MEG1, an enzyme capable of dephosphorylating the TCR ITAMs and regulating NF-κB, is dispensable for T cell development and/or T cell effector functions

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ABSTRACT

T cell receptor signaling processes are controlled by the integrated actions of families of protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases). Several distinct cytosolic protein tyrosine phosphatases have been described that are able to negatively regulate TCR signaling pathways, including SHP-1, SHP-2, PTPH1, and PEP. Using PTPase substrate-trapping mutants and wild type enzymes, we determined that PTPN4/PTP-MEG1, a PTPH1-family member, could complex and dephosphorylate the ITAMs of the TCR ζ subunit. In addition, the substrate-trapping derivative augmented basal and TCR-induced activation of NF-κB in T cells. To characterize the contribution of this PTPase in T cells, we developed PTPN4-deficient mice. T cell development and TCR signaling events were comparable between wild type and PTPN4-deficient animals. The magnitude and duration of TCR-regulated ITAM phosphorylation, as well as overall protein phosphorylation, was unaltered in the absence of PTPN4. Finally, Th1- and Th2-derived cytokines and *in vivo* immune responses to *Listeria monocytogenes* were equivalent between wild type and PTPN4-deficient mice. These findings suggest that additional PTPases are involved in controlling ITAM phosphorylations.

1. 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 (Call et al., 2004). TCR recognition of cognate peptide/MHC complexes on APCs results in a cascade of intracellular signaling processes (reviewed in Wange and Samelson, 1996). These intracellular signals are critical for T cell development, cytokine production, and/or proliferation. Seconds after TCR cross-linking, the cytoplasmic tail of CD3 ε undergoes a conformational change (Gil et al., 2002, 2005). This conformational change, along with simultaneous or subsequent activation of protein tyrosine kinases (PTKs), results in increased protein tyrosine phosphorylations (Gil et al., 2005; Minguet et al., 2007). The protein phosphorylation pathways commence once the Src-family PTKs, Lck and/or Fyn, are activated and re-localized to the CD3 subunits (Palacios and Weiss, 2004). There, the kinases phosphorylate the two tyrosine residues located within the ITAMs (reviewed in Cambier, 1995; Reth, 1989; Weiss, 1993). ITAMs are a conserved signaling motif and are present in one or more copies in the cytoplasmic tails of the CD3 γ, δ, ε and TCR ζ subunits. ITAMs comprise the sequence $YxxLx_{(6-8)}YxxL$, with the phosphorylation of the tyrosine residues providing the critical molecular determinant for TCR-mediated signal transmission (Pitcher and van Oers, 2003; Weiss and Littman, 1994). ITAMs also form the basis of

Abbreviations: PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; WT, wild type.

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antigen receptor signal transduction for the B cell receptor, selected activating NK cell receptors, and particular Ig-receptors (Humphrey et al., 2005; Reth, 1992; Weiss and Littman, 1994).

In T cells, the bi-phosphorylated ITAMs are bound by the tandem SH2 domains of ZAP-70, a Syk-family PTK (Hatada et al., 1995). ZAP-70 is catalytically activated upon tyrosine phosphorylation by Src PTKs (Chan et al., 1995; Wange et al., 1995). The signaling cascade continues with activated ZAP-70 phosphorylating several adaptor proteins, kinases and effector proteins (Jordan et al., 2003; Samelson, 2002). These latter molecules participate in the activation of multiple transcription factors, including NFAT, AP-1 and NF-κB, which results in the production of different cytokines (Huang and Wange, 2004; Li and Verma, 2002; Rao et al., 1997; Waldmann, 2006).

TCR-induced increases in protein tyrosine phosphorylations are transient, with most phosphoproteins returning to baseline levels within minutes of stimulation. The transient nature of phosphotyrosine induction is partly a consequence of changes in the enzymatic activity and/or distribution of protein tyrosine phosphatases (PTPases). PTPases are a family of enzymes that dephosphorylate tyrosine residues on a large number of TCR-regulated proteins. It is estimated that T cells express approximately 60 of the 107 potential PTPases identified in the human genome (Alonso et al., 2004; Gjorloff-Wingren et al., 2000; Mustelin et al., 2005). The identity of the PTPases that dephosphorylate components of the TCR signaling pathway have been elucidated by the characterization of PTPase-deficient or -mutant mice (Mustelin and Tasken, 2003; Veillette et al., 2002). Two key PTPases characterized in this manner include SHP-1 and SHP-2, homologous enzymes containing tandem SH2 domains followed by a catalytic domain (Tonks and Neel, 2001). SHP-1 is expressed specifically in hematopoietic cells and is mutated in motheaten mice. Thymocytes from these mutant mice have elevated levels of phosphoproteins compared to normal mice (Lorenz et al., 1996; Pani et al., 1996; Tsui et al., 1993). These analyses and biochemical characterizations of SHP-1 suggest that this PTPase dephosphorylates positive regulatory tyrosine residues in the catalytic domains of Src- and ZAP-70-family PTKs (Brockdorff et al., 1999). SHP-2, in contrast to SHP-1, is ubiquitously expressed and functions as a positive regulator of the Ras/ERK pathway following TCR signaling (Frearson and Alexander, 1998; Nguyen et al., 2006). One report suggested that SHP-2 can dephosphorylate the phospho-ITAMs of TCR ζ (Lee et al., 1998). Yet, neither SHP-1 nor SHP-2 has been conclusively shown to dephosphorylate ITAMs. Another PTPase implicated in TCR signaling is PEP, or Pest-domain Enriched Tyrosine Phosphatase. Mechanistically, a proline-rich domain of PEP constitutively associates with the SH3 domain of the PTK Csk, enabling the PEP/Csk complex to localize near the PTKs involved in TCR signaling (Cloutier and Veillette, 1996; Gjorloff-Wingren et al., 1999; Veillette et al., 2002). The targeted disruption of PEP results in increased numbers of effector/memory T cell subsets compared to wild type mice (Hasegawa et al., 2004). Yet, naïve T cells from these mice have similar TCR-induced protein tyrosine phosphorylation pathways as wild type mice, suggesting that other PTPases dephosphorylate the ITAMs.

Alternative approaches to identify PTPases that regulate signaling pathways include the use of substrate-trapping derivatives of PTPases to entrap phosphoproteins (Flint et al., 1997; Walchli et al., 2000). Using such PTPase screens, we identified PTPH1 (PTPN3) as a PTPase that interacts with and dephosphorylates TCR ζ, suggesting that PTPH1 is an essential regulator of TCR signaling (Sozio et al., 2004). This is consistent with transcriptional reporter assays in T cells, wherein the overexpression of PTPH1 inhibited TCR-induced NFAT activation (Han et al., 2000). Although mice containing a targeted deletion of PTPH1 exhibit enhanced growth, normal T cell development, TCR signaling events, and T cell effector functions is maintained (Bauler et al., 2007; Pilecka et al., 2007). 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, a PTPH1-family member that contains highly homologous N-terminal FERM-, central PDZ-, and C-terminal catalytic domains (Gu et al., 1991; Park et al., 2000). Transcriptional reporter assays in T cells containing elevated levels of PTPN4 suppressed TCR-induced NFAT and AP-1 activation (Han et al., 2000).

To determine whether PTPN4 negatively regulates TCR signaling events, we performed biochemical assays to characterize the functional interaction between PTPN4 and phospho-ζ. Findings from these experiments indicated that PTPN4 could interact with and dephosphorylate TCR ζ. In addition, a substrate-trapping derivative of PTPN4 potentiated NF-κB activation, even prior to TCR engagement. To fully define the contribution of PTPN4 in lymphocytes, we generated PTPN4-deficient mice. We report here that PTPN4 gene-disrupted mice had normal T cell development in the thymus and similar T cell subsets in the secondary lymphoid organs. T cells from these mice had normal TCR-induced signaling pathways. In addition, these mice exhibited a similar capacity to produce both Th1- and Th2-derived cytokines, such as IL-2, IL-4, IL-5, IL-13, and IFN-γ, following TCR/CD28 cross-linking. Finally, PTPN4-deficient mice had comparable abilities to clear primary *Listeria monocytogenes* infections as wild type littermates. These findings suggest that multiple PTPase-families are likely involved in the regulation of ITAM phosphorylations, providing for effective compensatory mechanisms in the absence of PTPN4.

2. Materials and methods

2.1. Antibodies

The 145-2C11 hybridoma (anti-CD3 ε) was obtained from American Type Culture Collection (ATCC). The 35.71 (anti-CD28) hybridoma was kindly provided by Dr. James Allison (Memorial Sloan-Kettering Cancer Center). Antibodies were purified from hybridoma culture supernatants with PA or PG affinity chromatography procedures (Harlow and Lane, 1988). C305.2 (anti-TCRβ) and 1F6 (anti-Lck) were obtained from Dr. Arthur Weiss (University of California, San Francisco). The following antibodies were used for Western blotting: anti-β-actin (4967; Cell Signaling Technologies), anti-FLAG (M2; Sigma–Aldrich), anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-IκBα (sc-371; Santa Cruz Biotechnology), anti-MAPK (Erk-1/2) and -phosphoMAPK (M8159; Sigma), anti-p42/44 (9102; Cell Signaling), anti-phospho-SAPK/JNK (9255; Cell Signaling), anti-SAPK/JNK (9252; Cell Signaling). The anti-TCR ζ (6B10.2) antibody has been previously described (van Oers et al., 1995). Anti-PTPN4-specific antibodies were generously provided by Dr. Philip Majerus (Washington University), or purchased from Orbigen (Orbigen, Inc.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig, goat anti-rabbit Ig (Bio-Rad Laboratories), or HRP-conjugated goat anti-mouse IgG2b (Invitrogen Corp.) were used as secondary antibodies. The following antibodies were utilized in multicolor flow cytometry (purchased from BD Pharmingen): APC-Cy7-B220, PerCP 5.5-CD4, PE-Cy7-CD8, FITC-CD25, PE-CD69, APC-Cy7-CD11b. Pacific Blue-CD3 was purchased from eBiosciences, and PE-Texas Red-CD69 and PE-Texas Red-CD62L were purchased from Invitrogen Corp. Cell populations were analyzed with either FACSCaliber or LSRII flow cytometers (Becton-Dickenson) using Cell Quest (BD) and/or FlowJo software (Treestar).