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The T Cell Protein Tyrosine Phosphatase Is a Negative Regulator of Janus Family Kinases 1 and 3

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Summary

Background: The immune response is regulated through a tightly controlled cytokine network. The counteracting balance between protein tyrosine kinase (PTK) and protein tyrosine phosphatase (PTP) activity regulates intracellular signaling in the immune system initiated by these extracellular polypeptides. Mice deficient for the T cell protein tyrosine phosphatase (TCPTP) display gross defects in the hematopoietic compartment, indicating a critical role for TCPTP in the regulation of immune homeostasis. To date, the molecular basis underlying this phenotype has not been reported.

Results: We have identified two members of the Janus family of tyrosine kinases (JAKs), JAK1 and JAK3, as bona fide substrates of TCPTP. Inherent substrate specificity in the TCPTP-JAK interaction is demonstrated by the inability of other closely related PTP family members to form an in vivo interaction with the JAKs in hematopoietic cells. In keeping with a negative regulatory role for TCPTP in cytokine signaling, expression of TCPTP in T cells abrogated phosphorylation of STAT5 following interleukin (IL)-2 stimulation. TCPTP-deficient lymphocytes treated with IL-2 had increased levels of tyrosine-phosphorylated STAT5, and thymocytes treated with interferon (IFN)-α or IFN-γ had increased tyrosine-phosphorylated STAT1. Hyperphosphorylation of JAK1 and elevated expression of iNOS was observed in IFN-γ-treated, TCPTP-deficient, bone marrow-derived macrophages.

Conclusions: We have identified JAK1 and JAK3 as physiological substrates of TCPTP. These results indicate a negative regulatory role for TCPTP in cytokine signaling and provide insight into the molecular defect underlying the phenotype of TCPTP-deficient animals.

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Background

Cytokines mediate a diverse array of physiological effects ranging from complex immunological functions such as lymphocyte and monocyte activation to lactation and growth hormone responses. Cytokine receptors propagate signaling through recruitment of the Janus family of cytoplasmic tyrosine kinases (JAKs), which are responsible for the propagation of signaling initiated by these extracellular polypeptides [1]. Activation of the JAKs results in tyrosine phosphorylation of a number of signaling molecules, including members of the STAT family of transcription factors [2]. Deregulation of cytokine function can have dramatic consequences, as exemplified by the generation of gene-targeted mice of JAK family members. JAK1-deficient mice die perinatally and are unable to nurse [3], while JAK2 gene-targeted mice display embryonic lethality due to anemia [4, 5]. TYK2 gene-targeted animals display defects in interferon (IFN)-α/β and -γ signaling as well as interleukin (IL)-12 responses [6, 7]. Finally, JAK3-deficient animals have a phenotype similar to human severe combined immunodeficiency (SCID), due to the lack of developed lymphocytes [8, 9]. These genetic studies have demonstrated the critical and nonredundant role that cytokine signaling and JAK tyrosine kinases play in both the development and function of the immune response; however, the regulation of JAK activity remains poorly understood.

Protein tyrosine phosphatases (PTPs) form a large superfamily of enzymes that play diverse roles in regulating cellular signaling events and homeostasis [10]. All members of this family are characterized by the presence of a 240 amino acid PTP domain. The T cell protein tyrosine phosphatase (TCPTP) is a member of this family of enzymes predominantly expressed in the hematopoietic lineage [11]. TCPTP null mice display severe defects in the hematopoietic compartment, resulting in death from anemia at 3–5 weeks of age [12]. These animals suffer from runting, splenomegaly, lymphadenopathy, as well as defects in lymphocyte proliferation and hematopoiesis, all of which are phenotypic characteristics that are in accordance with a defect in cytokine signaling. This pleiotropic phenotype observed in TCPTP-deficient mice led us to investigate the function of TCPTP in cytokine signaling.

Substrate identification is a crucial step in delineating the signaling pathways regulated by PTPs in vivo. To attain this goal, a substrate-based trapping approach can be employed to identify physiological targets of PTPs [13]. Catalytic domain mutants of PTPs can be generated by mutation of the aspartic acid residue, which acts as a general acid in the phosphate ester hydrolysis reaction. This mutation ablates the ability of PTPs to dephosphorylate target substrates but leaves

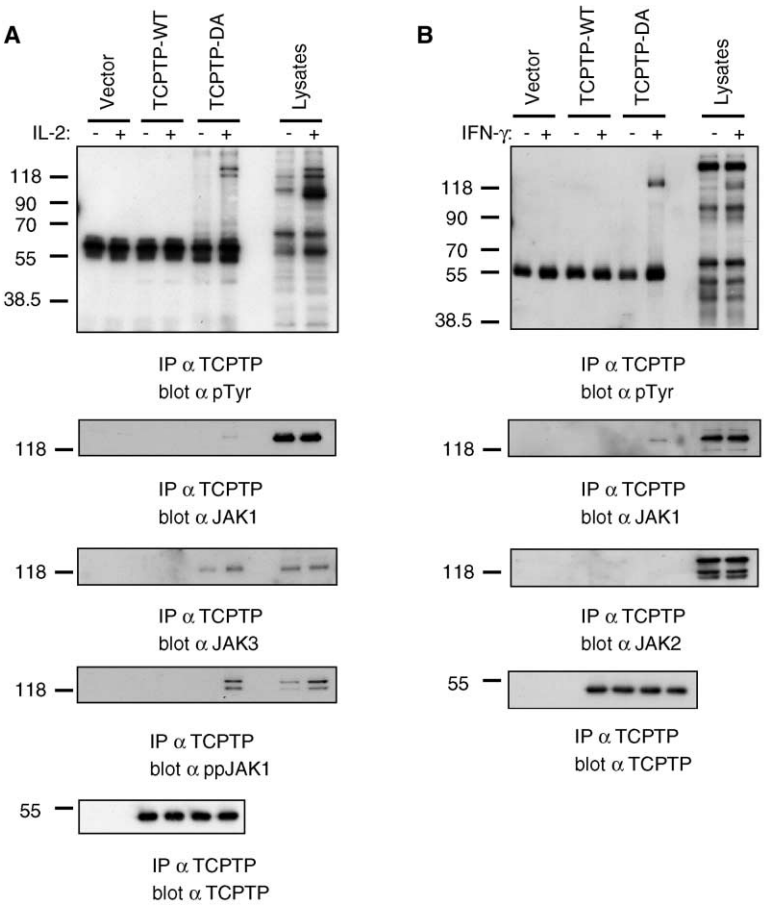

Figure 1. TCPTP Binds JAK1 and JAK3 in Cytokine-Stimulated Cells

(A) CTLL-2 cells were transfected with TCPTP-WT or -DA, cytokine depleted, and stimulated with IL-2 for 15 min. TCPTP immunoprecipitates were blotted with anti-phosphotyrosine antibodies to identify coimmunoprecipitating substrates. Membranes were stripped and reprobed with antibodies to JAK1, JAK3, and phospho-JAK1 (pY1022/1023). All experiments were blotted for TCPTP to ensure equal immunoprecipitation of the PTP.

(B) 293T cells were transfected with TCPTP-WT or -DA and stimulated for 15 min with IFN-γ. TCPTP immunoprecipitates were blotted with anti-phosphotyrosine and antibodies to identify coimmunoprecipitating substrates. Membranes were stripped and reprobed with antibodies against JAK1 and JAK2, and TCPTP to ensure equivalent expression of TCPTP.

substrate binding intact. Therefore, PTP-substrate complexes can be isolated by coimmunoprecipitation, and the substrates can subsequently be identified. Here, we use an in vivo substrate-trapping approach to identify substrates of TCPTP.

Results

JAK1 and JAK3 Are Substrates of TCPTP

The phenotype of TCPTP-deficient mice is consistent with a role for TCPTP in regulating cytokine signaling. Furthermore, the recent structural analysis of the highly related phosphatase PTP1B suggests that the preferred substrates of both TCPTP and PTP1B likely contain a tandem tyrosine motif such as that found in the activation loop of the JAK family of tyrosine kinases [14]. Since TCPTP is abundantly expressed in multiple hematopoietic cell types, and a significant fraction of the protein resides in the cytoplasm in hematopoietic cells (see Figure S1 in the Supplementary Material available with this article online), we asked whether JAK family members are substrates for TCPTP. We constructed either

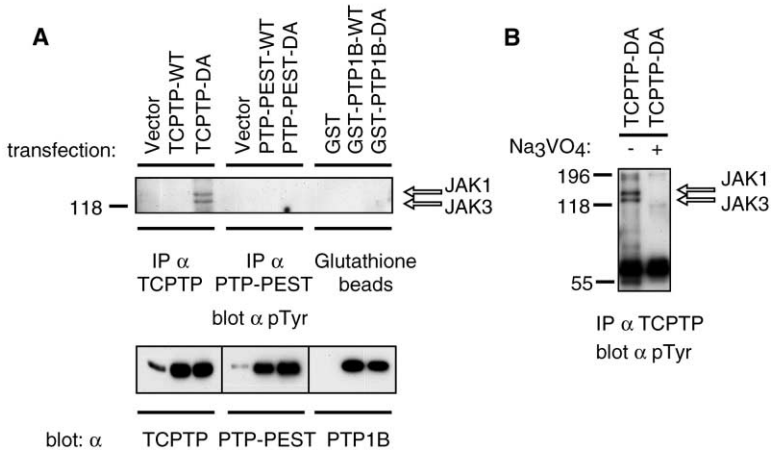

Figure 2. The Interaction of TCPTP and the JAKs Is Specific for the PTP Domain of TCPTP

(A) CTLL-2 cells were transfected with DA-trapping mutants for TCPTP, PTP-PEST, and PTP1B. IL-2-stimulated cells were processed for immunoprecipitation of the PTPs, and putative substrates were revealed by SDS-PAGE and anti-phosphotyrosine immunoblotting.

(B) CTLL-2 cells transfected with TCPTP-DA and stimulated with IL-2 in the presence or absence of 2 mM sodium orthovanadate, a potent competitive inhibitor of PTPs.

wild-type (WT) or D182A (DA) substrate-trapping mutants of TCPTP to isolate potential substrates by coimmunoprecipitation in vivo. In preliminary experiments, cotransfection of TCPTP-DA with JAK1, JAK2, JAK3, and TYK2, followed by immunoprecipitation of TCPTP and anti-phosphotyrosine blotting, indicated that TCPTP does bind to JAK family members (see Figure S2 in the Supplementary Material available with this article online).

To investigate the role of TCPTP in endogenous cytokine signaling, CTLL-2 cells, a cytotoxic T cell line dependent on IL-2 for growth, were transfected with TCPTP-WT, TCPTP-DA, or empty vector and stimulated with IL-2 to activate the IL-2 receptor as well as the downstream effectors JAK1 and JAK3. Putative substrates were revealed by immunoprecipitation of TCPTP and anti-phosphotyrosine immunoblotting to detect tyrosine-phosphorylated proteins bound by the TCPTP-DA-trapping mutant. In CTLL-2 cells stimulated with IL-2, two coimmunoprecipitating substrates were revealed by anti-phosphotyrosine immunoblotting (Figure 1A). Selective coimmunoprecipitation of these two substrates, from the numerous phosphoproteins present in the IL-2-stimulated lysates of these cells, indicates specificity in substrate recognition by TCPTP. Based upon the molecular weight of the coimmunoprecipitating bands and the observed inducible tyrosine phosphorylation of these proteins following IL-2 stimulation, we reasoned that these putative substrates might indeed be JAK1 and JAK3, both of which are activated after IL-2 receptor engagement. Reprobing of the blot from this experiment with anti-JAK1 and anti-JAK3 antibodies and anti-phospho-JAK1, which recognizes both activated JAK1 and JAK3, identified both JAK1 and JAK3 as inducibly coimmunoprecipitating with the TCPTP-DA substrate-trapping mutant (Figure 1A).

To examine the role of TCPTP in a distinct cytokine signaling pathway, we transfected human 293T cells with TCPTP-WT, TCPTP-DA, or empty vector. Following treatment with IFN-γ, which signals through JAK1 and JAK2, a single phosphotyrosine-containing protein was coimmunoprecipitated with TCPTP-DA. Subsequent Western blotting identified this band as JAK1 (Figure 1B). Notably, JAK2 was not coprecipitated with TCPTP, although it is activated downstream of IFN-γ.

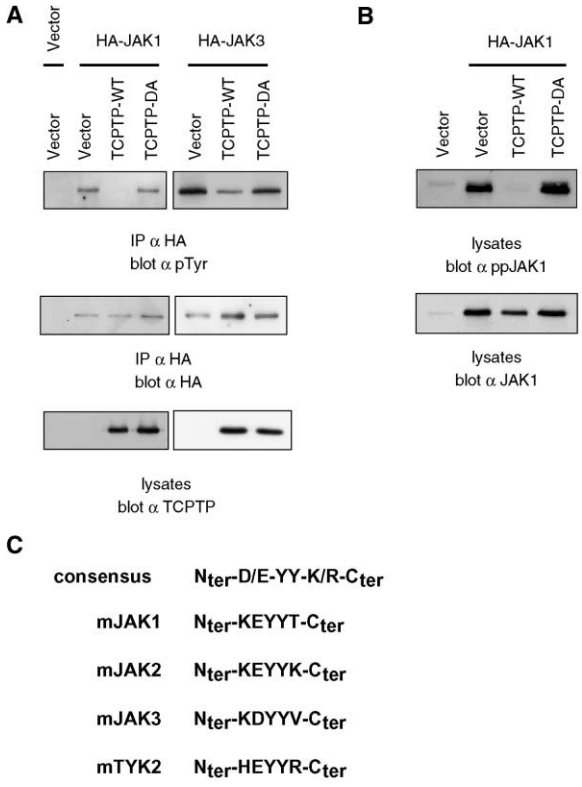

Figure 3. The Activation Loop of JAK1 Is the Molecular Target of TCPTP

(A) TCPTP dephosphorylates both JAK1 and JAK3 in vivo. HA-tagged JAK1 or JAK3 were cotransfected with TCPTP-WT or TCPTP-DA in COS7 cells. The JAKs were immunoprecipitated with anti-HA followed by anti-phosphotyrosine immunoblotting to determine the effect of TCPTP-WT or -DA on JAK phosphorylation levels. The bottom panel shows that both TCPTP-WT and -DA were equally expressed.

(B) JAK1 and either TCPTP-WT or -DA were cotransfected, and lysates, as in (A), were incubated with a pY1022/1023 phospho-specific JAK1 antibody that detects the tandem activation loop phosphotyrosines (Biosource).

(C) Primary amino acid sequence comparison of the tandem tyrosine residues found in all four JAK family members and the putative PTP1B/TCPTP consensus substrate recognition motif.

Specificity of the TCPTP-JAK Interaction

To determine whether the interaction of TCPTP with JAK1 and JAK3 is specific, or whether they are general substrates of other PTP family members, CTLL-2 cells were transfected with DA substrate-trapping mutants of PTP-PEST, a member of the PEST family of PTPs known to play a role downstream of antigen receptors [15]; PTP1B, a PTP that shares 74% sequence identity with TCPTP within the catalytic domain; or TCPTP. Following IL-2 stimulation, only TCPTP-DA formed a substrate-trapping complex with JAK1 and JAK3, while neither PTP-PEST nor PTP1B bound these putative substrates in vivo; although, PTP1B-DA did coimmunoprecipitate a distinct set of phosphotyrosyl proteins (Figure 2A, and data not shown).

To assess whether the interaction of TCPTP with JAK1 and JAK3 is mediated via the catalytic domain, we performed an IL-2-stimulated coimmunoprecipitation from transfected CTLL-2 cells in the presence or absence of 2 mM sodium orthovanadate, a potent competitive inhibitor of PTPs. In the presence of this inhibitor, all binding of phosphotyrosine proteins to TCPTP-DA was abolished, implicating the PTP domain as the mediator of the TCPTP-JAK interaction (Figure 2B).

We next tested whether JAK1 and JAK3, as putative substrates of TCPTP, are efficiently dephosphorylated in vivo. Cotransfection of JAK1 or JAK3 with TCPTP-WT resulted in substantial dephosphorylation of the kinases, while TCPTP-DA protected both JAKs from dephosphorylation (Figure 3A).

Recent structural and kinetic data describing the catalytic domain interaction of PTP1B, a close relative of TCPTP, complexed to the insulin receptor kinase activation loop phosphotyrosines, has been reported [14]. Ki-

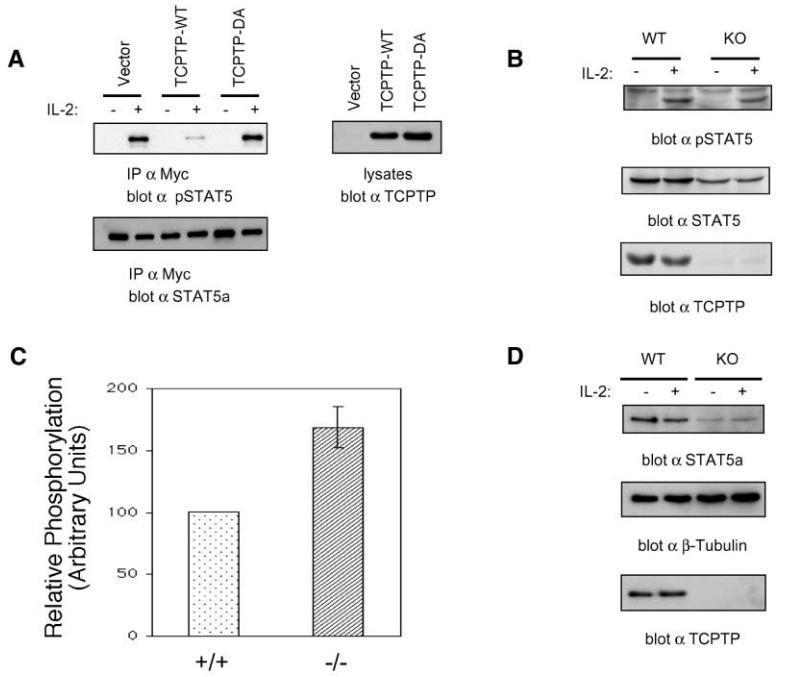

Figure 4. TCPTP Regulates Signaling Events Downstream of the IL-2 Receptor

(A) Cotransfection of Myc-STAT5a and TCPTP-WT or -DA in CTLL-2 cells, followed by IL-2 stimulation and immunoprecipitation of the Myc-tagged STAT5a, was performed to assess the effect of TCPTP on IL-2-induced STAT5 tyrosine phosphorylation with phospho-specific antibodies.

(B) Peripheral T cells from lymph node were isolated from wild-type (WT) and TCPTP null mice (KO) and stimulated with 1000 U/ml IL-2 for 15 min. Cell lysates were resolved by SDS-PAGE, and immunoblotting was performed with phosphotyrosine-specific STAT5 antibodies. The membrane was reprobed for STAT5 to assess loading of the STAT5 protein and with antibodies to TCPTP to verify the genotype of the cells used in the experiment.

(C) A graphic representation of STAT5 tyrosine phosphorylation levels obtained from IL-2-stimulated T cells from wild-type (+/+) or TCPTP-deficient mice (−/−) as shown in part (B) and quantified by densitometry. Shown is the mean of three independent experiments ± standard deviation. Data were analyzed by an unpaired, two-tailed Student's t test comparing the results of the wild-type, set to 100, and the knockout ($n = 3$, $p < 0.05$).

(D) Decreased levels of STAT5 in TCPTP-deficient T cells. Equivalent amounts of total protein lysate from primary T cells were separated by SDS-PAGE and immunoblotted with anti-STAT5 and anti-β-tubulin to confirm equivalent amounts of total protein in each sample.

netic evidence supports the structural model in which PTP1B preferentially binds to tandem phosphotyrosine motifs within polypeptides present in a consensus sequence of D/E-pYpY-K/R. JAK family members contain a similar activation loop motif; although, notably, JAK1 and JAK3 vary from this consensus and have a T or V residue, respectively, in place of the basic (K/R) residue, carboxy-terminal to the tandem tyrosines (Figure 3C). The high homology of the catalytic domains of TCPTP and PTP1B points to these phosphorylation sites as the likely molecular target of TCPTP. Using a site-specific antibody directed against the activation loop phosphotyrosines in JAK1 (pY1022/pY1023), we found that these sites were in fact dephosphorylated by TCPTP, as the phospho-specific anti-JAK1 did not detect JAK1 coexpressed with TCPTP-WT (Figure 3B). Dephosphorylation of the activation loop tyrosine residues by TCPTP is predicted to decrease JAK kinase activity, and therefore suggests a negative regulatory function for TCPTP in cytokine signaling.

STAT Phosphorylation Is Regulated by TCPTP

Binding of cytokines to their receptors leads to the activation of JAKs and subsequent tyrosine phosphorylation of STAT polypeptides, required for their dimerization and translocation to the nucleus where they activate target genes [2]. We investigated the effect of both TCPTP overexpression and loss of function on the downstream effector STAT5, in the IL-2 signaling pathway. CTLL-2 cells were cotransfected with a Myc-tagged STAT5a construct and either TCPTP-WT, TCPTP-DA, or vector alone. The cells were depleted of cytokine and subsequently stimulated with IL-2. Anti-Myc immunoprecipitates were blotted using a phosphotyrosine-specific STAT5 antibody, as an indication of the level of STAT5 activation. In the presence of vector alone, activation of STAT5 proceeded normally in response to IL-2 (Figure 4A). When cotransfected with TCPTP-WT, STAT5 activation was impaired, as observed by the decrease in tyrosine phosphorylation, while cotransfection of the TCPTP-DA mutant did not hinder the activation of STAT5. We also examined the effect of TCPTP loss of function on STAT5 phosphorylation in primary lymph node-derived T cells from either wild-type or TCPTP-deficient mice. Following IL-2 stimulation of primary T cells, lysates were immunoblotted with anti-phospho-STAT5 antibodies. In TCPTP-deficient primary T cells, we consistently observed hyperphosphorylation of STAT5 as compared to cells from wild-type control animals (Figures 4B and 4C). These results are consistent with a negative regulatory function for TCPTP in IL-2 signaling via dephosphorylation of JAK3 and JAK1. TCPTP-deficient T cells also contained reduced levels of STAT5 protein (Figure 4D), another indication of deregulated signaling in the absence of TCPTP.

Since we had observed that TCPTP-DA binds JAK1 in IFN-γ-stimulated 293T cells, we also investigated the consequence of loss of TCPTP activity on STAT1 tyrosine phosphorylation. Primary thymocytes, from both wild-type and TCPTP gene-targeted animals, were stimulated with IFN-γ. Immunoblotting with a phospho-STAT1 antibody revealed an increase in STAT1 phosphorylation in TCPTP-deficient thymocytes (Figure 5A). Similarly, increased levels of tyrosine-phosphorylated STAT1 were also found in TCPTP-deficient thymocytes

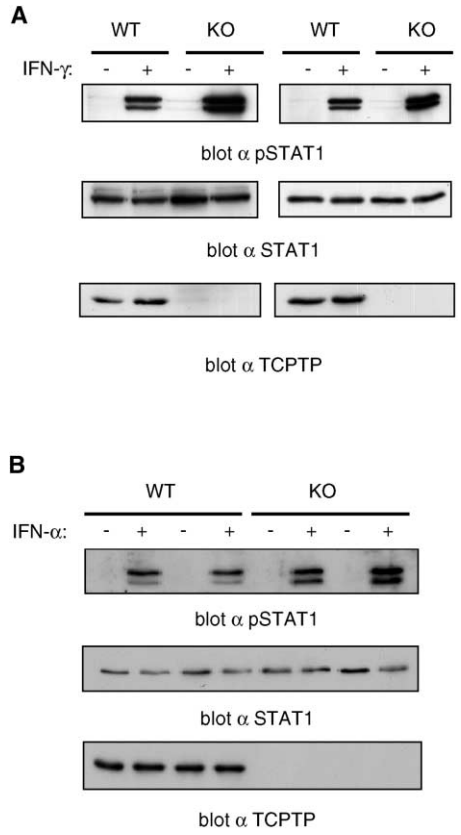

Figure 5. Hyperphosphorylation of STAT1 in TCPTP-Deficient Thymocytes

(A and B) Thymocytes from wild-type (WT) and TCPTP-deficient mice (KO) were stimulated with 1000 U/ml (A) IFN-γ and (B) IFN -α for 15 min. Activation of STAT1 was assessed with a phosphotyrosine-specific STAT1 antibody. Genotypes of the cells used were confirmed with anti-TCPTP immunoblotting. The results of two independent and representative experiments using cells derived from two individual TCPTP null animals and wild-type littermate controls are shown.

stimulated with IFN-α (Figure 5B). These results indicate that TCPTP negatively regulates a variety of cytokine signaling pathways in vivo as a consequence of its action on JAK1 and JAK3.

Hyperphosphorylation of JAK1 and Elevated Expression of iNOS in TCPTP-Deficient Macrophages

In order to obtain sufficient material to directly examine JAK phosphorylation status in the TCPTP null mice, as well as investigate the role of TCPTP in additional downstream events, we cultured bone marrow-derived macrophages (BMDMs) from both wild-type and knockout animals. BMDMs were stimulated with IFN-γ, a potent activator of macrophages, and both JAK1 and JAK2 were immunoprecipitated from cell extracts. Anti-phosphotyrosine immunoblotting of JAK1 immunoprecipitates revealed that the loss of TCPTP resulted in a significant increase in the tyrosine phosphorylation of JAK1 compared to wild-type control BMDM (Figures 6A and 6B). Consistent with the results in Figure 1B, anti-phosphotyrosine immunoblotting of JAK2 immunoprecipitates revealed that loss of TCPTP had no apparent effect on JAK2 phosphorylation levels (Figures 6A and 6C). These results indicate the substrate selectivity of TCPTP for JAK1 in vivo. To investigate the functional consequences of JAK1 hyperphosphorylation in the TCPTP-deficient macrophages, we examined the expression of iNOS, a primary target of IFN-γ signaling. Total cell lysates from primary BMDMs, derived from TCPTP-deficient or wild-type littermate controls treated with IFN-γ, were immunoblotted with anti-iNOS (Figure 6D). Expression of iNOS was consistently elevated in TCPTP-deficient macrophages, indicating hyperactivation of the IFN-γ signaling pathway.

Discussion

The molecular events that negatively regulate cytokine signaling pathways remain largely unknown. The SOCS and PIAS family of proteins, as well as the PTPs SHP-1 and CD45, have been reported to play a role in regulating cytokine signaling events [16]. SHP-1-deficient mice display gross hematopoietic abnormalities such as autoimmunity and macrophage hyperactivation that seem to indicate a negative role in cytokine signaling [17]. SHP-1 is known to play a well-characterized role in the negative regulation of antigen receptor signaling, as both T and B cells from SHP-1-deficient animals are hyperproliferative in vivo [18]. In SHP-1-deficient mice, hyperphosphorylation of JAK1 is observed in interferon signaling [19], and prolonged phosphorylation of JAK2 is observed after injection of growth hormone [20]. In the context of erythropoietin receptor signaling, recruitment of SHP-1 to the tyrosine-phosphorylated receptor chains enables its attenuation of JAK2 activation [21]. A direct catalytic domain interaction of SHP-1 with the JAKs has not been demonstrated, thereby leaving some uncertainty as to the molecular mechanism of SHP-1 negative regulation of cytokine signaling.

The transmembrane PTP CD45 has recently been implicated in cytokine signaling and as a regulator of the JAK family [22]. Mice homozygous for deletions in either exon-6 [23] or exon-9 [24] show reduced B cell maturation and a marked reduction in positive selection during thymic development. Antigen receptor-driven signals are defective in both T and B cells, as well as in IgE-promoted degranulation of mast cells [23, 24]. While cells from CD45 mutant mice show hyperactivation of all JAKs, this study failed to demonstrate a physical association of the PTP domain with the putative substrates in vivo as well [22]. CD45 may play a discrete role in cytokine signaling, but the phenotype of CD45-deficient animals suggests that antigen receptor signaling is most sensitive to the loss of this PTP and other PTPs are present to regulate cytokine signaling.

In this report, we have demonstrated a functional interaction of the catalytic domain of TCPTP with members of the JAK family of tyrosine kinases. Our results indicate that both JAK1 and JAK3 are specific TCPTP substrates. It is interesting to note that TCPTP may target certain members of the JAK family depending on the cytokine signaling pathway in question. We have observed a preferential interaction of TCPTP-trapping mutants with both JAK1 and JAK3 downstream of the IL-2 receptor. In BMDMs from TCPTP-deficient mice,

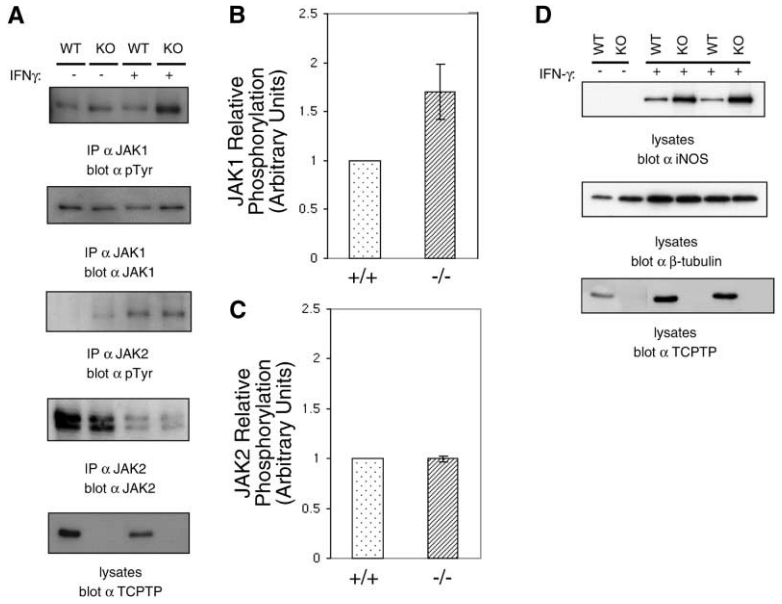

Figure 6. Primary Macrophages from TCPTP Null Mice Have Hyperphosphorylated JAK1 and Elevated Expression of iNOS

(A) BMDMs from wild-type (WT) and TCPTP null mice (KO) were cultured and stimulated with 1000 U/ml IFN-γ for 15 min. Cells were lysed, and JAK1 and JAK2 were immunoprecipitated and subjected to anti-phosphotyrosine immunoblotting. Membranes were stripped and reprobed with anti-JAK1 or anti-JAK2 to confirm equivalent immunoprecipitation. Lysates were immunoblotted for TCPTP to confirm the genotype.

(B and C) A graphic representation of (B) JAK1 (C) and JAK2 tyrosine phosphorylation from IFN-γ-stimulated BMDMs from wild-type (+/+) or TCPTP-deficient mice (−/−) as shown in part (A) and quantified by densitometry. Shown is the mean of three independent experiments ± standard deviation. Data were analyzed by an unpaired, two-tailed Student's t test comparing the results of the wild-type, set to 100, and the knockout ($n = 3$, $p < 0.1$).

(D) BMDMs from wild-type (WT) or TCPTP-deficient (KO) mice were stimulated with 100 U/ml IFN-γ for 20 hr. Total cell lysates were Western blotted with anti-iNOS. The results from two independent experiments, with cells derived from individual TCPTP wild-type and -deficient littermate controls, are shown.

JAK1 phosphorylation levels are increased after IFN-γ stimulation, while JAK2 phosphorylation appears to be unaffected. While we cannot preclude a role for TCPTP in regulating phosphorylation of JAK2 and TYK2, the in vivo analysis of both cultured cell lines and primary hematopoietic cells suggests that TCPTP does not have a general effect on all of the JAK family members.

Due to the high similarity within the respective catalytic domains of TCPTP and PTP1B, they were predicted to target similar substrates [14]. Recently, it has been demonstrated that JAK2 and TYK2 are substrates of PTP1B in fibroblasts [25]. It is interesting to note that, although both TCPTP and PTP1B appear to regulate members of the JAK family, the phenotypes of PTP1B and TCPTP-deficient mice are strikingly different [12, 26, 27]. PTP1B gene-targeted mice are resistant to diet-induced obesity and diabetes, while TCPTP-deficient mice display hematopoietic abnormalities. Our results, and previous reports, indicate that other factors, which may include tissue-specific expression patterns, subcellular localization [28], and additional higher order interactions, are involved in substrate recognition and the observed differences in substrate specificity.

These findings provide insight into the biochemical basis for the phenotype observed in the TCPTP-deficient mice. T cell secretion of IFN-γ during the immune response is known to play an important role in macrophage activation. Hypersensitivity of macrophages to IFN-γ would result in their hyperactivation and lead to the overproduction of inflammatory cytokines such as TNF-α and IL-1. Indeed, increased numbers of activated macrophages are observed in the spleen of TCPTP-deficient mice, which is consistent with an acute inflammatory response [11].

Conclusions

In this report, we have identified JAK1 and JAK3 as physiological substrates of TCPTP via a tandem in vivo substrate-trapping approach and biochemical analysis of primary cells from TCPTP gene-targeted mice. These results indicate a fundamental role for TCPTP in cytokine signaling that correlates well with the observed phenotype of the knockout animals and describes a novel mechanism in the regulation of the immune response.

Experimental Procedures

Materials

Unless otherwise specified, all materials were obtained from Sigma. RPMI, DMEM, and fetal bovine serum (FBS) were obtained from Wisent; recombinant murine IL-2 was obtained from Roche, while recombinant murine IFN-α, murine IFN-γ, and recombinant human M-CSF were obtained from Calbiochem; and human IFN-γ was obtained from GIBCO. Antibody 4G10 against phosphotyrosine; anti-JAK1, anti-JAK2, and anti-JAK3 antisera; and anti-iNOS and anti-PARP antibodies were obtained from Upstate Biotechnology. Anti-TCPTP antibodies 3E2 and 6F3 were purified from tissue culture supernatants of the respective hybridomas. Monoclonal antibodies anti-JAK1 and anti-TYK2 were obtained from BD Transduction Laboratories. All PCR reactions were performed using vent DNA polymerase (New England Biolabs) with primers synthesized by ACGT. Complete protease inhibitor tablets were obtained from Roche.

Cell Culture

CTLL-2 cells were routinely cultured in RPMI supplemented with 10% (v/v) FBS, 5 U/ml penicillin, 5 mg/ml streptomycin sulfate, 55 μM 2-mercaptoethanol (GIBCO-BRL), and 2 U/ml IL-2 at 37°C with 5% CO_2. COS-7 and 293T cells were cultured in DMEM containing 10% (v/v) FBS, 5 U/ml penicillin, and 5 mg/ml streptomycin sulfate.

Plasmids and Mutagenesis

All wild-type or mutant cDNAs were placed in the pEF-BOS expression vector (a kind gift Dr. Gary Koretzky, University of Pennsylvania) in the 5′-ClaI/XbaI-3′ sites. C-terminal Myc tags were introduced by including the epitope tag sequence in the antisense primer for PCR. The TCPTP-D182A mutant was constructed using PCR with the following mutagenic primers (mutation underlined): (+) 5′-TGAAACGAGAACCATATCTCAC-3′, (−) 5′-CTGGAACCCCAAAAGC TGGCC-3′; and the following terminal primers: (+)5′-TAAATCGATC CACCATGTCGGCAACCATCG-3′, (−) 5′-GTGTCTAGATTAGGTGT CTGTCAATCTTGG-3′. The same terminal primers were used for

pEF-TCPTP-WT. The PTP-PEST-WT cDNA was subcloned into the pEF-BOS vector at the 5′-ClaI/XbaI-3′ sites. The PTP-PEST-D199A mutant was created with the following primers: (+) 5′-GAATCGATT GGAGGATGGAGCAAGTGGAG-3′, (+) 5′-GTGAACTGGCCAGCC CATGATG-3′, (−) 5′-GTGGTACCTGTCACTGTCCTG-3′, and (−) 5′-CATCATGGGCTGGCCAGTTCAC-3′. The resulting PCR product was cloned into the PTP-PEST cDNA using a 5′-ClaI site and an internal HindIII site. The PTP1B WT and D181A cDNAs were amplified with the following primers: (+) 5′-GAGGATCCGAGATGGAAA AGGAGTTCG-3′ and (−) 5′-GAGCGGCCGCCTATGTGTTGCTGTT GAACAG-3′, and were subcloned into the pEBG vector at the 5′-BamHI/NotI-3′ sites. Constructs expressing JAK1, JAK3, and TYK2 were prepared by blunt ending the 5′ ends of the cDNAs with the T4 DNA polymerase (Roche), then digesting with XbaI. These modified cDNAs were ligated in to a 5′-ClaI-blunt ended/XbaI-3′-digested pEF-BOS vector. The integrity of all constructs was verified by fully sequencing the cDNA (Hospital for Sick Children, DNA sequencing facility).

Western Blotting

Protein complexes were resolved by 8% or 10% SDS-PAGE and transferred to PVDF membranes (Immobilon, Millipore). For phosphotyrosine, membranes were blocked for 60 min in 1% BSA in TBST. 4G10 was added at 1 μg/ml in blocking buffer for 60 min at room temperature. The membranes were washed three times, and the appropriate secondary was used for 45 min in blocking buffer. After repeating the above washes, the membranes were visualized via enhanced chemiluminescence (Amersham) and exposed to film (Kodak). For other antibodies, the blocking buffer used was 5% nonfat dried milk (Carnation) in TBST. Membranes were stripped in 62.5 Tris (pH 6.8), 2% SDS, 100 mM 2-mercaptoethanol and were washed extensively before use.

Transfection and Immunoprecipitation

CTLL-2 cells, at log phase growth, were washed twice with RPMI and resuspended at 20×10^6 cells/400 μL RPMI in a 0.4-cm electroporation cuvette (BioRad). Plasmid DNA was added at 40 μg and incubated with the cells for 10 min at room temperature. Cells were pulsed at 960 μF and 250V then chilled on ice for 10 min. Cells were resuspended in 20 ml CTLL-2 culture medium and grown for 16–18 hr as described above. Transfectants were then washed twice with PBS and depleted of cytokine for 4 hr. Transfectants were stimulated with 100 U/ml IL-2 for the indicated time points at 37°C. 293T cells were transfected using Lipofectamine (GIBCO-BRL) with 2 μg of the indicated plasmid DNA. After 20 hr, the cells were serum starved for 4 hr and stimulated with 1000 U/ml IFN-γ. In both experiments, the cells were quickly pelleted and washed with ice-cold PBS and lysed in HNMETG (50 mM HEPES [pH 7.5], 150 mM NaCl, 1.5 mM $MgCl_2$, 1.0 mM EGTA, 1.0% Triton X-100, 10% glycerol), without the addition of orthovanadate, for 20 min at 4°C with gentle rotation. Sodium orthovanadate was added to a final concentration of 2 mM for all other experiments. Lysates were cleared for 10 min at 4°C and 10,000 × g, and protein was quantitated by the method of Bradford (BioRad). A total of 1 mg lysate in 1 ml was precleared with either protein A or G-sepharose and normal rabbit or mouse IgG for 30 min at 4°C; then it was incubated with the indicated antibodies for 90 min prior to the addition of protein A or G-sepharose. The immune complexes were washed four times with HNMETG containing 0.1% Triton X-100 and eluted into 2× SDS sample buffer. SDS-PAGE, transfer, and Western blotting were performed as described above.

Isolation of Primary Cells

Primary thymocytes or lymph nodes were obtained from wild-type or TCPTP-deficient mice at days 14–18 after birth. The thymus or lymph nodes were removed and placed in RPMI supplemented with 10% (v/v) FBS, 5 U/ml penicillin, 5 mg/ml streptomycin sulfate, and 55 μM 2-mercaptoethanol (GIBCO-BRL). Cell suspensions were prepared by passing through a 70-μm nylon mesh. Primary cells were rested at 37°C for 4 hr prior to stimulation. Lymph node-derived T cells were stimulated with 1000 U/ml IL-2, and thymocytes were stimulated with 1000 U/ml IFN-α or IFN-γ for 15 min. Cells were washed once in ice-cold Hanks' balanced salt solution, then lysed in HNMETG buffer containing 10 mM NaF, 2 mM Na_3VO_4, and 420 mM NaCl to extract nuclear components. Lysates were resolved by SDS-PAGE and immunoblotted with anti-phospho-specific STAT1 or STAT5 antibodies. Data were analyzed by densitometry with the wild-type value set to 100 in each experiment. Bone marrow-derived macrophages were obtained by flushing the femurs of 14-day-old wild-type or TCPTP-deficient mice with DMEM containing 10% FBS and antibiotics. The cells were pelleted and resuspended in the above medium supplemented with 10 ng/ml M-CSF. Stromal cells and mature macrophages were removed by adherence to tissue culture plastic for 24 hr. The next day (day 1), nonadherent cells were resuspended at $1.5–2.0 \times 10^5$/ml M-CSF-containing medium and plated in 100-mm tissue culture plates. At day 4, the medium was removed and replaced with fresh growth medium containing M-CSF. At day 7, cells were depleted of M-CSF for 16–18 hr and stimulated with 1000 U/ml IFN-γ. For the induction of iNOS, BMDMs were incubated with or without 100 U/ml IFN-γ for 20 hr. Cells were lysed and subjected to immunopreciptiation and Western blotting as described.

Supplementary Material

Supplementary Material including additional Experimental Procedures and two supplementary figures is available at http://images.cellpress.com/supmat/supmatin.htm.

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