Original Article

T-cell protein tyrosine phosphatase (TCPTP) regulates phosphorylation of Txk, a tyrosine kinase of the Tec family

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Phosphorylation and dephosphorylation of intracellular enzymes, such as the T-cell-specific Tec family protein kinase Txk, are critically involved in the regulation of a number of cellular functions. Although PTPs located in the nucleus are limited, TCPTP is known to be one such nuclear phosphatase, due to its nuclear localization signal sequences. Here, we investigate the role of TCPTP in the regulation of Txk phosphorylation/dephosphorylation in Txk-transfected Cos7 and Jurkat cells.

Nuclear-type TCPTP (TC45) was present in both the nuclear and cytoplasmic compartments of Cos7 cells transfected with TC45. Cytoplasmic-type TCPTP (TC48) was localized in the cytoplasmic compartment of Cos7 cells transfected with TC48. We observed that expression of TC45 dephosphorylated Txk in the nuclei of Cos7 cells. TC48 expression did not dephosphorylate Txk; rather, it enhanced and sustained Txk phosphorylation in the nuclei of Cos7 cells.

Phosphorylation of Txk increased 60 minutes after lectin stimulation in Jurkat cells transfected with TCPTP-specific small interfering RNA (siRNA), which efficiently knocked down endogenous TCPTP.

TCPTP may play a crucial role in the regulation of Txk phosphorylation status in T-cells after stimulation.

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Introduction

Phosphorylated enzymes play crucial roles in the regulation of various cellular functions. Protein tyrosine phosphorylation is modulated by protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). In T-lymphocytes, several PTKs, including tyrosine kinase

expressed in hepatocellular carcinoma (Tec) family kinases such as Tec, inducible T-cell kinase (Itk), and Txk/resting lymphocyte kinase (Rlk), are expressed and involved in the T-cell receptor (TCR) signaling pathway[1].

We have reported that nuclear translocation of Txk regulated interferon (IFN)γ production in T-cells, forming a complex with poly (ADPribose) polymerase 1 (PARP1) and elongation factor 1 (EF1)α, a DNA repair protein and an aminoacyl-tRNA trafficking protein, respectively[2-4]. Self-phosphorylation of Txk (Y91) was essential for the trimer complex formation[5]. We found that the DNA binding activity of the protein complex was downregulated 1 hour after TCR stimulation[4]. Therefore, certain PTPs may be involved in the dephosphorylation of Txk in the nuclear compartment.

More than 100 PTPs are encoded in the human genome, and are organized into four classes. Class I, or classical PTPs, consist of receptor and non-receptor type PTPs, of which the vast majority of non-receptor type PTP are expressed in the cytosol[6, 7].

T-cell PTPs (TCPTPs) are recognized as major non-receptor type PTPs, and have a modular structure comprising an N-terminal catalytic domain and a non-catalytic C-terminal segment[8]. Although PTP localization in the cell nucleus is rare, TCPTP contains nuclear localization signal sequences and is known to be a nuclear phosphatase.

There are two alternative splicing isoforms of human TCPTP mRNA, a nuclear form (TC45) and a cytoplasmic form (TC48), each of which encode different C-terminals (Fig. 1A). Both isoforms have nuclear localization signal sequences (NLS) within the non-catalytic domain. TC45 is thus expected to localize within the nucleus.

TC48 has a hydrophobic domain in the C-terminus, which contains an endoplasmic reticulum (ER) targeting segment, and localizes mainly near the ER (Fig. 1A). The molecular weights of TC45 and TC48 are estimated to be 45kDa and 48kDa, respectively. TCPTP-deficient mice have been shown to have insufficient immune function[9].

Here, we studied the involvement of TCPTP in the phosphorylation regulation of Txk, an important kinase of activated T-cells.

Matelials and Methods

1)Cells and cell culture

Cos7 cells were routinely cultured at 37°C and 5 % CO_2 in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Carlsbad, CA) supplemented with 10 % heat-inactivated fetal bovine serum (FBS, Life Technologies), 100 units/ml penicillin (Life Technologies), and 100 μg/ml streptomycin (Life Technologies). Jurkat cells were cultured in RPMI 1640 (Life Technologies) supplemented with 10 % FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin.

2)Plasmid vectors and siRNA

Human Txk cDNA was kindly provided by Dr. G. W. Litman from the University of South Florida[10]. Txk cDNA was amplified by PCR and ligated into a mammalian expression vector, pAcGFP N1 (Clontech, Palo Alto, CA). Human cytoplasmic (NM_002828) and nuclear-type TCPTP (NM_080422) (Fig. 1A) cDNA were amplified from a Jurkat cell cDNA library by PCR. The PCR products were ligated into pDsRed monomer N1 (Clontech). Constitutively active Fyn expression vector (FynY531F, a constitutively active form of Fyn) was gifted from Dr. Toyoshima from the University of Tokyo. We conducted an inhibition assay of Jurkat cells using predesigned siRNA targeting TCPTP and scrambled siRNA (Origene, Rockville, MD).

3)Antibodies

We used the following antibodies for immunoblotting and immunoprecipitation: rabbit anti-green fluorescent protein (GFP) polyclonal antibody, mouse anti-GFP monoclonal antibody (JL-8, both from Clontech), anti-phosphotyrosine monoclonal antibody (4G10, Upstate, Lake Placid, NY), anti-human/mouse/rat TCPTP antibody (R & D systems, Minneapolis MN) and anti-beta actin monoclonal antibody (Sigma-Aldrich, St. Louis, MO).

4)Gene transfection into Cos7 and Jurkat cells

We transfected TC45-DsRed and TC48-DsRed plasmids with or without Txk-GFP plasmids into Cos7 cells in subconfluent condition, by lipofection using Lipofectamine 2000 or LTX (both from Life Technologies), following the manufacturer's recommendations. To activate Txk, constitutively active Fyn expression vector was simultaneously transfected into the cells. Txk-GFP plasmids or empty-GFP plasmids were transfected into Jurkat cells by electroporation using the Nucleofector® kit V and Nucleofector™ II system (Lonza, Basel, Switzerland), following the manufacturer's recommendations. Transfected Jurkat cells were cultured in selection medium consisting of RPMI1640, penicillin, streptomycin, 10 % FBS, and 0.5-1.3 mg/ml G418 (Life Technologies), for approximately 4 weeks. For knockdown experiments, siRNA targeting TCPTP and

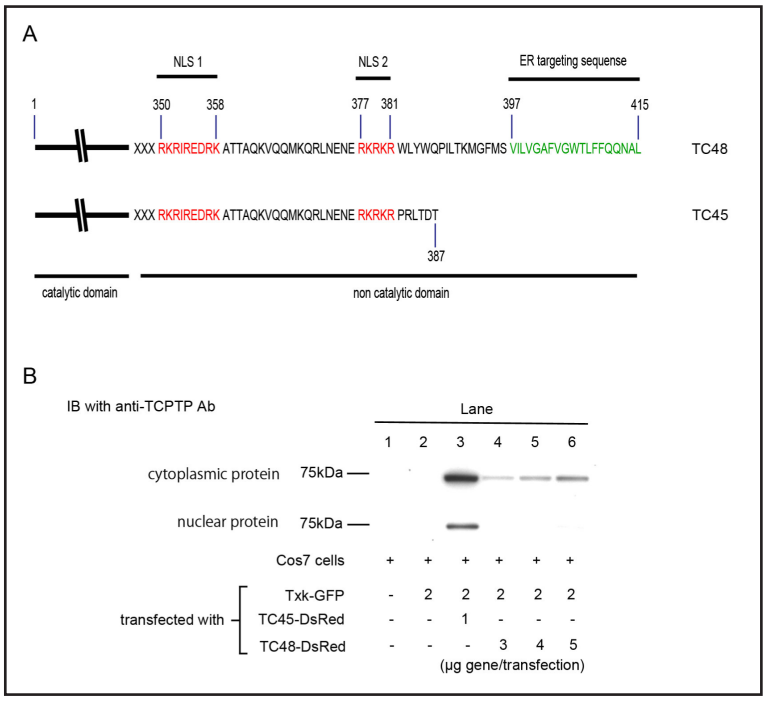

Fig. 1 Structure and cellular distribution of nuclear-type TCPTP (TC45) and cytoplasmic-type TCPTP (TC48)

(A)Domain structure of TCPTP. TCPTP has a modular structure comprised of an N-terminal catalytic domain and a non-catalytic C-terminal domain. There are two alternative splicing isoforms in human TCPTP mRNA, TC45 and TC48, each of which encodes different C-terminals. Both isoforms have nuclear localization signal sequences (NLS) within the non-catalytic domain. TC48 has a hydrophobic domain consisting of 19 amino acids in the C-terminus, which contains an endoplasmic reticulum (ER) targeting sequence. The molecular weight of TC45 and TC48 is estimated to be 45kDa and 48kDa, respectively.

(B)Cellular distribution of TCPTP was confirmed by immunoblotting (IB) with anti-TCPTP antibody in Cos7 cells. Cytoplasmic and nuclear fractions of Cos7 cells transfected with either TC45 or TC48 were collected separately. TC45-DsRed (45+28kDa) is distributed in both the cytoplasm and nucleus (lane 3). TC48-DsRed (48+28kDa) is localized exclusively in the cytoplasm (lane 4-6), confirming that TC45, but not TC48, localizes in the nucleus. Lane 1 was loaded with the nuclear and cytoplasmic fractions extracted from mock-transfected Cos7 cells.

scrambled siRNA was transfected into Txk-transfected Jurkat cells by electroporation using the Nucleofector™ II system. For activation, transfected Jurkat cells were supplemented with 5 μg/ml phytohaemagglutinin-L purified from red kidney bean (PHA-L, Roche Diagnostics, Mannheim, Germany) and 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich).

5)Immunoprecipitation and immunoblotting analysis

Whole cell lysates of Cos7 and Jurkat cells were extracted with lysis buffer (50 mM Tris-HCl, pH 7.4, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 % Nonidet P-40, phosphatase inhibitors and protease inhibitors).

Cytoplasmic fractions of transfected Cos7 cells were purified by collecting the supernatants of the cell extracts using buffer A (100 μl, 10 mM HEPES, pH 7.9, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.6 % Nonidet P-40, phosphatase inhibitors and protease inhibitors). The cell pellets were resuspended in buffer B (50 μl, 20 mM HEPES, pH 7.9, 0.42 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, phosphatase inhibitors and protease inhibitors), and the nuclear fractions were extracted. Protein concentration of the samples was measured by a BCA protein assay reagent kit (Thermo Fisher Scientific, Rockford, IL). All samples for immunoprecipitation and immunoblotting were analyzed in duplicate and triplicate, and obtained essentially the same results. Whole cell lysate and nuclear and cytoplasmic fractions were pre-cleared at 4°C using normal rabbit IgG coupled to protein G or protein A agarose beads (Santa Cruz Biotechnology, Dallas, TX). Pre-cleared samples were incubated with anti-GFP polyclonal antibody overnight and then immunoprecipitated with the agarose beads for three hours at 4°C. Beads were collected and washed three times with the lysis buffer above. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Life Technologies). The membranes were blocked with 3 % bovine serum albumin or with 5 % skim milk. Detection was conducted as previously described[4]. Histogram analysis of each blot was performed using Adobe Photoshop software CS5 version 12.0.4.

Results

1)Expression and subsequent cellular distribution of TC45 and TC48 in Cos7 cells

We first investigated the cellular distribution of TC45 and TC48 in Cos7 cells after transfection using an immunoprecipitation assay. Cytoplasmic and nuclear fractions were purified separately from Cos7 cells transfected with DsRed-tagged TC45 and TC48 expression

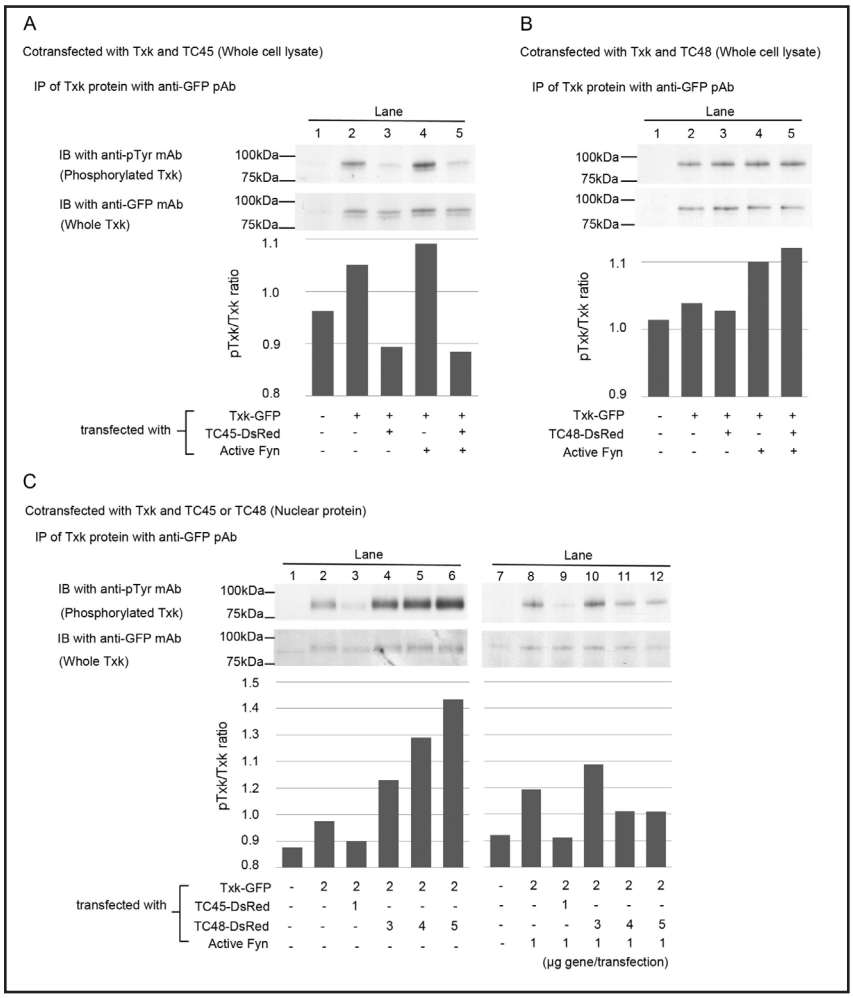

Fig. 2 Effects of TC45 and TC48 transfection on Txk phosphorylation in Cos7 cells

We analyzed the effects of TC45 and TC48 transfection on Txk phosphorylation by immunoprecipitation (IP) assays in Cos7 cells using whole cell lysates (A, B) and nuclear fractions (C). IB, immunoblotting.

(A)Txk phosphorylation in whole cell lysates of TC45-transfected Cos7 cells was evaluated. Phosphorylation of Txk is enhanced by co-transfection of constitutively active Fyn (lane 4). TC45 co-transfection dephosphorylates the activated Txk (lane 5). Moderate phosphorylation of Txk is detected in Txk-GFP-transfected Cos7 cells (lane 2), possibly due to the intrinsic kinase activity of Cos7 cells. Similarly, the phosphorylated Txk without constitutively activated Fyn is dephosphorylated by co-transfection of TC45 (lane 3). Lane 1 was loaded with whole cell lysates extracted from mock-transfected Cos7 cells.

(B)We demonstrated that TC48 does not dephosphorylate Txk in whole cell lysates of Cos7 cells.

(C)Txk phosphorylation in the nuclear fractions of TC45 and TC48 transfected Cos7 cells was evaluated. Phosphorylated Txk is detected in the nuclear fraction of Cos7 cells transfected with Txk (lane 2). The phosphorylated Txk is almost completely dephosphorylated in the nuclear fraction of Cos7 cells by co-transfection of TC45 (lane 3). Co-transfection with TC48 does not dephosphorylate Txk; rather TC48 enhances and sustains Txk phosphorylation in a dose-dependent manner (lane 4-6). Co-transfection with TC48 and constitutively active Fyn dephosphorylates Txk in a dose-dependent manner, possibly through the consumption of TC48 by the dephosphorylation of constitutively active Fyn (lane 10-12). Lanes 1 and 7 were loaded with the immunoprecipitated protein from the nuclear fraction extracted from mock-transfected Cos7 cells. We repeated each experiment several times, and confirmed that the results were reproducible (data not shown).

plasmids (Fig. 1B). We analyzed the distribution of TCPTP by immunoblotting with an anti-TCPTP antibody.

TC45 was present in both the cytoplasm and nucleus (Fig. 1B, lane 3). TC48 was localized exclusively in the cytoplasm (Fig. 1B, lanes 4-6), confirming that TC45, but not TC48, was localized in the nucleus. Confocal microscopic analysis of Cos7 cells transfected with their respective plasmids confirmed this distribution (data not shown).

2)Effect of TCPTP transfection on Txk phosphorylation in Cos7 cells

We analyzed the effects of TC45 and TC48 expression on Txk phosphorylation by immunoprecipitation assay using Cos7 whole-cell lysate and nuclear fractions. Txk-transfected Cos7 cells were co-transfected with TC45 and TC48. Constitutively active Fyn was co-transfected for Txk activation, cells were lysed, and supernatants were collected as whole-cell lysates. Phosphorylation of Txk was enhanced by co-transfection of constitutively active Fyn (Fig. 2A, lane 4). TC45 co-transfection dephosphorylated the activated Txk (Fig. 2A, lane 5). Moderate phosphorylation of Txk was detected from Txk-transfected Cos7 cells without constitutively active Fyn (Fig. 2A, lane 2). This may be due to the intrinsic kinase activity of Cos7 cells. Similarly, phosphorylated Txk without constitutively active Fyn was dephosphorylated by co-transfection of TC45 (Fig. 2A, lane 3).

We demonstrated that TC48 does not dephosphorylate Txk in Cos7 whole cell lysates (Fig. 2B). We evaluated Txk phosphorylation in the nuclear fractions of both TC45- and

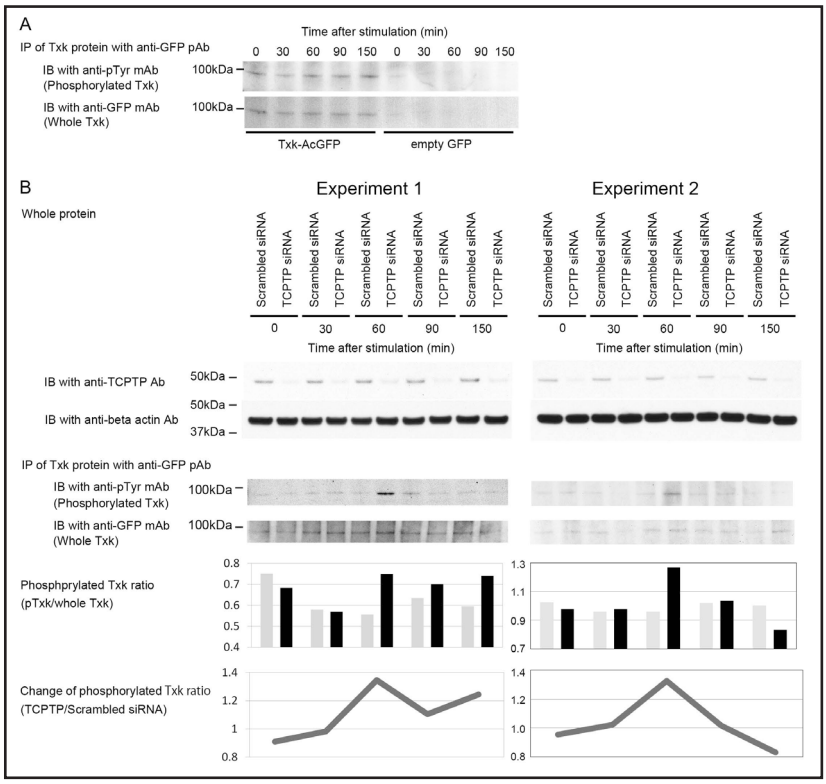

Fig. 3 Effect of TCPTP knockdown by small interfering RNA (siRNA) on Txk phosphorylation in Jurkat cells

Dephosphorylation of Txk in activated Jurkat cells transfected with Txk was investigated by utilizing the endogenous TCPTP of Jurkat cells. Cells were stimulated with PHA-L/PMA for 0, 30, 60, 90, or 150 minutes, and then whole cell lysates were analyzed. Representative results of two independent experiments are shown.

(A)Phosphorylation of Txk was detected by immunoblotting (IB) using anti-phosphotyrosine monoclonal antibody. Phosphorylation of Txk was continuously detected in Txk-GFP-transfected Jurkat cells, from 0 to 150 minutes after PHA-L/PMA stimulation, but not when transfected with empty-GFP vector.

(B)Immunoblotting of Jurkat whole cell lysates using anti-TCPTP polyclonal antibody indicated that knockdown of TCPTP reduced TCPTP protein expression (upper panels). Immunoprecipitation (IP) and anti-phosphotyrosine immunoblotting (IB) of Txk reveals phosphorylated Txk increased 60 minutes after PHA-L/PMA stimulation in TCPTP knockdown Jurkat cells (middle and lower panels).

TC48-transfected Cos7 cells (Fig. 2C). Phosphorylated Txk was detected in the nuclear fraction of Txk-transfected Cos7 cells (Fig. 2C, lane 2). Phosphorylated Txk was almost completely dephosphorylated in the nuclear fraction of Cos7 cells co-transfected with TC45 (Fig. 2C, lane 3). Co-transfection with TC48 did not cause dephosphorylation of Txk; rather, TC48 enhanced and sustained Txk phosphorylation in a dose-dependent manner (Fig. 2C, lanes 4-6). Interestingly, co-transfection of TC48 and constitutively active Fyn dephosphorylated Txk in a dose-dependent manner (Fig. 2C, lanes 10-12).

3)Effect of TCPTP downregulation by small interfering RNA (siRNA) on Txk phosphorylation in Jurkat cells

Txk dephosphorylation by endogenous TCPTP was observed in activated Txk-transfected Jurkat cells. Cells were stimulated with PHA-L/PMA for 0, 30, 60, 90, or 150 minutes, and whole cell lysates were analyzed.

Phosphorylation of Txk in Txk-transfected Jurkat cells was detected continuously from 0 to 150 minutes after PHA-L/PMA stimulation, but not in cells transfected with the empty vector (Fig. 3A).

TCPTP knockdown reduced TCPTP protein expression (Fig. 3B, upper panels, representative results of two independent experiments are shown). Phosphorylated Txk increased 60 minutes after PHA-L/PMA stimulation in TCPTP knockdown Jurkat cells (Fig. 3B, middle and lower panels).

Discussion

A majority of human Tec family PTKs, such as Btk[11, 12], Itk[13], and Txk[2, 14], translocate into the nucleus upon stimulation. We have previously reported that Txk forms a complex with PARP1 and EF1α in the nuclear compartment, which then can bind to the IFNγ promoter[4]. Txk plays a role in the progression of Th1 response in human T-cells[4]. In contrast to the substantial expression of PTKs in the nucleus of activated T-cells, only several PTPs, including TCPTP, exist in the nucleus among the 60 types of catalytically active PTPs expressed in T-cells[6, 7].

TC45 binds to nonspecific DNA[15] and promotes cell cycle progression[16]. TCPTP is predominantly expressed in the spleen and thymus[17], and TCPTP-deficient mice exhibit anemia, splenomegaly, lymphadenopathy, and thymic atrophy[9]. When T cells of TCPTP gene knockout mice were stimulated with mitogens, they did not show efficient proliferation[9, 18].

Recently, it was reported that TC45 dephosphorylates

signal transducer and activator of transcription (STAT) 1, 3, and 6, in the nuclear compartment[19-22]. STAT proteins are essential mediators for the differentiation of lymphocytes and antigen presenting cells. These results suggested that TC45 plays a crucial role in hematopoiesis and immune function[9]. Moreover, several genome-wide association studies (GWAS) revealed that the PTPN2 gene, encoding TCPTP, is associated with Crohn's disease[23], type 1 diabetes mellitus[24], and rheumatoid arthritis[25].

Stimulation of TCR in activated T-cells resulted in the production of IL-2, and induction of TCPTP expression did not affect IL-2 secretion in Jurkat cells[26]. On the contrary, a recent in vivo study indicated that TCPTP reduced antigen-induced T-cell response, using mice with T-cell-specific knockout of TCPTP[27]. TCPTP attenuated T-cell activation via the dephosphorylation of Lck and Fyn, both of which are members of the Src family of kinases[27].

We demonstrated that transfected TC45 dephosphorylated Txk in Cos7 cells, while expression of TC48 enhanced and sustained Txk phosphorylation in a dose-dependent manner when constitutively active Fyn was not transfected (Fig. 2C, lane 4-6). The underlying molecular mechanism of TC48-mediated Txk phosphorylation remains poorly understood. It is possible that the transfected TC48 in the cytoplasm dephosphorylates and inactivates several substrates, including intrinsic PTK and PTP, one of which may induce and sustain Txk phosphorylation in the nuclei of Cos7 cells.

In conclusion, this study demonstrated a possible association between TCPTP and Txk phosphorylation, and that TCPTP may play a crucial role in the activation of T-cells through the dephosphorylation of Txk.

Source of Funding

None

Conflict of interests

The authors declare no competing financial interests.

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