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Title: The nuclear isoform of protein tyrosine phosphatase TC-PTP regulates Interleukin-6-mediated signaling pathway through STAT3 dephosphorylation

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Running title: Dephosphorylation and deactivation of STAT3 by TC-PTP

ABSTRACT

In the previous study, we demonstrated that the nuclear isoform of T cell protein-tyrosine phosphatase (TC-PTP) dephosphorylated and deactivated signal transducer and activator of transcription 5a (STAT5a) and STAT5b, thereby negatively regulating prolactin (PRL)-mediated signaling pathway. In this study, we examined the involvement of the nuclear isoform of TC-PTP in Interleukin-6 (IL-6)-mediated signaling pathway. IL-6 is a multifunctional cytokine that plays important roles in the immune system, hematopoiesis, and acute phase reactions, and has also implicated in IL-6-related diseases. Here we demonstrate that IL-6-induced tyrosine-phosphorylation and activation of STAT3 were suppressed by overexpression of the nuclear isoform of TC-PTP in 293T cells. Tyrosine-phosphorylated STAT3 directly interacted with a substrate-trapping mutant of TC-PTP. Furthermore, retrovirus-mediated overexpression of the nuclear isoform of TC-PTP suppressed the IL-6-induced growth arrest of myeloid leukemia M1 cells. Endogenous TC-PTP complexed with STAT3 in the nucleus of M1 cells. These results strongly suggest that the nuclear isoform of TC-PTP may serve as a negative regulator of IL-6-mediated signaling pathway.

Key words: IL-6, T cell protein tyrosine phosphatase (TC-PTP), signal transducer and activator of transcription (STAT3), leukemia inhibitory factor (LIF)

INTRODUCTION

Interleukin-6 (IL-6) is a pleiotropic cytokine that regulates immune and inflammatory responses (1, 2), and also implicated in IL-6-related diseases (2). The IL-6 receptor is a heterodimeric complex, consisting of an IL-6 specific ligand-binding subunit, α chain, and a signal-transducing subunit, gp130. Structural analysis has revealed that both subunits belong to the cytokine receptor superfamily, and gp130 is shared by the receptors for ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M (OSM), and cardiotropin 1 (CT-1) (3, 4). The binding of IL-6 to α chain leads to the formation of receptor complexes, followed by tyrosine phosphorylation and activation of Janus protein tyrosine kinases (Jaks) and various cellular proteins, including gp130 itself. The activated Jaks, in turn, phosphorylate and activate latent signal transducer and activator of transcription (STAT) family of transcription factors (5, 6).

One member of the STAT family of proteins is STAT3, which is mainly activated by IL-6 family of cytokines, epidermal growth factor, and leptin (3, 4). Like other members of the STAT family, STAT3 is tyrosine-phosphorylated by Jaks, upon which it dimerizes, and translocates into the nucleus to activate target genes (5, 6).

Protein-tyrosine phosphatases (PTPs) are a large and structurally diverse family of enzyme that catalyze the dephosphorylation of tyrosine-phosphorylated proteins (7, 8). PTPs have been shown to participate as either positive or negative regulators of signal transduction in a wide range of physiological processes, which include cellular growth and proliferation, migration, differentiation, and survival (7, 8). Despite their important roles in a wide range of physiological and pathological processes, the mechanism by which PTPs exert their effects is often poorly understood.

T cell PTP (TC-PTP) is a ubiquitous intracellular PTP that was cloned originally from a peripheral human T-cell cDNA library (9, 10). Alternative splicing gives rise to two forms of TC-PTP that differ in the C termini, a 45-kDa isoform that is targeted to the nucleus and a 48-kDa isoform that localizes to the endoplasmic reticulum via a hydrophobic C-terminal region (11, 12, 13). In a recent study, it was shown that cellular stress causes reversible cytoplasmic accumulation of the 45-kDa isoform of TC-PTP (14). TC-PTP is found in higher amounts in lymphoid and hematopoietic cell lineages, suggesting that TC-PTP plays an important role in both hematopoiesis and immune function (9 10). Very interestingly, TC-PTP-deficient mice showed impaired bone marrow microenvironment and immune function (15). Especially, bone marrow of TC-PTP deficient mice failed to support the development of B cells and erythrocytes, but not granulocytes and macrophages.

More recently, we demonstrated that the nuclear isoform of TC-PTP dephosphorylated and deactivated STAT5a and STAT5b in transfected COS-7 cells as well as in mammary epithelial COMMA-1D cells, thereby negatively regulating the PRL-mediated signaling pathway (16) as well as PTP1B (17) which is structurally highly related TC-PTP comprises a subfamily of intracellular PTPs.

In this study, we examined the involvement of the nuclear isoform of TC-PTP in the IL-6-mediated signaling pathway. The data demonstrated that the nuclear isoform of TC-PTP was a potential negative regulator of IL-6-mediated signal transduction by dephosphorylating and deactivating STAT3. Of particular note is that overexpression of the nuclear isoform of TC-PTP was found to suppress the IL-6-induced growth arrest of myeloid leukemia M1 cells and endogenous TC-PTP complexed with STAT3 in the nucleus of M1 cells.

MATERIALS AND METHODS

Reagents and antibodies

Human recombinant IL-6 was a kind gift from Ajinomoto (Tokyo, Japan). Human recombinant soluble IL-6 receptor α (sIL-6R α) was a kind gift from Dr. Hirano (Osaka Univ., Osaka, Japan). Human recombinant LIF was purchased from INTERGEN (Purchase, NY). Expression vectors, Jak1, FLAG-tagged STAT3 and STAT3-LUC (18) were kindly provided by Dr. J. N. Ihle (St. Jude CRH., Memphis, TN) and Dr. T. Hirano (Osaka Univ., Osaka, Japan), respectively. Myc-tagged STAT3 mutants were generated by PCR methods and sequenced (primer sequences are available upon request). Anti-HA, anti-Myc, anti-STAT3 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phosphotyrosine (PY20) was purchased from Pharmingen (San Diego, CA). Anti-FLAG M2 antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-TC-PTP 3E2 monoclonal antibody was a kind gift from Dr. M. L. Tremblay (McGill Univ., Montreal, Canada) (15).

Cell culture, transfections, luciferase assays, and cell growth assays

M1, a murine myeloid leukemia cell line was cultured in RPMI 1640 containing 10% fetal calf serum (FCS). Human embryonic kidney carcinoma cell line, 293T was maintained in DMEM containing 10% FCS and transfected by the standard calcium precipitation protocol. Luciferase assay was performed as described (19). The cells were harvested and lysed in 100 μ l of PicaGene Reporter Lysis Buffer (Toyo Ink, Tokyo, Japan) and assayed for luciferase and β -galactosidase activities according to the manufacturer's instructions.

Luciferase activities were normalized to the β -galactosidase activities. Cell growth was determined by Cell Counting Kit-8 (Wako Chemicals, Tokyo, Japan) according to the manufacturer's instructions.

***In Vitro* Dephosphorylation Assay**

GST fusion proteins containing full-length nuclear isoform of TC-PTP was described previously (16). STAT3 immune complexes prepared from 293T cells that had been transfected with STAT3 together with Jak1 were processed and incubated with indicated GST fusion proteins as previously described (17).

Retrovirus-mediated gene delivery

HA-tagged nuclear isoform of TC-PTP in pLXSN retroviral vector (16) introduced into PLATE-E ecotropic packaging cells (20). M1 cells were infected with the retrovirus-containing culture medium and then selected in the presence of G418 (1 mg/ml) for 2 wk. To eliminate clonal deviation, G418-resistant polyclonal cells were used for subsequent experiments.

Immunoprecipitation and immunoblotting

The immunoprecipitation and Western blotting were performed as described previously (21). 293T cells were harvested and lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 0.15 M NaCl, containing 1% NP-40, 1 μ M phenylmethylsulfonyl fluoride and 10 μ g/ml each of aprotinin, pepstatin and leupeptin). M1 cells (2×10^8 cells) were stimulated with IL-6 (1 μ g/ml) for 30 min. Nuclear extracts were prepared as described previously (22). Nuclear extracts in the

above lysis buffer containing 0.5% NP-40 were immunoprecipitated with anti-STAT3 antibody. The immunoprecipitates from cell lysates were resolved on 5-20% SDS-PAGE and transferred to Immobilon filter (Millipore, Bedford, MA). The filters were then immunoblotted with each antibody. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

RESULTS AND DISCUSSION

TC-PTP dephosphorylates and deactivates IL-6-activated STAT3 in 293T cells

In our previous study, we showed that the nuclear isoform of TC-PTP dephosphorylated PRL-activated STAT5a and STAT5b (13). To examine whether the nuclear isoform of TC-PTP has any effects on IL-6-stimulated STAT3 activation, we first assessed changes in tyrosine-phosphorylation of STAT3, which trigger its activation, in 293T cells. In these experiments, 293T cells were stimulated with IL-6 in the presence of soluble IL-6 receptor α (sIL-6R α), which was shown to potentiate the biological activities of IL-6 (23). Wild-type (WT) or catalytically inactive Asp/Ala (D/A) or Cys/Ser (C/S) forms of TC-PTP were co-transfected with FLAG-tagged STAT3 into 293T cells. Thirty minutes after stimulation of IL-6/soluble IL-6R α or another IL-6 family cytokine, LIF, cells were lysed, and STAT3 was immunoprecipitated and subjected to immunoblotting with antiphosphotyrosine antibody. Upon co-expression of TC-PTP WT, ligand-induced tyrosine phosphorylation of STAT3 was almost abolished, compared with that of mock transfectants (Fig. 1, A and B).

Dephosphorylation of STAT3 was not observed when the cells were co-transfected with TC-PTP C/S or TC-PTP D/A, suggesting that phosphatase activity of TC-PTP is essential for the dephosphorylation of STAT3. Comparable expression of TC-PTP WT and mutants was confirmed by immunoblotting with anti-HA antibody (Fig. 1, A and B, lower panels).

To further examine whether TC-PTP has any effects on IL-6-induced transcriptional activation, we utilized the transient transfection assay. The STAT3-mediated transcriptional responses were measured by using STAT3-LUC, in which the α 2-macroglobulin promoter

(17) drives expression of the luciferase (LUC) reporter gene. 293T cells were transfected with STAT3-LUC and treated with IL-6 plus sIL-6R α or LIF and LUC activities were determined. As shown in Fig. 1, C and D, IL-6 or LIF stimulated STAT3-LUC activity, which was inhibited by a dominant negative STAT3 in a dose-dependent manner (data not shown). When cells were co-transfected with TC-PTP WT, STAT3-LUC activity decreased in a dose-dependent manner compared with that of mock or mutant transfectants (Fig. 1, C and D). These results indicate that TC-PTP dephosphorylates STAT3 and negatively regulates IL-6- or LIF-mediated STAT3 transcriptional activation in 293T cells.

STAT3 and TC-PTP physically interact in 293T cells

One of the mechanisms that are consistent with the data described above is direct dephosphorylation of STAT3 by the nuclear isoform of TC-PTP, which trigger its deactivation. We first tested this possibility by co-immunoprecipitation experiments. Expression vectors encoding FLAG-tagged STAT3 and HA-tagged TC-PTP WT or a substrate trapping mutant, TC-PTP D/A, were transiently transfected into 293T cells together with or without Jak1. As previously described (24), this catalytically inactive mutant retains the ability to bind substrates and forms stable enzyme-substrate complexes. Cells were lysed and subjected to immunoprecipitation with anti-FLAG antibody. Immunoprecipitates were then used in Western analysis with anti-HA antibody. As shown in Figure 2A, STAT3 interacted with only TC-PTP D/A in the presence of Jak1, suggesting that tyrosine phosphorylation of STAT3 by Jak1 is a prerequisite for STAT3-TC-PTP D/A interactions.

To delineate the domains in the STAT3 that mediate the protein-protein interactions between STAT3 and TC-PTP D/A, co-immunoprecipitation experiments were performed

with a series of mutant STAT3 proteins (Fig. 2B). As shown in Figure 2 C, when most of the N-terminal domain, the coiled-coil domain or the DNA-binding domain was deleted, STAT3 did not interact with TC-PTP D/A. However, STAT3 (494-750), which encodes the part of the SH2 domain and the transcriptional activation domain, interacted with TC-PTP D/A. These data suggest that the C-terminal region containing the critical tyrosine phosphorylation site, which is indispensable for its activation, is required for STAT3 to interact with TC-PTP.

STAT3 is a specific substrate of TC-PTP

To further confirm dephosphorylation action of TC-PTP on STAT3, recombinant glutathione-S-transferase (GST) fusion protein containing full-length nuclear isoform of TC-PTP WT was expressed in *Escherichia coli* and purified as described previously (15). 293T cells that had been transfected with FLAG-tagged STAT3 together with Jak1, and phosphorylated STAT3 was immunoprecipitated. The indicated amounts of the recombinant GST-TC-PTP WT fusion protein and GST were added to the immune complexes and incubated at 37 °C for 30 min. As shown in Fig. 3A, the tyrosine phosphorylation level of STAT3 was reduced to approximately 70% by 3 µg TC-PTP WT, and incubation with 10 µg of the fusion protein resulted in complete dephosphorylation of STAT3 (Fig 3). Incubation of the immune complexes with empty GST resulted in no reduction in tyrosine phosphorylation level of STAT3, suggesting that STAT3 is a specific substrate of TC-PTP.

TC-PTP suppresses IL-6-induced growth arrest of myeloid leukemia M1cells

To examine the role of TC-PTP in IL-6-mediated signaling under more physiological conditions, TC-PTP was introduced into myeloid leukemia M1 cells by a retroviral infection system. Myeloid leukemia M1 cells can be induced for growth arrest and terminal differentiation into macrophages in response to IL-6 or LIF (3, 17). The nuclear isoform of HA-tagged TC-PTP cDNA was ligated into a retroviral vector and introduced into M1 cells. Cells were selected in G418-supplemented cell culture medium and then directly used for subsequent experiments. Similar amounts of HA-tagged TC-PTP and mutants were expressed in M1 cells (Fig. 4A). The mock- or catalytically inactive TC-PTP-transfected M1 cells underwent cell death after treatment with IL-6 for 3 days. However, in the cells expressing TC-PTP WT, IL-6-induced growth arrest was inhibited by 50 % compared with that of mock transfectants (Fig. 4B). These results strongly suggest that the nuclear isoform of TC-PTP regulates IL-6 signaling pathway in M1 cells.

To further confirm that endogenous TC-PTP interact with STAT3 *in vivo*, co-immunoprecipitation experiments were performed using nuclear extracts obtained from untransfected M1 cells that were either left untreated or were treated with IL-6. As shown in Fig. 4C, anti-STAT3 immunoprecipitate from nuclear extracts of M1 cells contained the TC-PTP protein. Interestingly, after treatment of IL-6, the enhanced TC-PTP protein band was observed in the anti-STAT3 immunoprecipitate of nuclear extracts. These results also suggest that endogenous TC-PTP interact and form a complex with STAT3 in nucleus of M1 cells.

Conclusions

IL-6 is a pleiotropic cytokine with a wide range of biological activities such as support of hematopoiesis, regulation of acute phase reactions, and generation of immune responses (1). Uncontrolled hyperproduction of IL-6 causes plasmacytosis, hyper-gamma-globulinemia, thrombocytosis, mesangial cell proliferation of the kidney as well as inflammatory symptoms which are frequently observed in autoimmune diseases (2). Therefore, interference with IL-6 signal transduction may be useful for autoimmune disease therapy. Recent studies have documented the interaction of a large number of intracellular proteins with the effector molecules STAT3 to influence IL-6 signaling (25-34). We have shown here the involvement of the nuclear isoform of TC-PTP in IL-6 signaling pathway. IL-6-induced tyrosine-phosphorylation and activation of STAT3 were suppressed by overexpression of TC-PTP WT in 293T cells. Tyrosine-phosphorylated STAT3 directly interacted with a substrate-trapping mutant of TC-PTP and was in vitro dephosphorylated by recombinant GST-TC-PTP WT protein. Furthermore, retrovirus-mediated overexpression of the nuclear isoform of TC-PTP suppressed the IL-6-induced growth arrest of myeloid leukemia M1 cells. Endogenous TC-PTP complexed with STAT3 in the nucleus of untransfected M1 cells.

Recent studies identified several molecules that regulate IL-6 signaling. Suppressors of cytokine signaling (SOCS-1), which is induced by various cytokines including IL-6, suppresses Jak-STAT signaling by direct interaction with Jak kinases (25, 26, 27). Protein inhibitor of activated STAT3 (PIAS3) inhibits DNA binding activity of activated Stat3 and subsequent gene expression (28). Several PTPs are shown to play an important role in the regulation of IL-6 signaling pathway. However, no nuclear protein tyrosine phosphatase has been shown to involve in the regulation of IL-6 signaling pathway. Src homology

2-containing phosphatase 2 (SHP-2) involved in the down regulation of Jak-STAT signaling activated by several cytokines (29, 30, 31). Furthermore, upon IL-6 stimulation, SHP-2 also acted as an adaptor associating with Grb2 and activated mitogen-activated protein kinase (MAPK) (29, 32). A cytosolic isoform of PTP ϵ , PTP ϵ C, is also shown to reveal an inhibitory effect on STAT3 activation induced by IL-10 as well as by IL-6 (33, 34). We demonstrated that a classical PTP, PTP1B negatively regulated the PRL-mediated signaling pathway by dephosphorylating and deactivation of STAT5a and STAT5b in mammary epithelial cells as well as the nuclear isoform of TC-PTP (15, 16). Jak2 and Tyk2, but not Jak1 were also demonstrated to be substrates of PTP1B (36). PTP1B is well known to be responsible for negatively regulating insulin signaling by dephosphorylating the phosphotyrosine residues of the insulin receptor kinase (37, 38). PTP1B knockout study identified PTP1B as a promising target for anti-diabetes/obesity drug discovery (39). Recently, TC-PTP deficient mice were developed (18). The phenotype of TC-PTP deficient mice was not lethal in utero, but all homozygous mutant mice died by 3-5 week of age, which showed runting, splenomegaly, and lymphadenopathy. Homozygous mice showed specific defects in bone marrow, B cell lymphopoiesis, and erythropoiesis, as well as impaired T and B cell functions, suggesting that TC-PTP plays an important role in both hematopoiesis and immune function. However, the precise reason for bone marrow B lymphopoietic and erythropoietic failure in these mice is still unknown. Considering that IL-6 stimulates B lymphopoiesis and STAT5 acts in the downstream of erythropoietin receptor, these observations suggest that TC-PTP may be a key regulator of signal transduction processes in the downstream of hematopoietic cytokines such as IL-6 and erythropoietin.

In this study, we for the first time demonstrated that the nuclear isoform of TC-PTP is an important regulator of STAT3 function in the downstream of IL-6 signaling in M1 cells and

thereby may have critical roles in the progression of IL-6-related diseases. More detailed understanding of the interaction between STAT3 and TC-PTP is therefore important as this new information may provide new therapeutic approaches for these conditions.

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FIGURE LEGENDS**Fig. 1. TC-PTP dephosphorylates and deactivates IL-6-activated STAT3 in 293T cells.**

(A) and (B) 293T cells (1×10^7) were transfected with FLAG-tagged STAT3 (7.5 μg) together with HA-tagged TC-PTP wild-type (WT) or catalytically inactive Asp/Ala (D/A) or Cys/Ser (C/S) forms of TC-PTP, empty vector (5 μg). 48 h after transfection, cells were starved for 12h, followed by treatment with or without IL-6 (100ng/ml) and soluble IL-6 receptor α chain (sIL-6R α , 100ng/ml)(A) or LIF (100ng/ml) (B) for 30 min. Cell lysates were then immunoprecipitated with an anti-FLAG antibody or anti-HA antibody, and immunoblotted with an anti-phosphotyrosine antibody (anti-PY, upper panel), an anti-FLAG antibody (middle panel) or, an anti-HA antibody (lower panel).

(C) and (D) 293T cells were transfected with STAT3-LUC (0.4 μg each) and/or indicated amounts (15 to 150ng) of TC-PTP WT, or TC-PTP D/A, TC-PTP C/S, empty vector. 48 h after transfection, cells were stimulated for an additional 12h with or without IL-6 (100ng/ml) and sIL-6R α (100ng/ml)(C) or LIF (100ng/ml)(D), and LUC activities were determined. The results are presented as the fold induction of luciferase activity from triplicate experiments, and the error bars represent the standard deviations.

Fig. 2. STAT3 and TC-PTP physically interact in 293T cells.

(A) 293T cells (1×10^7) were transfected with FLAG-tagged STAT3 (7.5 μg) together with or without HA-tagged TC-PTP WT or TC-PTP D/A (5 μg) in the presence or absence of Jak1 (1 μg). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-FLAG antibody or anti-HA-antibody, and immunoblotted with an anti-HA antibody

(upper panel) or an anti-FLAG antibody (middle panel), an anti-HA antibody (lower panel). (B) Domain structure of STAT3 and mutant fragments are schematically shown. The arrow indicates tyrosine phosphorylation site on tyrosine 705, which is critical for its activation. (C) Mapping the TC-PTP interaction domain of STAT3. 293T cells (1×10^7) were transfected with STAT3 (1–137) or STAT3 (138–319), STAT3 (320–493), STAT3 (494–750) (10 μ g) and HA-tagged TC-PTP D/A (7.5 μ g), together with Jak1 (1 μ g). 48 h after transfection, cells were lysed and immunoprecipitated with an anti-HA antibody, and immunoblotted with anti-Myc antibody (upper panel) or anti-HA antibody (middle panel). Total cell lysates (20 μ g) were blotted with anti-Myc antibody (lower panel). The asterisks indicate the migration position of the STAT3 deletion mutants.

Fig. 3. STAT3 is a specific substrate of TC-PTP.

293T cells (1×10^7) were transfected with FLAG-tagged STAT3 (7.5 μ g), together with Jak1 (1 μ g). 48 h after transfection, cells were and then subjected to an *in vitro* dephosphorylation assay as described in *MATERIALS AND METHODS*. After termination of the incubation, proteins were separated by SDS-PAGE and analyzed with an anti-phosphotyrosine antibody (PY). The same blot was reprobed with anti-FLAG antibody after stripping.

Fig. 4. TC-PTP suppresses IL-6-induced growth arrest of myeloid leukemia M1 cells.

(A) M1 cells were retrovirally infected with empty vector or HA-tagged TC-PTP WT, TC-PTP C/S, TC-PTP D/A mutant and selected in the cell culture medium supplemented with G418 (1 mg/ml). Polyclonal clones for each were lysed and immunoprecipitated with an

anti-HA antibody, followed by immunoblotting with an anti-HA antibody.

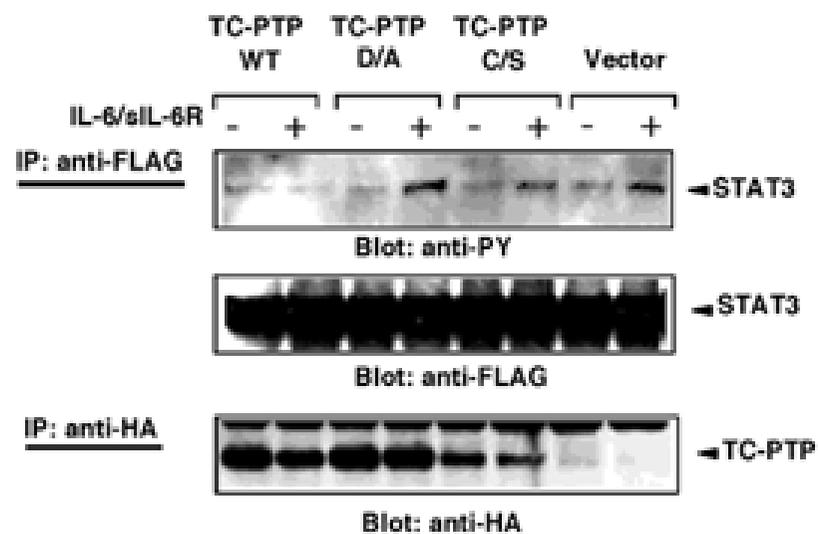
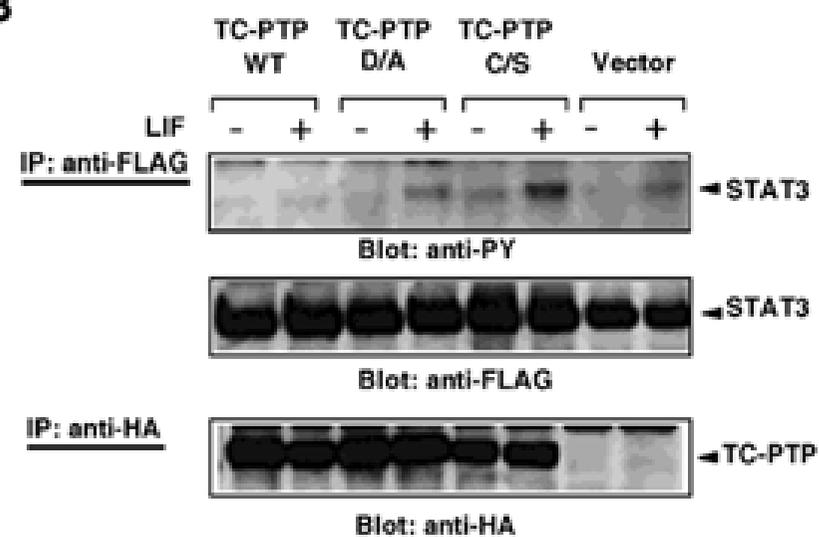
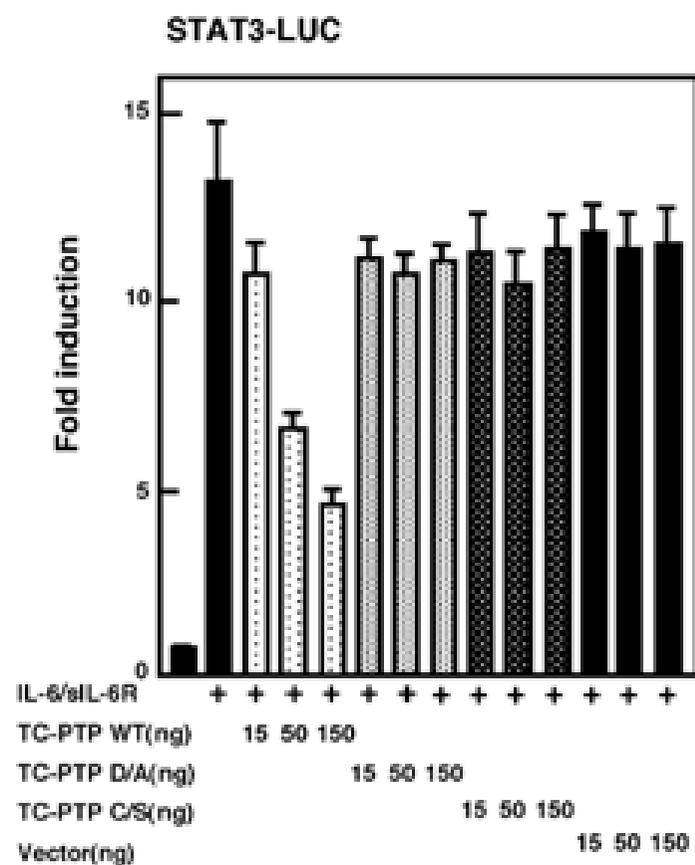
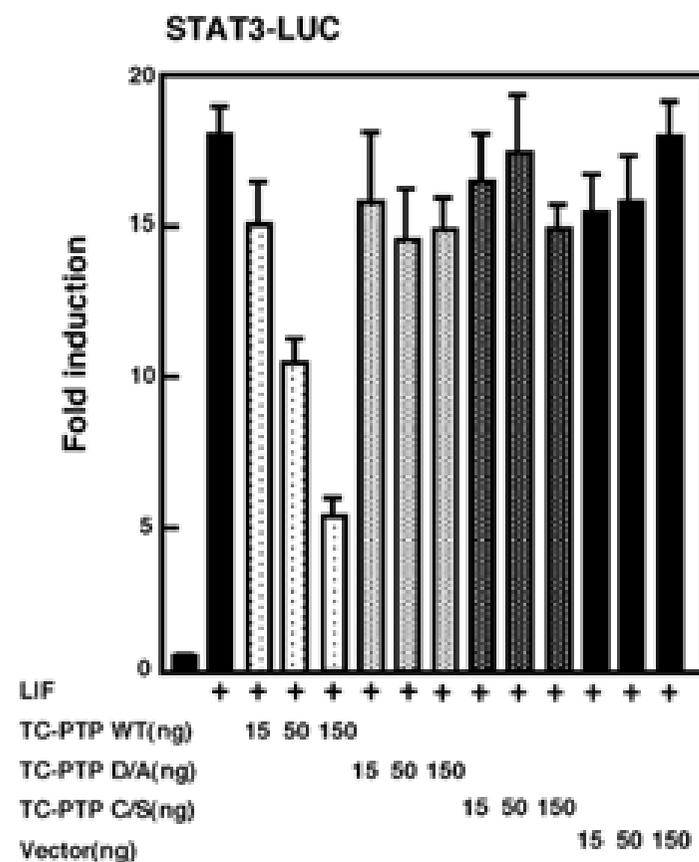
(B) M1 transfectants were cultured with or without IL-6 as indicated for 3 days. Cell growth was determined by Cell Counting Kit-8 as described in *MATERIALS AND METHODS*.

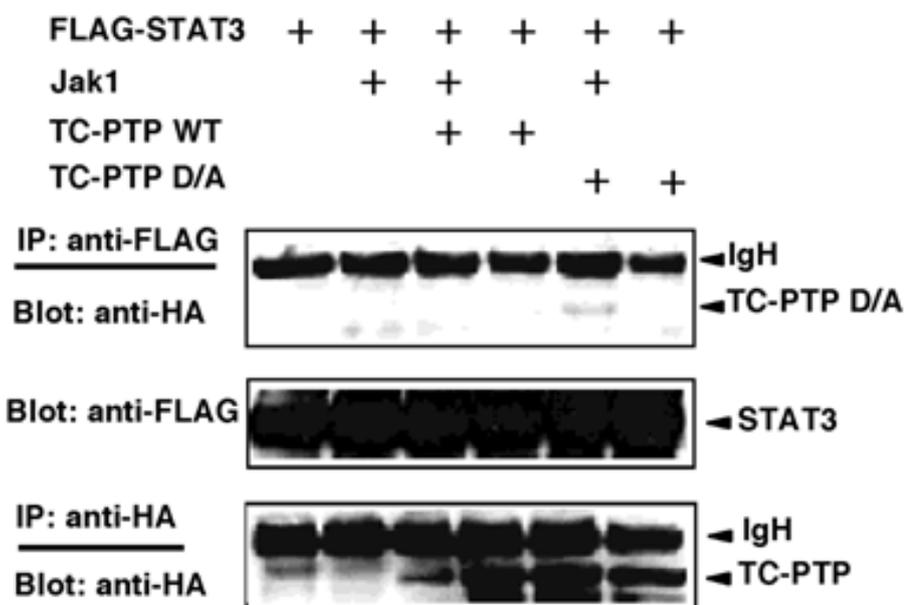
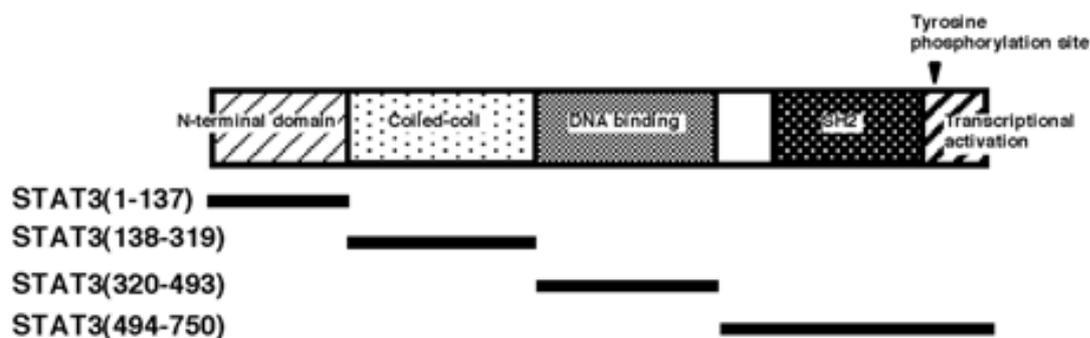
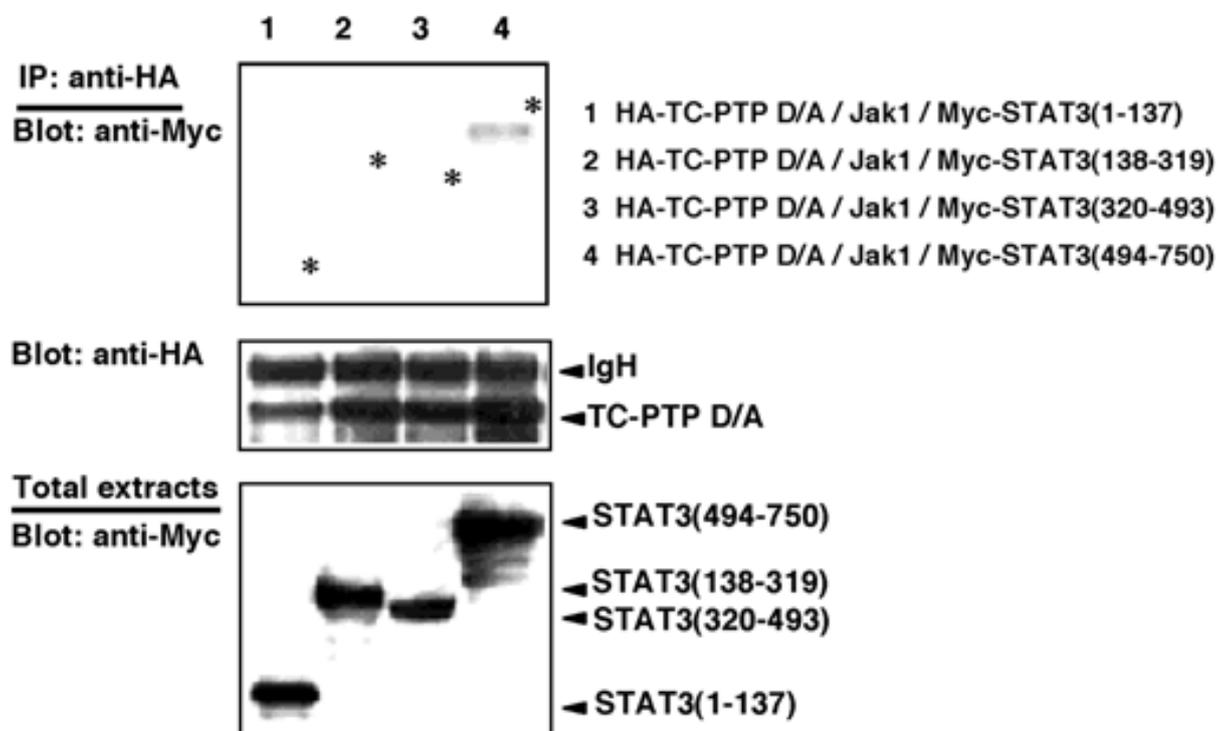
M1-stable clones were treated with IL-6 at various concentrations indicated for 2 days, and DNA synthesis was then measured by thymidine incorporation. The results are presented from three independent experiments, and the error bars represent the standard deviations.

(C) M1 cells (2×10^8 cells) were either left untreated, or treated with IL-6 (1 $\mu\text{g/ml}$) for 30 min.

Nuclear extracts were prepared as described in *MATERIALS AND METHODS*. Nuclear extracts were lysed and immunoprecipitated with either control IgG or anti-STAT3 antibody as indicated, followed by immunoblotting with anti-TC-PTP 3E2 antibody (upper panel).

The same blot was reprobed with anti-FLAG antibody after stripping (lower panel).

A**B****C****D****Fig. 1**

A**B****C****Fig. 2**

GST (μg)

0

3.0

10.0

GST-TC-PTP WT (μg)

3.0

10.0



STAT3

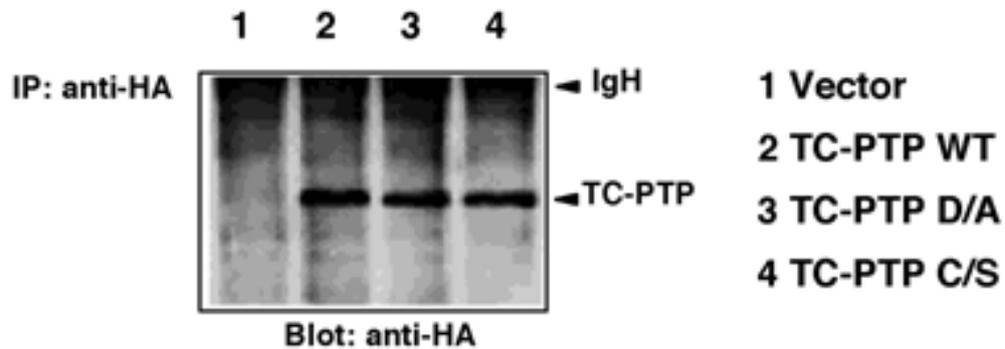
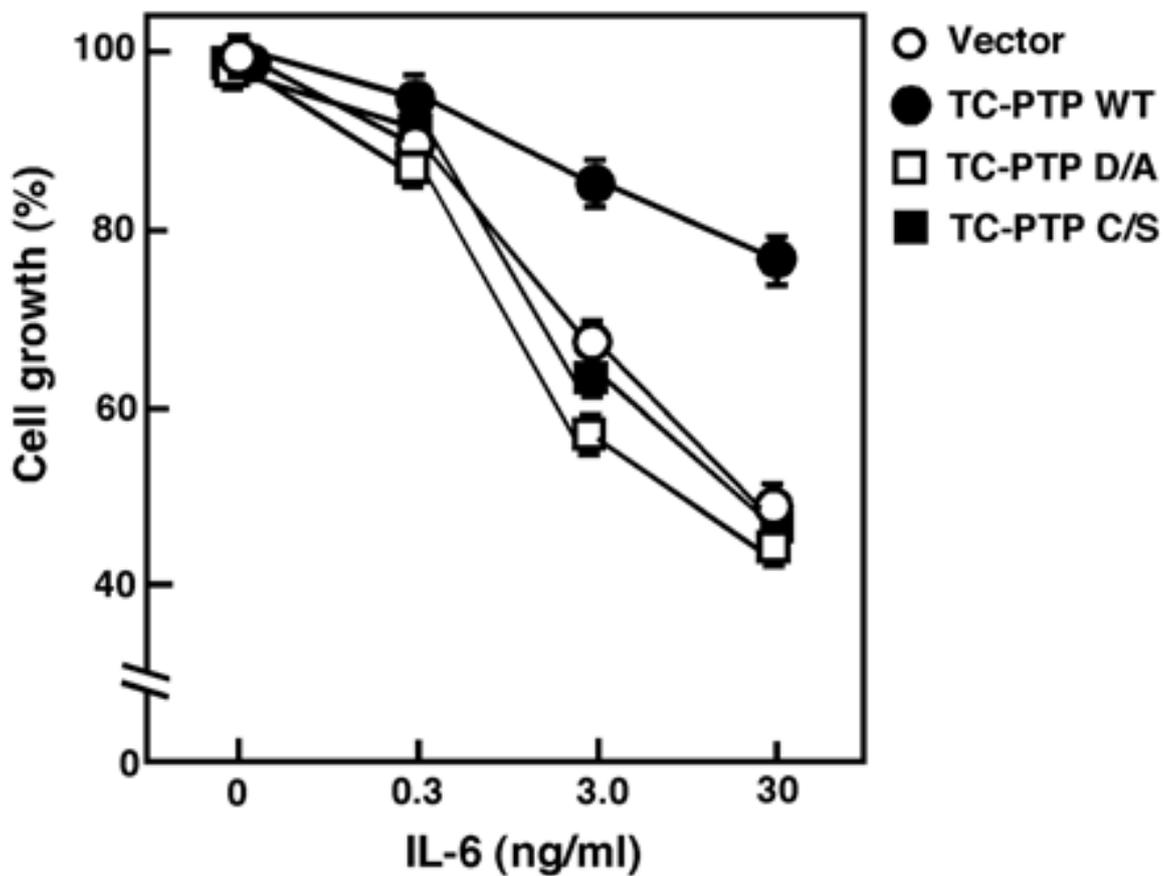
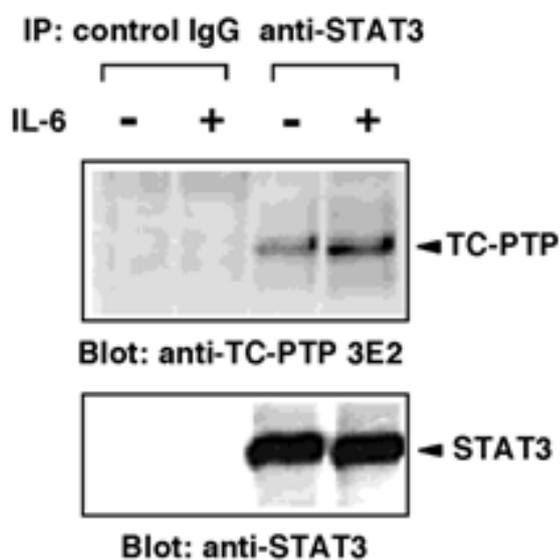
Blot: anti-PY



STAT3

Blot: anti-FLAG

Fig. 3

A**B****C****Fig. 4**