Identification of a Nuclear Stat1 Protein Tyrosine Phosphatase

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Upon interferon (IFN) stimulation, Stat1 becomes tyrosine phosphorylated and translocates into the nucleus, where it binds to DNA to activate transcription. The activity of Stat1 is dependent on tyrosine phosphorylation, and its inactivation in the nucleus is accomplished by a previously unknown protein tyrosine phosphatase (PTP). We have now purified a Stat1 PTP activity from HeLa cell nuclear extract and identified it as TC45, the nuclear isoform of the T-cell PTP (TC-PTP). TC45 can dephosphorylate Stat1 both in vitro and in vivo. Nuclear extracts lacking TC45 fail to dephosphorylate Stat1. Furthermore, the dephosphorylation of IFN-induced tyrosine-phosphorylated Stat1 is defective in TC-PTP-null mouse embryonic fibroblasts (MEFs) and primary thymocytes. Reconstitution of TC-PTP-null MEFs with TC45, but not the endoplasmic reticulum (ER)-associated isoform TC48, rescues the defect in Stat1 dephosphorylation. The dephosphorylation of Stat3, but not Stat5 or Stat6, is also affected in TC-PTP-null cells. Our results identify TC45 as a PTP responsible for the dephosphorylation of Stat1 in the nucleus.

A wide variety of cytokines and growth factors activate intracellular signaling events involving Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). Ligand activation of receptor-associated JAKs leads to tyrosine phosphorylation of the receptor chains, creating docking sites for STATs. In turn, STATs become phosphorylated on tyrosine, dimerize, and translocate to the nucleus to activate transcription (8). A precise regulation of both the magnitude and duration of JAK activity and of STAT activation is essential for the cytokine orchestration of many biological processes, and dysregulation of the JAK-STAT pathway has pathological implications (4, 33). Negative regulation of the JAK-STAT signaling pathway occurs through several distinct mechanisms. Cessation of signaling from the cell surface occurs through degradation of the receptor-ligand complex via the ubiquitin-proteasome pathway (5, 13) and through induction of the suppressor of cytokine signaling (SOCS) family of proteins, which inhibit the activity of JAKs (14). In addition, several protein tyrosine phosphatases (PTPases), including SHP-1, SHP-2, CD45, and PTP-1B, can dephosphorylate either cytokine receptors or JAKs (17, 23, 33). PTPases that can dephosphorylate STATs in the cytoplasm have also been described (1, 36). STATs are rapidly exported back into the cytoplasm and taken part in subsequent activation-inactivation cycles (3, 9, 12, 13, 22, 26). The identity of the nuclear Stat1 phosphatase, however, had remained uncovered. Other STATs also undergo rapid activation-inactivation cycles (5, 20, 35), suggesting that nuclear STAT dephosphorylation may be a general phenomenon.

Here we report the purification of a nuclear Stat1 phosphatase, which we identified as TC45, the nuclear isoform of the ubiquitously expressed T-cell PTP (TC-PTP). We show that TC45 can dephosphorylate Stat1 in vitro and in vivo. Consistently, analysis of TC-PTP-null mouse embryonic fibroblasts (MEFs) revealed impaired dephosphorylation of Stat1 in the nucleus. The dephosphorylation of Stat3, but not Stat5 or Stat6, was also affected in TC-PTP-null cells.

**MATERIALS AND METHODS**

**Plasmid constructs.** pGEX-Stat1 was constructed by inserting the Stat1a cDNA into pGEX2T. PATH-v-Abl contains the kinase domain of v-Abl as a trpE fusion gene and a Tet r gene. The coding regions of the human TC45, mouse TC45, and mouse TC48 cDNAs were PCR amplified from IMAGE consortium clones 1894955, 604362, and 3602983, respectively, and cloned into pGEX4T-1, pEBB, pCDNA3, pBabeuro, and/or pBabeuroFlag.

**Cell culture and reagents.** 293T and U2OS cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS) and PenStrep. DNA transfections were performed by the CaPO4 method, and clones stably expressing hTC45 were selected in 0.4 mg of G418 per ml. TC-PTP wild-type (7/1, 11/1) and TC-PTP-null (4/7, 14/7) MEF cell lines were maintained in DMEM containing 10% FBS as described previously (16). 4/TC45 and 4/ were derived from 4 cells after infection with retrovirus containing pBabeuro-hTC45 and phabeuro, respectively, followed by single-colony selection in 2.5 μg of puromycin per ml. Retrovirus was prepared by transfection of amphotropic dNX cells and utilized as described previously (24). 14/TC45 and 14/ pools were established by retroviral infection using pBabeuroFlag-mTC45 and pBabeuroFlag-mTC48, respectively, and analyzed after 1 to 2 weeks of puromycin selection. Human gamma interferon (INF-γ) (a gift from Amsen) and murine INF-γ (Peprotech) were used at 5 ng/ml. Staurosporine (Sigma) was used at 0.25 to 0.5 μM as indicated.

**Bacterial expression and purification of tyrosine-phosphorylated Stat1.** pGEX-Stat1 and PATH-v-Abl plasmids were cotransformed into the bacterial strain RR1 and selected on plates containing ampicillin, tetracycline, and 20 μg of tryptophan per ml (W). Expression of the glutathione S-transferase (GST)—
Stat1 fusion protein was induced as previously described (29), but with the following modifications. An overnight culture of RR1 (containing the pGEX-STAT1 and pATH-v-Abl plasmids) grown in Luria broth supplemented with ampicillin (100 μg/ml), tetracycline (100 μg/ml), and W (20 μg/ml) was diluted 1:50 into 1 liter of the same medium and grown for 3 h at 30°C followed by another 1.5 h in the presence of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The cells were then pelleted, resuspended in 1 liter of modified M9 medium (1× M9 salts, 0.5% [w/vol] Casamino Acids [Sigma A-2247], 0.1 mM CaCl₂, 0.2% glucose, 10 μg of thiamine B1 per ml) with IPTG, but without W, to induce the expression of the TyrE-v-Abl fusion protein (18), and cultured for another 1.5 h at 30°C. The cells were pelleted, resuspended in 20 ml of ice-cold lysis buffer (phosphate-buffered saline [PBS], 50 mM EDTA, 1% Triton X-100, 1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 1 μg of pepstatin A per ml) and sonicated. GST-p-Stat1 was bound to glutathione agarose beads (Sigma), and after extensive washing eluted in elution buffer (10 mM glutathione, 50 mM Tris [pH 8.0], 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.067% dialyzed against buffer A containing 1.1 M ammonium sulfate). The active fractions eluting from 1.1 to 1.8 M NaCl were pooled and dialyzed against buffer B (50 mM HEPES [pH 7.5], 5% glycerol, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF) containing 50 mM NaCl. This was loaded onto a 5-ml Blue Sepharose Fast Flow column (C10/10; Pharmacia), and bound proteins were eluted with a linear gradient of 1.4 to 0 M ammonium sulfate in buffer B. Collected fractions were assayed for PTP activity, and active fractions eluting at 1.0 to 0.9 M ammonium sulfate were pooled and dialyzed against buffer B (50 mM HEPES [pH 7.5], 5% glycerol, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF) containing 50 mM NaCl. This was loaded onto a 5-ml Blue Sepharose Fast Flow column (C10/10; Pharmacia), and bound proteins were eluted with a linear gradient of 50 mM to 2.5 M NaCl in buffer B. The active fractions eluting from 1.1 to 1.8 M NaCl were dialyzed in buffer containing 1.1 M sodium sulfate and 1.2 M NaCl to 1 M ammonium sulfate. Active fractions containing p-Stat1 and 2.5 μl of HeLa cell nuclear extract (ne) were incubated at 30°C for various periods as indicated. The reaction mixtures were analyzed by Western blotting with anti-p-Stat1(Y701) antibody. (D) In vitro Stat1 PTPase assay performed as in panel C with inclusion of 0.5 mM sodium orthovanadate (OV) as indicated.

**RESULTS**

Establishment of an in vitro Stat1 PTPase assay. In order to identify the nuclear Stat1 phosphatase, we set out to purify the enzymatic activity by biochemical fractionation with tyrosine-phosphorylated Stat1 as a substrate in a PTPase assay. Tyrosine-phosphorylated Stat1 (p-Stat1) was generated and purified from bacteria coexpressing Stat1 and the kinase domain of v-Abl. Analysis of the purified p-Stat1 protein by Western blotting with a phospho-Stat1(Y701)-specific antibody demonstrated that it was phosphorylated on tyrosine 701, the functionally important tyrosine residue (Fig. 1A and B). The decreased level of p-Stat1 phosphorylation of p-Stat1 occurred in a time-dependent manner under these conditions (Fig. 1C). The decreased level of p-Stat1 phosphorylation was due to a tyrosine phosphatase activity, since the inclusion of the tyrosine phosphatase inhibitor orthovanadate prevented the dephosphorylation of p-Stat1 (Fig. 1D).

**Purification and identification of a nuclear Stat1 phosphatase**
tase. Using the established in vitro PTPase assay, we purified a Stat1 PTPase to near homogeneity from HeLa cell nuclear extract through a series of chromatographic fractionations (Fig. 2A). Silver-staining analysis of fractions from the MonoS column revealed the presence of a 45-kDa protein, the abundance of which was in correlation with the detected Stat1 PTPase activity (Fig. 2B, top and middle panels), suggesting that this protein may be the enzyme responsible for the observed Stat1 dephosphorylation.

We explored the possibility that the purified 45-kDa protein may be an already known nuclear PTPase. The human TC-PTP gene encodes two distinct protein products as a result of alternative splicing: TC45 (or TC-PTPa), a 45-kDa protein located mainly in the nucleus, and TC48 (or TC-PTPb), a 48-kDa endoplasmic reticulum (ER)-associated protein (6, 7, 15, 21). To test whether the purified 45-kDa protein is TC45, protein blot analysis of the MonoS fractions was performed with a monoclonal antibody against TC-PTP. The 45-kDa protein in these fractions was specifically recognized by the anti-TC-PTP antibody (Fig. 2B, bottom panel). While both TC45 and TC48 were detected in HeLa whole-cell extract, only TC45 was present in the nuclear fraction from which it was subsequently purified (Fig. 2C).

To confirm that TC45 is indeed responsible for the dephosphorylation of Stat1, TC45 was removed from HeLa nuclear extract by using anti-TC-PTP antibody (Fig. 2D, top panel). p-Stat1 was not dephosphorylated when incubated with the TC45-depleted nuclear extract. In contrast, p-Stat1 was dephosphorylated by nuclear extract that had been pretreated with control anti-IgG antibody (Fig. 2D, bottom panel). These results demonstrate the specificity of the in vitro Stat1 PTPase assay and indicate that TC45 is the phosphatase present in HeLa nuclear extract that is responsible for the dephosphorylation of Stat1.

TC45 can dephosphorylate Stat1 in vitro and in vivo. To test if TC45 can directly dephosphorylate Stat1, we conducted an in vitro PTPase assay with p-Stat1 and bacterially produced GST-TC45 immobilized on glutathione beads. p-Stat1 was readily dephosphorylated by GST-TC45 (Fig. 3A). Dephosphorylation...
of p-Stat1 by GST-TC45 was inhibited by orthovanadate, demonstrating the specificity of the reaction.

To examine if TC45 can affect IFN-\(\gamma\)-induced Stat1 phosphorylation in vivo, 293T cells were cotransfected with Flag-Stat1 and increasing amounts of TC45. Transfected cells were then treated with IFN-\(\gamma\) and harvested for protein blot analysis. Coexpression of Flag-Stat1 with TC45 resulted in a dose-dependent decrease in the levels of IFN-\(\gamma\)-induced tyrosine-phosphorylated Stat1 (Fig. 3B). In addition, a human osteosarcoma cell line stably expressing TC45 (U2OS-TC45) was established. Protein extracts from U2OS-TC45 and the parental U2OS cells treated with IFN-\(\gamma\) for various periods of time were prepared and analyzed by protein blotting with anti-p-Stat1 antibody. The amount of IFN-\(\gamma\)-induced tyrosine-phosphorylated Stat1 in the U2OS-TC45 cell line was reduced over the entire course of treatment compared to that of the parental cell line (Fig. 3C). These data suggest that TC45 can dephosphorylate tyrosine-phosphorylated Stat1 in vitro and in vivo.

Analysis of Stat1 signaling in wild-type and TC-PTP-null MEFs. We next used TC-PTP-null MEFs to confirm the role of TC45 in Stat1 dephosphorylation. Wild-type (7+/+) and TC-PTP-/- (4/-) MEF cells were treated with IFN-\(\gamma\) for various periods, and whole-cell extracts were analyzed by Western blotting with anti-p-Stat1 antibody. In the 7+/+ cells, the level of tyrosine-phosphorylated Stat1 reached a maximum at 15 min of IFN-\(\gamma\) stimulation and then gradually declined thereafter (Fig. 4A). In contrast, in the mutant 4/- cells, tyrosine-phosphorylated Stat1 continued to accumulate and persisted at higher levels for at least 2 h of IFN-\(\gamma\) stimulation. Since the overall level of tyrosine-phosphorylated Stat1 is determined by the balance of phosphorylation and dephosphorylation events, prolonged Stat1 phosphorylation in TC-PTP-/- cells may result from either an increase in JAK kinase activity or a decrease in phosphatase activity towards Stat1. To specifically monitor the rate of Stat1 dephosphorylation, we employed a previously described pulse-chase strategy (12, 13). Staurosporine, a protein kinase inhibitor, was added to cells pretreated with IFN-\(\gamma\), blocking the continuous phosphorylation of Stat1 by JAKs. Residual levels of preactivated Stat1 were then determined at several later time points. In wild-type 7+/+ cells, Stat1 was almost completely dephosphorylated after a 20-min staurosporine chase (or 30 min of IFN-\(\gamma\) treatment). In contrast, tyrosine dephosphorylation of Stat1 was completely blocked in the mutant 4/- cells (Fig. 4B). Fractionation of wild-type and mutant MEF cells treated for 30 min with IFN-\(\gamma\) followed by a staurosporine chase revealed defective Stat1 dephosphorylation in both the nuclear and cytoplasmic fractions (Fig. 4C). Similar results were obtained when an independent pair of TC-PTP wild-type (11+/+) and mutant (14/-) MEF cell lines were analyzed (Fig. 4C). A defect in Stat1 dephosphorylation after IFN-\(\beta\) stimulation was also observed (data not shown).

Nuclear extracts from wild-type 7+/+ and mutant 4/- MEFs were also examined for their ability to dephosphorylate p-Stat1 in vitro. Consistently, nuclear extract from wild-type 7+/+ cells, but not mutant 4/- cells, was able to dephosphorylate p-Stat1 (Fig. 4D). Taken together, these data suggest that TC45 plays a major role in the dephosphorylation of Stat1 in the nucleus.

Reconstitution of TC-PTP-null MEFs with TC45, but not TC48, rescues the defect in Stat1 dephosphorylation. To demonstrate that the observed defect in Stat1 dephosphorylation in TC-PTP-null cells is indeed due to the lack of TC45 protein, we performed reconstitution analysis. An expression vector encoding TC45 was introduced into 4/- cells and a stable
FIG. 5. Reconstitution of TC-PTP-null MEFs with TC45, but not TC48, rescues the defect in Stat1 dephosphorylation. (A) Whole-cell extracts (75 μg/lane) from 7+/+ and 4−/− and 4−/−TC45 cells were analyzed by Western blotting with the 3E2 monoclonal anti-TC-PTP antibody. (B) 7+/+, 4−/−, 4−/−, and 4−/−TC45 cells lines were stimulated with IFN-γ for 30 min followed by a staurosporine chase (0.25 μM) for various periods as indicated. Whole-cell extracts were analyzed by Western blotting with the p-Stat1 antibody. Stripped blots were reprobed with anti-actin antibodies as indicated. (C) Puromycin-resistant pools of 14−/− cells retrovirally infected with pBabeuroFlag-TC45 (14−/−TC45) or pBabeuroFlag-TC48 (14−/−TC48) were analyzed for expression with a Flag antibody. (D) Staurosporine-chase experiment as described above with the 14−/−TC45 and 14−/−TC48 pools.

clone, 4−/−TC45, was used for further analysis. Western blot analysis indicated that the level of TC45 expression in 4−/−TC45 cells was about 20% of that in the wild-type 7+/+ MEF cells (Fig. 5A). Staurorosporine-chase analysis was performed on 4−/−TC45 cells together with the control cell line 4−/− as well as the parental 4−/− and wild-type MEFs. Reconstitution of 4−/− cells with TC45 (4−/−TC45), but not empty vector (4−/−), was sufficient to rescue the defect in Stat1 dephosphorylation (Fig. 5B). Similar results were obtained with several other TC45-reconstituted 4−/− cells (not shown). We conclude that TC45 is required for the tyrosine dephosphorylation of Stat1.

To ensure that the dephosphorylation of Stat1 by TC45 is a nuclear event, mutant 14−/− MEF cells were infected with retrovirus expressing either Flag-TC45 or Flag-TC48 and selected for drug resistance. Pools of drug-resistant cells were used for staurorosporine-chase analysis to examine the dephosphorylation of Stat1 after IFN treatment. Unlike TC45, TC48 was unable to rescue the defect in Stat1 dephosphorylation (Fig. 5D), even though TC48 was expressed at a higher level than TC45 in these pools (Fig. 5C). Similar results were obtained when 4−/− cells were used for this experiment (data not shown). TC48 was functional in these cells, because it rescued mitogen-induced cyclin D1 expression (16) as efficiently as TC45 (data not shown). Since TC48 is identical to TC45, except for the presence of a distinct COOH-terminal sequence that targets it to the ER, these results provide further evidence to support the in vivo specificity of TC45 in Stat1 dephosphorylation.

Analysis of the specificity of TC45 in STAT dephosphorylation. We next examined the involvement of TC45 in the inactivation of other STAT proteins. Wild-type and mutant MEFs were treated with interleukin-6 (IL-6), growth hormone, or IL-4 followed by a staurosporine chase to examine the dephosphorylation of Stat3, Stat5, and Stat6, respectively (Fig. 6). The dephosphorylation of IL-6-activated Stat3 was defective in 4−/− cells as compared to wild-type 7+/+ cells (Fig. 6A). Stat3 is also activated by IFN-γ in MEFs (11). A similar defect in Stat3 dephosphorylation was observed in TC-PTP-null MEFs after IFN-γ treatment (data not shown). In contrast, we observed no difference in the rates of Stat5 or Stat6 dephosphorylation in wild-type and mutant MEFs (Fig. 6B and C).

To validate the observed specificity of TC45 in STAT dephosphorylation, primary thymocytes isolated from wild-type and TC-PTP−/− mice were examined by staurorosporine-chase analysis. The lack of the TC-PTP gene resulted in a reduced rate of Stat1 dephosphorylation after IFN-α treatment, while
A.

Samples were analyzed as in panel without addition of staurosporine to demonstrate the effect of stauro-

antibodies. (B) Wild-type thymocytes were treated with IL-2 with or 

tively. The stripped blots were reprobed with anti-Stat1 and anti-Stat5 

respectively, followed by a staurosporine chase. Whole-cell extracts 

these thymocytes is demonstrated (Fig. 7B). The percentages 

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The transient nature of cytokine signaling is in part accom-

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and B). The in vitro PTPase assay used in this study is specific, 

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with TC-PTP-null MEFs or primary thymocytes. In the ab- 

sence of TC45, the dephosphorylation of Stat1 is defective. 

The TC-PTP gene is ubiquitously expressed, and alternative 

splicing of the last exon produces two distinct protein products. 

TC48 is targeted to the ER via its hydrophobic COOH termi-

nus, while TC45 lacks this hydrophobic COOH terminus and is 

largely localized to the nucleus due to the unmasking of a 

bipartite nuclear localization signal (15). It should be noted 

that a small fraction of TC45 is also present in the cytoplasm. 

In addition, under certain conditions, such as mitogen stimu-

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TC48 is unlikely to be involved in this process, since there is no 
detectable TC48 in MEFs (Fig. 5A), and reconstitution of 

TC-PTP-null cells with TC48 failed to rescue the defect in 

Stat1 dephosphorylation. Thus, the low level of TC45 present in 

the cytoplasm is likely involved in Stat1 dephosphorylation.

The rate of Stat5 dephosphorylation after IL-2 treatment was 

not affected (Fig. 7A). As a control, the effect of addition of 

staurosporine on IL-2-induced Stat5 phosphorylation levels in 

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Our results suggest the existence of other PTPases in STAT 

dephosphorylation. Although TC45 is clearly the primary 

phosphatase responsible for the dephosphorylation of Stat1, 

dephosphorylation was observed after prolonged IFN stimula-

tion of TC-PTP-null MEFs (Fig. 4C). The identity of such 

minor Stat1 PTPase(s) is currently unknown. Our data suggest 

that TC45 is also involved in Stat3 dephosphorylation. During 

final preparation of the manuscript, Aoki et al. reported that 

TC-PTP dephosphorylates Stat5A and Stat5B in vitro and in 

vivo when overexpressed in COS-7 or mammary epithelial cells 

(2). We examined the dephosphorylation of Stat5 after growth 

hormone and IL-2 stimulation in TC-PTP-null MEFs and pri-

mary thymocytes, respectively, and observed no difference in 

Stat5 dephosphorylation. Other potential PTPs that might be 

responsible for the dephosphorylation of Stat5 in these cells 

are PTP1B and Shp2 (1, 36). The dephosphorylation of Stat6 

was also unaffected in TC-PTP-null cells, although basal levels of 

Jak1 and Jak2 phosphorylation were slightly higher in TC-PTP-null 

cells than in the wild-type cells (data not shown).

DISCUSSION

The transient nature of cytokine signaling is in part accom-

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These results support the conclusion that TC45 displays spec-

ificity in the dephosphorylation of STATs in vivo.

FIG. 7. Analysis of Stat1 and Stat5 dephosphorylation in wild-type 

and TC-PTP-null thymocytes. (A) Primary thymocytes isolated from 

wild-type and TC-PTP-null mice were treated for 15 min with 500 U of 
murine IFN-α (Calbiochem) and 80 U of IL-2 per ml (Invitrogen), 

respectively, followed by a staurosporine chase. Whole-cell extracts 

were analyzed as in panel A.

B.

The stripping of the last exon produces two distinct protein products. 

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