

Arginine Methylation of STAT1 Regulates Its Dephosphorylation by T Cell Protein Tyrosine Phosphatase*

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Transcriptional induction by interferons requires the tyrosine and serine phosphorylation of the STAT1 transcription factor as well as its amino-terminal arginine methylation. Here we show that arginine methylation of STAT1 controls the rate of STAT1 dephosphorylation by modulating its interaction with PIAS1 and the nuclear tyrosine phosphatase TcPTP. Inhibition of STAT1 arginine methylation, or mutation of STAT1 Arg-31, results in a prolonged half-life of STAT1 tyrosine phosphorylation. This effect appears to be mediated by an increased binding of PIAS1 to STAT1 in the absence of STAT1 arginine methylation and a concomitant decrease in the association of STAT1 with TcPTP. Furthermore, inhibitors of arginine methylation require the presence of PIAS1 to exert their negative regulatory effect on the dephosphorylation of STAT1.

STAT¹ (signal transducers and activators of transcription) proteins are a family of latent cytoplasmic transcription factors that are activated by a large number of extracellular signals such as growth factors or cytokines (1). Activation of STAT proteins requires their tyrosine phosphorylation, and STATs 1, 3, and 5 also require carboxyl-terminal serine phosphorylation for maximal transactivation (2, 3). The amino terminus is one of the best conserved regions among the STAT proteins and is necessary in the regulation of STAT1 nuclear localization (4), interaction with the STAT1 inhibitor PIAS1 (5) and the transcriptional coactivator cAMP-response element-binding protein (CREB)-binding protein (6), and dephosphorylation of STAT1

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¹ The abbreviations used are: STAT, signal transducers and activators of transcription; Jak, Janus tyrosine kinase; PIAS, protein inhibitor of activated STAT; TcPTP, T cell protein tyrosine phosphatase; MEF, mouse embryonic fibroblast; IFN, interferon; GST, glutathione S-transferase; PIPES, 1,4-piperazinediethanesulfonic acid; ISG, interferon-stimulated gene; MTA, methylthioadenosine; MDA, *N*-methyl-2-deoxyadenosine.

Tyr-701 (7). Numerous proteins have been identified as negative regulators of the Jak/STAT pathway, including suppressor of cytokine signaling proteins (8), the PIAS family of nuclear STAT inhibitors (8, 9), or tyrosine phosphatases that target membrane-proximal events such as receptor phosphorylation or Jak kinase activity (10, 11). However, the identity of the nuclear tyrosine phosphatase(s) that inactivate STAT proteins in the nucleus has remained elusive (12, 13).

TcPTP was one of the first tyrosine phosphatases identified, and although widely expressed in different tissues, it is particularly abundant in hematopoietic cells and placenta (14). TcPTP-deficient mice suffer from severe splenomegaly and die between 3 and 5 weeks of age. Two differentially spliced mRNAs account for the presence of a cytoplasmic 48-kDa form and a 45-kDa nuclear-targeted protein (14). This nuclear isoform of TcPTP was recently identified as the elusive STAT1 nuclear tyrosine phosphatase (15). Since TcPTP is constitutively expressed and appears to require no further interferon-mediated post-translational modification for its activity, we reasoned that the Tyr-701 dephosphorylation of STAT1 by TcPTP might be regulated on the level of protein-protein interaction.

MATERIALS AND METHODS

Cell Culture—TcPTP-deficient cells were provided by Dr. M. Tremblay and have been described previously (15, 16). Primary human fibroblasts and MEF, HeLa, and HEK293T cells were maintained in Dulbecco's modified Eagle's medium, and U266 cells were cultured in RPMI 1640 medium. All media were supplemented with penicillin and streptomycin and 10% fetal bovine serum.

Reagents and Plasmids—IFN α and IFN β were generous gifts from Hoffman LaRoche and Biogen Inc., respectively. Staurosporine, *N*-methyl-2-deoxyadenosine, adenosine, and homocysteine were purchased from Sigma and were dissolved in culture medium. GST-STAT1 and GST-STAT1(R31E) have been described previously (17). FLAG-PIAS1 and FLAG-PIAS3 expression plasmids were a generous gift of Dr. K. Shuai. The TcPTP expression plasmid has been described previously (26).

Cell Extracts—To generate whole cell lysates, cells were lysed on the plates with lysis buffer (1 ml) containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 50 mM NaF, 10 mM β -glycerophosphate, 1 mM sodium vanadate, and 1 mM phenylmethylsulfonyl fluoride. For co-immunoprecipitation experiments, cells were lysed in buffer containing 100 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 0.1% Triton X-100, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 1 mM vanadate. Lysates were centrifuged at 13,000 rpm for 5 min, and protein concentration was determined by the Lowry method (Bio-Rad Protein Assay). Cytoplasmic/nuclear extracts were prepared by Dounce homogenizing cells in Buffer A (20 mM HEPES, pH 7.9, 10 mM KCl, 1 mM MgCl₂, 10% glycerol, and 0.1% Nonidet P-40), and sedimentation of nuclei was done by centrifugation at 1000 rpm for 5 min. The supernatant (=cytoplasmic extract) was removed, and nuclei were extracted with Buffer A containing 300 mM NaCl (=nuclear extract).

Immunoprecipitation and Immunoblotting—Cell lysates were pre-cleared with protein G-Sepharose for 30 min and incubated with pre-immune serum or antiserum to STAT1 and protein G-Sepharose overnight at 4 °C. GST-STAT1 was isolated using GSH-agarose. After SDS-PAGE and transfer onto polyvinylidene difluoride membrane, proteins were detected with anti-STAT1 (Transduction Labs), anti-phospho-STAT1 Tyr-701 (New England Biolabs), anti-TcPTP (Sigma), or anti-PIAS1/3 antibodies (Santa Cruz Biotechnology). For dimethylarginine immunoprecipitations, cell lysates were incubated with monoclonal mono-/dimethylarginine (Abcam) or an isotype control antibody overnight at 4 °C. Immunoreactive proteins were resolved by SDS-PAGE, and blots were immunoblotted with monoclonal STAT1 antibodies. All blots were developed with horseradish peroxidase-conjugated secondary antibodies and ECL (Amersham Biosciences).

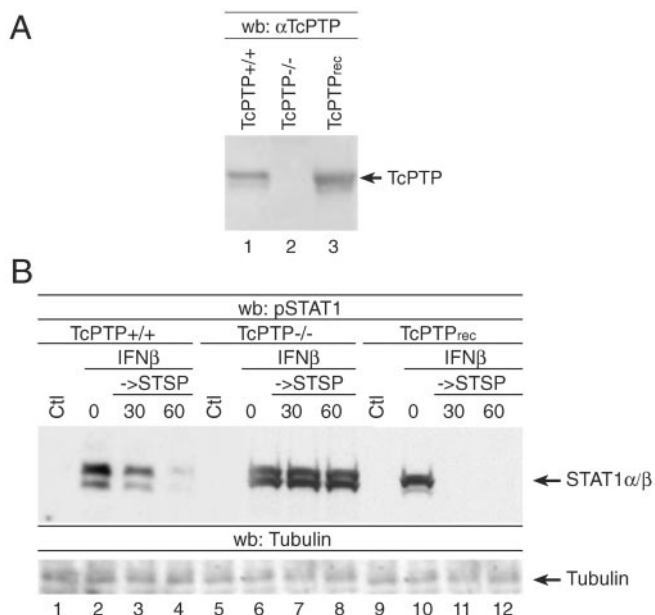


FIG. 1. TcPTP is the nuclear STAT1 tyrosine phosphatase. *A*, expression levels of TcPTP. Total cell extracts from wild-type, TcPTP^{-/-}, and reconstituted MEF cells were analyzed by Western blotting using anti-TcPTP antibody. *B*, STAT1 tyrosine dephosphorylation requires TcPTP. Wild-type, TcPTP^{-/-}, and reconstituted MEF cells were stimulated with IFNβ (1000 units/ml) for 30 min prior to addition of staurosporine (500 nM), and incubation was continued for the indicated time periods. Nuclear extracts were analyzed by Western blotting using anti-phospho-STAT1 antibody. The blot was then reprobbed for tubulin to ensure equal protein loading. *wb*, Western blot; *rec*, reconstituted; *STSP*, staurosporine; *Ctl*, control; *pSTAT1*, phospho-STAT1.

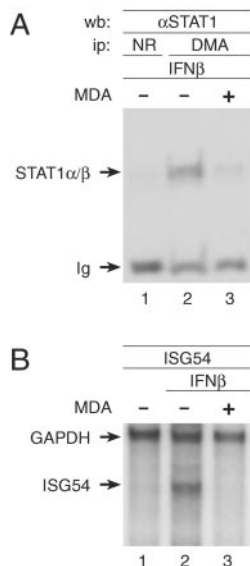


FIG. 2. Effects of arginine methyltransferase inhibitors on STAT1-mediated transcription. *A*, MDA inhibits STAT1 arginine methylation. U266 cells were preincubated with adenosine (300 μM) + homocysteine (300 μM) + *N*-methyl-2-deoxyadenosine (300 μM) for 3 h (this treatment is labeled “MDA” in the figure). Whole cell extracts were subject to immunoprecipitation with dimethylarginine (DMA)-specific antibodies, and the isolated proteins were analyzed by Western blotting using anti-STAT1 antibody. *B*, MDA inhibits STAT1-mediated transcription. U266 cells were pretreated with the arginine methylation inhibitors as in *A* prior to stimulation with IFNβ (1000 units/ml) for 2 h, and ISG54 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were determined by RNase protection assay. *wb*, Western blot; *ip*, immunoprecipitation; *NR*, non-reactive (isotype control).

RNase Protection Assays—Total RNA was isolated using TRIzol reagent. ³²P-Labeled riboprobes were generated by *in vitro* transcription using T3, T7, or SP6 RNA polymerase (Promega). Labeled riboprobe

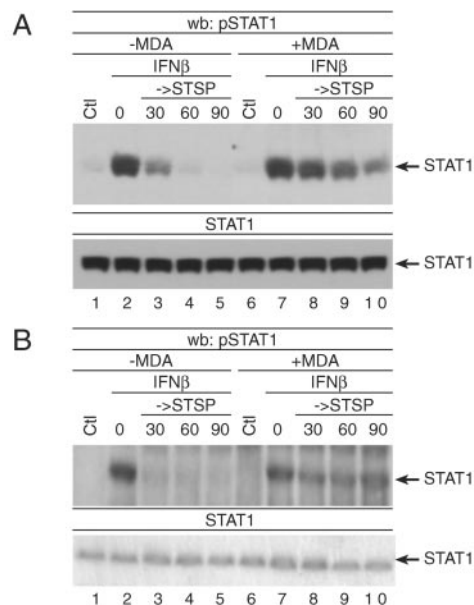


FIG. 3. Effects of arginine methylation on STAT1 tyrosine dephosphorylation. *A*, MDA treatment of U266 cells prevents STAT1 tyrosine dephosphorylation. U266 cells were left untreated or were subjected to MDA pretreatment as in Fig. 2*A*. Cells were then stimulated with IFNβ (1000 units/ml) for 30 min prior to addition of staurosporine (500 nM), and incubation was continued for the indicated time periods. Cell extracts were analyzed by Western blotting using anti-phospho-STAT1 antibody, and the blot was reprobbed for STAT1 levels. *B*, MDA treatment of fibroblasts prevents STAT1 tyrosine dephosphorylation. Treatment was the same as in *A*, but primary human foreskin fibroblasts were used in the experiment. *wb*, Western blot; *STSP*, staurosporine; *pSTAT1*, phospho-STAT1; *Ctl*, control.

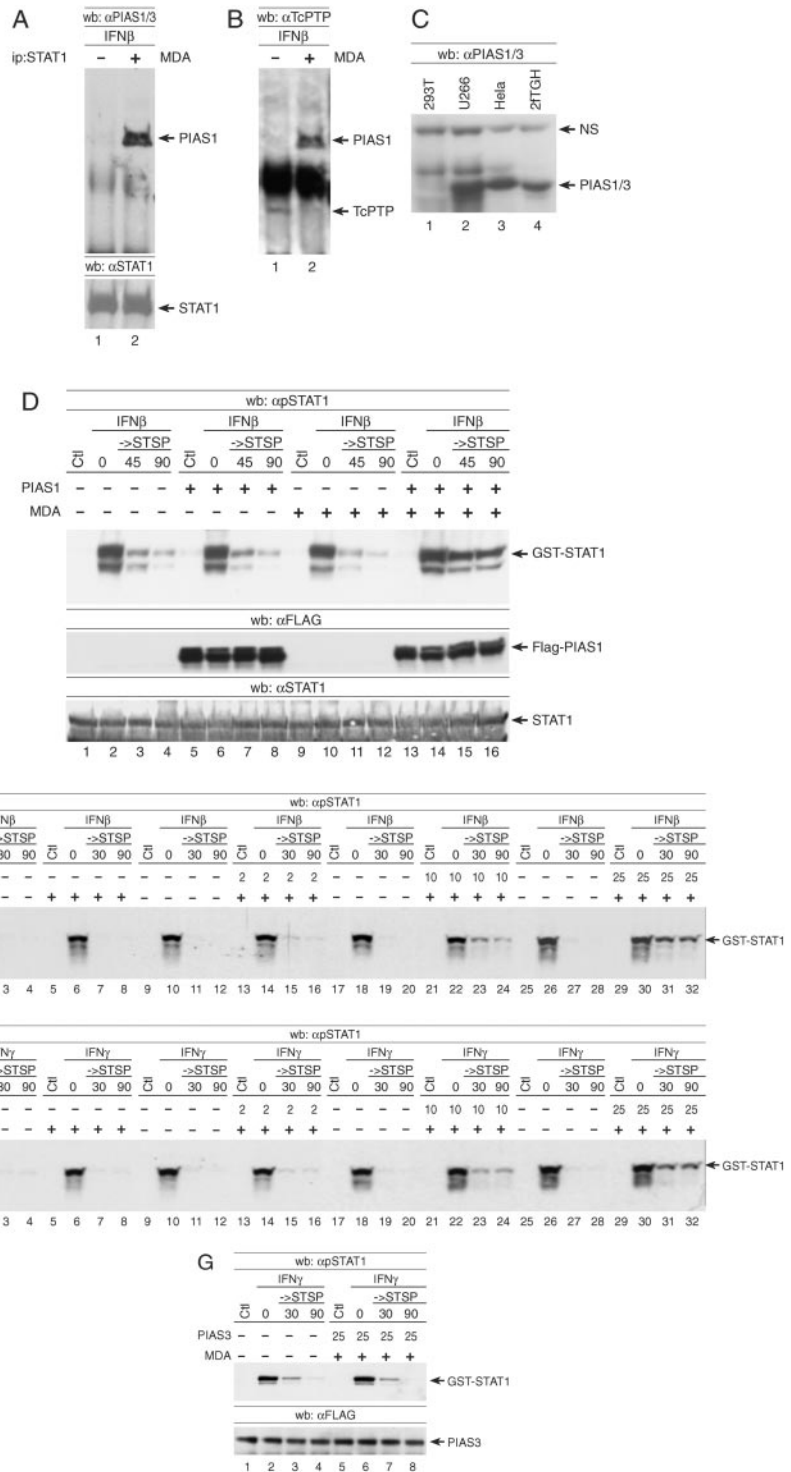
and 10 μg of RNA were incubated in hybridization buffer (4:1 formamide and 5× stock; 5× stock was 200 mM PIPES, pH 6.4, 2 M NaCl, 5 mM EDTA) overnight at 56 °C prior to digestion with T1 RNase. Protected fragments were separated by electrophoresis on a 4.5% polyacrylamide, urea gel.

RESULTS AND DISCUSSION

TcPTP Is the Major Nuclear STAT1 Tyrosine Phosphatase—Hoeve *et al.* (15) recently described the 45-kDa nuclear splice variant of TcPTP as the nuclear tyrosine phosphatase responsible for the dephosphorylation of STAT1. As shown in the pulse-chase experiments in Fig. 1*B*, a complete lack of Tyr-701 dephosphorylation of IFNβ-stimulated nuclear STAT1 is observed in TcPTP-deficient MEF cells over a 1-h period compared with wild-type cells (Fig. 1*B*, lanes 2–4 versus 6–8). When TcPTP^{-/-} cells were stably reconstituted with the 45-kDa form of TcPTP (Fig. 1*A*, lane 3), the rate of STAT1 dephosphorylation could be restored to that of wild-type cells (Fig. 1, lanes 10–12), testifying to the fact that TcPTP accounts for the majority of nuclear phosphatase activity directed against STAT1.

***N*-Methyl-2-deoxyadenosine (MDA) Treatment Inhibits STAT1 Arginine Methylation and ISG Induction**—Previously we have shown that methylation of Arg-31 of STAT1 modulates interferon-induced gene transcription (17). Inhibition of STAT1 arginine methylation by methylthioadenosine (MTA) increased the binding of PIAS1 to STAT1. We therefore wanted to test whether arginine methylation of STAT1 would also affect its interaction with TcPTP and consequently alter its rate of dephosphorylation. As MTA is no longer commercially available, we used an alternative strategy to inhibit STAT1 arginine methylation. Incubation of U266 cells with adenosine and DL-homocysteine in the presence of the *S*-adenosylhomocysteine hydrolase inhibitor MDA results in the cellular accumulation of

FIG. 4. PIAS1 and TcPTP compete for STAT1 binding. **A**, MDA treatment of U266 cells facilitates PIAS1 binding to STAT1. U266 cells were left untreated or were subjected to MDA pretreatment as in Fig. 2A prior to stimulation with IFN β (1000 units/ml) for 30 min. STAT1 was immunoprecipitated, and isolated proteins were probed for the presence of PIAS1. The upper part of the blot was probed for STAT1 to ensure equal immunoprecipitation and protein loading. **B**, MDA treatment of U266 cells inhibits TcPTP binding to STAT1. The blot shown in **A** was reprobed using antibodies against TcPTP. **C**, analysis of PIAS1 expression levels. Total cell extracts from 293T, U266, HeLa, and 2fTGH cells (25 μ g) were analyzed by Western blotting for the presence of PIAS1. **D**, MDA treatment requires PIAS1 to inhibit STAT1 dephosphorylation. 293T cells were transiently transfected with GST-STAT1 with or without FLAG-PIAS1 (as indicated) and were either left untreated or subjected to MDA pretreatment as in Fig. 2A. Cells were then stimulated with IFN β (1000 units/ml) for 30 min prior to addition of staurosporine (500 nM), and incubation was continued for the indicated time periods. Cell extracts were analyzed by Western blotting using anti-phospho-STAT1 antibody, and the blot was reprobed for GST-STAT1 and FLAG-PIAS1 expression. **E**, 293T cells were transiently transfected with 2 μ g of GST-STAT1 and either 2, 10, or 25 μ g of FLAG-PIAS1 as indicated and stimulated as in **D**. Cell extracts were analyzed by Western blotting using anti-phospho-STAT1 antibody. **F**, same as **E** except that 5 ng/ml IFN γ was used for stimulation. Cell extracts were analyzed by Western blotting using anti-phospho-STAT1 antibody. Expression levels of GST-STAT1 and FLAG-PIAS1 in **E** and **F** were verified by probing blots with anti-STAT1 and anti-FLAG antibodies as in **D** (data not shown). **G**, same as **F**, lanes 25–32, except that FLAG-PIAS3 was transfected. Expression levels of GST-STAT1 (data not shown) and FLAG-PIAS3 (bottom panel) were verified by probing blots with anti-STAT1 and anti-FLAG antibodies as in **D**. *wb*, Western blot; *ip*, immunoprecipitation; *NS*, non-specific; *Ctl*, control; *STSP*, staurosporine; *pSTAT1*, phospho-STAT1.



S-adenosylhomocysteine, a potent inhibitor of protein-arginine methyltransferases (18). This regimen (from here on referred to as “MDA treatment”) resulted in the complete inhibition of STAT1 arginine methylation (Fig. 2A, lane 2 versus 3) and consequential abrogation of the IFN β -induced transcription of the *ISG54* gene (Fig. 2B, lane 2 versus 3) as we had previously observed after MTA treatment of cells.

STAT1 Arginine Methylation Modulates Its Tyrosine Dephosphorylation—To evaluate the role of arginine methylation in STAT1 tyrosine dephosphorylation, we performed pulse-chase experiments in U266 cells in the absence or presence of the methylation inhibitors described above. Cells were treated for

30 min with IFN β followed by the addition of the kinase inhibitor staurosporine and continued incubation for the indicated time periods in the presence of IFN β . While MDA treatment had no effect on the initial level of STAT1 tyrosine phosphorylation achieved after 30 min of stimulation with IFN β (Fig. 3A, lanes 2 and 7), the inhibition of arginine methylation resulted in a significant delay in the dephosphorylation of Tyr-701 (Fig. 3A, lanes 3–5 versus 8–10). This effect is not restricted to U266 cells as a similar delay in STAT1 Tyr-701 dephosphorylation in the absence of its arginine methylation was observed when primary human fibroblasts were used in the experiments (Fig. 3B, lanes 3–5 versus 8–10).

PIAS1 Mediates the Effect of Arginine Methylation Inhibitors on the Dephosphorylation of STAT1—To investigate whether the lack of Arg-31 methylation *per se* accounts for the absence of TcPTP association or whether a competitive binding of PIAS1 prohibited STAT1 interaction with TcPTP, we decided to explore a possible function for PIAS1 in the delayed Tyr-701 dephosphorylation in the absence of arginine methylation. Unfortunately, PIAS1-deficient cells have not yet been reported. We therefore decided to screen several cell lines for their expression levels of PIAS1 to identify cells that express only minimal amounts of PIAS1 protein. 293T cells (Fig. 4C, lane 1) express only very low levels of PIAS1 compared with U266, HeLa, or 2FTGH fibrosarcoma cells (Fig. 4C, lanes 2, 3, and 4, respectively). When 293T cells were treated with MDA, virtually no delay in STAT1 Tyr-701 dephosphorylation was observed compared with cells with intact arginine methylation (Fig. 4D, lanes 2–4 versus 10–12). Transient introduction of PIAS1 into the 293T cells *per se* also did not alter the rate of STAT1 tyrosine dephosphorylation (Fig. 4D, lanes 6–8). However, when 293T cells that were transiently transfected with PIAS1 were subjected to treatment with the methylation inhibitors, a significant delay in STAT1 Tyr-701 dephosphorylation occurred (Fig. 4D, lanes 14–16).

To further demonstrate a competitive, negative regulatory effect of PIAS1 on STAT1 dephosphorylation, increasing amounts of PIAS1 cDNA (0 μ g, lanes 1–8; 2 μ g, lanes 9–16; 10 μ g, lanes 17–24; or 25 μ g, lanes 25–32) were cotransfected with GST-STAT1 (2 μ g), and pulse-chase-type experiments after IFN β stimulation were performed in the absence or presence of the methylation inhibitors. As shown in Fig. 4E, a PIAS1 concentration-dependent inhibition of STAT1 dephosphorylation was only observed in the presence of MDA, whereas no such effect was evident in the absence of the methylation inhibitor.

As IFN β activates STAT proteins other than STAT1 that can form heterodimers with tyrosine-phosphorylated STAT1, it seemed possible that such heterodimer formation exerts an influence over the kinetics of STAT1 dephosphorylation. We therefore performed similar STAT1 dephosphorylation experiments using IFN γ for stimulation of the cells since this interferon triggers exclusively the phosphorylation of STAT1. The effects of PIAS1 and arginine methylation on STAT1 dephosphorylation rates observed after IFN γ stimulation (Fig. 4F) parallel exactly the results obtained with the use of IFN β (Fig. 4E).

To further demonstrate the specificity of PIAS1 in mediating the effects of the methylation inhibitors on STAT1 dephosphorylation, we also used PIAS3, which interacts with STAT3 but not with STAT1, in these experiments. As shown in Fig. 4F, in contrast to PIAS1, PIAS3 is unable to alter the rate of STAT1 dephosphorylation in the presence of the methylation inhibitors.

These results demonstrate that the presence of PIAS1 is essential in order for the methylation inhibitors to exert their negative regulatory effects. Furthermore, these findings also exclude the possibility that the MDA treatment might controversially affect the enzymatic activity of TcPTP.

Arginine methylation has received increasing attention over the last year as several recent reports have illustrated a novel role for this post-translational modification in regulating protein-protein interaction and transcriptional induction (17, 19–25). STAT1 was the first transcription factor reported to require arginine methylation for its function (17). Interestingly,

while STAT1 is unable to bind DNA in the absence of its arginine methylation, the lack of this modification causes a significant delay in STAT1 tyrosine dephosphorylation. Nevertheless, interference with the arginine methylation status of STAT1 has no effect on the initial level of STAT1 Tyr-701 phosphorylation obtained after IFN α/β stimulation.

Mutation of STAT1 Arg-31 results in decreased binding to TcPTP² as well as to PIAS1 (17). In contrast, the results presented in Fig. 4, A and B, indicate that inhibition of STAT1 arginine methylation results in increased association with PIAS1 with a concomitant decrease in TcPTP binding, suggesting a possible competition of these two proteins for binding to STAT1. These results demonstrate that Arg-31 is a critical residue for binding of STAT1 to both PIAS1 and TcPTP, while methylation of the Arg-31 residue alters the relative preference for one of the two proteins.

In summary, our data support a model in which arginine methylation of STAT1 regulates its interaction with PIAS1 and consequently with the nuclear tyrosine phosphatase TcPTP and thereby modulates the duration of STAT1 tyrosine phosphorylation.

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² W. Zhu, T. Mustelin, and M. David, unpublished observation.