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Arginine Methylation of STAT1 Modulates IFNα/β-Induced Transcription

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Summary

Transcriptional induction by interferons requires the tyrosine and serine phosphorylation of STAT transcription factors. The N-terminal region is highly homologous among the STAT proteins and surrounds a completely conserved arginine residue. Here we demonstrate arginine methylation of STAT1 by the protein arginine methyl-transferase PRMT1 as a novel requirement for IFNα/β-induced transcription. Methylthioadenosine, a methyl-transferase inhibitor that accumulates in many transformed cells, inhibits STAT1-mediated IFN responses. This inhibition arises from impaired STAT1-DNA binding due to an increased association of the STAT inhibitor PIAS1 with phosphorylated STAT1 dimers in the absence of arginine methylation. Thus, arginine methylation of STAT1 is an additional posttranslational modification regulating transcription factor function, and alteration of arginine methylation might be responsible for the lack of interferon responsiveness observed in many malignancies.

Introduction

STAT (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 (Schindler and Darnell, 1995). Seven mammalian STAT family members have been described, and they feature high amino acid homology over several distinct regions. Common to all STAT proteins is an SH2 domain and a conserved tyrosine residue whose phosphorylation in response to receptor activation mediates the homo- or heterodimerization of STAT proteins via phosphotyrosine/SH2 domain interaction (Heim et al., 1995). Dimerized STATs can then translocate to the nucleus where they bind to conserved enhancer elements and activate transcription of immediate early response genes. In addition to tyrosine phosphorylation, STATs 1, 3, and 5 require also phosphorylation on a C-terminal serine residue to achieve maximal transactivation potential (David et al., 1995b; Wen et al., 1995). The N terminus is one of the best conserved regions among the STAT proteins and has therefore been the target of intense investigation. Deletion of the N terminus has implicated it in the STAT1 dephosphorylation on tyrosine 701 (Shuai et al., 1996), in the interaction with the transcriptional coactivator CBP (Korzus et al., 1998), and in the regulation of its nuclear localization (Strehlow and Schindler, 1998).

Phosphorylation of the conserved tyrosine and serine residues is the only known posttranslational modification of STAT proteins. Tyrosine phosphorylation of STAT1 via the interferon receptor requires the activity of the Janus Kinase (JAK) family of tyrosine kinases (Velazquez et al., 1992; Muller et al., 1993; Watling et al., 1993), whereas serine phosphorylation is thought to be facilitated by members of the MAP kinase family (David et al., 1995a). Therefore, it is not surprising that the intracellular domain of the interferon receptors was found to provide docking sites for proteins known to be involved in STAT1 phosphorylation events (David et al., 1995a, 1995b, 1996a, 1996b). Interestingly, a yeast two-hybrid screen recently revealed the interaction of the protein arginine methyl-transferase PRMT1 with the IFNAR1 chain of the IFNα/β receptor (Abramovich et al., 1997). PRMT1 was first isolated through its interaction with BTG1 and TIS21, two genes associated with cell quiescence (Lin et al., 1996). Abrogation of PRMT1 expression by antisense technology decreased the antiviral and antiproliferative abilities of type I interferons, suggesting a role for PRMT1 in IFNα/β receptor mediated signaling events. In addition, a recent report described the isolation of a novel JAK binding protein 1 (JBP-1) with arginine methyl-transferase activity (Pollack et al., 1999).

In the present study, we show that PRMT1 associates with STAT1 and methylates STAT1 on Arg-31. Mutational analysis and the use of the protein methyl-transferase inhibitor 5′-methyl-thioadenosine (MTA) revealed that methylation of STAT1 is required for transcriptional activation. Furthermore, we provide evidence that the presence of excess MTA found in certain cancer cells is sufficient to inhibit the arginine methylation of STAT1, consequently impairing interferon-mediated gene induction and antiproliferation.

Results

PRMT1 Associates with and Methylates STAT1

The N termini of the mammalian STAT proteins display high sequence homology and surround a highly conserved arginine residue (Arg-31) found in all seven mammalian STATs (Figure 1A). It was previously shown that mutation of Arg-31 of STAT1, as well as antisense abolition of the protein arginine methyl-transferase PRMT1, result in alterations in the antiproliferative effects of IFNβ (Shuai et al., 1996; Abramovich et al., 1997). We therefore reasoned that PRMT1 might exert control over STAT1

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A

												31								
STAT1	L	Y	D	D	S	.	F	P	M	E	I	R	Q	Y	L	A	Q	W	L	E
STAT2	L	Y	S	H	S	L	L	P	V	D	I	R	Q	Y	L	A	V	W	I	E
STAT3	L	Y	S	D	S	.	F	P	M	E	L	R	Q	F	L	A	P	W	I	E
STAT4	F	Y	D	D	N	.	F	P	M	E	I	R	H	L	L	A	Q	W	I	E
STAT5a	L	Y	G	Q	.	H	F	P	I	E	V	R	H	Y	L	A	Q	W	I	E
STAT5b	L	Y	G	Q	.	H	F	P	I	E	V	R	H	Y	L	S	Q	W	I	E
STAT6	L	Y	V	D	.	.	F	P	Q	H	L	R	H	L	L	G	D	W	L	E

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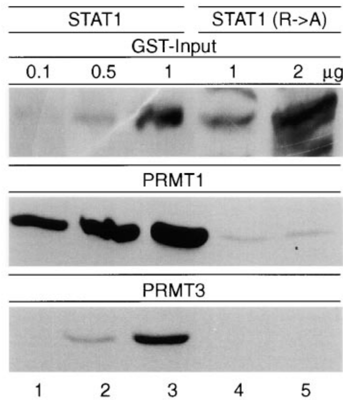

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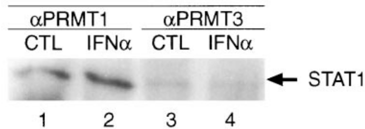

Figure 1. STAT1 and PRMT1 Interaction

(A) Alignment of amino acid sequences of the NH2 termini of STAT proteins Arg-31 of STAT1 is highly conserved among all seven STAT proteins.

(B) In vitro methylation of STAT1 on Arg-31. The indicated amounts of STAT1(NH2)-GST (lanes 1–3) or STAT1(NH2)-GST (Arg-31→Ala) (lanes 4 and 5) were subjected to in vitro methylation assays using recombinant PRMT1 (middle panel) or recombinant PRMT3 (bottom panel). The input of STAT1-GST proteins was verified by silver stain (top panel).

(C) STAT1 associates with PRMT1. HeLa cells were left untreated (lanes 1 and 3) or incubated with IFNα (1000 U/ml). PRMT1 (lanes 1 and 2) and PRMT3 (lanes 3 and 4) were immunoprecipitated and resolved proteins were immunoblotted for STAT1.

function by utilizing Arg-31 of STAT1 as a target for methylation.

To test this hypothesis, we used a GST fusion protein encompassing the first 129 aa of STAT1 for in vitro methylation assays using recombinant PRMT1 or PRMT3 (Figure 1B). Recombinant PRMT1 achieved a robust methylation of STAT1 (Figure 1B, middle panel, lanes 1–3), whereas the related PRMT3 (Tang et al., 1998) yielded only a significantly lower methylation of STAT1 (bottom panel, lanes 1–3). To prove that Arg-31 was indeed the target for methylation by PRMT1, we introduced an Arg-31 to Ala mutation in the STAT1(NH2)-GST fusion protein to be used in the in vitro methylation assay. As anticipated, the elimination of Arg-31 resulted in the complete loss of the methylation of the fusion protein by either PRMT1 or PRMT3 (middle and bottom panel, lanes 4 and 5). It is important to note that the STAT1 (NH2/R→A) mutant still contains seven other arginine residues. In addition, numerous arginines can be found in the GST portion of the fusion protein. Nevertheless, only Arg-31 can serve as a target for methylation. These results not only identify Arg-31 of STAT1 as a key residue for methylation, but also demonstrate substrate specificity of the two arginine methyl transferases.

PRMT1 has been shown to associate with the IFNα/β receptor (Abramovich et al., 1997) and our results suggested STAT1 could be a substrate for PRMT1. We therefore attempted to demonstrate a direct interaction of STAT1 and PRMT1. Extracts from untreated or IFNα-treated HeLa cells were subject to immunoprecipitation with either anti-PRMT1, anti-PRMT3, or rabbit preimmune serum, and resolved immunoprecipitates were immunoblotted for the presence of STAT1. As shown in Figure1C, STAT1 associated with PRMT1 in a ligand-independent manner (lanes 1 and 2). In contrast, STAT1 was not detectable in immunoprecipitates obtained with PRMT3 serum (lanes 3 and 4) or with preimmune serum (data not shown). Thus, the selectivity of the in vivo association apparently confirms the specificity observed in the in vitro STAT1 arginine methylation assays.

STAT1 Is Methylated on Arg-31 In Vivo

Our data clearly established STAT1 as an in vitro substrate for arginine methylation by PRMT1. In order to demonstrate that STAT1 is indeed arginine methylated in vivo, we subjected cell lysates derived from untreated or IFNα-treated cells to immunoprecipitation using monoclonal antibodies against dimethylarginine (DMA). The isolated proteins were then analyzed for the presence of STAT1 by immunoblotting. STAT1 appeared to be methylated even in untreated cells. However, the amount of STAT1 immunoprecipitated with the DMA antibody appears to increase after stimulation with IFNα (Figure 2A, lanes 1 and 2). No STAT1 was detected in immunoprecipitates obtained with an appropriate isotype control antibody (data not shown).

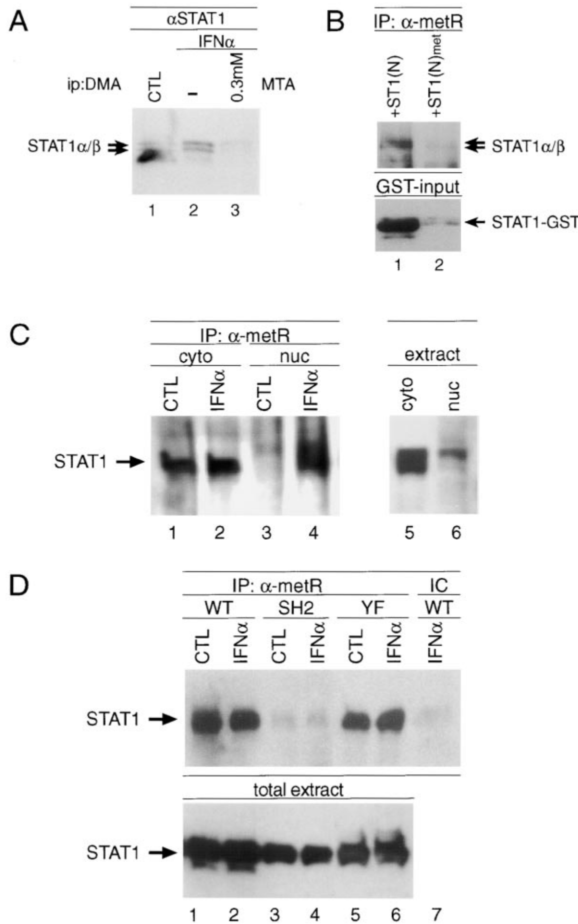

Figure 2. STAT1 Is Methylated on Arg-31 In Vivo

(A) MTA prevents STAT1 arginine methylation. U266 cells were treated with IFNα without (lane 2) or with (lane 3) pretreatment with 0.3 mM MTA for 1 hr, and lysates were subject to immunoprecipitation with DMA antibodies. Resolved proteins were immunoblotted for the presence of STAT1.

(B) Only methylated STAT1(NH2)-GST competes for STAT1 binding to DMA antibodies. Cell lysate derived from 2fTGH cells was subjected to immunoprecipitation with mono-/dimethylarginine antibodies in the presence of either STAT1(NH2)-GST (lane 1) or STAT1(NH2)-GST that had been methylated in vitro by recombinant PRMT1 (lane 2). Resolved proteins were immunoblotted for the presence of STAT1. The amount of STAT1(NH2)-GST added to the cell lysate prior to incubation is shown in the lower panel.

(C) Cytoplasmic/nuclear distribution of methylated STAT1: 2fTGH cells were treated with 1000 U/ml IFNα for 30 min, and cytoplasmic (lanes 1 and 2) and nuclear (lanes 3 and 4) extracts were subjected to immunoprecipitation with mono-/dimethylarginine antibodies. 1% of each extract was also loaded (lanes 5 and 6), and the resulting blots probed for STAT1 (cyto = cytoplasmic extract; nuc = nuclear extract).

(D) Requirements for STAT1 arginine methylation: 293 cells were transfected with HA-tagged wild-type STAT1 (lanes 1 and 2), SH2mut-STAT1 (lanes 3 and 4), or Y701F-STAT1 (lanes 5 and 6). Whole cell extracts were loaded directly (lower panel), or subjected to immunoprecipitation with mono-/dimethylarginine antibodies (upper panel, lanes 1–6) or isotype control (IC) antibodies (lane 7), and the resulting blots probed with anti-HA antibodies.

5′-methyl-thioadenosine (MTA) has been reported as a specific inhibitor of protein methyl transferases (Williams-Ashman et al., 1982), and previous studies showed that total protein methylation was reduced in cells incubated with MTA (Maher, 1993). We therefore tested whether MTA would inhibit arginine methylation of STAT1. As anticipated, pretreatment of cells with MTA prior to IFNα stimulation significantly reduced the amount of STAT1 that is immunoreactive with DMA antibodies (Figure 2A, lane 3). To demonstrate the specificity of the antibodies, we performed the immunoprecipitation with the DMA antibodies also in the presence of either unmethylated or in vitro methylated STAT1(NH2)-GST. As shown in Figure 2B, only the methylated form of STAT1(NH2)-GST (lane 2) was able to compete with STAT1 for binding to the DMA antibody, even though a substantially larger amount of the unmethylated protein was added to the lysates (lane 1, lower panel).

In order to determine whether the methylated form of STAT1 would preferentially localize to the nucleus, we generated cytoplasmic and nuclear extracts from untreated or IFNα-stimulated 2fTGH cells. Analysis of the amount of STAT1 immunoprecipitated with the DMA antibodies revealed that approximately 60%–70% of the methylated STAT1 can be found in the nucleus after IFNα stimulation (Figure 2C, compare lanes 2 and 4). Strikingly, only approximately 5%–10% of the total STAT1 protein can be found in the nuclear extracts of IFNα−treated cells (lanes 5 and 6). Thus, the stoichiometry of methylation is significantly higher in the nuclear pool of tyrosine-phosphorylated STAT1 when compared to the cytoplasmic fraction. We next wanted to test whether tyrosine phosphorylation of STAT1 was a prerequisite for its arginine methylation. 293T cells were transiently transfected with HA-tagged forms of either wild-type STAT1, STAT1 harboring a nonfunctional SH2 domain, or carrying a mutation in the site of tyrosine phosphorylation. Probing of the DMA immunoprecipitates with anti-HA antibodies illustrated that tyrosine phosphorylation of STAT1 is not required for its arginine methylation (Figure 2D, lanes 5 and 6). Interestingly, a functional SH2 domain appears to be necessary for STAT1 to undergo arginine methylation (lanes 3 and 4). These results not only demonstrate that tyrosine phosphorylation is not a prerequisite for arginine methylation of STAT1, but also confirm that the methylation occurs in the cytoplasm since STAT1 (Y701F) is unable to translocate to the nucleus.

To further verify Arg-31 as the site of STAT1 arginine methylation, we performed mass-spectrometrical analysis on STAT1 immunoprecipitated from IFNα-stimulated U266 cells. Isolated STAT1 was subject to digestion by V8 protease, and the masses of the resulting peptide fragments were determined by MALDI-TOF (data not shown). A fragment with a mass of 1319.74 was identified that corresponded to the peptide spanning amino acids 30 to 39 (**IRQYLAQWLE**). Two additional masses at 1333.71 and 1347.68 were detected that conform with the mono- and dimethylated form of this peptide. These results clearly demonstrate that STAT1 is arginine methylated in vivo, and confirm Arg-31 as the site of this modification.

Mutation of Arg-31 Alters the Transcriptional Potential of STAT1

Since we had identified Arg-31 as the site of methylation in vitro and in vivo, we decided to test the effect of mutations in this residue on IFNα-mediated gene tran-

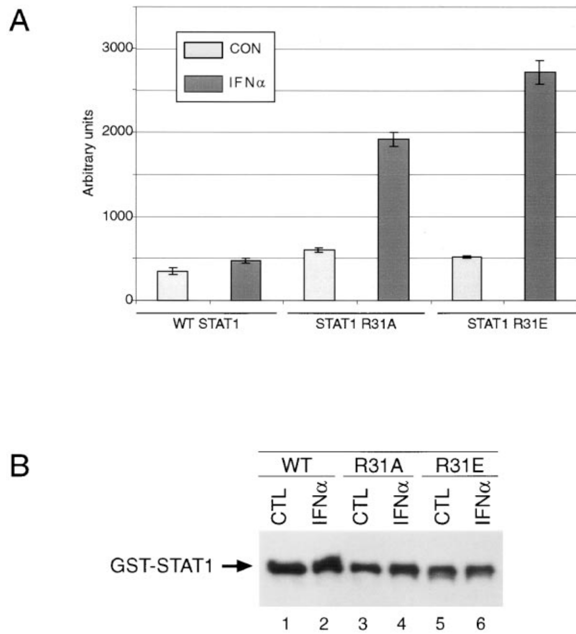

Figure 3. Effects of Arg-31 Mutations on STAT1 Transcriptional Activity

(A) 293 cells were transiently transfected with IRF-1-luciferase and GST-fusions of either wild-type STAT1, R31A-STAT1, or R31E-STAT1. Transcriptional response towards 1000 U/ml IFNα in three independent experiments is shown.

(B) Lysates from (A) were probed for STAT1 to verify expression levels of the individual STAT1 mutants.

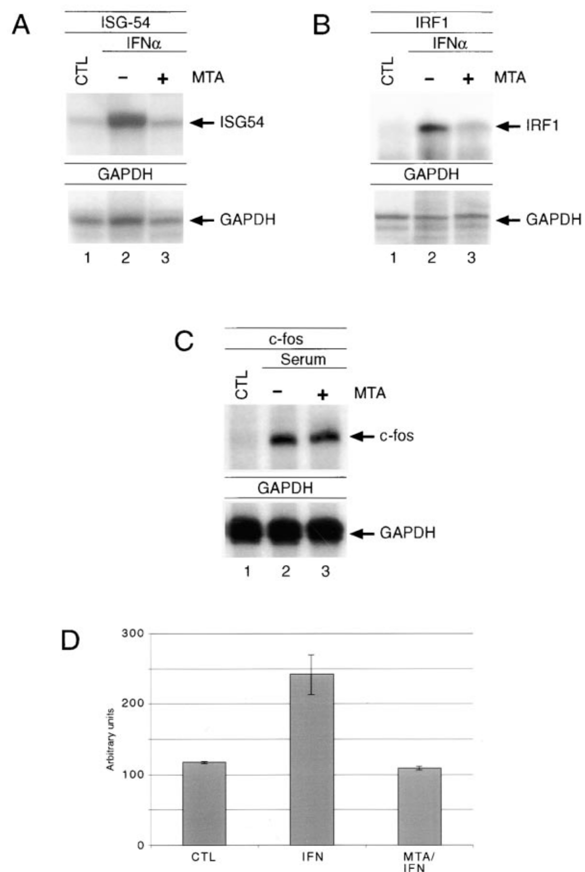

Figure 4. Effects of MTA on STAT1-Mediated Gene Transcription

(A) MTA inhibits IFNα-mediated *ISG54* induction. HeLa cells were left untreated (lane 1), or treated with IFNα (1000 U/ml) for 2 hr either in the absence (lane 2) or presence of 0.3 mM (lane 3) MTA. Total RNA was isolated, and the amounts of *ISG54* (top panel) and *GAPDH* (bottom panel) mRNA were determined by RNase protection assay.

(B) MTA inhibits IFNα-mediated *IRF-1* induction.

Same as (A), but a riboprobe corresponding to human *IRF-1* was used in the RNase protection assay.

(C) MTA does not inhibit serum induction of c-*fos*. Quiescent HeLa cells were stimulated with serum for 20 min in the absence (lane 2) or presence of 0.3 mM (lane 3) MTA. Total RNA was isolated, and the amounts of c-*fos* (top panel) and *GAPDH* (bottom panel) mRNA were determined by RNase protection assay.

(D) MTA inhibits IFNα-mediated induction of IRF-1-luciferease. 293 cells were transfected with IRF-1-luciferase and wild-type STAT1, and the effect of 0.3 mM MTA on the transcriptional response towards 1000 U/ml IFNα was analyzed.

scription. We generated STAT1 mutants in which Arg-31 was replaced with either alanine or glutamic acid, but unfortunately found that these substitutions render the protein unstable (data not shown). However, we were able to overcome this problem by fusing GST to the N terminus of the mutant STAT1 proteins. Reporter assays using IRF-1-luciferase showed that wild-type GST-STAT1 yielded a 1.4-fold induction in response to IFNα (Figure 3A), which was less when compared to STAT1 without GST (data not shown). Nevertheless, mutation of the positively charged Arg-31 with the hydrophobic alanine resulted in a significantly increased ability of this GST-STAT1 mutant to promote IFNα-mediated transcription. This substitution might mimic the removal of the positive charge of Arg-31 by the methylation reaction. An ever stronger increase in the transcriptional response was observed when Arg-31 was replaced with the acidic glutamic acid. Equal expression of all GST-STAT1 proteins was confirmed by probing the 293T cell lysates with anti-GST antisera (Figure 3B).

Inhibition of Arginine Methyl-Transferase Activity Decreases STAT1-Mediated Transcription

Since our data supported a role for arginine methylation in STAT1 function, we decided to investigate the potential effects of the methylation inhibitor MTA on the capacity of STAT1 to induce gene transcription. Cells were pretreated with 0.3 mM MTA prior to incubation with IFNα for an additional 2 hr. Total RNA was isolated and hybridized to riboprobes corresponding to the interferon-inducible *ISG54* and *IRF-1* genes. As shown in Figures 4A and 4B, IFNα stimulation resulted in an increase in the levels of *ISG54* and *IRF-1* mRNA (lanes 1 and 2), genes which are controlled by a STAT1/STAT2 heterodimer or a STAT1 homodimer, respectively. This expression was significantly reduced in the presence of 0.3 mM MTA (lanes 3). As it had been previously reported that histones are also subject to methylation (Chen et al., 1999), we wanted to exclude that reduced methylation of histones was responsible for the observed inhibition of STAT1-mediated gene transcription by MTA. Strikingly, MTA did not effect the serum induction of *c-fos*, a STAT1-independent induction (Figure 4C). In addition,

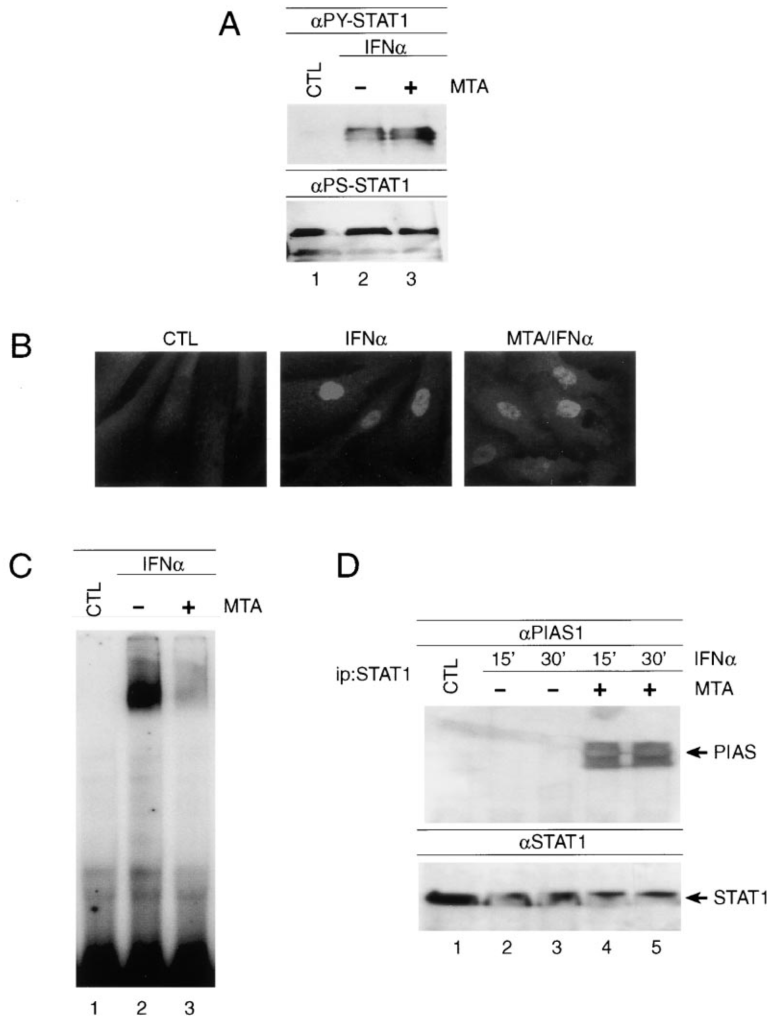

Figure 5. Effects of MTA on STAT1 Phosphorylation, Translocation, and DNA Binding

(A) MTA does not effect STAT1 Tyr-701 or Ser-727 phosphorylation. U266 cells were treated with IFNα (1000 U/ml) for 30 min (lane 2), or pretreated with 0.3 mM MTA (lane 3) for 2.5 hr prior to IFNα treatment. Whole cell extracts were immunoblotted with STAT1 (phosphoTyr-701) (top panel) or STAT1 (phosphoSer-727) antibody (bottom panel).

(B) MTA does not inhibit STAT1 nuclear translocation.

Primary human foreskin fibroblasts were left untreated (left panel), stimulated with IFNα (1000 U/ml) for 30 min (middle panel), or pretreated with 0.3 mM MTA for 2.5 hr prior to IFNα stimulation. Subcellular localization of STAT1 was determined with a monoclonal antibody against STAT1.

(C) U266 cells were treated with IFNα in the absence (lane 2) or presence (lane 3) of 0.3 mM MTA, and EMSAs were performed using the GRR element as a probe.

(D) U266 cells were treated for the indicated times with IFNα in the absence (lanes 2 and 3) or presence (lanes 4 and 5) of 0.3 mM MTA. STAT1 was immunoprecipitated, and isolated proteins were probed for the presence of PIAS1. The blot was subsequently reprobed for STAT1 to ensure equal protein loading.

MTA was also able to exert its inhibitory effect when transcription was analyzed by means of an IRF-1-luciferase reporter assay (Figure 4D).

Arginine Methylation Alters STAT1 DNA Binding by Regulating PIAS Association

Since tyrosine and serine phosphorylation of STAT1 are absolute prerequisites for its transactivating capabilities (Shuai et al., 1993; Wen et al., 1995), we stimulated cells for 30 min with IFNα without or with preincubation with 0.3 mM MTA, and immunoblotted the resulting cell lysates with antibodies specific for STAT1 (phosphoTyr-701) (Figure 5A, upper panel) and STAT1 (phosphoSer-727) (lower panel). Our results illustrate that there is no obvious defect in the IFNα-induced tyrosine phosphorylation of STAT1 due to pretreatment with MTA. Similarly, phosphorylation of Ser-727, though constitutive, was not affected by the presence of MTA.

To ensure that MTA did not affect the ability of STAT1 to translocate to the nucleus, we determined the subcellular localization of STAT1 by immunohistochemistry using a monoclonal STAT1 antibody (Figure 5B). Concomitant with the intact phosphorylation of STAT1, we found that the nuclear translocation of STAT1 was not affected by the presence of MTA.

STAT1-regulated transcription relies on binding to defined response elements within the promoters of STAT1-regulated genes. We therefore analyzed whether MTA treatment would alter the DNA binding of STAT1 after IFNα stimulation. EMSAs using the STAT1-specific GRR element as a probe revealed that MTA pretreatment reduced the ability of tyrosine phosphorylated STAT1 to bind DNA (Figure 5C, lanes 2 and 3). To ensure that MTA did not prohibit STAT1 DNA interactions by direct interference with the in vitro binding conditions, we added MTA directly to cell lysates from cells treated with IFN only. Under those circumstances, MTA had no effect on STAT1 DNA binding (data not shown). The effect of MTA was preserved in the presence of the protein synthesis inhibitor cycloheximide (data not shown), excluding the possibility of induction of an inhibitor by the MTA pretreatment.

The reduction in STAT DNA binding we observed after MTA treatment is reminiscent of the actions of members of the protein inhibitor of activated STATs (PIAS) family (Chung et al., 1997; Liu et al., 1998). Addition of recombinant PIAS1 protein to cell extracts derived from IFN-treated cells interfered with the DNA binding of STAT1 (Liu et al., 1998). PIAS1 was found to specifically associate with STAT1, but not with other STAT proteins. Fur-

thermore, a recent report demonstrated that the interaction of PIAS1 with STAT1 occurs at the N terminus where Arg-31 is located (Liao et al., 2000). Therefore, we investigated if the inhibition of STAT1 DNA binding in the presence of MTA was due to an increase in PIAS1 association. STAT1 immunoprecipitates obtained from cells treated with IFNα in the absence or presence of MTA were analyzed for the presence of PIAS1 protein. Whereas PIAS1 was essentially undetectable in STAT1 immunoprecipitates from IFNα-stimulated cells (Figure 5D, lanes 2 and 3), a dramatic increase in PIAS1 binding to STAT1 was observed when MTA was present during the stimulation (lanes 4 and 5). No PIAS1 protein was detected when preimmune serum was used for immunoprecipitation (data not shown). Increased PIAS1 association was not due to upregulation of PIAS1 protein (data not shown). This result suggests that inhibition of STAT1 arginine methylation by MTA facilitates an interaction with the STAT1 inhibitor PIAS1.

Accumulated MTA in Transformed Cells Inhibits STAT1 Methylation

MTA is not only a pharmacological agent used to inhibit arginine methylation in vitro, but is actually generated in vivo as a metabolic intermediate in the conversion of putrescine to spermidine and of spermidine to spermine (Williams-Ashman et al., 1982). Methyl-thioadenosine phosphorylase (MTAP), an enzyme in the salvage pathway for adenine nucleotide and in methionine biosynthesis, efficiently catalyses the breakdown of cellular MTA to almost undetectable levels (Kamatani et al., 1981). Due to their genomic proximity, MTAP is often found homozygously codeleted with the p16 tumor suppressor gene locus in a large number of cancers (Nobori et al., 1996). Since MTAP is the sole enzyme responsible for MTA cleavage, we reasoned that MTAP deficiency could result in sufficient accumulation of MTA to cause an inhibition of endogenous protein methyl transferases. To test this hypothesis, we decided to analyze the effects of interferon on the MTAP-deficient human lung carcinoma cells A549-MTAP(−/−), and their MTAP-reconstituted counterpart A549-MTAP(+/+) (Nobori et al., 1996).

First, we wanted to verify that the interferon-induced STAT1 phosphorylation pathway was intact. We therefore treated A549-MTAP(−/−) or A549-MTAP(+/+) cells with IFNα or IFNβ for 30 min, and analyzed for the levels of STAT1 phosphoTyr-701 or STAT1 phosphoSer-727 by Western blot (Figure 6A). As anticipated from the experiments applying exogenous MTA, we detected no differences in STAT1 Tyr-701 or Ser-727 phosphorylation between the A549-MTAP(−/−) and A549-MTAP(+/+) cells. Similarly, immunofluorescence of A549-MTAP(−/−) and A549-MTAP(+/+) cells displayed equal nuclear accumulation of STAT1 in response to IFNα and IFNβ stimulation (data not shown).

As we hypothesized that the A549-MTAP(−/−) cells may accumulate MTA, we tested if the cytosolic MTA concentration in A549-MTAP(−/−) cells was indeed adequate to inhibit in vitro methylation reactions. We first determined the activity of endogenous PRMT1 in A549-MTAP(−/−) or A549-MTAP(+/+) cell lysates by in vitro methylation assays using STAT1(NH2)-GST as a substrate. Whereas the addition of lysate from MTAP(+/+) cell lysates resulted in a pronounced arginine methylation of STAT1-GST, no methylation could be detected when A549-MTAP(−/−) cell lysate was used (Figure 6B). Interestingly, Western blotting of the same lysates indicated the A549-MTAP(−/−) cells expressed even higher levels of PRMT1 than their reconstituted counterparts, suggesting a compensatory reaction of the cells in response to the inhibition of endogenous protein arginine methylation reactions by accumulated MTA (Figure 6B, lower panel).

To further assure that the observed differences in the in vitro methylation reactions were not due to a potential defect in the activity of endogenous PRMT1, we repeated the experiments with recombinant PRMT1. The addition of increasing amounts of A549-MTAP(+/+) lysate had no detectable effect on STAT1(NH2)-GST methylation (Figure 6C, lanes 2, 4, and 6). In contrast, the presence of an increasing amount of lysates derived from A549-MTAP(−/−) cells resulted in a drastic, dose-dependent inhibition of STAT1(NH2)-GST methylation (lanes 1, 3, and 5). To verify that the inhibitory activity present in the lysate derived from A549-MTAP(−/−) cells was indeed MTA, we incubated these cell lysates without and with recombinant, purified MTAP prior to addition of the lysate to the in vitro methylation reactions. As shown in Figure 6D, preincubation of the cell lysate with rMTAP reduced the inhibitory activity of the A549-MTAP(−/−) cell lysates.

These results demonstrate that the absence of MTAP and consequential accumulation of MTA do not inhibit STAT1 Tyr-701 or Ser-727 phosphorylation, but have a dramatic effect on the in vitro methylation of STAT1 (NH2)-GST.

Characterization of the Interferon Response in A549-MTAP(−/−) Cells

Our earlier biochemical analysis using exogenous MTA suggested a role for arginine methylation in STAT1-mediated transcription. Therefore, the possibility of a deficiency in the expression of STAT1-driven genes in A549-MTAP(−/−) cells was investigated. A549-MTAP(−/−) or A549-MTAP(+/+) cells were treated with IFNβ, and total RNA was analyzed for the presence of *IRF-1* and *GAPDH* mRNA. Only RNA isolated from IFNβ-stimulated A549-MTAP(+/+), but not from A549-MTAP(−/−) cells, contained detectable amounts of *IRF-1* mRNA (Figure 7A, compare lanes 2 and 4). In conformity with the mRNA levels, IRF-1 protein was only found induced in A549-MTAP(+/+) (Figure 7B, lanes 6 to 10), but not in A549-MTAP(−/−) cells (lanes 1 to 5) following stimulation by IFNβ.

The obvious defect in the expression of an interferon-induced gene that contributes significantly to the antiproliferative function of this cytokine led us to consider the possibility that A549-MTAP(−/−) cells may display defects in the biological response to IFN. To test this hypothesis, we employed proliferation assays on A549-MTAP(−/−) and A549-MTAP(+/+) cells. Although both cell lines exhibited the same growth rate when left untreated, the A549-MTAP(−/−) cells displayed a marked defect with regards to their antiproliferative response toward Type I IFNs. Compared to the A549-MTAP(+/+) cells, A549-MTAP(−/−) cells were significantly less af-

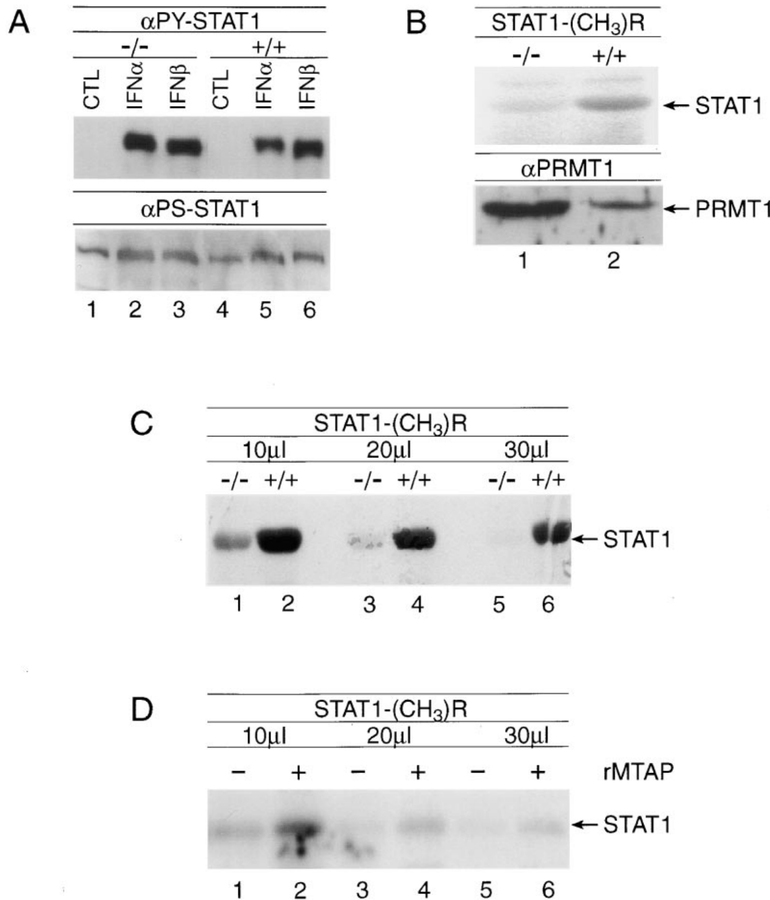

Figure 6. Effect of MTAP Deletion on STAT1 Function

(A) Intact STAT1 (Tyr-701) or (Ser-727) phosphorylation in MTAP-deficient cells. A549-MTAP(−/−) or A549-MTAP(+/+) cells were left untreated (lanes 1 and 4), or treated for 30 min with 1000 U/ml IFNα (lanes 2 and 5) or IFNβ (lanes 3 and 6). Equal amounts of protein were immunoblotted with either STAT1 (phosphoTyr-701) (top panel) or STAT1 (phosphoSer-727) antibody (bottom panel).

(B) Reduced STAT1 arginine methylation in A549-MTAP(−/−) cell lysates.

Lysates (30 μl) of A549-MTAP(−/−) (lane 1) or A549-MTAP(+/+) (lane 2) cells were added to an in vitro methylation reaction using GST-STAT1 as substrate (top panel). Lysates from both cell lines were resolved on SDS-PAGE and immunoblotted for endogenous PRMT1 (bottom panel).

(C) A549-MTAP(−/−) lysates inhibit STAT1 methylation by recombinant PRMT1.

10 μl (lanes 1 and 2), 20 μl (lanes 3 and 4), or 30 μl (lanes 5 and 6) total cell lysate derived from A549-MTAP(−/−) or A549-MTAP(+/+) were added to an in vitro methylation reaction utilizing recombinant PRMT1 and GST-STAT1 as a substrate.

(D) MTAP treatment of A549 cell lysate reduces its inhibitory activity.

A549 cell lysate was incubated without (lanes 1, 3, and 5) or with recombinant MTAP (lanes 2, 4, and 6) for 30 min at 30°C, and the indicated amount of the lysates was added to the in vitro methylation reactions as in (C).

fected in their growth by IFNα or IFNβ at any of the concentrations tested (Figure 6B).

These observations strongly support our model that MTAP deficiency will lead to a sufficiently high accumulation of MTA to result in inhibition of STAT1 methylation and cause diminution of the biological responses to IFNs.

Discussion

Investigations into STAT1 regulation have focused heavily on posttranslational amino acid modification by phosphorylation. In particular, phosphorylation of Tyr-701 has been found to be essential for dimerization, nuclear translocation, and DNA binding of STAT proteins (Schindler and Darnell, 1995). In addition, concomitant phosphorylation of Ser-727 results in a significant increase in the transactivating potential of STAT1 (Wen et al., 1995). It is also thought that the N terminus of STAT1 mediates interactions with other regulatory proteins that exert a modifying effect on STAT phosphorylation. As such, the deletion of the first 62 amino acids of STAT1 results in delayed inactivation and an alteration in the antiproliferative response toward interferon (Shuai et al., 1996).

Alignment of N termini of the seven mammalian STATs reveals a region of high homology and an invariant arginine at position 31, suggesting functional importance of this residue. Using an in vitro methylation assay, we demonstrated that GST-STAT1(NH2) was efficiently methylated on Arg-31, particularly in the presence of recombinant PRMT1. Intriguingly, the sequence surrounding Arg-31 bears little resemblance to the proposed glycine-rich consensus sequence observed in other arginine-methylated proteins (Clarke, 1993; Gary and Clarke, 1998). Although the GST-STAT1(NH2) construct contained 20 additional arginine residues, mutation of the Arg-31 to alanine completely abolished methylation. This was quite surprising since in an in vitro system utilizing high levels of recombinant enzyme, one might expect less specificity than in vivo. The specificity of the methylation reaction was also illustrated by the fact that PRMT1 was significantly more efficient in catalyzing STAT1 methylation than PRMT3. Indeed, coimmunoprecipitation studies detected an interaction between STAT1 and PRMT1, but not PRMT3. These results demonstrated that Arg-31 of STAT1 is an efficient substrate for methylation, and PRMT1 is capable of mediating this reaction. Immunoprecipitations of STAT1 with dimethyl arginine antibodies, as well as mass-spectrometric analysis, confirmed that STAT1 is indeed methylated on Arg-31 in vivo. Through the use of cytoplasmic and nuclear extracts, as well as several STAT1 mutants, we were able to show that methylation of STAT1 occurs in the cytoplasm and requires a functional STAT1-SH2 domain, but is independent of STAT1 tyrosine phosphorylation. It is interesting to note that only 5–10% of the total cellular STAT1 was found in the nucleus after IFNα

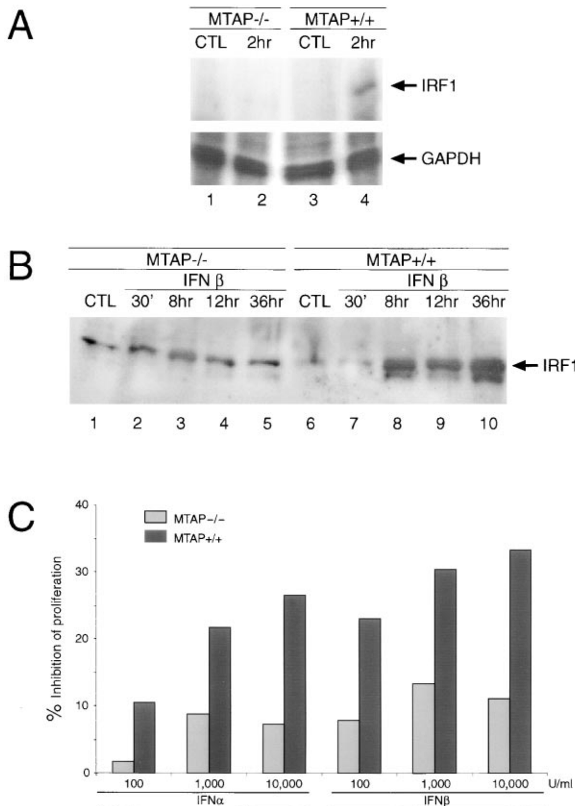

Figure 7. Reduced IFN Responsiveness in A549-MTAP(−/−) Cells

(A) A549-MTAP(−/−) lack IFN-induced IRF-1 mRNA. A549-MTAP(−/−) (lanes 1 and 2) or A549-MTAP(+/+) cells (lanes 3 and 4) were left untreated (lanes 1 and 3), or treated with 1000 U/ml IFNβ (lanes 2 and 4) for the indicated times. Total RNA was isolated, and RNase protection assays were performed using IRF-1 and GAPDH as probes.

(B) A549-MTAP(−/−) lack IFN-induced IRF-1 expression. A549-MTAP(−/−) (lanes 1–5) or A549-MTAP(+/+) cells (lanes 6–10) were left untreated (lanes 1 and 6), or treated with 1000 U/ml IFNβ (lanes 2–5 and 7–10) for the indicated times. Lysates were resolved by SDS-PAGE and immunoblotted for IRF-1.

(C) Reduced antiproliferative effect of IFNα/β in A549-MTAP(−/−) cells.

A549-MTAP(−/−) or A549-MTAP(+/+) cells were cultured in the presence of 100, 1000, or 10,000 U/ml of IFNα or IFNβ. A nonradioactive cell proliferation assay was performed four days after plating, and a representative of three identical experiments is shown above.

stimulation, indicating that only a small fraction of STAT1 undergoes ligand-induced tyrosine phosphorylation and nuclear translocation. Nevertheless, more than 60% of the methylated form of STAT1 was found in the nucleus, suggesting that the stoichiometry of methylation is significantly higher in the nuclear pool of tyrosine phosphorylated STAT1.

To investigate the biological importance of arginine methylation in STAT1-mediated gene induction, we utilized the methyl-transferase inhibitor MTA, which completely abrogated IFNα/β-induced gene transcription without affecting STAT1 tyrosine or serine phosphorylation. Arginine methylation of STAT1 is found at substantial levels in unstimulated cells, but is nevertheless sensitive to inhibition by MTA. The importance of Arg-31 in IFNα-mediated gene transcription is further illustrated by mutational analysis. Methylation of this positively charged amino acid results in the elimination of the positive charge and an increase in hydrophobicity. Indeed, replacement of arginine 31 with an uncharged hydrophobic alanine residue results in increased transcriptional activity. Moreover, substitution of arginine with the acidic and negatively charged glutamic acid further increased the ability of STAT1 to promote transcription.

Although inhibition of STAT1 arginine methylation by MTA had no effect on its tyrosine phosphorylation, STAT1 interaction with DNA was significantly impaired in the absence of STAT1 methylation. Loss of IFN-induced STAT1 DNA binding is reminiscent of the action of the PIAS family of STAT inhibitors whose presence has been demonstrated to inhibit STAT DNA binding (Chung et al., 1997; Liu et al., 1998). Indeed, inhibition of arginine methylation by MTA allows for an association between STAT1 and the PIAS1 protein that inhibits STAT DNA binding and induced transcription.

MTA is the substrate of methyl-thioadenosine phosphorylase (MTAP), a catabolic enzyme in the purine salvage pathway. MTAP has been the focus of several cancer-related studies as its gene is near the p16 locus and is codeleted in at least 85% of p16−/− human cancers (Fitchen et al., 1986; Smaaland et al., 1987; Carson et al., 1988; Traweek et al., 1988; Nobori et al., 1991, 1993; Della Ragione et al., 1992, 1993, 1995; Zhang et al., 1996). As cellular MTA levels are regulated by MTAP degradation, we investigated the possibility that MTAP(−/−) cells could accumulate a sufficient concentration of endogenous MTA to inhibit cellular protein arginine methylation activity. When added to an in vitro methylation assay, A549-MTAP(−/−) cell lysates completely inhibited STAT1 arginine methylation by endogenous or recombinant PRMT1 while A549-MTAP(+/+) lysates did not alter STAT1 methylation. Moreover, the inhibitory capacity of A549-MTAP(−/−) lysates was reduced following pretreatment with purified MTAP. We conclude that MTA concentrations in MTAP(−/−) cells are sufficient to inhibit protein arginine methylation. Interestingly, the level of endogenous PRMT1 appears elevated in MTAP(−/−) cells and may be a compensatory mechanism for the inhibition of arginine methylation caused by excess MTA.

Examination of the interferon-induced expression of IRF-1 in MTAP(−/−) and MTAP(+/+) cells at the RNA and protein level demonstrated a substantial induction of IRF-1 in MTAP(+/+) cells that is starkly absent in MTAP(−/−) cells. It thus appears that the lack of arginine methylation of STAT1 in MTAP(−/−) cells produces a significant decrease in STAT1-induced transcription of IRF-1 and possibly other tumor suppressor genes. This difference in IRF-1 induction parallels a greater antiproliferative effect of IFNα/β in MTAP(+/+) cells compared to MTAP(−/−) cells. Thus, the inhibition of STAT1 arginine methylation by increased amounts of intracellular MTA seems to inhibit the biological effects of interferon.

The role of arginine methylation in signal transduction events has not been widely explored. Levels of cellular arginine methylation have been shown to rise following NGF stimulation of PC12 cells (Cimato et al., 1997) and lipopolysaccharide treatment of B cells (Law et al., 1992). Defined substrates for arginine methylation in-

clude FGF (Burgess et al., 1991; Pintucci et al., 1996), nucleolin (Najbauer et al., 1993), and fibrillarin (Najbauer et al., 1993), as well as hnRNP A1 (Shen et al., 1998) and hnRNP A2 (Nichols et al., 2000) where arginine methylation is implicated in hnRNP nuclear localization. Additionally, a recent study reported the isolation of a coactivator–associated arginine methyl transferase 1 (CARM1) whose activity enhanced the transcriptional activity of certain nuclear hormone receptors, but only when expressed with members of the p160 family of coactivators (Chen et al., 1999). Interestingly, arginine methylation within a proline-rich region in Sam68, a proposed adaptor for src kinases, was shown to prohibit interaction with SH3 domains while maintaining the ability of Sam68 to interact with WW domains (Bedford et al., 2000). Thus, arginine methylation of Sam68 modulates its interactions with SH3- and WW- containing proteins. We envision a similar scheme for STAT-PIAS interactions where arginine methylation obstructs the interaction of STAT1 with its inhibitor PIAS1. Indeed, the amino-terminal domain of STAT1 (aa 1–191) where arginine 31 is located is necessary for association with PIAS1. It is interesting to note that similar regions of STAT1 are required for PIAS1 interaction and for tyrosine dephosphorylation; it is therefore possible that these events occur in a coordinated manner.

In summary, arginine methylation is an additional STAT1 modification, one that appears deficient in MTAP(−/−) cells due to accumulation of a biological inhibitor of arginine methyl transferases. Since MTAP is frequently deleted in cancers, its presence might prove useful as a prognostic marker for interferon therapy.

Experimental Procedures

Cell Culture

Primary human fibroblasts, HeLa, HEK293, and A549 cells were maintained in DMEM, and U266 cells were cultured in RPMI1640. All media was supplemented with penicillin and streptomycin, and 10% fetal bovine serum (10% horse serum for A549 cells).

Interferons and Reagents

IFNα and IFNβ were generous gifts from Hoffman LaRoche and Biogen Inc., respectively. ^{3}H-S-Adenosyl-methionine was obtained from ICN Pharmaceuticals. 5′-methyl-thioadenosine (MTA) was purchased from Sigma and dissolved in culture medium.

Plasmids

GST-STAT1 and GST-STAT1(R31A) have been described previously. GST-STAT1(R31E) was generated by site-directed mutagenesis using QuickChange (Stratagene). Plasmids encoding GST fusion proteins of STAT1 amino acids 1–129, and the same region with the Arg-31→Ala mutant, were generated by excision of a BamHI/SspI fragment from pEBG GST-STAT1 and pEBG GST-STAT1 (Arg→Ala), respectively, and ligation into the BamHI/SmaI sites of pGEX-KG (Pharmacia), which had been modified to accept the BamHI/SmaI insert. GST-PRMT1 and GST-PRMT3 were previously described (Lin et al., 1996).

In Vitro Methylation Reactions

Two μg GST-PRMT1 or GST-PRMT3 were incubated with 10–15 μg of (GST-STAT1–129) or (GST-STAT1–129 Arg-31→Ala) and 2 μl of [^{3}H] Ado-Met (NEN Life Science, 0.55 mCi/ml) raised to a final volume of 80 μl with PBS and incubated for 90 min at 37°C. Reactions were terminated by the addition of SDS-loading buffer. A549 and A549-MTAP reconstituted cell lysates were obtained by scraping cells into PBS followed by sonication and centrifugation.

Cell Extracts and Electrophoretic Mobility Shift Assay (EMSA)

To generate whole cell lysates, cells were lysed on the plates with lysis buffer (1 ml) containing 20 mM Hepes pH 7.4, 1% TX-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium-vanadate, and 1 mM PMSF. For coimmunoprecipitation experiments, cells were lysed in buffer containing 100 mM NaCl, 50 mM TRIS pH 7.5, 1 mM EDTA, 0.1% TX-100, 10 mM NaF, 1 mM PMSF, and 1 mM vanadate. Lysates were centrifuged at 13,000 rpm for 5 min and protein concentration was determined by Lowry (BioRad Protein Assay). Cytoplasmic/nuclear extracts were prepared by douncing cells in Buffer A (20 mM HEPES pH 7.9, 10 mM KCl, 1 mM $MgCl_2$, 10% Glycerol, 0.1% NP40) and sedimentation of nuclei by centrifugation at 1000 rpm for 5 min. Supernatant (=cytoplasmic extract) was removed, and nuclei were extracted with Buffer A containing 300 mM NaCl (=nuclear extract). EMSAs were performed using whole cell extracts and an end-labeled oligonucleotide corresponding to the GRR sequence of the *FcγRI* gene (Wilson and Finbloom, 1992).

Immunoprecipitation and Immunoblotting

Cell lysates were precleared with Protein G-Sepharose for 30 min, and incubated with preimmune sera or antisera to PRMT1, PRMT3, or STAT1 and protein G-Sepharose overnight at 4°C. After SDS-PAGE and transfer onto PVDF membrane, proteins were detected with anti-STAT1 (Transduction Labs), anti-phosphoSTAT1 Y701 (New England Biolabs), anti-phosphoSTAT1 Ser-727 (Upstate Biotechnology, Inc.), anti-IRF-1, or anti-PIAS1 antibodies (Santa Cruz). For dimethyl-arginine immunoprecipitations, cell lysates were incubated with monoclonal mono/dimethyl-arginine or dimethyl-arginine antibodies (Abcam) or an isotype control antibody overnight at 4°C. For antigen-block experiments, the cell lysate was divided in half, and unmethylated or in vitro methylated STAT1(NH2)-GST was added prior to the dimethyl-arginine antibodies. Immunoreactive proteins were resolved by SDS-PAGE, and blots were immunoblotted with monoclonal STAT1 antibodies. All blots were developed with HRP-conjugated secondary antibodies and ECL (Amersham).

Proliferation Assays

A549- and A549-MTAP reconstituted cells were seeded at 100 cells/well with indicated amounts of IFNα or IFNβ or left untreated for three days before analysis with a Non-radioactive Cell Proliferation Assay (Promega). Assays were performed according to manufacturer's instructions.

Immunofluorescence

Cells were seeded onto coverslips and incubated overnight at 37°C in DMEM containing 10% FBS. After treatment, coverslips were rinsed with PBS followed by one wash with PIPES buffer. Cells were fixed in methanol at room temperature and nuclei permeabilized with 0.5% Nonidet P-40/PB. Coverslips were washed three times with PBS, blocked with 10% goat serum, and incubated with anti-STAT1 antibodies (Transduction Laboratory). Cells were rinsed four times with PBS prior to incubation with Cy3-conjugated secondary antibody. After washing, coverslips were mounted onto glass slides in 50% glycerol/PBS.

RNase Protection Assays

Total RNA was isolated using TRIzol Reagent. ^{32}P-labeled riboprobes were generated by in vitro transcription using T3, T7, or SP6 RNA polymerase (Promega). Labeled riboprobe 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.

Luciferase Assays

Cells were transiently transfected with either ISRE or IRF-1 firefly luciferase reporter plasmids and STAT1 constructs, as indicated in the figure legends, using Superfect (Qiagen). Renilla luciferase under the control of the thymidine kinase promoter was cotransfected as an internal standard. Firefly and renilla luciferase activity were

determined using Promega's Dual Luciferase Kit according to the manufacturer's instructions, and firefly luciferase activity was normalized to renilla luciferase activity.

Mass-Spectrometric Analysis

U266 cells were treated with IFNα for 30 min, STAT1 was immunoprecipitated from the lysates and resolved on 7.5% SDS-PAGE. The excised gel band was subject to in-gel digestion with Glu-C (Promega). Extracted peptides were purified using a reversed-phase microcolumn (C18 Zip-tip, Millipore) and analyzed on a MALDI-TOF mass spectrometer (PE Voyager DE-STR) using α-cyano-4-hydroxycinnamic acid as matrix.

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