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Arginine Methylation of NIP45 Modulates Cytokine Gene Expression in Effector T Lymphocytes

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Summary

Posttranslational modification of proteins within T cell receptor signaling cascades allows T lymphocytes to rapidly initiate an appropriate immune response. Here we report a role for arginine methylation in regulating cytokine gene transcription in the T helper lymphocyte. Inhibition of arginine methylation impaired the expression of several cytokine genes, including the signature type 1 and type 2 helper cytokines, interferon γ, and interleukin-4. T cell receptor signaling increased expression of the protein arginine methyltransferase PRMT1, which in turn methylated the nuclear factor of activated T cells (NFAT) cofactor protein, NIP45. Arginine methylation of the amino terminus of NIP45 modulated its interaction with NFAT and resulted in augmented cytokine production, while T cells from mice lacking NIP45 had impaired expression of IFNγ and IL-4. Covalent modification of NIP45 by arginine methylation is an important mechanism of regulating the expression of NFAT-dependent cytokine genes.

Introduction

Signal transduction pathways utilize posttranslational modifications to translate changes in the extracellular milieu into environment-sensitive gene expression in a timely and efficient fashion. Phosphorylation of serine, threonine, and tyrosine residues and protein ubiquitination have been widely studied (Freiman and Tjian, 2003; Roose and Weiss, 2000). Although methylation of arginine residues was discovered over 30 years ago, it has only recently aroused substantial interest (McBride and Silver, 2001). Two types of protein arginine methyltransferases (PRMT) have been subclassified based on the symmetry of their reaction products. Both type I (PRMT1, PRMT3, CARM1 [PRMT4], PRMT6) and type II (PRMT5) methyltransferases induce monomethylation of arginine residues as a reaction intermediate, but type I protein arginine methyltransferases generate asymmetric dimethyl-arginine residues, while type II PRMTs catalyze the formation of symmetric dimethyl-arginine residues (Frankel et al., 2002; McBride and Silver, 2001). PRMT2, despite sequence similarity to PRMT1, has not been demonstrated to have enzymatic activity (Qi et al., 2002; Scott et al., 1998). Arginine methylation regulates subcellular localization (Shen et al., 1998; Yun and Fu, 2000) and modulates protein-protein interactions. For example, arginine methylation of the Sam68 proline-rich region prevents interaction with its SH3 domain binding partners, Fyn, Lck, and Itk, without altering affinity to WW domains (Bedford et al., 2000), while Stat1 arginine methylation in response to IFNα/β signaling prevents its interaction with the PIAS1 inhibitor, thereby regulating Stat1 transcriptional ability (Mowen et al., 2001). PRMT1-deficient mice are embryonic lethal, and CARM1-deficient mice die late during embryogenesis or perinatally, demonstrating a requirement for arginine methylation by these PRMTs in developmental processes (Pawlak et al., 2000; Yadav et al., 2003).

T helper (Th) lymphocytes can be divided into two major subsets based on unique functions and cytokine profiles. Th1 cells produce IFNγ, IL-2, TNFα, and TNFβ and are responsible for cell-mediated immunity. Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13 cytokines that are vital in the humoral immune response to extracellular pathogens (Murphy and Reiner, 2002; Szabo et al., 2003). The balance between the two Th subsets determines the outcome of an immune response to a foreign agent, be it a virus, parasite, allergen, autoantigen, or tumor antigen. Overexpression of Th2 cytokines can lead to allergy and asthma, while an overactive Th1 response may result in autoimmunity (Glimcher and Murphy, 2000). Because of their opposing roles in immune function, it is critical that the development of Th1/Th2 populations be tightly regulated. Engagement of three classes of surface receptors, the T cell receptor (TCR), and costimulatory and cytokine receptors, directs Th lineage commitment. The cytokine environment stimulates intracellular processes which direct the epigenetic programming of the T helper precursor cell (Thp) toward the Th1 or Th2 lineage (O'Garra, 1998). Th1 development relies on the presence of IFNγ and IL-12, while the development of Th2 cells requires IL-4 (Glimcher and Murphy, 2000).

Antigen binding to the TCR of Th1 and Th2 cells, a process mimicked by anti-CD3 stimulation or by treatment with phorbyl myristate acetate (PMA) and the calcium ionophore ionomycin (I), activates the quiescent T cell to transcribe cytokine genes. TCR stimulation induces a number of known signaling pathways. For example, TCR ligation induces calcium mobilization resulting in activation of the phosphatase calcineurin (Clipstone and Crabtree, 1992). Calcineurin dephosphorylates nuclear factor of activated T cell (NFAT) proteins allowing NFAT translocation to the nucleus (Beals et al., 1997a; Crabtree and Olson, 2002; Timmerman et al., 1996). There are five NFAT family members, NFATc1–5 (Hogan et al., 2003; Northrop et al., 1994), three of which, NFATc1–3, are expressed in the lymphoid system (Rengarajan et al., 2000). While NFATs are not expressed in a Th subset-specific manner, they are required for the transcription of multiple cytokine genes as evidenced by the global impairment in cytokine production in mice lacking both NFATc1 and NFATc2

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(Peng et al., 2001). The balance of NFAT proteins likely determines the spectrum of cytokines produced by Th1 or Th2 cells (Rengarajan et al., 2002b). NFATs heterodimerize with a number of other nuclear proteins that serve to modify their activity. Within the nuclear compartment, NFATs associate with AP-1 factors and with IRF-4 to activate gene transcription (Rao et al., 1997; Rengarajan et al., 2002a; Ullman et al., 1993). Another NFAT binding partner is NIP45, a nuclear protein that dramatically augments NFAT-driven IL-4 production (Hodge et al., 1996a). The mechanism by which this occurs, however, has remained obscure.

In the present study, we show that arginine methylation plays an important role in cytokine production by T helper cells. PRMT1 is a highly expressed protein arginine methyltransferase in T helper cells, and its expression is induced by TCR signals. We demonstrate that increased PRMT1 expression causes activation of IFNγ and IL-4 gene transcription. One target of PRMT1 activity is NIP45. Arginine methylation of NIP45 acts to modulate the interaction between NIP45 and NFAT, resulting in alterations of cytokine gene expression.

Results

Inhibition of Arginine Methylation Impairs T Helper Cytokine Production

To test whether protein arginine methylation plays a role in Th cytokine production, we made use of 5′-methylthioadenosine (MTA), an inhibitor of protein methyltransferases (Williams-Ashman et al., 1982). Incubation of cells with MTA reduced the total amount of cellular protein methylation (Maher, 1993). Thp were stimulated under Th1- or Th2-differentiating conditions. Pretreatment with MTA prior to PMA/I (P/I) stimulation led to a decrease in select cytokine mRNAs. We observed decreases in IFNγ and IL-2 transcripts in Th1 cells and IL-4, IL-5, IL-6, and IL-13 transcripts in Th2 cells. IL-10 and IL-9 mRNA levels in Th2 cells were not affected by MTA, demonstrating that the observed decrease in select cytokine mRNAs was not due to general transcriptional attenuation (Figure 1A). Th1 or Th2 cells left unstimulated or treated with MTA alone did not induce cytokine transcripts (data not shown). MTA treatment did not nonspecifically inhibit other signaling processes induced by P/I stimulation as revealed by immunoblotting MTA-treated cell lysates with anti-phospho-p44/42 MAP kinase antibodies (Figure 1A). Inhibition of methyltransferase activity reduced the number of IFNγ-producing Th1 cells and IL-4-producing Th2 cells (Figure 1B). MTA treatment of Th1 and Th2 cells did not result in increased cell death as measured by annexin V and propidium iodide staining (data not shown). Thus, PRMT-mediated arginine methylation may regulate Th cell cytokine production.

As PRMTs could control levels of cytokine RNA through altering promoter activation or affecting RNA stability, we tested the effect of MTA treatment on the activity of a Th2-selective region of the IL-4 promoter (-760 to $+68$). This portion of the IL-4 promoter is responsive to transactivation by the Th2-specific factor, c-Maf, in conjunction with NFAT (Rengarajan et al., 2000; Szabo et al., 1993; Wenner et al., 1997). Jurkat cells, a human T cell line, were transfected with an IL-4 luciferase reporter and NFATc2 and c-Maf expression vectors. Coexpression of NFATc2 and c-Maf greatly induced IL-4 promoter activity under P/I stimulation conditions; however, pretreatment with MTA prior to P/I stimulation inhibited NFATc2 and c-Maf-driven IL-4 promoter transactivation (Figure 1C), indicating that the decrease in IL-4 RNA in Th2 cells occurred at least partly at the transcriptional level.

NIP45 Is a Potential PRMT Target in T Helper Cells

The effects of MTA on IFNγ and IL-4 expression were reminiscent of an NFAT-interacting factor we had previously isolated (Hodge et al., 1996a). NFAT interacting protein (NIP) 45 (Kd) is a cofactor in NFATc2/c-Maf-driven IL-4 promoter activity and endogenous IL-4 expression (Figure 2A and Hodge et al., 1996a). We have subsequently observed a similar synergy between NIP45 and the Th1-specific transcription factor T-bet (Szabo et al., 2000) in transactivating the IFNγ promoter (Figure 2A). A preliminary analysis of NIP45-deficient mice, whose production and characterization will be described in detail elsewhere, revealed a decrease in levels of both IL-4 and IFNγ from TCR-stimulated NIP45$^{-/-}$ T cells (Figure 2B). Further, the amino terminus (aa 6–32) of NIP45 contains eleven arginine residues within RXR and RG motifs shown to be favorable for methylation by type I PRMTs (McBride and Silver, 2001) (Figure 2C). We tested whether recombinant PRMT1 could methylate NIP45, NFATc2, T-bet, or c-Maf by performing in vitro methylation assays with immunoprecipitates of these factors. PRMT1 methylated NIP45 but not ΔN-NIP45 in which amino acids 1–32 are deleted (Figure 2D, lanes 2 and 3, left panel). Importantly, NIP45 immunoprecipitates were not methylated when recombinant PRMT1 was absent, indicating that methyltransferase activity was not simply coimmunoprecipitating with NIP45 (Figure 2D, lanes 2 and 4, left panel). Additionally, we did not detect any specifically methylated proteins in NFATc2, c-Maf, or T-bet immunoprecipitates (compare to vector-only immunoprecipitates, Figure 2D, lanes 5, 6, 8, 9, 10, and 11, left panel). Among the factors tested, NIP45 is the only potential PRMT1 target (Figure 2D, lanes 2, 6, 9, and 11, left panel).

PRMT1 Is Highly Expressed in T Helper Cells

To determine which PRMT(s) was the target of MTA inhibition, the expression pattern of PRMTs in Th cells was determined. Thp, Th1, and Th2 cell lysates were subjected to immunoblot analysis for PRMT1, PRMT2, PRMT3, CARM1, PRMT5, and PRMT6. CARM1 and PRMT6 were expressed at low levels although CARM1 was not detected at the Thp stage. Notably, CARM1 and PRMT6 were only detected by immunoblot with higher amounts of protein lysates (at least 30 μg, data not shown). PRMT2, PRMT3, and PRMT5 were expressed at moderate levels in Thp, Th1, and Th2 cell lysates (Figure 3A). Induction of CARM1 expression in Th1 and Th2 cells was observed. Because PRMT1 expression seemed robust in Th cells, we studied it further. Indeed, PRMT1 protein levels were slightly induced in

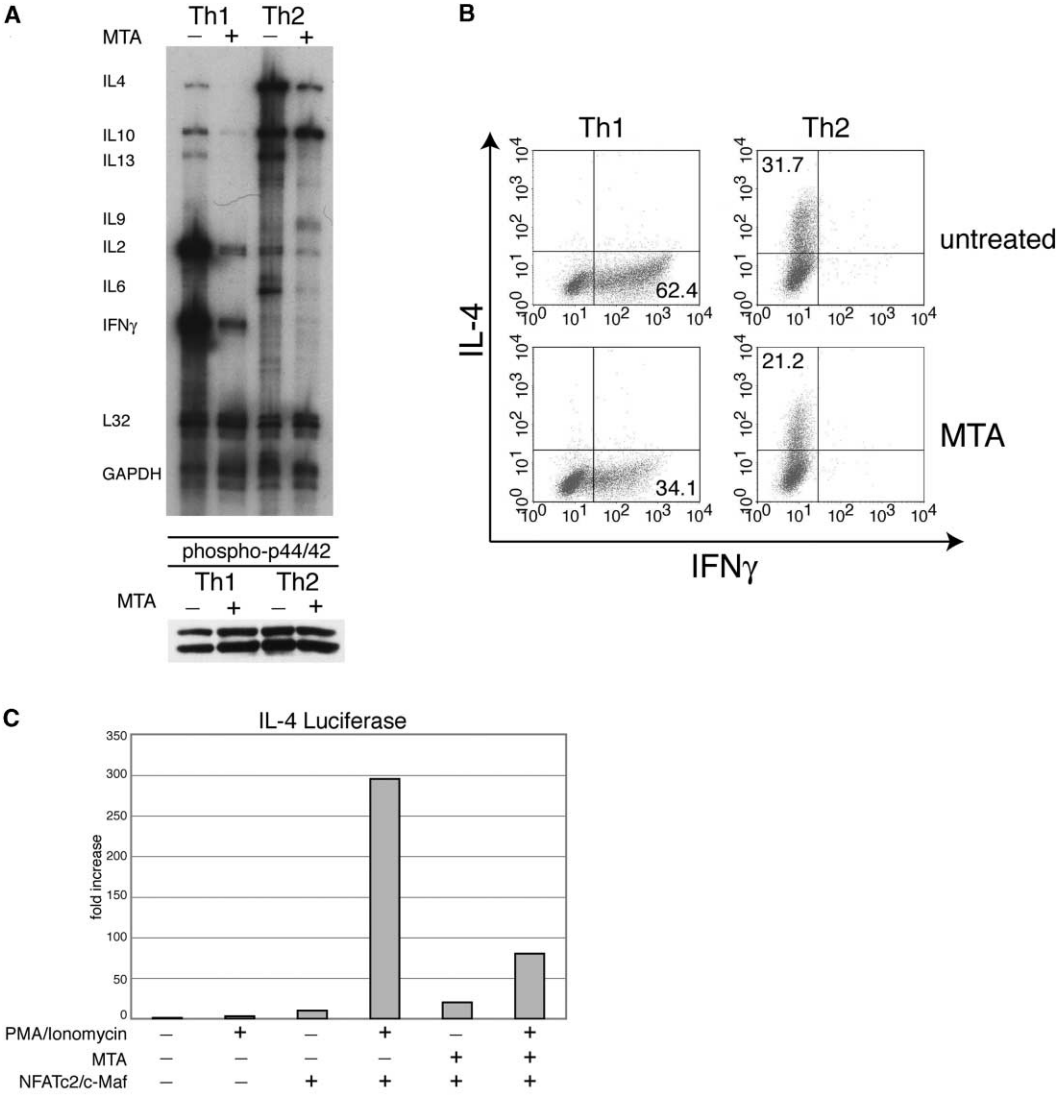

Figure 1. Effects of MTA on Th Cytokine Production

(A) Naive Thp cells were grown under Th1 or Th2 conditions for 7 days. Th1 and Th2 cells were treated with P/I for 3 hr or pretreated with 1 mM MTA for 60 min prior to P/I stimulation. RNA was analyzed by RNase protection. L32 and GAPDH served as internal controls. Protein lysates from Th1 and Th2 cells untreated or pretreated with MTA for 2 hr prior to 5 min P/I stimulation were immunoblotted with anti-phospho p44/42 MAP kinase antibodies.

(B) Th1 and Th2 cells were treated as in (A) and intracellular cytokine analysis was performed.

(C) Jurkat cells were transfected with IL-4 reporter vector (2.5 μg) and expression vectors for c-Maf (2.5 μg), NFATc2 (2.5 μg), and PRMT1 (10 μg) along with a TK-Renilla luciferase vector (5 ng) as an internal control. Transfectants were left unstimulated or pretreated with 1 mM MTA before a 6 hr P/I stimulation. Luciferase values were calculated relative to TK-Renilla luciferase internal controls and are expressed relative to unstimulated reporter activity. Similar results were obtained in at least three independent experiments.

Th1 and Th2 cells following stimulation with plate-bound anti-CD3 (Figure 3B). Since PRMT1 protein levels were regulated by TCR stimulation in Th1 and Th2 cells, we determined the kinetics of PRMT1 transcript expression following anti-CD3 stimulations. Th cells were left unstimulated or stimulated with anti-CD3/anti-CD28 antibodies for the indicated times. PRMT1 transcripts were present in unstimulated Th cells (d0) and were markedly upregulated 24 hr after TCR stimulation (d1) (Figure 3C). After 3 and 5 days in culture, PRMT1 transcripts declined to levels slightly above baseline Thp expression, and when day 5 cultures were restimulated with anti-CD3, PRMT1 transcripts were again upregulated (sample D6R, Figure 3B). Upregulation of PRMT1 transcripts was observed by 6 hr after anti-CD3/anti-CD28 polyclonal antibody stimulation and was cyclosporin A sensitive (Figure 3D), indicating that PRMT1 expression was induced through TCR signaling in an NFAT-dependent manner.

These data support earlier findings that PRMT1 accounts for the majority of PRMT activity in mammalian cells (Tang et al., 2000) as further evidenced by a greater

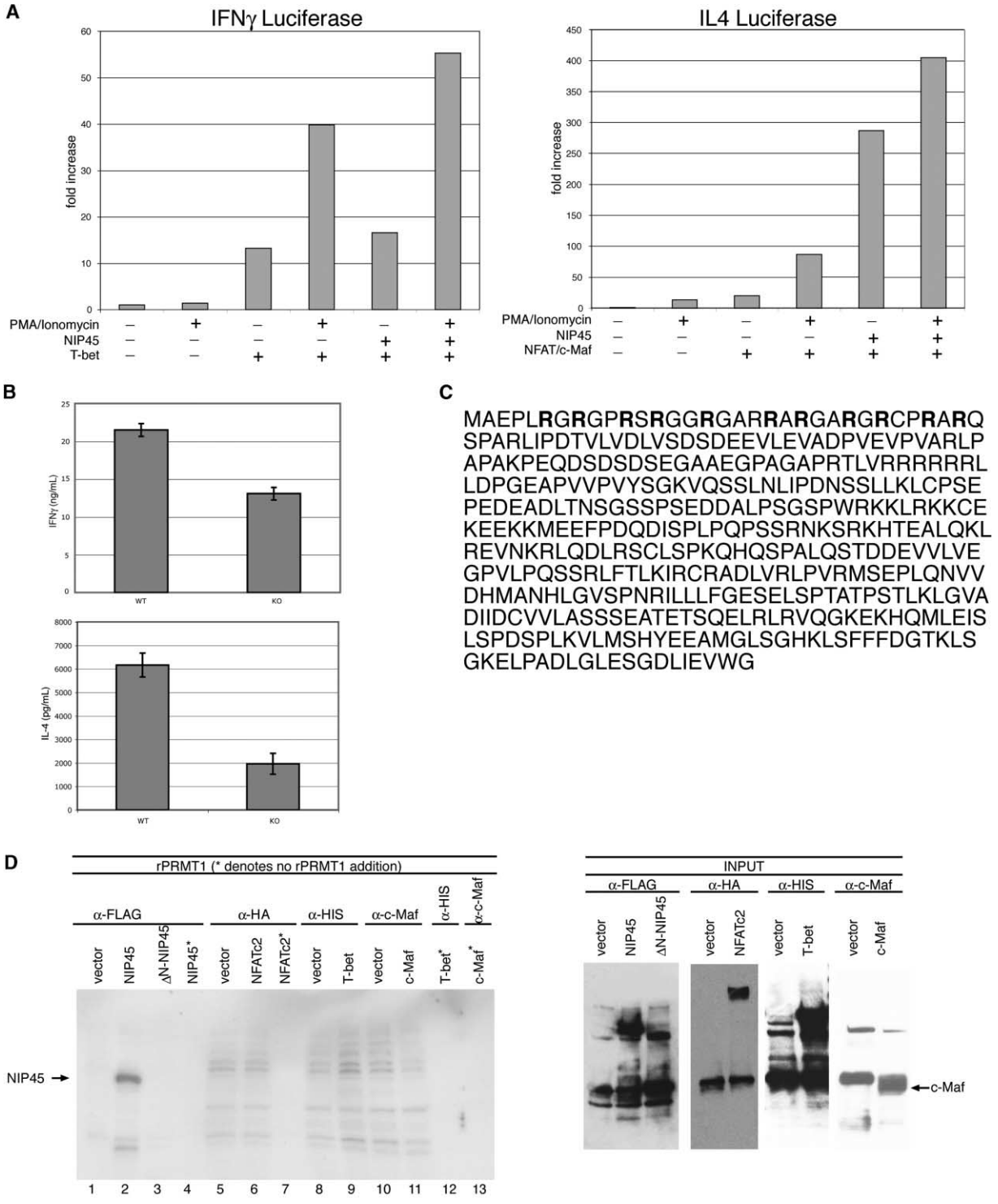

Figure 2. NIP45 Also Augments Transcription from IFNγ and IL-4 Promoters

(A) Jurkat cells were transfected with the 9.2 kb IFNγ luciferase reporter (5 μg) and expression vectors for T-bet (5 μg) and NIP45 (5 μg) or with the IL-4 luciferase reporter (2.5 μg) and expression vectors for NFATc2 (2.5 μg), c-Maf (2.5 μg), and NIP45 (5 μg). Cells were unstimulated or stimulated with P/I for 6 hr prior to luciferase assays. Luciferase units were normalized to TK-Renilla luciferase activity. Results are representative of at least three independent experiments.

(B) Naive Thp cells from WT and NIP45 KO mice were stimulated with anti-CD3 + anti-CD28 under Th1- or Th2-skewing conditions. On day 7, cells were restimulated with anti-CD3, and 24 hr cell supernatants were analyzed by ELISA to measure IFNγ and IL-4 concentrations. Data are representative of at least three independent experiments.

(C) The amino terminus (aa 6–32) of NIP45 contains 11 arginine residues within consensus motifs for arginine methylation. Putative methylated arginines are indicated in bold.

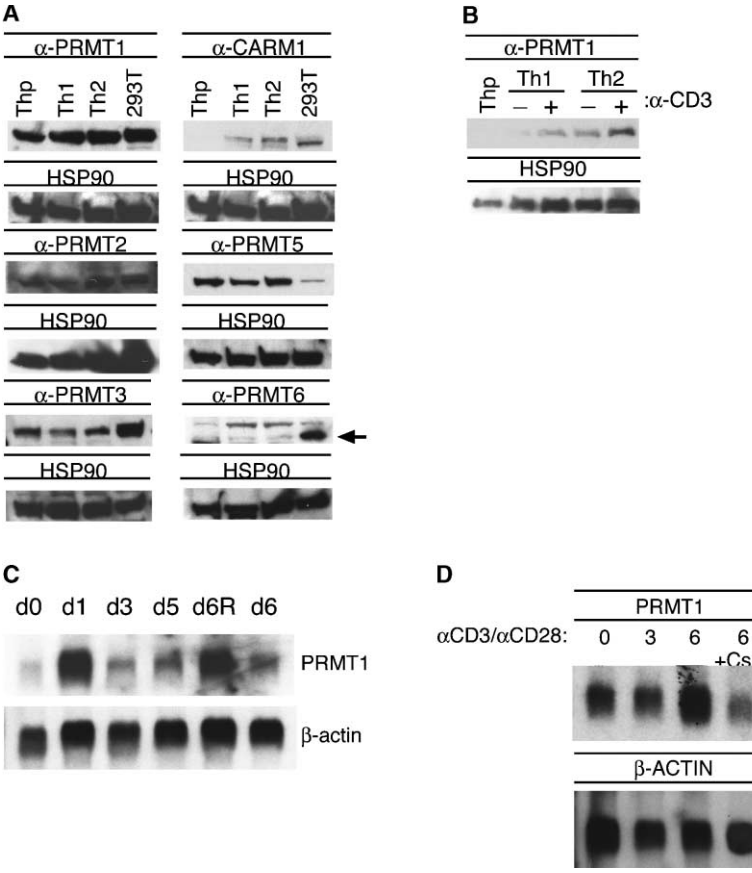

Figure 3. PRMT1 Is Highly Expressed in T Helper Cells

(A) PRMT expression in Th cells. Thp, Th1, Th2, and 293 cell protein lysates (30 μg) were analyzed by Western blot with antibodies to PRMT1, PRMT2, PRMT3, CARM1, PRMT5, and PRMT6. The location of PRMT6 is indicated by an arrow. Equal loading was confirmed using an antibody to HSP90.

(B) PRMT1 expression in Th cells. Protein lysates (10 μg) from Th1 and Th2 cells unstimulated or stimulated with plate-bound anti-CD3 for 1 day were analyzed by Western blot.

(C) PRMT1 transcripts are upregulated by TCR stimulation. T helper cells from Balb/c mice were unstimulated or stimulated with plate-bound anti-CD3 and anti-CD28 for 1 day or 3 days. For D6R samples, cells from day 5 were restimulated with anti-CD3 for 24 hr. RNA was prepared and analyzed by Northern blot with a probe to PRMT1. Nothern blots were reprobed with a β-actin probe to assess loading.

(D) TCR-induced PRMT1 expression is rapid and cyclosporin A (CsA) sensitive. T helper cells isolated from the lymph nodes of Balb/c mice were untreated or pretreated with CsA for 1 hr prior to stimulation with anti-CD3/anti-CD28 for 0, 3, and 6 hr. RNA was harvested, and Northern blot analysis for PRMT1 expression was performed. Equal loading was determined by reprobing with a β-actin probe.

than 50% reduction in the level of asymmetric dimethylarginine in PRMT$^{-/-}$ ES cells (Pawlak et al., 2000). Thus, PRMT1 seemed likely to be the primary methyltransferase responsible for modulating TCR-regulated cytokine production.

PRMT1 Augments IFNγ and IL-4 Promoter Activity

Since MTA inhibited the expression of IFNγ and IL-4, the signature Th1 and Th2 cytokines, we investigated whether PRMT1 could induce IFNγ and IL-4 promoter activity on its own or in conjunction with Th transcription factors. Jurkat cells were cotransfected with a 9.2 kb IFNγ luciferase reporter construct and PRMT1 alone or together with T-bet (Szabo et al., 2000). Transfection of PRMT1 alone did not transactivate the IFNγ promoter even with P/I stimulation. As described previously, provision of T-bet induced IFNγ promoter activity under unstimulated and P/I stimulation conditions (Figure 4A). Cotransfection of PRMT1 and T-bet resulted in an enhancement of T-bet-regulated IFNγ reporter activity only in the context of P/I stimulation (Figure 4A). Jurkat cells transfected with PRMT1 exhibited little induction of the IL-4 promoter over baseline even with P/I stimulation (Figure 4B) while coexpression of NFATc2 and c-Maf greatly induced IL-4 luciferase activity (Figure 4B). Similar to T-bet, provision of PRMT1 along with NFATc2 and c-Maf substantially augmented IL-4 promoter activity under P/I stimulation conditions (5-fold over NFATc2/c-Maf only) (Figure 4B).

The IL-4 promoter contains several composite NFAT/AP-1 sites. This led us to investigate whether PRMT1 coactivation is mediated through NFAT or AP-1 factors as both NFAT and JunB are involved in the regulation of IL-4 promoter activity (Li et al., 1999). c-Maf, also a member of the AP-1 family, synergizes with JunB to induce IL-4 promoter activity, likely as a result of cooperative DNA binding (Li et al., 1999). To test whether PRMT1 augmented JunB/c-Maf IL-4 promoter transactivation, we cotransfected PRMT1, JunB, and c-Maf with the IL-4 luciferase reporter. Expression of JunB or c-Maf alone with the IL-4 promoter had little effect on P/I-stimulated reporter activity (20- to 30-fold compared to 20-fold vector control); however, as previously reported, under P/I stimulation, cotransfection of JunB and c-Maf led to a 50-fold increase compared to vector control. PRMT1 expression alone, as shown in Figure 3B, had no effect on promoter activity or on JunB/c-Maf-induced IL-4 reporter activity (Figure 4C). In contrast, PRMT1

(D) NIP45 is methylated, but ΔN-NIP45, NFATc2, T-bet, and c-Maf are not. 293 cells were transfected with expression vectors for NIP45, ΔN-NIP45, NFATc2, T-bet, and c-Maf, which were immunoprecipitated from cell extracts. Immunoprecipitates were subjected to in vitro methylation by recombinant PRMT1 (left panel). Westerns were performed on immunoprecipitate samples (right panel). c-Maf expression is indicated by an arrow.

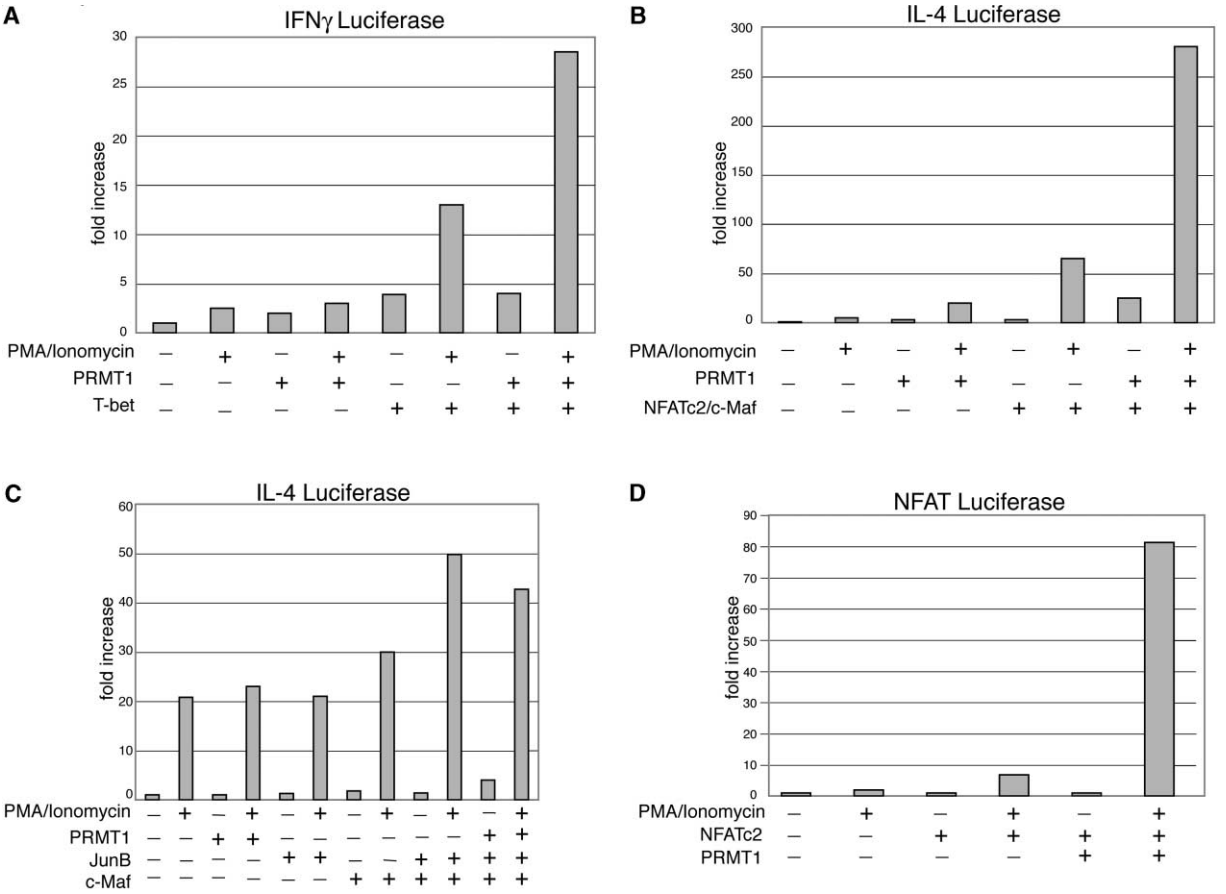

Figure 4. PRMT1 Regulates the IFNγ and IL-4 Promoters

(A) Jurkat cells were transfected with the 9.2 kb IFNγ luciferase reporter (5 μg) and expression vectors for T-bet (5 μg) and PRMT1 (10 μg). Cells were unstimulated or stimulated with P/I for 6 hr prior to luciferase assays.

(B) Jurkat cells were transfected with the IL-4 luciferase reporter (2.5 μg), and expression vectors for NFATc2 (2.5 μg), c-Maf (2.5 μg), and PRMT1 (10 μg). Cells were treated as in (A).

(C) Jurkat cells were transfected with the IL-4 luciferase reporter (2.5 μg) and expression vectors for JunB (2.5 μg), c-Maf (2.5 μg), and PRMT1 (10 μg). Cells were treated as in (A).

(D) Jurkat cells were transfected with a luciferase reporter driven by NFAT consensus sites and expression vectors for NFATc2 (2.5 μg) and PRMT1 (10 μg). Cells were treated as in (A).

Luciferase units were normalized to TK-Renilla luciferase activity. Results are representative of at least three independent experiments.

synergized with NFATc2 to augment NFATc2 transactivation of an NFAT reporter element by approximately 10-fold (Figure 4D). Thus, PRMT1-induced transcriptional enhancement occurs with both the IFNγ and IL-4 promoters and, at least in the case of IL-4 promoter induction, is mediated through the NFAT transcription factor.

NIP45 Is Methylated by PRMT1

To test whether other PRMTs could methylate NIP45, we compared the ability of PRMT1, PRMT3, CARM1, and PRMT5 to methylate a bacterially expressed GST-NIP45 fusion protein. In this assay, only PRMT1 was capable of methylating NIP45 (Figure 5A, lane 2, upper panel). However, PRMT1, PRMT3, and CARM1 immunoprecipitates methylated histones while PRMT5 immunoprecipitates methylated myelin basic protein (MBP) (Figure 5A, bottom panel). Methylation of NIP45 observed in immunoprecipitates from cell lysates could be due to the methyltransferase activity of another PRMT that is recruited by PRMT1. We tested whether PRMT1 could directly methylate NIP45 using recombinant proteins. As shown in Figure 5B, recombinant PRMT1 methylated bacterially expressed GST-NIP45 (lane 2) whereas CARM1 did not (lane 5), although CARM1 did methylate histone H3 (lane 7) as described (Chen et al., 1999). These data suggest that PRMT1 methylation of NIP45 is direct and specific. To prove that the amino terminus of NIP45 was the target for methylation by PRMT1, we created a GST-ΔN-NIP45 fusion protein in which amino acids 1–32 were deleted. Elimination of the amino terminus resulted in a loss of PRMT1-induced methylation (Figure 5B, lane 3). Methylation of proteins in vitro by PRMT1 correlates well with their methylation by PRMT1 in vivo (Pawlak et al., 2002). To test this notion directly, we transfected wild-type (wt) and PRMT1$^{-/-}$ ES cells with NIP45 and then performed Western blot analysis

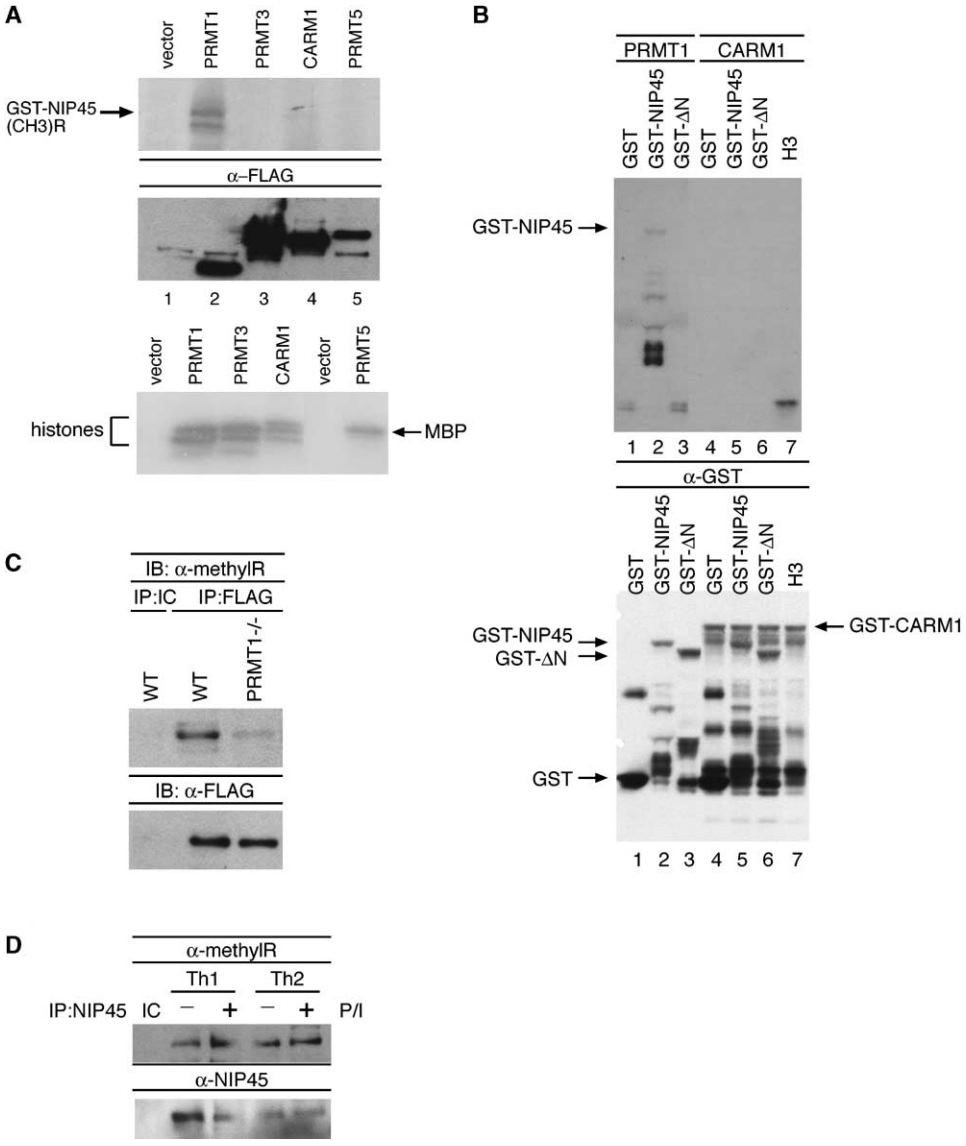

Figure 5. NIP45 Is Methylated by PRMT1

(A) PRMT1, PRMT3, CARM1, and PRMT5 proteins were immunoprecipitated from 293 cell extracts using anti-Flag agarose, and in vitro methylation assays were performed with recombinant GST-NIP45. Western blot analysis was performed with anti-Flag. Calf thymus histones and MPB were used as positive controls for methyltransferase activity.

(B) PRMT1-His and GST-CARM1 were used in in vitro methylation assays with GST, GST-NIP45, and GST-ΔN-NIP45 (GST-ΔN). Histone H3 was used as a positive control for CARM1 activity. Input was verified by probing with anti-GST.

(C) Wt ES cells and PRMT1$^{-/-}$ ES cells were transfected with control vector or Flag-tagged NIP45. Lysates were immunoprecipitated with anti-Flag agarose. Resolved proteins were immunoblotted with anti-dimethyl-arginine (asymmetric) antibody. Immunoblots were probed with anti-Flag to determine equal loading.

(D) Th1 and Th2 lysates were immunoprecipitated with monoclonal antibodies against NIP45 or the appropriate isotype control. Immunoblots were probed with a polyclonal anti-dimethyl-arginine (asymmetric) antibody and were reprobed with a polyclonal NIP45 antibody.

using an antibody specific for asymmetrically methylated arginines within RG repeats similar to those found in the amino terminus of NIP45. Methylation of NIP45 was greatly reduced in PRMT1$^{-/-}$ ES cells (Figure 5C, upper panel). To determine whether NIP45 was methylated in Th cells, endogenous NIP45 was immunoprecipitated from Th1 or Th2 cell lysates and immunoblotted with the anti-methylated arginine antibody. NIP45 was methylated in both Th1- and Th2-skewed lysates, and its methylation status did not change after P/I stimulation (Figure 5D, upper panel). We conclude that NIP45 is a substrate of PRMT1, the amino terminus of NIP45 is necessary for methylation by PRMT1, and NIP45 is methylated in vivo.

Association between NIP45 and PRMT1

To examine the specificity of the PRMT1/NIP45 interaction, GST-NIP45 and GST-ΔN-NIP45 fusion proteins

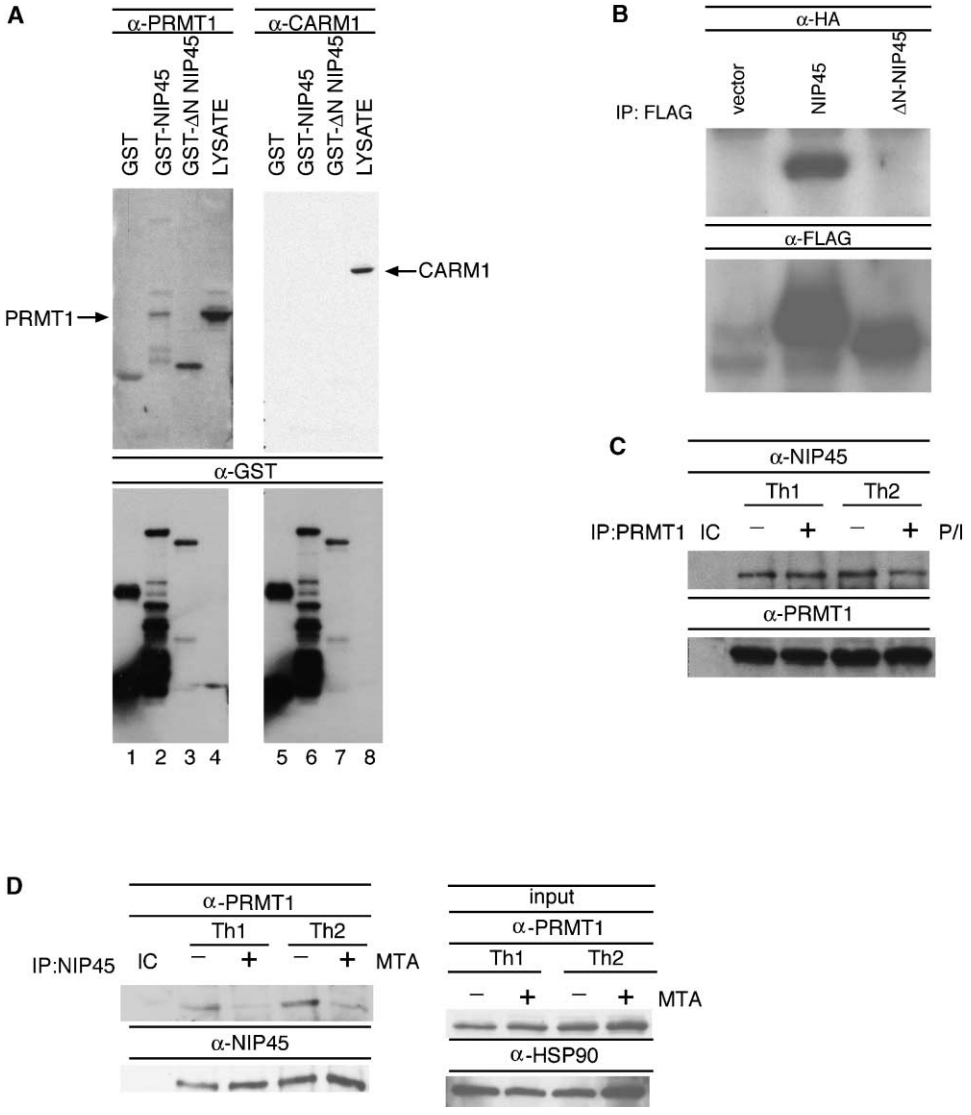

Figure 6. NIP45 and PRMT1 Interact

(A) Jurkat cell lysates were used in pull-down assays with 25 μg of recombinant GST, GST-NIP45, and GST-ΔN-NIP45. PRMT1 or CARM1 in pull-down samples was determined by immunoblotting with anti-PRMT1 and anti-CARM1 antibodies. Immunoblots were reprobed with anti-GST antibodies to determine equal loading.

(B) Flag-NIP45 or Flag-ΔN-NIP45 or vector control and HA-PRMT1 expression constructs were transfected in 293 cells. Lysates were immunoprecipitated with anti-Flag agarose, and interaction with PRMT1 was determined by probing immunoblots with a monoclonal anti-HA antibody and reprobing with anti-Flag.

(C) Lysates from unstimulated or 60 min P/I-stimulated Th1 or Th2 cells were immunoprecipitated with anti-PRMT1 or the appropriate isotype control. Interaction with endogenous NIP45 was determined by immunoblotting with anti-NIP45 monoclonal antibodies and reprobed with anti-PRMT1.

(D) Th1 and Th2 cells were either pretreated with 1 mM MTA for 2 hr or untreated prior to 60 min P/I stimulation. NIP45 immunoprecipitates were tested for PRMT1 association by immunoblotting with anti-PRMT1. Blots were reprobed with anti-NIP45 antisera. Whole-cell extracts were immunoblotted for PRMT1 and HSP90.

were used in pull-down assays. Similar to the in vitro methylation results, PRMT1 but not CARM1 associated with full-length NIP45 (Figure 6A, lanes 2 and 6, upper panel). Furthermore, the interaction between NIP45 and PRMT1 was dependent on the amino terminus of NIP45 (Figure 6A, lane 3). To parallel the pull-down assays, 293T cells were transfected with HA-PRMT1, FLAG-NIP45, and FLAG-ΔN NIP45 mutant. PRMT1 coimmunoprecipitated with full-length NIP45 but not with the amino-terminal deletion mutant (Figure 6B), indicating that not only is this region a potential site of PRMT1 methylation but that it is also necessary for a physical NIP45/PRMT1 interaction. Thus, the selectivity and amino terminus dependence of the NIP45 interaction with PRMT1 in vivo mirrors the specificity observed in the in vitro methylation assays.

To evaluate the physiologic significance of the PRMT1 and NIP45 interaction, we performed coimmunoprecipi-

tation assays using Th1 or Th2 cell lysates unstimulated or stimulated with P/I. Indeed, PRMT1 and NIP45 proteins associate with each other in Th cells, and this association does not change under conditions mimicking TCR stimulation (Figure 6C, upper panel). Additionally, inhibition of methyltransferase activity by MTA abrogated the association between PRMT1 and NIP45, suggesting that this may be one mechanism by which MTA treatment of Th1 and Th2 cells impairs cytokine gene expression (Figure 6D).

The Amino Terminus of NIP45 Is Important for Its Function

Since the amino terminus of NIP45 is methylated and important for interaction with PRMT1, we asked whether the arginine methylation domain of NIP45 within the amino terminus was necessary for NIP45 function as an NFAT cofactor. To determine the association ability of recombinant ΔN-NIP45 and full-length NIP45 with NFAT, pull-down assays with HA-NFATc2-transfected Jurkat cell lysates were performed. The ΔN-NIP45 mutant had reduced NFATc2 binding ability, demonstrating that the amino terminus of NIP45 was important for its interaction with NFAT (Figure 7A). To assess the cofactor function of ΔN-NIP45, Jurkat cells were transfected with different combinations of PRMT1, NFATc2, c-Maf, NIP45, and ΔN-NIP45 to test their effects on IL-4 promoter activity. As above, PRMT1 greatly enhanced NFATc2 and c-Maf IL-4 promoter transactivation (Figure 7B, lanes 4 and 10). Also, as shown previously, NIP45 augmented NFATc2 and c-Maf-induced IL-4 promoter activity (lanes 5 and 6). Not surprisingly, given the reduced ability of ΔN-NIP45 to interact with NFAT, deletion of the NIP45 amino terminus reduced the transactivation ability of NIP45 under unstimulated and P/I stimulation conditions (lanes 7 and 8). To ensure that deletion of the NIP45 amino terminus did not alter its subcellular localization, we expressed ΔN-NIP45 fused to green fluorescent protein in fibroblasts and found that nuclear localization was still intact (data not shown). Thus, the impaired function of ΔN-NIP45 was not due to aberrant cellular localization. When NIP45 and PRMT1 were cotransfected with NFATc2 and c-Maf, reporter activity was not stimulated beyond that seen with just PRMT1, NFATc2, and c-Maf (lanes 10 and 12). However, when ΔN-NIP45 was expressed in conjunction with PRMT1, NFATc2, and c-Maf, the dramatic augmentation of IL-4 promoter activity by PRMT1 was lost (lanes 10 and 14). Therefore, the amino terminus of NIP45 is necessary for potent NIP45-induced augmentation of NFAT transactivation and is responsible for PRMT1 coactivation of the IL-4 promoter. Indeed, NFATc2, NIP45, and PRMT1 form a ternary complex as evidenced by the fact that PRMT1 and NFATc2 association is substantially promoted by the presence of full-length NIP45 but much less so by the presence of ΔN-NIP45 (Figure 7C). These data suggest that the amino terminus of NIP45 may serve a dual function in supporting NFAT interaction and recruiting PRMT1 to the NFAT transcription-activating complex.

Next, we wanted to determine the function of arginine methylation in regulating NIP45 activity. Arginine methylation has previously been shown to regulate subcellular localization and protein-protein interactions. Because ΔN-NIP45 is still localized to the nucleus, it was unlikely that arginine methylation of the amino terminus of NIP45 was necessary for its presence in the nucleus. Since NIP45 binds to NFATc2 and the amino terminus of NIP45 is important for this association (Hodge et al., 1996a), we tested the effects of MTA treatment on the interaction between NIP45 and NFATc2. NIP45 and NFATc2 were coexpressed in 293 cells, which were left untreated or treated with MTA. Inhibition of arginine methylation reduced the association between NIP45 and NFATc2 (Figure 7D), suggesting that the function of arginine methylation of NIP45 is to modulate this interaction.

Discussion

Here we have demonstrated an important role for arginine methylation in controlling TCR-initiated cytokine gene expression. We show that the arginine methyltransferase, PRMT1, is situated downstream of the TCR in progenitor Th cells. Blockade of PRMTs with methyltransferase inhibitors diminished cytokine production. Upon receptor occupancy, TCR-transmitted signals induce PRMT1 to work in concert with Th cell transcription factors, especially members of the NFAT family, to augment cytokine production. One major substrate of PRMT1 is not NFAT itself but rather the NFAT-interacting nuclear protein NIP45, previously shown to greatly enhance NFAT-driven IL-4 production by an unknown mechanism (Hodge et al., 1996a). PRMT1 specifically methylates arginine residues in the amino terminus of NIP45, a modification that facilitates its association with NFAT and hence explains its ability to alter cytokine gene expression.

A contribution of arginine methylation to signal transduction pathways has been previously demonstrated. Levels of cellular arginine methylation rise following NGF stimulation of PC12 cells (Cimato et al., 1997) and lipopolysaccaride (LPS) treatment of B cells (Law et al., 1992). LPS treatment of a murine macrophage cell line induced methylation of the RNA binding protein HuR (Li et al., 2002). PRMT1 also interacts with the IFNAR1 chain of the IFNα/β receptor (Abramovich et al., 1997) and abrogation of PRMT1 expression disrupted the antiproliferative and antiviral abilities of IFNα/β (Abramovich et al., 1997). Our studies are the first to place arginine methylation downstream of TCR signaling pathways. We have uncovered one means—facilitation of protein-protein interaction—through which arginine methylation functions to control cytokine gene expression. However, there may well be additional means by which TCR signaling utilizes arginine methylation as a control mechanism. For example, we have not determined whether the enzymatic activity of PRMT1 or other PRMTs is regulated by TCR stimulation. PRMT1 may itself be subject to posttranslational modifications or its subcellular localization altered by TCR signaling. TCR-induced localization of NFAT or cofactors like NIP45 to actively transcribed cytokine loci may serve to recruit PRMT1 to participate in chromatin remodeling.

In these studies, we have established the NIP45/NFAT interaction as a target for PRMT1 action on the IL-4 promoter. PRMT1 also increased, while MTA inhibited,

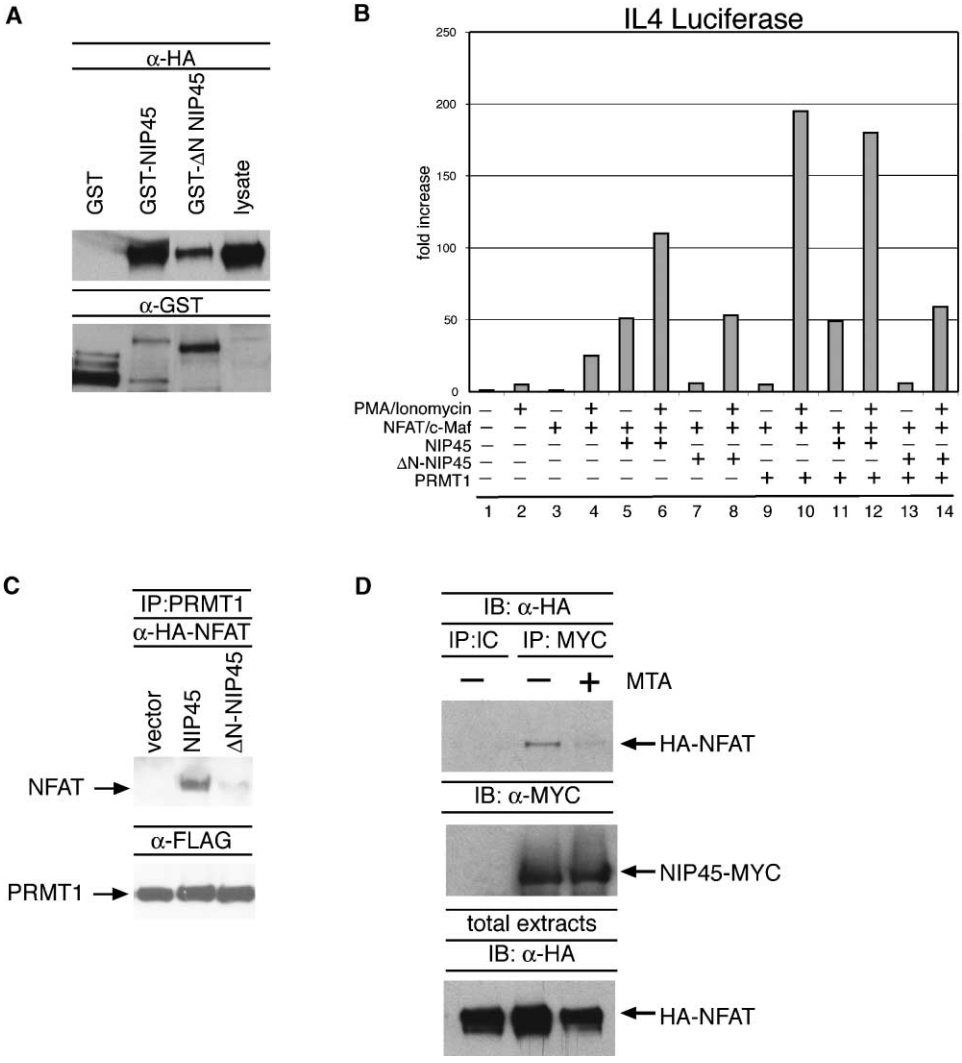

Figure 7. MTA Inhibits NIP45 and NFATc2 Interaction

(A) HA-NFAT transfected Jurkat cell lysates were used in pull-down assays with recombinant GST, GST-NIP45, and GST-ΔN-NIP45. Immunoblots were probed with an anti-HA antibody to detect NFAT and reprobed with anti-GST antibodies.

(B) Jurkat cells were transfected with the IL-4 promoter (2.5 μg) and expression vectors encoding NFATc2 (2.5 μg), c-Maf (2.5 μg), NIP45 (5 μg), ΔN-NIP45 (5 μg), and PRMT1 (10 μg). Cells were treated as in Figure 2A. Luciferase units were normalized to TK-Renilla luciferase activity. Results are representative of at least three independent experiments.

(C) 293 cells were transfected with HA-NFATc2 and Flag-PRMT1 along with vector only, Flag-NIP45, or Flag-ΔN-NIP45. Lysates were immunoprecipitated with anti-PRMT1 antibodies and immunoblotted with anti-HA antibodies to test for NFAT coimmunoprecipitation and reblotted with anti-Flag.

(D) 293 cells were transfected with HA-NFATc2 and NIP45-MycHis expression vectors and left untreated or treated with MTA. Lysates were immunoprecipitated with anti-Myc agarose or isotype control. Immunoblots were probed with anti-HA antibody and reprobed with anti-Myc (9E10) antibody.

IFNγ promoter activity, but to date we have been unsuccessful in revealing an interaction between NIP45 and T-bet. Since the IFNγ gene is also regulated by NFAT (Glimcher and Murphy, 2000; Hodge et al., 1996b; Kiani et al., 2001; Peng et al., 2001), it is possible that IFNγ gene sensitivity to arginine methylation is NFAT/NIP45 mediated. Indeed, all of the cytokines whose expression was altered by inhibition of arginine methylation are known to be regulated by NFATs while the cytokines whose expression was unaffected are not known NFAT target genes. Nevertheless, there are likely to be other partners for NIP45 as well as other substrates for PRMT1 in Th cells. For example, NIP45 also binds to TRAF2, an interaction which represses IL-4 production (Lieberson et al., 2001). One explanation for this reduction may be that unmethylated NIP45 has an increased affinity for TRAF2 over NFAT, resulting in sequestering of NIP45 away from NFAT. It is likely that there are additional targets of PRMT activity in Th cells. Indeed, MTA treatment of NIP45$^{-/-}$ Th cells further reduces the levels of IFNγ and IL-4 transcripts, demonstrating the existence of additional PRMT targets involved in Th cytokine production (K.A.M., unpublished data). Stat1 is a known target of PRMT1 activity in the context of IFNα/β signal-

ing (Mowen et al., 2001), and this may also be true for IFNγ signaling, key in driving Th1 function, in Th cells. Similarly, Stat6, which contains the same conserved arginine residue as Stat1, may be arginine methylated in the context of IL-4 R signaling. Arginine methylation of histones has been correlated with protranscriptional activity (Chen et al., 1999; Ma et al., 2001; Strahl et al., 2001; Wang et al., 2001) although this has not been demonstrated as of yet for cytokine genes. Nevertheless, it is possible that arginine methylation serves to maintain or stabilize the chromatin configuration at cytokine loci.

A number of interesting questions remain about the role of methyltransferases in Th and other cell types. Although we have focused our attention on PRMT1, other PRMTs singly or in concert, may participate in regulating Th cell genes. Arginine methylation may alter cytokine gene expression in other cell types. IFNγ is secreted by CD8 T cells, natural killer cells, and dendritic cells while IL-4 is produced by NK T cells and mast cells. Along these lines, we have recently observed impaired cytokine production from NIP45$^{-/-}$ mast cells as well as from Th cells (K.A.M., unpublished data). It will be important to identify the membrane receptors in non-T cells responsible for inducing arginine methylation as well as to identity the relevant methyltransferases and to investigate the effects of PRMT family members on the immune system by gene targeting or siRNA-mediated knockdown. Unfortunately, we were unable to assess the role of PRMT1 in vivo since neither the available PRMT1$^{-/-}$ ES cell line nor its wt counterpart was able to reconstitute the lymphoid system. Additional attempts at creating knockdown PRMT1 T cell lines also have not been successful, reflecting, perhaps, the obligate role of PRMT1 in cell survival. Finally, the rapid TCR-mediated, CsA-sensitive induction of PRMT1 constitutes an intriguing positive feedback loop model where NFAT upregulates PRMT1 which in turn methylates NIP45 thereby increasing NIP45/NFAT association. Interestingly, like PRMT1, NIP45 is also upregulated in Th1 and Th2 cells (Lieberson et al., 2001), although the signaling pathways responsible are as yet unknown.

Arginine methylation is a complement to known signaling pathways emanating from the TCR that lead to rapid cytokine production in Th1 and Th2 cells. Understanding the role of posttranslational modifications, like arginine methylation, of proteins key in regulating cytokine production will give us novel targets for controlling aberrant cytokine expression in disease states exacerbated by an inappropriate Th1 or Th2 response.

Experimental Procedures

Mice and Cell Culture

BALB/c mice were purchased from Taconic. Th cells were isolated by magnetic bead selection (Miltenyi Biotech). Naive Th cells were sorted by FACS (MoFlo, Becton Dickinson) to obtain CD4$^+$CD62L^{hi} populations. T cells were cultured in RPMI and stimulated with plate-bound 1 μg/ml anti-CD3 (2C11) plus 2 μg/ml anti-CD28 (Pharmingen). For Th1 skewing, 5 ng/ml rIL-12 (Pharmingen) and 10 μg/ml anti-IL-4 (11B11) were added to the primary culture, and for Th2 skewing, 10 ng/ml rIL-4 (Pharmingen) and 10 μg/ml anti-IFNγ (R4/GA2). For P/I stimulations, cells were incubated in 50 ng/ml PMA and 1 mM ionomycin (Calbiochem). MTA (Sigma) was dissolved in culture media. Jurkat cells were grown in RPMI complete media. 293 cells were grown in DMEM. D3H wt ES cells and PRMT1$^{-/-}$ ES cells were the generous gift of H. Earl Ruley (Pawlak et al., 2000).

Plasmids, Transfections, and Luciferase Assays

Expression vectors for NFATc2, c-Maf (Rengarajan et al., 2002a), T-bet (Szabo et al., 2000), and HA-PRMT1 (Koh et al., 2001) were described. pcDNA JunB was a generous gift of T. Jackson and A. Wurster. Flag-tagged PRMT1, PRMT3, CARM1, and PRMT5 expression vectors were the generous gift of Richard B. Gaynor (Kwak et al., 2003). For pcDNA NIP45 Myc-His, NIP45 cDNA was inserted into EcoRI and XhoI sites of pcDNA 3.1A (Invitrogen). Flag-NIP45 was created by excising NIP45 cDNA with EcoRI and XhoI from pcDNA NIP45 Myc-His and ligated into EcoRI and SalI sites of pFlagCMV 6c (Sigma). Flag-ΔN-NIP45 was created by amplifying from pcDNA NIP45 Myc-His to delete amino acids 1–32 and inserted into EcoRI and SalI sites of pFlag CMV6c. Oligonucleotides were as follows: 5′-GATTCCAGGCTCATTCCAGACACCG-3′ and 5′-CTCGAGTCAGCCCCAGACTTCGATG-3′. pGEX-NIP45 and pGEX-ΔN-NIP45 were generated by ligating EcoRI and XhoI excision and PCR products as in above into EcoRI and XhoI sites of pGEX-4T-1 (Amersham). The IL-4 and IFNγ luciferase reporters were described (Szabo et al., 2000). pNFAT-luciferase (4× NFAT) was obtained from Stratagene. Jurkat cells were transfected using a BioRad electroporator (280 V, 975 μF, 5×10^6 cells/0.4 ml). Thymidine kinase promoter-driven Renilla luciferase was used as an internal control. Luciferase activity was determined using Promega's Dual Luciferase Kit. 293T cells were transfected using Effectene (Qiagen).

RNase Protection Assay and Northern Analysis

RNA was prepared using RNeasy (Qiagen). Cytokine RNA levels were analyzed using the RiboQuant multiprobe kit, mck-1, (Pharmingen). For Northern blots, hybridization was performed in Ultra-Hyb (Ambion) using ^{32}P-labeled Prmt1 (created by EcoRI/BamHI excision of pSG5 HA-Prmt1), and β-actin cDNA was a generous gift of S. Miaw.

Intracellular Cytokine Staining and FACS Analysis

Intracellular cytokine staining was performed as described (Szabo et al., 2000) and analyzed using a FACSCalibur.

Antibodies, Immunoprecipitations, and Immunoblots

For whole-cell lysates, lysis buffer containing 20 mM HEPES (pH 7.4), 1% TX-100, 100 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, 1 mM PMSF, 1 mM vanadate, and complete protease inhibitor tablets (Roche) was used. For coimmunoprecipitation experiments, lysis buffer containing 100 mM NaCl, 50 mM TRIS (pH 7.5), 1 mM EDTA, 0.1% TX-100, 10 mM NaF, 1 mM PMSF, 1 mM vanadate, and complete protease inhibitor tablets was used. Antibodies used for immunoprecipitations were as follows: anti-Flag agarose (Sigma) for Flag-NIP45, Flag ΔN-NIP45 Myc His, Flag-PRMT1, Flag-PRMT2, Flag-PRMT3, Flag-CARM1, and Flag-PRMT5, anti-HA agarose (Santa Cruz) for HA-NFAT, anti-His agarose (Santa Cruz) for T-bet, anti-c-Maf, and anti-PRMT1 (Santa Cruz). Primary antibodies were: anti-phospho p44/42 (Cell Signaling), monoclonal anti-PRMT1 (Abcam), polyclonal anti-PRMT1 (Santa Cruz), anti-PRMT2 (Imgenex), anti-PRMT3 (Upstate), anti-CARM1 (Upstate), anti-PRMT5 (Imgenex), anti-PRMT6 (Imgenex), anti-HSP90 (Santa Cruz), anti-Flag (Sigma), anti-HA (Babco), anti-His (Sigma), anti-c-Maf (Santa Cruz), anti-dimethyl-arginine, asymmetric (ASYM24) (Upstate), and anti-GST (Calbiochem). NIP45 antibodies were described previously (Lieberson et al., 2001).

Protein Purification and Pull-Down Assays

Recombinant GST-CARM1, GST-PRMT1, GST-NIP45, and GST-ΔN-NIP45 were prepared as described (Mowen and David, 2001). GST-PRMT1 was thrombin (Amersham) cleaved and concentrated using Micron 10 filter device (Millipore). PRMT1-His was prepared the same except Ni beads (Invitrogen) were used. For pull-downs, Jurkat cells were lysed in 20 mM HEPES (pH 7.4), 0.1% NP-40, 50 mM NaCl, 50 mM NaF, 10 mM β-glycerophosphate, and 1 mM PMSF and diluted to 1 mg/ml. A 50% slurry of GSH beads and 25 μg bait were added to 1 ml of Jurkat whole-cell lysates and incubated overnight at 4°C.

In Vitro Methylation Assays

For transcription factor methylation assays, immunoprecipitates were washed four times with lysis buffer and once with methylation reaction buffer (20 mM Tris [pH 8], 200 mM NaCl, 0.4 mM EDTA) and resuspended in methylation reaction buffer, 1 μg GST-PRMT1, and 6 μM S-adenosyl-[*methyl*-^{3}H]methionine. Reactions were incubated for 90 min at 30°C and stopped with SDS sample buffer. SDS-PAGE gels were fixed with 10% acetic acid/10% methanol, washed, and incubated with Amplify (Amersham) before exposing to Hyperfilm MP at −80°C. Methylation reactions with immunoprecipitated PRMTs were performed using 5 μg of glutathione-eluted GST-NIP45 as a substrate. Methylation reactions with GST-NIP45, GST-ΔN-NIP45, PRMT-His, and GST-CARM1, reactions were performed with 15 μg of glutathione-eluted substrate and 5 μg of bead-bound enzyme. Reactions were resolved by SDS-PAGE in 10% NuPAGE Bis-Tris gel (Novex) with the MOPS running buffer. Gels were transferred to PVDF, blocked in 5% non-fat dry milk, and soaked for 30 min in Amplify. The blot with Whatman paper soaked in Amplify behind it was exposed to Hyperfilm MP.

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