Asymmetric arginine dimethylation of RelA provides a repressive mark to modulate TNFα/NF-κB response

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Edited by George R. Stark, Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH, and approved March 10, 2016 (received for review November 14, 2015)

Nuclear factor kappa B (NF-κB) is an inducible transcription factor that plays critical roles in immune and stress responses and is often implicated in pathologies, including chronic inflammation and cancer. Although much has been learned about NF-κB–activating pathways, the specific repression of NF-κB is far less well understood. Here we identified the type I protein arginine methyltransferase 1 (PRMT1) as a restrictive factor controlling TNFα-induced activation of NF-κB. PRMT1 forms a cellular complex with NF-κB through direct interaction with the Rel homology domain of RelA. We demonstrate that PRMT1 methylates RelA at evolutionary conserved R30, located in the DNA-binding L1 loop, which is a critical residue required for DNA binding. Asymmetric R30 dimethylation inhibits the binding of RelA to DNA and represses NF-κB target genes in response to TNFα. Molecular dynamics simulations of the DNA-bound RelA:p50 predicted structural changes in RelA caused by R30 methylation or a mutation that interferes with the stability of the DNA–NF-κB complex. Our findings provide evidence for the asymmetric arginine dimethylation of RelA and unveil a unique mechanism controlling TNFα/NF-κB signaling.

arginine methylation | genes | TNFα | NF-κB

Immune responses are often associated with inflammation that evolved in higher organisms as a defense mechanism to protect them from infection and tissue injury (1); however, inappropriate control of inflammatory responses may give rise to chronic inflammation that leads to various pathologies, including cancer (2). Tumor necrosis factor α (TNFα) is a major proinflammatory cytokine that controls systemic inflammation and the acute-phase reaction. TNFα activates a rapid transcription of genes regulating inflammation, primarily through activation of nuclear factor kappa B (NF-κB) (3). Some of these genes act to suppress TNFα-induced apoptosis, whereas others promote inflammation (4). Thus, the outcome of inflammatory response downstream of TNFα signaling is largely dependent on the genetic program regulated by NF-κB.

NF-κB is a family of transcription factors formed by homodimerization or heterodimerization of related Rel proteins, including RelA, p105/p50, p100/p52, c-Rel, and RelB (5). Among these, RelA- and c-Rel–containing NF-κB dimers are engaged mainly downstream of the canonical signaling induced by TNFα. Treatment of cells with TNFα leads to activation of the IκB kinase (IKK) complex, resulting in phosphorylation and subsequent degradation of IκB proteins, which normally restrain inactive NF-κB in the cytoplasm (6). This promotes the accumulation of NF-κB in the nucleus, where it binds to κB DNA consensus sequences and activates target genes. Although NF-κB is regulated by complex formation with IκBs (7) and controlled nuclear-cytoplasmic shuttling (8, 9), additional mechanisms are needed to ensure the proper specificity, magnitude, and timing of NF-κB responses. Those include the interactions of NF-κB with various cofactors and heterologous DNA-binding proteins and various posttranslational modifications of Rel proteins and histones surrounding NF-κB target genes (10, 11).

Over the past decade, multiple covalent modifications of the transactivating RelA subunit, including phosphorylation, acetylation, O-linked β-*N*-acetylglucosamination, sumoylation, nitrosylation, and degradation-promoting ubiquitination, have been described to regulate transcriptional activity, DNA binding, and target gene specificity of NF-κB (11). Recent work of several laboratories has identified methylation as another regulatory RelA modification that controls gene expression downstream of TNFα and IL-1β signaling. Several histone lysine methyltransferases, including SET7/9, SETD6, and NSD1, have been shown to both positively and negatively regulate NF-κB through direct monomethylation or dimethylation of distinct lysine residues in RelA (12–15). Recent studies also have shown that RelA is methylated by the type II protein arginine methyltransferase 5 (PRMT5), which catalyzes the synthesis of ω-N^G-monomethylarginine (MMA) and symmetric ω-N^G,N'^G-dimethylarginine (SDMA) (16, 17).

The molecular aspects and functional consequences of RelA lysine methylation have been studied extensively, whereas the regulation of NF-κB by arginine methylation is just beginning to emerge. In addition to PRMT5, several type I PRMTs, including PRMT1, PRMT2, PRMT4, and PRMT6, have been shown to coregulate NF-κB target genes (18–22); however, it remains unclear whether Rel proteins are also modified by type I PRMTs that methylate arginines asymmetrically, thereby leading to the formation of asymmetric ω-N^G,N^G-dimethylarginine (ADMA).

Accumulating data indicate that both PRMT5 and a group of asymmetric type I PRMTs, in particular PRMT1, frequently share common recognition sequences and can compete for the same arginine residues. The interplay between type I and II arginine methylation is well documented in transcriptional regulation. Asymmetric dimethylation of histone H4R3 by PRMT1 has been linked to the activation of genes (23), whereas symmetric

Significance

The transcription factor nuclear factor kappa B (NF-κB) plays a vital role in cellular immune, stress, and proliferative responses by regulating genes. To maintain correct execution of the genetic program and to prevent pathologies (e.g., chronic inflammation), the activation of NF-κB has to be transient and precisely controlled. We describe a unique inhibitory mechanism that mediates NF-κB–dependent regulation of inflammation-associated genes and uses arginine methylation of the RelA subunit of NF-κB by protein arginine methyltransferase 1. This modification reduces the DNA-binding affinity of RelA, altering the recruitment to gene promoters and the transcriptional potency of NF-κB. This may be developed into an approach to inhibit the pathological functions of NF-κB.

Author contributions: T.V. designed research; A.R., J.E.F., L.K., and T.V. performed research; L.K., H.H.L., K.R.L., L.A.H., and T.V. analyzed data; and L.A.H. and T.V. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1522372113/-/DCSupplemental.

dimethylation of H4R3 by PRMT5 is associated with repression (24). In addition to histones, both types of arginine methylation regulate other proteins involved in the initiation and elongation steps of transcription. Similar to histone H4R3 methylation, PRMT1 and PRMT5 antagonize each other and differentially regulate E2F-1 by producing asymmetrically and symmetrically dimethylated forms of E2F-1, respectively (25). Transcriptional elongation factor SPT5 also can be methylated at a single arginine residue in its RNApol II-binding domain by PRMT1 and PRMT5, resulting in inhibition of the elongation-promoting activity of SPT5 at the promoters of interleukin-8 (*IL-8*) and NF-κB inhibitor α (*NFKBIA*) genes (26).

Given that type I and II PRMTs often share common substrates, it is possible that, along with providing symmetric dimethylation, methyl groups can modify RelA asymmetrically as a part of complex and context-specific NF-κB regulation. Here we present evidence that the RelA subunit of NF-κB is a direct target of PRMT1. We demonstrate that PRMT1 forms a cellular complex with RelA and asymmetrically dimethylates the conserved R30 residue in the RelA DNA-binding L1 loop. The asymmetric dimethylation of RelA by PRMT1 is enhanced after prolonged treatment of cells with TNFα and, in contrast to the symmetric modification (16, 17), negatively regulates the DNA binding and transcriptional activity of RelA. We also find that down-regulation of PRMT1 enhances the expression of NF-κB target genes after TNFα stimulation by facilitating RelA recruitment to specific promoters. Taken together, our findings reveal a previously unidentified posttranslational modification of RelA, namely asymmetric arginine dimethylation, which regulates NF-κB in response to TNFα.

Results

The Rel Homology Domain of RelA Interacts with PRMT1 and Is a Substrate for Arginine Methylation. Given that type I PRMTs are known to control NF-κB target genes (18–22), we investigated whether Rel proteins are modified by asymmetric arginine dimethylation. We tested PRMT1, PRMT4, and PRMT6, all of which are present in the nucleus and modify histones, for their ability to methylate the RelA subunit of NF-κB. The bacterially expressed His-Rel homology domain (RHD) of RelA (amino acids 1–306) was incubated with affinity-purified GST-PRMTs in the presence of ^{3}H-S-adenosine-methionine (^{3}H-SAM) as a donor of methyl groups (Fig. 1*A*). Calf thymus core histones were used to control PRMT activities. Among the PRMTs tested, only PRMT1 was able to specifically methylate the RHD of RelA in vitro.

To obtain further evidence that RelA is targeted by PRMT1, we tested the associations between these proteins in vivo and in vitro. For this, HEK 293T cells were cotransfected with myc-RelA and His-myc-PRMT1 or control expression vectors, and PRMT1-specific protein complexes were purified using nickel-nitrilotriacetic acid (Ni-NTA) agarose. As shown in Fig. 1*B*, RelA was specifically copurified with PRMT1, suggesting complex formation between these proteins in vivo. To assess which region in RelA interacts with PRMT1, we used C-terminal deletion mutants of RelA lacking the linker region and/or the transactivation domain (TAD) (Fig. 1*B*, *Lower*). Analysis of RelA mutants revealed that the deletion of linker and/or TAD did not impair binding to PRMT1-specific cellular complexes. Consistent with these results, bacterially expressed GST-PRMT1 interacted with the RHD of RelA in an in vitro GST pull-down assay (Fig. 1*C*). This indicates a direct interaction between the N-terminal RHD of RelA and PRMT1. The association between RelA and PRMT1 was detected in unstimulated cells, and the level of coimmunoprecipitated PRMT1 was increased on TNFα treatment (Fig. 1*D*). We conclude that this interaction is dynamic and might be regulated in a stimulus-dependent manner.

PRMT1 Methylates RelA at Evolutionary Conserved R30. To identify putative PRMT1 consensus sites, we analyzed the protein sequence of RelA with two web-based bioinformatics tools, MeMo 2.0 (27) and MASA 1.0 (28). MeMo uses a support vector machine (SVM) algorithm, whereas MASA combines SVM with structural characteristics of proteins to identify methylation sites. In silico analysis of

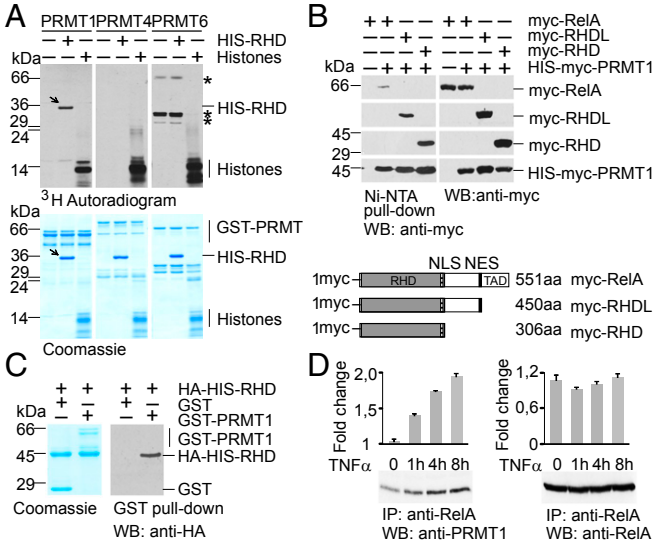

Fig. 1. PRMT1 interacts with the RelA subunit of NF-κB and methylates the RHD of RelA. (*A*) Recombinant PRMTs were incubated with histones or His-RelA (amino acids 1–306) in the presence of ^{3}H-SAM, resolved by SDS/PAGE, and analyzed by autoradiography. Arrows indicate methylated RelA; asterisks represent automethylated PRMT6 peptides. (*B*) PRMT1 forms a complex with RelA in vivo. (*Upper*) Full-length and deletion variants of RelA were expressed in HEK 293T cells and copurified with ectopic His-PRMT1. (*Lower*) The variants of RelA are shown schematically. The RHD, TAD, and nuclear import (NLS) and export (NES) signals are indicated. (*C*) PRMT1 physically binds to the RHD of RelA. In vitro GST pull-down assays were performed using the HA-His-RHD with GST or GST-PRMT1 purified from bacteria. The precipitated proteins were analyzed by immunoblotting with anti-HA antibody. (*D*) HEK 293T cells were stimulated with 20 ng/mL of TNFα for the indicated times. Endogenous RelA complexes were immunoprecipitated from the cell lysates and analyzed for the presence of PRMT1. Intensities of the immunoblot signals were quantified using ImageJ 1.49.

the RelA sequence by MeMo revealed four putative consensus sites for arginine methylation (Fig. S1*A*, *Left*): R30, R35, and R236, located within the RHD of RelA, and R330, located outside of the RHD. Analysis with MASA identified R30, R149, R187, and R236 as potential modification sites with an SVM score above the threshold of 0.5 (Fig. S1*A*, *Left*). To increase the stringency of prediction, we excluded R35, R149, R187, and R330, because they were confirmed by only one of these programs. Further analysis using MASA also excluded R236, because it was characterized by a low value of the solvent-accessible surface area surrounding the predicted methylation site (Fig. S1*A*, *Left*). Thus, the use of different prediction algorithms identified R30 as a prominent residue potentially methylated in RelA.

Interestingly, the same residue has been identified previously in vivo symmetrically dimethylated by PRMT5 (16), further supporting our in silico prediction. This prompted us to investigate whether R30 resides within a more general methylation consensus sequence that could be methylated by PRMT1. Sequence alignment revealed that R30 of RelA is highly conserved throughout all Rel proteins, including the viral oncoprotein v-Rel (Fig. S1*A*, *Right*). Moreover, the R30 residue and its surrounding RxxRxRxxC motif are evolutionarily conserved in species ranging from the hemichordate worm *Saccoglossus kowalevskii* to *Homo sapiens*. Dorsal, the *Drosophila* homolog of RelA, also appears to have a putative methylation site (albeit a lysine) in the same position.

We next performed LC-ESI-MS/MS analysis to identify PRMT1-specific methylation sites in RelA ex vivo. His-RelA was ectopically expressed alone or coexpressed with PRMT1 in HEK 293T cells followed by affinity purification using Ni-NTA agarose (Fig. S1*B*). MS analysis revealed a dimethylation-specific 28-Da mass shift detected at R30 in the ^{21}VEIIEQPKQRGM^{32} peptide (Fig. 2*A*). To further verify the R30 methylation by PRMT1, each

or all arginine residues present in RxxRxRxxC motif were substituted with lysine. A single point mutation at R30 (R30K) or a triple mutation at R30, R33, and R35 (RTK) completely abolished the His-RelA peptide (amino acids 1–107) methylation by PRMT1 (Fig. 2*B*), whereas single-substitution R33K or R35K had no detectable effect. Analysis of the crystal structure of RelA:p50 dimer bound to the κB DNA sequence of the IFNβ promoter (29) confirmed that R30 is located in the exposed DNA-binding L1 loop region of RelA, whereas R33 and R35 are less sterically accessible for methylation (Fig. S2). Similarly, methylation of the RG motif in the context of the RGxRxR sequence by PRMT1 has been shown for dishevelled 3 (Dvl3) (30).

To investigate R30 methylation in RelA in vivo, we generated antibodies that specifically recognize asymmetric dimethylation at R30 (Fig. S3 *A* and *B*). As shown in Fig. 2*C*, the methylation-specific antibodies immunoprecipitated ectopically expressed wild-type RelA protein but not its mutated R30A version, indicating asymmetric dimethylation of R30 in vivo as well. Furthermore, in vivo experiments revealed that R30 methylation could be stimulated by TNFα, suggesting a possible regulatory role of this modification in agonist-induced NF-κB responses.

PRMT1-Targeted R30 Is Critical for the DNA-Binding and Transactivation Function of RelA. The N-terminal RxxRxRxxC motif of RelA is a functional element of the DNA-binding L1 loop that provides a DNA sugar-phosphate backbone and base-specific contacts (31, 32). Although R30 does not directly contact DNA (Fig. S2), it is located within the RxxRxRxxC motif of the L1 loop in close proximity to the DNA-contacting R33 and R35. This prompted us to investigate whether PRMT1-targeted R30 is important for the function of the L1 loop. For this, the wild-type RHDs of RelA, R30K, and RTK mutants were purified from *Escherichia coli* and analyzed for their ability to bind DNA by an electrophoretic mobility shift assay (EMSA) and a fluorescence-based thermal-shift assay. Although the R30K substitution did not change the positive charge at this position, the mutation decreased the thermal stability of the RHD and inhibited DNA binding substantially (Fig. 3*A* and Fig. S4). Inhibition was also observed for the RTK mutant, in which R30 and two other critical DNA-binding residues were mutated.

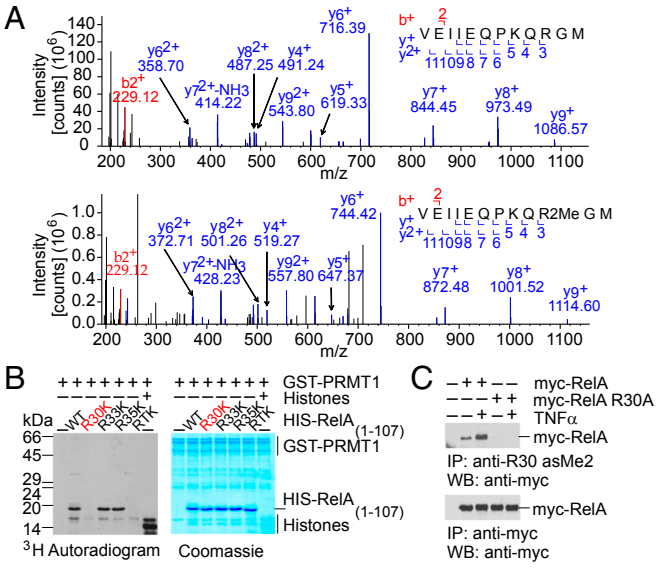

Fig. 2. PRMT1 methylates RelA at R30 located within the DNA-binding L1 loop of the RHD. (*A*) Human RelA was analyzed by MS for the presence of methylarginine residues. Shown are MS/MS spectra of the nonmethylated (*Upper*) and dimethylated (*Lower*) peptide ^{21}VEIIEQPKQRGM^{32} containing R30. (*B*) In vitro methylation assay with GST-PRMT1 and wild-type or mutant RelA (amino acids 1–107) in the presence of ^{3}H-SAM. The RTK mutant harbors substitutions of R30, R33, and R35 to lysine. (*C*) TNFα stimulates RelA methylation at R30 in vivo. HEK 293T cells expressing myc-RelA or myc-RelA R30A were stimulated with TNFα for 8 h, and RelA methylation was analyzed by immunoprecipitation with anti-R30 asMe2, followed by immunoblotting using anti–c-myc (9E10) antibodies.

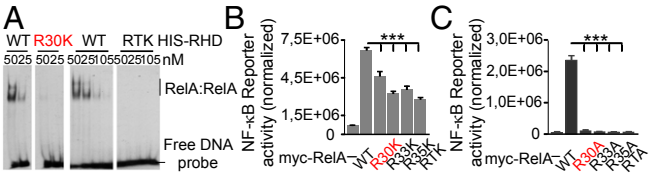

Fig. 3. R30 mutations inhibit the DNA-binding and reporter activity of RelA. (*A*) EMSA of wild-type and mutated RelA proteins using the κB consensus oligonucleotide. Distinct parts of the same gel are separated by white spaces. (*B* and *C*) HEK 293T cells were transfected with 2×(κB)-luciferase reporter and wild-type or mutated myc-RelA constructs. The luciferase activity is presented as normalized mean values ± SD of triplicates. RTA and RTK possess substitutions of R30, R33, and R35 to alanine and lysine, respectively.

Given that DNA binding is important for the transcriptional activity of RelA, we assumed that the R30 mutation might affect RelA-dependent transcription. To test this, the mutant RelA proteins R30K, R33K, R35K, or RTK or similar mutants possessing R-to-A substitutions were transiently expressed in HEK 293T cells and analyzed by luciferase assays (Fig. 3 *B* and *C* and Fig. S5*A*). Consistent with our EMSA results, the R30K mutation decreased RelA transcriptional activity compared with the wild-type protein (Fig. 3*B*). A similar suppression of RelA was caused by R33K, R35K, and RTK mutations. Moreover, substitution of R30 to alanine, which is associated with the loss of a positive charge, resulted in almost complete inhibition of RelA reporter activity (Fig. 3*C*). Comparable inhibition of the reporter was observed for the R33A, R35A, and RTA mutants as well. These data reveal that, similar to R33 and R35, the R30 residue is structurally and functionally important for the RelA DNA binding and transcriptional activity.

Methylation of RelA by PRMT1 Represses DNA Binding and Reporter Activity of NF-κB. Because R30 is critical for the L1 loop function, we studied whether RelA methylation by PRMT1 affects the binding of NF-κB to DNA and transcriptional activity. For this, purified His-RHD of RelA was incubated with recombinant GST-PRMT1 in the presence or absence of SAM. Both samples were verified for equal amounts of RelA protein and then tested for DNA binding by EMSA (Fig. 4*A* and Fig. S5*B*). The analysis showed that methylated RelA had lower affinity to a κB consensus oligonucleotide compared with that of nonmethylated protein.

To obtain more molecular details on how the R30 methylation affects RelA DNA binding, we performed molecular dynamics simulations of the DNA-bound RelA:p50 dimer. We found that monomethylation and asymmetric R30 dimethylation preserved the DNA-protein complex over 100 ns but altered local hydrogen bonding (Fig. 4*B*); for instance, a loss of free H-bond donor functions was observed for the R30 amino acid side chain on monomethylation and asymmetric dimethylation. This predicted a reduction in H-bond formation with acceptor residues, such as E279, T191, and E193. In contrast, H bonding of R30 with D277 and K28 residues was enhanced on methylation. The replacement of R30 by alanine, a mutation inhibiting RelA (Fig. 3*C*), was also found to affect H bonding similar to that observed for methylation.

To evaluate the effect of RelA methylation on NF-κB activity, we established HEK 293T cells with stable expression of PRMT1-specific shRNA or control shRNA targeting GFP mRNA. Cells expressing shRNA against PRMT1 were characterized by a 70–80% decrease in PRMT1 mRNA and protein levels compared with cells transduced with the control virus (Fig. S6*A*). The methylation of ectopic RelA was substantially reduced (~90%) on the silencing of PRMT1 (Fig. 4*C*), suggesting that PRMT1 is the major methyltransferase mediating RelA asymmetric dimethylation at R30 in vivo.

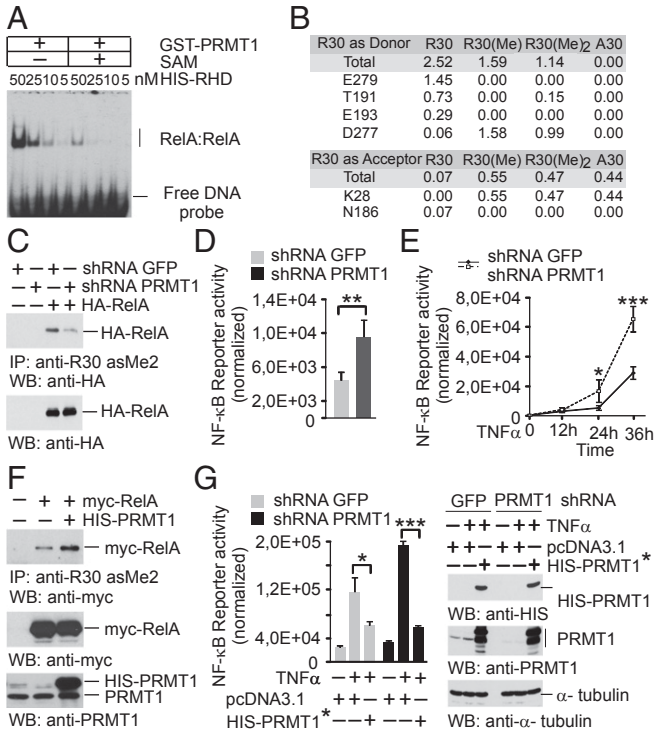

Fig. 4. RelA methylation by PRMT1 inhibits the DNA-binding and transcriptional activity of NF-κB. (*A*) EMSA with nonmethylated and in vitro methylated RelA was performed as shown in Fig. 3*A*. (*B*) Hydrogen-bonding occupancy of nonmethylated, monomethylated, and asymmetrically dimethylated R30 and A30 in molecular dynamics simulations. (*C*) HEK 293T cells expressing shRNAs were transfected with control vector or an HA-RelA construct. RelA methylation was analyzed as in Fig. 2*C*, followed by immunoblotting using anti-HA antibody. (*D*) Control and PRMT1 knockdown HEK 293T cells were transfected with 2×(κB)-luciferase reporter. Luciferase activity was measured in cell lysates and normalized to protein content. (*E*) Cells were transfected with the NF-κB reporter and stimulated with 20 ng/mL TNFα for 40 min. Luciferase activity was measured at the indicated time points. (*F*) HEK 293T cells were cotransfected with myc-RelA and His-PRMT1–expressing plasmids. R30 methylation was analyzed as above. (*G*) Cells were transfected with control or shRNA-resistant His-PRMT1* constructs. (*Left*) Luciferase assays were performed as described in *E*. (*Right*) The expression of His-PRMT1 was controlled by immunoblotting. Values are means ± SD of triplicates. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

We next studied the effect of PRMT1 depletion on NF-κB activity by using a 2×(κB)-luciferase reporter. PRMT1-silenced cells showed elevated levels of the basal, TNFα-induced, or RelA-induced expression of luciferase compared with the control cells (Fig. 4 *D* and *E* and Fig. S6*B*). The ectopic expression of shRNA-resistant PRMT1 had opposite effects and decreased the activity of NF-κB in control and PRMT1-depleted cells. This correlated with an enhanced asymmetric dimethylation of R30 observed on overexpression of PRMT1 (Fig. 4 *F* and *G*). The inhibition of NF-κB activity was also seen in $RelA^{-/-}$ mouse embryonic fibroblasts (MEFs) when the ectopic wild-type RelA was coexpressed with PRMT1. The mutation of R30 to lysine produced similar repression of NF-κB independent of PRMT1 expression (Fig. S6*C*). We confirmed these findings by using an alternative NF-κB reporter that contained the κB element from the *CXCL10* gene promoter located upstream of the luciferase coding sequence (Fig. S6*D*). Taken together, these results indicate that PRMT1 inhibits NF-κB reporter activity in vivo, consistent with the lower DNA binding observed for methylated RelA in vitro.

PRMT1 Regulates NF-κB Target Genes in Response to TNFα. To address the physiological role of PRMT1-mediated RelA methylation, we compared basal and TNFα-induced expression of NF-κB target genes in control and PRMT1 knockdown cells. On silencing of PRMT1, the overall expression of *NFKBIA, TNFα*, and TNFα-induced protein 3 (*A20*) mRNAs was enhanced in response to TNFα (Fig. 5*A* and Fig. S6*E*), suggesting that PRMT1 down-regulates NF-κB target genes. Interestingly, *NFKBIA* expression was affected by PRMT1 deficiency only at later time points of TNFα stimulation, whereas activation of *TNFα* and *A20* genes was enhanced throughout the entire treatment in PRMT1-depleted cells. This finding is consistent with the differential regulation of NF-κB target genes by a PRMT1-dependent mechanism that may act in a promoter-specific manner. We did not find any significant difference in the basal expression of tested genes between the control and knockdown cells. This suggests that in the context of native chromatin, PRMT1 represses the agonist-induced transcriptional activity of NF-κB rather than critically controlling the overall inactive state of this transcription factor.

PRMT1 Dynamically Associates with TNFα-Inducible Promoters of NF-κB Target Genes and Counteracts RelA Binding. To gain further insight into RelA regulation by asymmetric dimethylation, we examined detailed R30 methylation kinetics in response to TNFα (Fig. 5 *B* and *C*). As before, normally growing cells showed weak asymmetric dimethylation of RelA; treatment with TNFα had little or no effect on R30 methylation at early time points, but a substantial increase was observed after 8 h. Methylation of R30 was absent or reduced when the PRMT1 expression was diminished, however (Fig. 5*C*). The enhanced methylation of R30 was correlated with a rapid decrease in the amount of RelA bound to the *NFKBIA* and *TNFα* promoters and coincided in time with the termination of TNFα response (Fig. 5 *A* and *D* and Fig. S7*A*). This effect is consistent with the inhibition of RelA DNA binding by PRMT1-mediated methylation observed in our in vitro and in vivo studies (Fig. 4*A* and Fig. S7*B*), implying that PRMT1 may negatively control NF-κB recruitment to genes activated by TNFα.

To investigate this, we analyzed the binding of RelA or PRMT1 to κB regions of *NFKBIA* and *TNFα* genes in control and PRMT1 knockdown cells treated with TNFα. As expected, there was little binding of RelA to the *NFKBIA* and *TNFα* promoters under basal conditions, and the level of chromatin-bound RelA increased rapidly in the response of control cells to TNFα (Fig. 5*D*). Promoter occupancy by RelA remained high during the first 4 h of TNFα treatment, and then declined to almost basal levels after 24 h. In contrast, PRMT1 was found to reside on the *NFKBIA* promoter even before treatment, and its binding decreased gradually following TNFα stimulation. PRMT1 binding to *TNFα* differed from that seen for *NFKBIA*. After stimulation, we observed rapid clearance of PRMT1 from the *TNFα* promoter, followed by its reappearance after 4 h that coincided in time with NF-κB inactivation. Inducible RelA recruitment to *NFKBIA* was seen in knockdown cells as well, but PRMT1 depletion decreased the dissociation of RelA from the DNA after 12 h and 24 h of TNFα treatment. This was accompanied by a lower abundance of PRMT1 at the promoter compared with control cells. The PRMT1 signal detected in knockdown cells originated from the remaining protein, as shown in Fig. S6*A*.

Reduced PRMT1 occupancy on *TNFα* correlated with enhanced RelA binding to the κB region under both basal and stimulated conditions, suggesting that PRMT1 might control DNA binding of NF-κB in untreated cells as well. The binding of RelA was not sufficient to activate *TNFα* in the absence of stimulus but was correlated with a greater *TNFα* transcription, observed already at the early time points of stimulation (Fig. 5 *A* and *D*). These data suggest counteraction between the PRMT1 promoter occupancy and RelA binding to the regulatory elements of several NF-κB target genes.

Discussion

Previous studies have implicated a critical role of IκBα degradation/resynthesis in the control of NF-κB. IκBα inhibits the DNA binding of NF-κB and promotes the nuclear export of IκBα–NF-κB complexes, providing negative feedback regulation

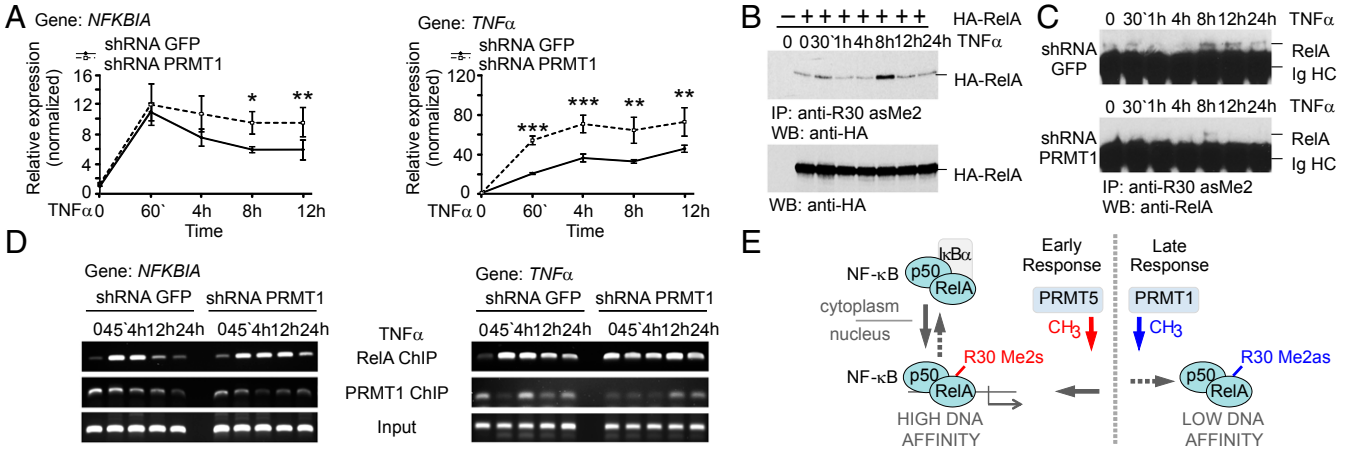

Fig. 5. PRMT1 regulates NF-κB target genes in response to TNFα. (*A*) *NFKBIA* and *TNFα* gene-specific mRNAs were quantified at the indicated time points after TNFα stimulation using qRT-PCR and the comparative ddCt method. Values are means ± SD of at least three experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. (*B* and *C*) The time course of methylation of ectopic (*B*) or endogenous RelA (*C*) in response to TNFα was analyzed as in Fig. 2*C*, followed by immunoblotting using anti-HA or anti-RelA (C20) antibodies, respectively. (*D*) ChIP analysis of promoter occupancy by RelA and PRMT1 in TNFα-treated control and PRMT1 knockdown cells. (*E*) Model for PRMT1-mediated regulation of NF-κB. In addition to IκB-mediated repression, NF-κB is negatively regulated by methylation of the RelA subunit that is catalyzed by PRMT1. Asymmetric R30 dimethylation in RelA inhibits the transcription of TNFα-induced genes by reducing the DNA binding of NF-κB. Symmetric R30 dimethylation, reported previously by Wei et al. (16), is seen as an NF-κB–activating mark. It is postulated that symmetric and asymmetric RelA dimethylation may represent a specific on/off switch mechanism modulating cytokine-induced NF-κB responses.

(7). Other mechanisms target mainly late NF-κB responses and include interactions of the RelA subunit with corepressor proteins, such as Twist1/2 (33), PIAS1 (34), and IκBζ (35). These regulatory mechanisms also include covalent modifications of RelA and its coregulators, such as IKKα-induced phosphorylation of RelA and PIAS1 (34, 36) or PDLIM2-, COMMD1-, and SOCS1-dependent RelA ubiquitination (37, 38), as well as the proteasomal degradation of promoter-bound NF-κB (39). The presence of multiple NF-κB–deactivating mechanisms suggests that the inhibitory function of IκBα is required but not sufficient for the broad range of NF-κB regulation.

Here we report a unique inhibitory mechanism that regulates NF-κB target genes in response to TNFα. This mechanism involves the asymmetric dimethylation of RelA by PRMT1 (Fig. 5*E*), which inhibits the binding of NF-κB to gene promoters, thereby reducing its transcriptional activity. To explain this effect at the molecular level, we first identified R30 as a PRMT1 methylation site located within the RxxRxRxxC motif of the RelA DNA-binding L1 loop. We then showed that R30 is an important structural residue that not only constitutes a part of the L1 loop, but also critically contributes to its DNA-binding function. Using recombinant RelA proteins purified from *E. coli*, an organism that lacks arginine methylation, we showed that the R30K mutation per se destabilizes the RHD and inhibits DNA binding (Fig. S4). We concluded that these effects were caused by a change in the amino acid chemistry at this position, which argues for a structural role of R30.

In the crystal structure, R30 is exposed at the surface between the N and C terminal Ig-like subdomains of the RHD (Fig. S2) and can form H bonds with several residues, namely T191, E193, and N186 located within the L3 loop, as well as E279 and, to a lesser extent, D277 from the Lβfßg loop (40). Mutation of R30 to alanine completely abrogated these interactions. Moreover, we found that monomethylation and asymmetric dimethylation of R30 altered H bonding, similar to that observed for the inactivating R30A mutation (Fig. 4*B*). The loss of H bonds will likely destabilize the interactions between the L1 loop and the rest of the N-terminal domain, thereby increasing the flexibility of the L1 loop as well as possibly altering the side chain positioning of R33, R35, and other DNA-contacting residues. Such structural changes are expected to disrupt DNA binding by the RHD and may possibly explain the inhibition caused by the R30 methylation or mutation.

In addition to dimethylation of R30, MS analysis revealed monomethylation of at least one additional arginine (R236) located in the C terminus of the RHD (Fig. S1*C*). The predicted low structural accessibility of R236 (Fig. S1*A*) may possibly explain its monomethylated state, although dimethylation of this residue by PRMT1 cannot be excluded. Interestingly, R236 is adjacent to IκBα-interacting and dimerization interfaces of the RHD (41), raising the possibility that PRMT1 might regulate different functions of RelA through modification of distinct arginines.

PRMT1 functions both to activate and to repress genes (23, 42) by methylating histone H4 and different nonhistone proteins associated with chromatin functions. We have identified PRMT1 as a negative regulator of several NF-κB target genes. Depletion of PRMT1 increased late *NFKBIA* expression and enhanced overall *TNFα* and *A20* activation by TNFα (Fig. 5*A* and Fig. S6*E*). This correlated with the enhanced binding of RelA to gene promoters on TNFα stimulation. We also observed a dynamic association/dissociation of PRMT1 with/from chromatin characterized by different kinetics depending on the specific gene promoters (Fig. 5*D*). The chromatin association of PRMT1 was not dependent on RelA and also could be detected before TNFα treatment. Moreover, promoter occupancy by PRMT1 was counteracted with the presence of RelA, supporting the inhibitory effect of PRMT1. In agreement with our findings, the inhibition of *NFKBIA* and *IL-8* genes by promoter-associated PRMT1 and PRMT5 has been described previously, but different regulatory mechanisms have been proposed (26). It has been shown that PRMT1 and PRMT5 methylate SPT5 and repress its promoter association and interaction with RNApol II, resulting in a lower rate of transcriptional elongation. It also has been reported that under basal conditions, the ubiquitin ligase activity of TNF receptor-associated factor 6 is impaired by PRMT1-mediated methylation, resulting in inhibition of NF-κB (22). It is likely that PRMT1-mediated regulation of NF-κB target genes requires methylation of other proteins involved in transcription or upstream of NF-κB.

The RelA L1 loop, which is critical for nucleotide sequence recognition and binding, has been shown to be monomethylated at K37 by SET7/9 (12) and symmetrically dimethylated at R30 by PRMT5 (16). These modifications cause an increase in the RelA affinity to DNA and activate NF-κB, in contrast with the inhibitory asymmetric R30 dimethylation by PRMT1 identified in our study. Thus, the RxxRxRxxC motif is modified by different protein lysine and arginine methyltransferases, notably SET7/9, PRMT1, and PRMT5,

resulting in methylation-specific marks with distinct biological consequences. Similarly, the asymmetric and symmetric dimethylation of H4R3 by PRMT1 and PRMT5 has either stimulating or repressing effects on genes (23, 24). Evidence has been provided indicating that different effector molecules, such as CBP/p300 and DNMT3a, are recruited to the same histone residue by the presence of either ADMA or SDMA. The exact interplay between these modifications in the control of NF-κB remains unclear, however. Symmetric and asymmetric R30 dimethylation might be counteracting and occurring at different stages of NF-κB responses. Indeed, we found that asymmetric R30 dimethylation is enriched at later time points (Fig. 5 *B* and *C*), whereas symmetric dimethylation of this residue seems to be induced shortly after cytokine treatments (16), supporting their distinct regulatory functions. In this context, it could be hypothesized that symmetric/asymmetric R30 dimethylation represents a specific on/off switch mechanism for adjusting cytokine-induced NF-κB responses (Fig. 5*E*).

In summary, the present study has identified asymmetric arginine dimethylation as a regulatory posttranslational modification of RelA and PRMT1 as a major PRMT responsible for this modification. We report a previously unknown function for PRMT1 in the regulation of TNFα signaling that points to PRMT1 as a restrictive factor controlling NF-κB activity (Fig. 5*E*). This model is supported by observations that PRMT1 knockdown enhanced the expression of NF-κB target genes by facilitating RelA recruitment to their promoters in response to TNFα. In light of these findings, selective targeting of PRMT1 may be seen as an opportunity to specifically modulate NF-κB–mediated inflammatory and immune responses.

Materials and Methods

Antibodies, protein purification, methylation assays, and molecular dynamics simulations are described in *SI Materials and Methods*. Virus production and transduction of cells were done as reported previously (9). Quantitative RT-PCR and thermal shift assays were done using SYBR Green- and SYPRO Orange-based detection methods with a PikoReal 96 Real-Time PCR System (Thermo Fisher Scientific). EMSA and luciferase assays have been described previously (9, 18).

ACKNOWLEDGMENTS. We thank Didier Trono for retroviral constructs; Johanna E. Mayrhofer, Christoph Dotter, and M. Imran Khan for technical assistance; and Ilja Vietor for critical comments on the manuscript. We acknowledge the High-Performance Computing platform at the University of Innsbruck for providing access to the Leo-II computer cluster. This work was supported by Austrian Science Fund Grant P24251 (to T.V.). J.E.F. is the recipient of a DOC fellowship from the Austrian Academy of Sciences.

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