Arginine methylation regulates IL-2 gene expression: a role for protein arginine methyltransferase 5 (PRMT5)

Stéphane RICHARD[1], Mélanie MOREL and Patrick CLÉROUX

Terry Fox Molecular Oncology Group and the Bloomfield Center for Research on Aging, Lady Davis Institute for Medical Research, Sir Mortimer B. Davis Jewish General Hospital, and Departments of Oncology and Medicine, McGill University, Montréal, Québec, Canada H3T 1E2

Arginine methylation is a post-translational modification resulting in the generation of aDMAs (asymmetrical ω-N^G,N^G-dimethylated arginines) and sDMAs (symmetrical ω-N^G,N'^G-dimethylated arginines). The role of arginine methylation in cell signalling and gene expression in T lymphocytes is not understood. In the present study, we report a role for protein arginine methylation in regulating IL-2 (interleukin 2) gene expression in T lymphocytes. Leukaemic Jurkat T-cells treated with a known methylase inhibitor, 5′-methylthioadenosine, had decreased cytokine gene expression, as measured using an NF-AT (nuclear factor of activated T-cells)-responsive promoter linked to the luciferase reporter gene. Since methylase inhibitors block all methylation events, we performed RNA interference with small interfering RNAs against the major PRMT (protein arginine methyltransferases) that generates sDMA (PRMT5). The dose-dependent decrease in PRMT5 expression resulted in the inhibition of both IL-2- and NF-AT-driven promoter activities and IL-2 secretion. By using an sDMA-specific antibody, we observed that sDMA-containing proteins are directly associated with the IL-2 promoter after T-cell activation. Since changes in protein arginine methylation were not observed after T-cell activation in Jurkat and human peripheral blood lymphocytes, our results demonstrate that it is the recruitment of methylarginine-specific protein(s) to cytokine promoter regions that regulates their gene expression.

Key words: arginine methylation, interleukin 2 (IL-2), Jurkat T-cells, protein arginine methyltransferase 5 (PRMT5), small interfering RNA (siRNA), T-cell receptor signalling.

INTRODUCTION

Many proteins rely on post-translational modifications for proper function. The phosphorylation of serine, threonine and tyrosine residues is a key event in most signalling pathways, where the interplay between kinases and phosphatases rapidly adjusts the phosphate status of molecules in response to signals. There is no comparable dynamic regulation known for protein methylation. Arginine methylation is a post-translational modification that adds monomethyl or dimethyls to the guanidine nitrogen atoms of arginine [1,2]. There are two major classes of enzymes responsible for protein arginine methylation: type I enzymes promote the formation of aDMAs (asymmetrical ω-N^G,N^G-dimethylated arginines) and type II enzymes catalyse the formation of sDMA (symmetrical ω-N^G,N'^G-dimethylated arginines). Since it was recently observed that monomethylarginine is deimidated by peptidyl arginine deiminases, arginine methylation is a reversible post-translational modification [3,4].

Saccharomyces cerevisiae contains one major type I PRMT (protein arginine methyltransferase), namely HMT1, which was identified, in a screen for mutants that cause synthetic lethality, with a temperature-sensitive mutant allele of NPL3, an hnRNP (heterogeneous nuclear ribonucleoprotein) [5]. Independently, hmt1 was identified as a homologue of the major mammalian type I enzyme PRMT1 [6]. Research on this organism unveiled the first biological role for arginine methylation: shuttling of the RNA-binding protein Npl3p between the nucleus and cytoplasm requires hmt1 activity [7]. Moreover, hmt1 activity has recently been shown to regulate mRNA export [8]. The mechanism by which this post-translational modification regulates protein shuttling is not clear, but it has been shown that methylation of Npl3p interferes with its phosphorylation, and this in turn indirectly prevents Npl3p from interacting with the nuclear import receptor Mtr10p [9,10].

Yeast two-hybrid screens have implicated PRMT1 in interactions with signalling molecules and nuclear factors. Two members of a family of mitogen-induced proteins, TIS21 and BTG1, interact with PRMT1 [11]. Similarly, ILF3 (interleukin enhancer binding factor 3) or NF110, a nuclear factor proposed to be involved in the regulation of transcription and DNA repair, binds PRMT1 [12]. The activity of PRMT1 is modulated by these interactions with ILF3, TIS21 and BTG1. PRMT1 also interacts with the cytoplasmic domain of the interferon-α/β cytokine receptor, and lowering the methyltransferase expression using antisense oligonucleotides suppressed interferon growth-inhibitory effects [13]. A null mutation of PRMT1 was generated in mice, and homozygous mutant embryos die shortly after implantation, failing to develop beyond E6.5. PRMT1 is not required for cell viability, and mutant ES cell lines have been established [14]. PRMT2 displays high similarity to HMT1 and PRMT1 and contains an SH3 domain [5]. PRMT2 has been identified as a co-activator for the oestrogen receptor [15]. PRMT3 was discovered to interact with PRMT1 in a yeast two-hybrid screen [16]. PRMT3 has a C2H2 zinc-finger motif that, similar to the SH3 domain in PRMT2, may determine its substrate specificity. Recently, PRMT3 has been shown to function in rRNA processing [17]. Studies of the crystal structure of the PRMT3 catalytic core have revealed an AdoMet-binding domain and a barrel-like structure, with the active site situated in a pocket between these two domains [18]. The active site is highly conserved between all PRMTs.

Abbreviations used: aDMA, asymmetrical ω-N^G,N^G-dimethylated arginine; CARM1, co-activator-associated arginine methyltransferase 1; ChIP, chromatin immunoprecipitation; IL-2, interleukin 2; ILF3, interleukin enhancer binding factor 3; MTA, 5′-deoxy-5′-methylthioadenosine; NIP45, nuclear factor of activated T-cells-interacting protein of 45 kDa; NF-AT, nuclear factor of activated T-cells; PBL, peripheral blood lymphocyte; PRMT, protein arginine methyltransferase; sDMA, symmetrical ω-N^G,N'^G-dimethylated arginine; siRNA, small interfering RNA.

[1] To whom correspondence should be addressed, at Lady Davis Institute for Medical Research, 3755 Côte Ste-Catherine Road, Montréal, Québec, Canada H3T 1E2 (email stephane.richard@mcgill.ca).

CARM1 (co-activator-associated arginine methyltransferase 1) was identified as a co-activator-associated arginine methyltransferase I for nuclear receptors [19], myogenic transcription factor MEF2C [20] and β-catenin [21]. CARM1-null animals die perinatally, and have dysregulated gene expression by nuclear receptors and T-cell defects [22,23]. PRMT1 also has co-activator activity and functions synergistically with CARM1 [21,24,25]. The ability of PRMT1 to methylate histone H4 and that of CARM1 to methylate histone H3 [26,27] suggest that these enzymes contribute to the histone 'code' [28]. Indeed, the methylation of histone H4 by PRMT1 facilitates subsequent acetylation by p300, probably influencing chromatin remodelling [25,29]. ChIP (chromatin immunoprecipitation) analysis has shown that histone H3 becomes methylated *in vivo* on Arg^{17} at the oestrogen receptor-regulated pS2 gene [30] and at a genome-integrated MMTV (murine-mammary-tumour virus) reporter [31]. CARM1 has also been shown to methylate the poly(A)$^+$-binding protein PABP1 [32] and the transcriptional cofactors CBP (CREB-binding protein)/p300 (where CREB stands for cAMP-response-element-binding protein) [33] and HuR [34]. Thus CARM1 may regulate multiple aspects of the gene-specific activation including histone methylation, acetylation as well as general transcript integrity.

The major mammalian PRMT that generates sDMA is JBP1 (Janus kinase binding protein 1) [35], also known as pICln-binding protein [36], which has been renamed as PRMT5. PRMT5, which is the mammalian homologue of yeast Skb1 and Hsl7p, was identified, in a yeast two-hybrid screen, as a JAK-interacting protein [35]. PRMT5 [37] has been shown to have methyltransferase activity towards MBP (myelin basic protein), histones H2A and H4 [35] as well as Sm proteins B, B′, D1, D3 and LSm4 [38,39]. PRMT5 methylates arginine-glycine (RG)-rich sequences in these substrates [37], and forms oligomers [40]. The function of PRMT5 is unknown but it has been shown to exist in a 20 S complex called methylosome [38,39]. PRMT5-null animals have not been reported so far and the physiological consequences of reduced PRMT5 levels are unknown. PRMT5 has been identified in promoter complexes, where it has been proposed to function as a transcriptional repressor by methylating histones [41,42] and the transcription elongation factor SPT5 [43]; however, a role in T-cells remains unknown. PRMT6 was identified by searching the human genome and its substrates are unknown [44] and, recently, we have identified HIV Tat as its first substrate [45]. PRMT7 was identified in a screen to identify drug sensitivity genes [46] and has monomethylarginine-specific activity [47].

In the present study, we have uncovered a role for PRMT5 in the expression of IL-2 (interleukin 2). Our results demonstrate that inhibition of methylation in Jurkat T lymphocytes with methylase inhibitors impaired the IL-2- and NF-AT (nuclear factor of activated T-cells)-driven responses. Successful knockdown of PRMT5 gave similar results. In addition, we observed that sDMA-containing protein(s) are specifically associated with the IL-2 promoter after T-cell activation. Our results demonstrate that arginine methylation is a necessary post-translational modification in T-cells for the recruitment of transcription factors and other components during cytokine gene expression.

MATERIALS AND METHODS

Protein analysis

ASYM24, SYM11, anti-PRMT1, anti-PRMT5 and anti-Sam68 (AD-1) antibodies have been described previously [48–51] and anti-PRMT7 was generated using the peptide antigen KIFCSRANPTTGSVEWLEEDEHYD. These antibodies as well as the anti-phosphotyrosine (4G10) were obtained from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Anti-PRMT3 and anti-CARM1 were raised in rabbits with keyhole limpet haemocyanin-conjugated peptides (KAGEALKGKVTVHKNKKDPRSL and KTMGGPAISMASPMSIPTNTMHYGS respectively). The anti-α-actin monoclonal antibody was purchased from Sigma–Aldrich.

Jurkat cells or PBLs (peripheral blood lymphocytes) (2.5×10^6 cells) were lysed in 200 μl of SDS/PAGE sample buffer, and 30 μl was separated by SDS/PAGE, transferred on to nitrocellulose membranes and immunoblotted with anti-PRMT1, -PRMT3, -CARM1, -PRMT5, -PRMT7, -SAM68 (loading control), -phosphotyrosine (4G10), -ASYM24 and -SYM11 antibodies. Anti-Sam68 and α-actin were immunoblotted to ensure equal loading. The immunoblots were visualized by using a goat anti-mouse or anti-rabbit secondary antibody conjugated with horseradish peroxidase (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.) and chemiluminescence (PerkinElmer, Boston, MA, U.S.A.).

PBL isolation

Heparinized whole blood was centrifuged at 900 ***g*** for 10 min. Then, the buffy coat was removed and mixed with an equal volume of PBS. The mixture was layered over Ficoll-Paque™ PLUS (Amersham Biosciences) and centrifuged for 10 min at 1000 ***g***. The interface was then harvested, pelleted and washed twice with PBS. The pelleted cells were resuspended in Lympho-Kwik® and incubated for 20 min at 37 °C. After mixing the cells, 0.2 ml of PBS was layered on top of it and the preparation was mixed for 2 min at 2000 ***g***. The pellet was washed three times with PBS and then resuspended in medium.

RNA interference

siRNAs (small interfering RNA) were purchased from Dharmacon Research (Lafayette, CO, U.S.A.). PRMT5 siRNA was derived from the sequence with accession no. XM_033433 (nt 1598–1620). The fluorescein-labelled luciferase GL2 duplex was used as control siRNA (no. D-1120-05; Dharmacon Research). The SUPER plasmid was a gift from R. Agami (Netherlands Cancer Institute, Amsterdam, The Netherlands). pSUPER 5 were generated by ligation of the annealed oligos (5′-gatccccCCGCTATTGCACCTTGGAattcaagagaTTCCAAGGTGCAATAGCGGtttttggaaa-3′ and 5′-agcttttccaaaaaCCGCTATTGCACCTTGGAAtctcttgaaTTCCAAGGTGCAATAGCGGggg-3′) into pSUPER previously digested with BglII and HindIII. The uppercase letters represent the targeting sequences. Total RNA was extracted with TRIzol® (Invitrogen) and analysed by reverse transcriptase–PCR using the following primer pairs: PRMT5, 5′-GGACCTCATTGTACAGCTTGGAG-3′ and 5′-CTCTCAGTACCAGCAGGCCATC-3′; glyceraldehyde-3-phosphate dehydrogenase, 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′.

Transfections and T-cell activation

Exponentially growing Jurkat T-cells (kindly provided by G. Crabtree, Stanford University Medical School, Stanford, CA, U.S.A.) were washed and resuspended in RPMI 1640 (2×10^7 cells/ml). Cells (0.5 ml) were added to an Eppendorf tube containing 5 μg of firefly luciferase reporter plasmid (NF-ATLuc or IL-2Luc, a gift from G. Crabtree), 1 μg of transfection efficiency reporter pRL-TK (Promega, Madison, WI, U.S.A.) and 60–240 pmol of control or PRMT5-specific siRNA. For pSUPER experiments, 1–10 μg of pSUPER or pSUPER 5 replaced the siRNA and the total pSUPER DNA was adjusted to 10 μg with empty pSUPER. The DNA/cell mixture was electroporated in a

0.4 cm electrode gap cuvette (Bio-Rad Laboratories, Hercules, CA, U.S.A.) with a single pulse of 280 V and 950 μF using a Gene Pulser II (Bio-Rad Laboratories). The cells were left on ice for 15 min and transferred to a 6 cm Petri dish containing 6 ml of complete medium and incubated at 37 °C in a humidified 5 % CO_2 incubator. After 48 or 72 h, the cells were harvested, counted and resuspended in complete medium for activation.

The stimulation of Jurkat T-cells for luciferase gene assays was performed as follows: first, 250 μl of goat anti-mouse antibody (Zymed, San Francisco, CA, U.S.A.) at a concentration of 10 μg/ml was added to each well of a 24-well plate and incubated for 2 h at room temperature (22 °C) on the day before activation. The antibody was then removed and replaced by 250 μl/well of 1 μg/ml anti-CD3 UCHT1 monoclonal antibody and the plate was incubated overnight at 4 °C (Immunotech, Marseille, France). Subsequently, the excess antibody was removed and replaced by 10^6 cells in 1 ml of complete medium for the indicated times. For CD3 plus PMA stimulation, 25 ng/ml PMA was added to the cells for the indicated time. The PMA/ionomycin stimulation was performed by resuspending 10^6 cells in 1 ml of complete medium containing a final concentration of 25 ng/ml PMA and 1 μM ionomycin. After the activation, the cells were incubated for 6 h at 37 °C and the cells were harvested and the firefly (*Photinus pyralis*) and pansy (*Renilla reniformis*) luciferase levels were determined using the Dual-Luciferase Reporter assay system (Promega). For the immunoblotting experiments, the anti-CD3 and anti-CD3/CD28 activation of the Jurkat cells and PBLs respectively was prepared by resuspending 2.5×10^6 cells in 50 μl of complete medium in an Eppendorf tube. Then, 4 μg/ml anti-CD3 UCHT1 monoclonal antibody and/or 0.5 μl of anti-CD28 (catalogue no. 340975, Becton Dickinson) was added. The cells were incubated on ice for 15 min followed by incubation at 37 °C for 5 min, 30 min and 3 h. The cells were lysed by adding 150 μl of boiling 2 × SDS/PAGE sample buffer.

For methylase inhibitor treatment, Jurkat T-cells were transfected as described above with NF-ATLuc and pRL-TK. The cells were either treated with 250 μM of a methylase inhibitor [MTA (5′-deoxy-5′-methylthioadenosine)] or mock-treated with DMSO 24 h post-transfection. The dose of MTA was increased to 750 μM 8 h later, and the cells were incubated overnight. The cells were then activated with anti-CD3 antibody for 6 h in the presence of a total dose of 750 μM MTA (or an equivalent volume of DMSO). The cells were activated as described above.

HEK-293 cells (human embryonic kidney 293 cells) were transfected in 24-well plates using Lipofectamine™ Plus (Invitrogen). The cells were either transfected with pSUPER, pSUPER 1 (containing PRMT1 siRNA) or pSUPER 5 (containing PRMT5 siRNA). At 24 h intervals, up to 96 h, the cells were harvested and analysed by reverse transcriptase–PCR or by immunoblotting.

Flow cytometry

Jurkat T-cells transfected either with the fluorescein-labelled luciferase GL2 duplex (no. D-1120-05; Dharmacon Research) or with a non-effective siRNA (negative control) were incubated for 48 h and then resuspended in 1 ml of RPMI 1640. FITC expression was analysed by flow cytometry.

IL-2 production assay

Jurkat T-cells transfected by electroporation as described previously with either FI-siGL2 or si5 were cultured for 24 h in 96-well plates in the presence or absence of 25 ng/ml PMA and 1 μM ionomycin. The supernatants were harvested and IL-2 was detected by ELISA (Pierce Biotechnology, Rockford, IL, U.S.A.) according to the manufacturer's instructions.

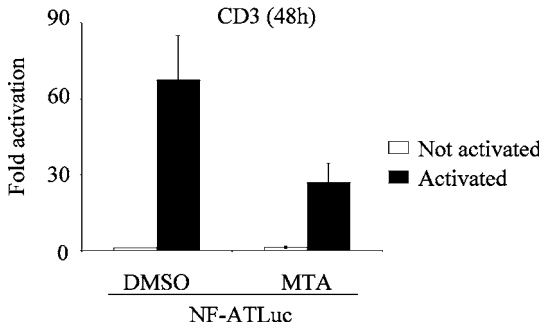

Figure 1 Methylation is important for NF-AT-driven promoter activity

Methylase inhibitor treatment on Jurkat T-cells transfected with NF-ATLuc and pRL-TK for 48 h, then activated or not on a 24-well plate with anti-CD3 for 6 h. Results are expressed as the means ($n > 3$) for the fold activation compared will non-activated DMSO-treated control.

ChIP assay

The ChIP experiments were performed essentially as described in [52]. Briefly, unactivated Jurkat cells and Jurkat cells activated for 1 h with CD3 (5×10^6 cells) were cross-linked with 1 % formaldehyde for 15 min at room temperature. The immunoprecipitation buffer was 20 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.01 % SDS, 1.0 % Triton X-100 and 1.2 mM EDTA with protease inhibitors, in a total volume of 1.2 ml. The precleared extract was split into two equal portions; one was used for control preimmune serum and the other portion was incubated with the test antibody. Out of the total extract, 1/60 was saved for the input. After the immunoprecipitation, the beads were washed four times with buffer and once with buffer supplemented with 500 mM NaCl and a final detergent wash. Each the ChIP experiment was repeated several times with 15–20 % deviation. Anti-PRMT5 and anti-SYM11 antibodies were used for the immunoprecipitations (Upstate Biotechnology). The primers used in PCR were the IL-2 promoter (forward, 5′-ACTGTTTCATACAGAAGGCGTTAA, and reverse, TGGGGGTGTCAAAATGTTTTACAT) and β-actin (+140 to +585). Input DNAs were diluted 1:5 and 2 μl was utilized for PCR. The DNA fragment products were amplified in 30 cycles and this was established as the linear range of PCR.

RESULTS

To determine whether methylation was required for T-cell gene activation, the normalized activity of an NF-AT luciferase reporter was examined in anti-CD3-stimulated Jurkat T-cells treated with the methylation inhibitor MTA. The NF-AT luciferase reporter that we used harbours three copies of the NF-AT-binding site (−286 to −257 of human IL-2) linked to the minimal human IL-2 promoter (−72 to +47) driving firefly luciferase [53]. The normalization was achieved by the co-transfection of pRL-TK, which is a plasmid expressing the *R. reniformis* luciferase under the control of the herpes simplex thymidine kinase promoter providing a constitutive expression. Jurkat T-cells pretreated with MTA showed a significant 2-fold decrease in response to a 6 h anti-CD3 stimulation compared with mock DMSO-treated cells, suggesting that methylation is required for gene expression in T-cells (Figure 1).

Total cell extracts from anti-CD3-stimulated Jurkat T-cells were lysed, separated by SDS/PAGE and immunoblotted with anti-phosphotyrosine (4G10), anti-aDMA-specific (ASYM24; [50]) and anti-sDMA-specific (SYM11; [51]) antibodies. As expected, a rapid increase in phosphotyrosine was observed (Figure 2A,

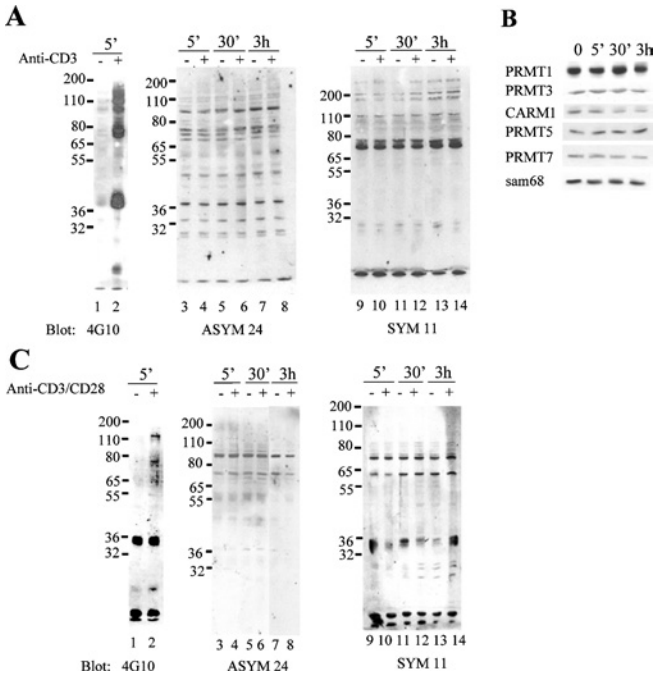

Figure 2 Arginine methylation and PRMT expression are not altered after T-cell activation

(**A**) Jurkat cells were left unactivated or activated for 5 min, 30 min or 3 h with anti-CD3 and lysates were prepared from non-activated (−) and activated (+) cultures with 2 × SDS/PAGE buffer. The proteins were analysed by immunoblotting with antibodies directed against phosphotyrosine (4G10, lanes 1 and 2), aDMA (ASYM24, lanes 3–8) or sDMA (SYM11, lanes 9–14). Migration of the molecular-mass standards is expressed in kDa. (**B**) The cell extracts activated in (**A**) were also immunoblotted with anti-PRMT1, -PRMT3, -CARM1, -PRMT5, -PRMT7 and -Sam68 (loading control) antibodies. (**C**) Human peripheral blood lymphocytes were left unactivated or activated for 5 min, 30 min or 3 h with anti-CD3 and anti-CD28 antibodies and the cell extracts were analysed as in (**A**).

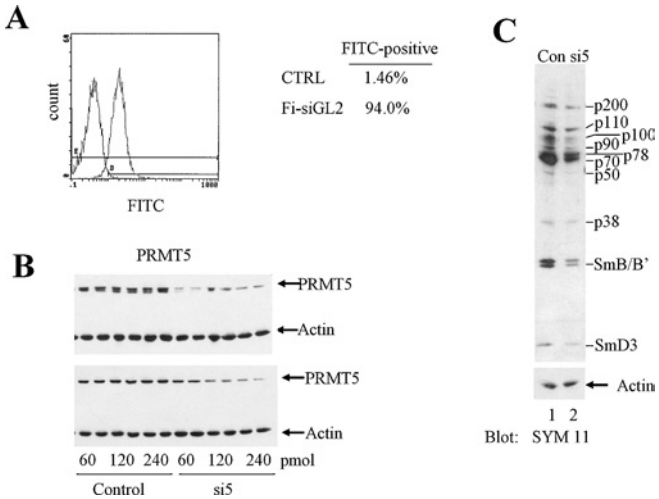

Figure 3 SiRNA knockdown of PRMT5 in Jurkat T-cells

(**A**) Jurkat T-cells transfected with a non-effective siRNA (left peak) or with fluorescein-labelled luciferase GL2 duplex (control siRNA, FI-siGL2; right peak) were harvested 48 h post-transfection and analysed by flow cytometry to determine the amount of FITC-positive cells. (**B**) Western blots from lysates of Jurkat T-cells transfected by electroporation with increasing amounts of FI-siGL2 and si5 harvested at 48 h (top panels) and 72 h (bottom panels) post-transfection. For each concentration of siRNA in pmol, there are two lanes: the mock-activated sample is on the left and the anti-CD3-activated sample is on the right. The expression of actin and Sam68 were used as loading control. (**C**) A representative SYM11 immunoblot is shown comparing the levels of symmetrical methylation of Jurkat T-cells transfected with Fi-siGL2 (con, lane 1) or si5 (lane 2) after 48 h treatment. The expression of actin is shown for equivalent loading.

lane 2), clearly demonstrating T-cell activation. Many proteins were detected with the dimethylarginine-specific antibodies but no major difference in the overall arginine methylation or in the expression of the known PRMTs was observed up to 3 h after anti-CD3 stimulation (Figures 2A and 2B). Similar results were obtained using PBLs stimulated with anti-CD3 and anti-CD28 antibodies (Figure 2C). PBLs had fewer methylated proteins compared with Jurkat cells, suggesting that transformed cells may have higher levels of protein arginine methylation. PBLs did contain, however, several additional sDMA-containing proteins (p34 and p60) in comparison with Jurkat cells. Anti-actin and Sam68 antibodies were used to visualize equivalent loading.

To optimize delivery conditions of siRNA in Jurkat T-cells, we electroporated the cells with fluorescein-tagged control siRNAs and examined the percentage of cells that received the siRNAs by flow cytometry. The left peak of Figure 3(A) represents T-cells that received a scrambled tag-free siRNA, whereas the right peak represents cells that received FITC-tagged siRNAs. Approx. 94 % of the cells was FITC-positive, as indicated by the quantity of cells that have an increase in fluorescence (Figure 3A). These results indicate that siRNA have a unique propensity to enter cells by electroporation that plasmids do not possess. Similar observations were made by McManus et al. [54] by using the double-positive immature T-cell line E10. We then tested the capacity of siRNAs directed against PRMT5 to decrease the expression of this methyltransferase at the level of mRNA and protein, 48 and 72 h after the introduction of the siRNA. PRMT5 was chosen as it represents a major enzyme generating sDMA. PRMT5 protein levels were decreased in a dose-dependent manner (Figure 3B). Moreover, the mRNA for PRMT5 was also decreased in a similar manner, as visualized by reverse transcriptase–PCR (results not shown). The knockdown of PRMT5 is predicted to decrease the amount of sDMA-containing proteins. Indeed, a decrease was visualized by using SYM11 (Figure 3C), further confirming that PRMT5 knockdown was achieved. Densitometric analysis revealed that the Sm D3 methylated epitope was decreased by 2.0-fold and the Sm B/B′ doublet decreased 3.3-fold after PRMT5 siRNA treatment (Figure 3C). The intensity of the SYM11 signal for the higher molecular mass proteins was also decreased by approx. 2–3-fold (Figure 3C).

We next examined whether the knockdown of PRMT5 affected the expression of NF-AT and IL-2 luciferase-driven reporter genes. Jurkat T-cells were electroporated with an NF-AT luciferase reporter plasmid, an expression vector for *Renilla* to insure equal transfection efficiencies along with siRNA directed against PRMT5; 48 and 72 h after electroporation, cells were activated with anti-CD3 antibodies for 6 h and firefly luciferase activity was read and normalized to *Renilla* luciferase. The luciferase gene activation was stimulated approx. 60–80-fold with anti-CD3 stimulation and, as expected, PMA/ionomycin induced approx. 200-fold induction (Figure 4A). Anti-CD3 stimulation of the NF-AT reporter construct was inhibited significantly with higher doses (240 pmol) of PRMT5 siRNA for 48 or 72 h, but not with an equivalent amount of control siRNA (Figure 4B). We also confirmed the RNA interference data by using a vector-based approach. The PRMT5 siRNA sequence was expressed as a hairpin under the control of the histone H1 promoter in the pSUPER plasmid [55]. Jurkat T-cells co-transfected with pSUPER 5 also showed a dose-dependent decrease in NF-AT luciferase activity (Figure 4C), further confirming that PRMT5 expression is required

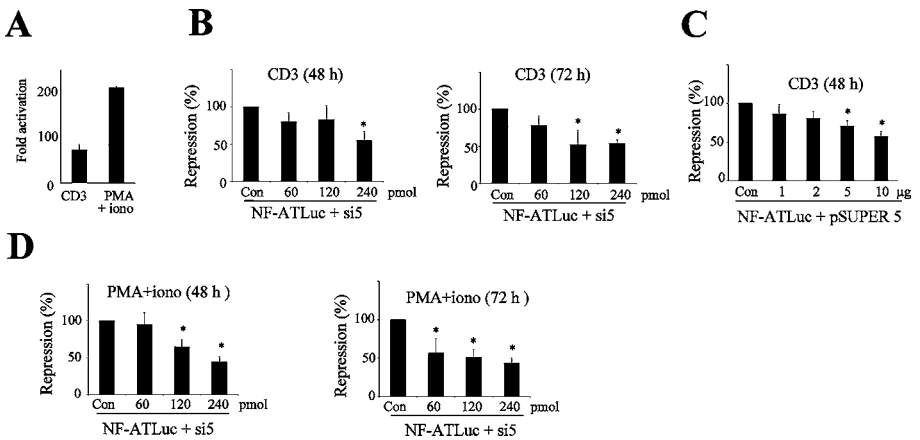

Figure 4 PRMT5 knockdown inhibits NF-AT promoter-driven gene expression

(**A**) Jurkat T-cells were transfected with NF-ATLuc, pRL-TK and stimulated with either anti-CD3, PMA/ionomycin and the fold activation calculated from the unstimulated cells. (**B**) Jurkat T-cells transfected with NF-ATLuc, pRL-TK and different concentrations of the control FI-siGL2 (Con) and si5 for 48 h (left panel) or 72 h (right panel), and then activated on a 24-well plate with anti-CD3 for 6 h. The results are expressed as the mean percentage ($n > 3$) of repression of the control FI-siGL2 at the same concentration. (**C**) Jurkat T-cells were transfected with NF-ATLuc, pRL-TK and different concentrations of pSUPER 5 for 48 h and then activated with anti-CD3. The results are expressed as the mean percentage ($n > 3$) of repression of the control with an equal amount of DNA. Statistical evaluation was calculated by paired Student's t test. $^*P < 0.05$ differ significantly from control. pSUPER (10 μg) was used as control and the increasing amounts of pSUPER 5 were equalized to a total amount of 10 μg with pSUPER. (**D**) Jurkat T-cells were transfected with NF-ATLuc, pRL-TK and different concentrations of FI-siGL2 (Con) and si5 for 48 h (left panel) or 72 h (right panel), and then activated on a 24-well plate with PMA and ionomycin for 6 h. The results are expressed as the mean percentage ($n > 3$) of repression of the control FI-siGL2 at the same concentration.

for NF-AT luciferase reporter expression after CD3 cross-linking. We next investigated whether PRMT5 siRNA could inhibit NF-AT luciferase reporter activity stimulated with PMA + ionomycin. A statistically significant dose-dependent inhibition was observed at 48 and 72 h after treatment with 120 and 240 pmol of PRMT5 siRNA (Figure 4D). Finally, we examined the ability of the PRMT5 siRNA to abrogate the activity of an expression vector containing the entire enhancer/promoter sequence -326 to $+47$ of the human *IL-2* gene. A dose-dependent inhibition after anti-CD3 cross-linking and PMA treatment was observed in cells treated with PRMT5 siRNA (Figure 5A). These results are consistent with the NF-AT data observed in Figure 4. To confirm further that the down-regulation of PRMT5 expression affects IL-2 expression, we examined the ability of PRMT5 siRNA to influence IL-2 secretion. Jurkat T-cells that received control or PRMT5 siRNA were activated with PMA/ionomycin and the levels of IL-2 protein secreted from the cells were measured by using an ELISA. In cells treated with 240 pmol of PRMT5 siRNA, a significant decrease in the secreted IL-2 of approx. 35–40 % was measured compared with cells treated with control siRNA (Figure 5B). The decrease in secreted IL-2 protein further demonstrates that decrease of PRMT5 abrogates IL-2 gene expression.

We examined whether PRMT5 or methylated proteins were directly associated with the IL-2 promoter by ChIP assays. Chromatin was prepared from unstimulated and CD3-stimulated Jurkat cells and immunoprecipitations were performed with pre-immune normal rabbit serum, anti-PRMT5 antibodies or the sDMA-specific SYM11 antibodies. PRMT5 was not associated with the IL-2 promoter nor with β-actin (Figure 6A). These results suggested that the PRMT5 transcriptional effect is indirect. Thus either PRMT5 influences a non-transcriptional event and/or PRMT5 methylates a transcriptional regulator that will regulate IL-2 gene expression. To examine the latter possibility, we immunoprecipitated chromatin with SYM11. Interestingly, SYM11 detected an sDMA-containing protein(s) that associated directly with the IL-2 promoter only in activated T-cells (Figure 6), but not with the β-actin gene. These results suggest that an arginine-methylated protein(s) is recruited to the IL-2 promoter after T-cell signalling.

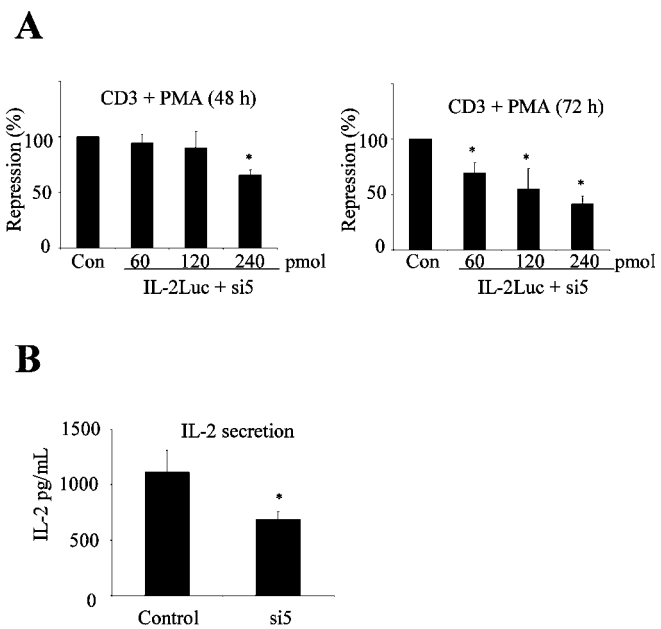

Figure 5 PRMT5 knockdown inhibits IL-2 promoter-driven gene expression

(**A**) Jurkat T-cells transfected with IL-2Luc (where the luciferase is under the control of the complete IL-2 promoter/enhancer region), pRL-TK and various amounts of FI-siGL2 (Con) and si5 were activated in a 24-well plate with anti-CD3 + PMA for 6 h at 48 h (left panel) and 72 h (right panel) post-transfection. The results are expressed as the mean percentage ($n > 3$) of repression of the control FI-siGL2 at the same concentration. (**B**) Transfection with si5 affects IL-2 secretion. Jurkat T-cells were transfected with FI-siGL2 (control) or si5 and, 48 h later, were stimulated with PMA/ionomycin for 24 h. The supernatants were analysed for the presence of IL-2 by ELISA and are expressed in terms of pg/ml. This experiment represents the average of $n > 7$. Statistical evaluation was calculated by paired Student's t test. $^*P < 0.05$ differ significantly from control.

DISCUSSION

We present evidence that protein arginine methylation is necessary for IL-2 gene activation. Jurkat T-cells treated with MTA, a known

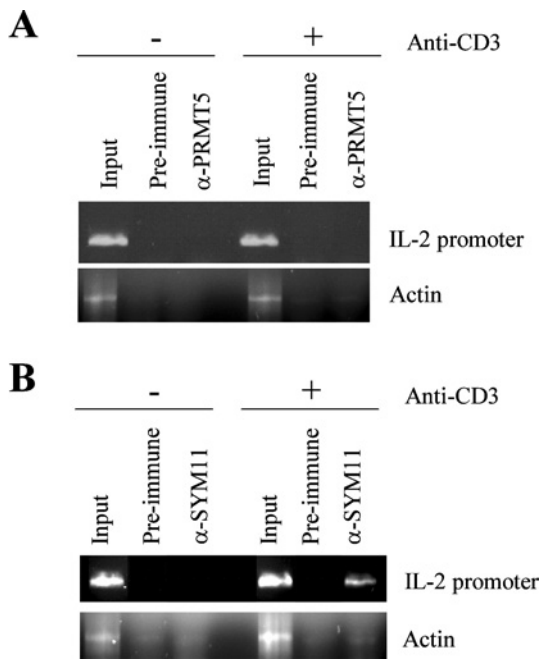

Figure 6 sDMA-containing proteins are directly involved in IL-2 gene expression

ChIP assays were conducted using cross-linked chromatin from either anti-CD3 activated for 1 h or unactivated Jurkat T-cells as described in the Materials and methods section with either preimmune (PI) or immune (I) anti-PRMT5 (**A**) or anti-SYM11 (**B**) antibodies as indicated. The eluted or the input DNA was PCR-amplified with human IL-2 promoter primers or with the β-actin gene and separated by agarose-gel electrophoresis.

methylase inhibitor, had decreased cytokine gene expression. Similarly, the dose-dependent decrease in PRMT5 expression resulted in the inhibition of IL-2-driven promoter activities. In addition, Jurkat cells treated with PRMT5 siRNA secreted smaller amounts of IL-2 after activation. Early events such as the induction of phosphotyrosine and the activation of the mitogen-activated protein kinase were unaffected by the PRMT5 siRNA treatment (results not shown). By using ChIP assays, we detected sDMA-containing protein complexes directly associated with the IL-2 gene promoter in activated T-cells. Our results demonstrate that arginine methylation participates in the recruitment of transcriptional complexes to the IL-2 promoter.

By immunoblotting resting and activated Jurkat T-cell extracts with anti-dimethylarginine-specific antibodies (ASYM24 and SYM11), we observed many aDMA- and sDMA-containing proteins. These observations are consistent with our recent proteomic analysis in HeLa cells that demonstrates that many proteins contain dimethylarginines [51]. A high level of basal arginine methylation was observed in resting T-cells (Figure 2) and, after T-cell activation, this level of arginine methylation remained increased, making it difficult to observe further increases that may occur after activation. The absence of global changes in dimethylarginine-containing proteins was also observed with PMA + ionomycin treatment (results not shown). Jurkat cells displayed more methylated proteins than PBLs, and it remains to be determined whether this will be a property of transformed lymphocytes. Our antibodies are specific for arginine-glycine repeats and only recognize a subset of methylated proteins. It is probable that arginine methylation of certain proteins occurs after T-cell activation, but these will have to be identified using other strategies such as stable isotope labelling by amino acids in cell culture.

The observation that siRNA interferes with PMA/ionomycin-stimulated gene activation implies that arginine methylation is required for the calcium-calcineurin-NF-AT and/or PKC-Jun/Fos pathways. Moreover, based on the results obtained with the ChIP assays, it is probable that the effect mediated by PRMT5 is directly at the level of transcription. PRMT5 might methylate a transcription factor or a co-regulator that is required for IL-2 gene expression and this methylated protein(s) may be directly recognized by SYM11 antibodies in the ChIP assays (Figure 6). Few substrates of PRMT5 involved in transcriptional regulation have been identified. It is known that histones [35] and the transcriptional elongation factor SPT5 are PRMT5 substrates [43]. PRMT5 has been observed to function as a transcriptional repressor of the cyclin E1 promoter [42] and Myc [41]. In addition, PRMT5 has been shown to prevent the association of the transcriptional elongation factor SPT5 with RNA polymerase II [43]. Evidence also exists that PRMT5 functions as a transcriptional activator, as microarray analysis has identified approx. 50 gene products that are down-regulated by the inhibition of PRMT5 expression [56]. Our results demonstrate that PRMT5 functions as a transcriptional activator of IL-2 gene expression. Since PRMT5 was not associated with the IL-2 gene promoter, PRMT5 most probably exerts its effects indirectly through a methylated protein(s). Overexpression of PRMT5 did not significantly increase NF-AT gene activation (results not shown), suggesting that the PRMT5 substrates may already be fully methylated. The transcription factors that activate the NF-AT reporter plasmids in our experiments are known to be NF-AT family members and activator protein 1 complexes [57,58]. Since it is well known that PRMT5 prefers to methylate RG repeats [37], we searched the sequences of several transcription factors known to interact with the IL-2 promoter. We also searched for RG repeats in the signalling proteins required for IL-2 gene expression. Three potential methylation sites were identified: one GRG motif is present in the human NF-ATc protein (accession number U08015), GRG followed by GRGRG sequences are present in the transcription factor Oct-2 (accession number M36653) and several GRG repeats were found in NIP-45 (accession number U76759).

Arginine methylation has been shown to regulate the localization of several proteins [1]. Thus arginine methylation by PRMT5 may ultimately regulate the shuttling of the NF-AT family members by interfering with their import. The import/export of RNA-binding proteins including Sam68, hnRNP A2, Npl3p and Hrp1p has been shown to be regulated by arginine methylation [7,9,50,59]. Little is known about the mechanism by which arginine methylation regulates protein localization. For Npl3p, it was shown that arginine methylation prevented the phosphorylation of a composite import signal recognized by the nuclear import receptor Mtr10p [9]. In addition, arginine methylation may modulate the activity of signalling proteins, leading to the activation of transcription factors. For example, the activity of calcineurin [60], the phosphatase required to initiate the nuclear translocation of cytoplasmic NF-AT, may be modulated.

NF-45 and NF-90 were isolated as a complex that binds to ARRE-2 (antigen recognition response element 2) in the IL-2 promoter [61] and have been shown to function as RNA-binding proteins [62]. NF45 (NM_004515) contains a GRGRG sequence and the various isoforms of NF90 (NM_012218, NM_004516, NM_153464 and AF141870) have the following RG-rich sequence: RGRGRGSIRGRGRGRG. These are excellent sites of methylation by PRMT5. Since arginine methylation was shown to affect protein–protein interactions [63], PRMT5 could methylate one or both of these factors and modulate their heterodimerization and their ability to regulate their function in RNA metabolism. Interestingly, the NF110 isoform (or ILF3) of NF90 is a substrate

that interacts with PRMT1 [12]. It was shown to be part of a complex responsible for recruiting PRMT1 to the YY1-activated promoter [64]. NF90 has also been shown to translocate in the cytoplasm after T-cell activation, where it binds stabilization elements within the 3′-untranslated region of the IL-2 mRNA [65]. We predict that arginine methylation may also regulate IL-2 mRNA stability. However, the absence of untranslated regions from the luciferase gene prevented us from assaying this particular function. Thus the effect that we observe probably influences the cascades leading to transcriptional activation. In the IL-2 secretion assay, arginine methylation may be regulating the transcriptional activation and mRNA stabilization.

While our manuscript was under review, Mowen et al. [66] reported that NIP45 (NF-AT-interacting protein of 45 kDa) is a substrate of PRMT1 and that arginine methylation regulates the cytokine response. They have reported that PRMT1 protein expression is increased after activation, but that NIP45 arginine methylation is constitutive. A summary of their results has been discussed by us previously [67]. Our present study as well as the study by Mowen et al. [66] clearly demonstrates a role for arginine methylation in regulating the cytokine response. The challenge will be to identify specific substrates that have increased or decreased methylated arginines after T-cell activation and to integrate arginine methylation within the known signalling cascades.

In conclusion, we have uncovered a role for PRMT5 and symmetrical dimethylarginine in the expression of IL-2. Our results demonstrate that inhibition of methylation in Jurkat T lymphocytes with methylase inhibitors or PRMT5 siRNA impaired IL-2 gene expression. In addition, we observed that sDMA-containing protein(s) are specifically associated with the IL-2 promoter after T-cell activation as detected by ChIP assays. Our results demonstrate that arginine methylation is a necessary post-translation modification in T-cells for the recruitment of transcription factors and other components during cytokine gene expression.

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