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The AT-hook of the Chromatin Architectural Transcription Factor High Mobility Group A1a Is Arginine-methylated by Protein Arginine Methyltransferase 6*

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Riccardo Sgarra[‡1], Jaeho Lee[§1], Michela A. Tessari[‡], Sandro Altamura[‡], Barbara Spolaore[¶], Vincenzo Giancotti[‡], Mark T. Bedford[§], and Guidalberto Manfioletti[‡2]

From the [‡]Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, University of Trieste, 34127 Trieste, Italy, [§]Science Park-Research Division, M. D. Anderson Cancer Center, The University of Texas Smithville, Texas, 78957, and [¶]CRIBI Biotechnology Centre, University of Padua, 35131 Padua, Italy

The HMGA1a protein belongs to the high mobility group A (HMGA) family of architectural nuclear factors, a group of proteins that plays an important role in chromatin dynamics. HMGA proteins are multifunctional factors that associate both with DNA and nuclear proteins that have been involved in several nuclear processes, such as transcriptional regulation, viral integration, DNA repair, RNA processing, and chromatin remodeling. The activity of HMGA proteins is finely modulated by a variety of post-translational modifications. Arginine methylation was recently demonstrated to occur on HMGA1a protein, and it correlates with the apoptotic process and neoplastic progression. Methyltransferases responsible for these modifications are unknown. Here we show that the protein arginine methyltransferase PRMT6 specifically methylates HMGA1a protein both *in vitro* and *in vivo*. By mass spectrometry, the sites of methylation were unambiguously mapped to Arg[57] and Arg[59], two residues which are embedded in the second AT-hook, a region critical for both protein-DNA and protein-protein interactions and whose modification may cause profound alterations in the HMGA network. The *in vivo* association of HMGA and PRMT6 place this yet functionally uncharacterized methyltransferase in the well established functional context of the chromatin structure organization.

HMGA1a belongs, together with its isoform HMGA1b and the highly related protein HMGA2, to the high mobility group A (HMGA)[3] family of non-histone chromosomal proteins (1, 2). HMGA family members are considered proto-oncogenes and, when overexpressed in cell line models, are able to transform cells or increase their malignancy. In addition, transgenic mice overexpressing HMGA develop different tumors (3–5).

HMGA proteins contain about 100 amino acid residues and have three DNA-binding domains called AT-hooks that mediate their ability to interact with the narrow minor groove of AT-rich DNA sequences (6). By binding to DNA and/or transcription factors, HMGA proteins can organize the assembly of nucleoprotein-DNA transcriptional complexes (called enhanceosomes) at the level of enhancers or promoters activating or repressing transcription of a large number of mammalian genes. For this reason, they are referred to as architectural transcription factors (6, 7). In addition to transcriptional regulation, HMGA proteins are involved in other nuclear processes, such as viral integration, RNA processing, DNA repair, and chromatin structural organization and remodeling, indicating HMGA as highly connected nodes in the chromatin protein network (3, 6, 8).

HMGA proteins are subjected to a variety of post-translational modifications (PTMs) that modulate their multi-interacting property with both DNA and proteins (9–15). HMGA1a and HMGA1b are constitutively phosphorylated by casein kinase 2 at the two or three serine residues of the C-terminal end (16). Cell cycle-dependent phosphorylation by p34/cdc2 kinase has been detected on Thr[52] and Thr[77] (numbers refer to human HMGA1a) residues, flanking the second AT-hook and shown to cause a strong decrease in the DNA-binding affinity (17, 18). Likewise p34/cdc2, phosphorylation by protein kinase C on Thr[20], Ser[43], and Ser[63] caused a significant reduction of DNA-binding affinity (13). Acetylation of Lys[64] and Lys[70] are critical for the stability of the enhanceosome assembled on the *IFN-β* gene (19, 20). In particular, acetylation of Lys[70] by p300 CBP-associated factor (PCAF)/GCN5 results in the stabilization, whereas that of Lys[64] by p300/CBP (CREB-binding protein) causes destabilization of the enhanceosome. Methylation is the most recent HMGA PTM reported (9–14). Arg[25] within the first AT-hook of HMGA1a has been found methylated (monomethylated) in tumor cell lines, reaching in some samples up to 50% of total HMGA1a protein content. Methylation on this residue is modulated during the apoptotic process reaching highest levels at later stages, with the formation of apoptotic bodies (14). More recently, dimethylation on the same Arg[25] residue has been reported by another group (21). An extensive study on both HMGA1a and HMGA1b proteins reported a high level of PTMs, and in particular, dimethylation of arginine and lysine residues was increased in breast cancer cells with higher metastatic potential (9, 10). Altogether, these data lead authors to suggest the existence of a PTM "code" for HMGA proteins similar to that reported for histones (10, 11). Although the kinases and acetyltransferases that are able to modify HMGA proteins have been identified, enzymes responsible for arginine and lysine methylation are just starting to be discovered (22).

Protein (lysine and arginine) methylation is an emerging type of PTM that has added a new dimension to the signal transduction field (23, 24).

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dipartimento di Biochimica, Biofisica e Chimica delle Macromolecole, Università di Trieste, Via L. Giorgieri 1, 34127 Trieste, Italy. Tel.: 39-040-5583675; Fax: 39-040-5583694; E-mail manfiole@units.it.

[3] The abbreviations used are: HMGA, high mobility group A; PRMT, protein arginine methyltransferase; PTM, post-translational modification; *IFN-β*, interferon β; CREB, cAMP-response element-binding protein; HIV, human immunodeficiency virus; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione *S*-transferase; NPM, nucleophosmin; PABP1, poly(A)-binding protein 1; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; HEK, human embryonic kidney; [^{3}H]AdoMet, ^{3}H-labeled *S*-adenosyl-L-methionine; GAR, glycine arginine-rich.

At variance from histones, where the prevalent modification is lysine methylation (25), in HMGA proteins, arginine methylation seems particularly relevant (9, 11). Arginine methylation has been implicated in signal transduction, RNA metabolism, transcriptional regulation, and DNA repair (23, 24). Arginine residues are methylated by protein arginine methyltransferases (PRMTs). To date, two distinct PRMT activities have been found in mammalian cells; both types catalyze the formation of ω-N^G-monomethyl arginine as an intermediate. Type I PRMT activity is defined by the formation of asymmetric ω-N^G,N^G-dimethylarginine residues, whereas type II activity is defined by the formation of symmetric ω-N^G,N'^G-dimethylarginine residues. Currently, known type I enzymes include PRMT1, PRMT3, coactivator-associated arginine methyltransferase 1 (CARM1)/PRMT4, PRMT6, and PRMT8 (24). No activity has been demonstrated for PRMT2 (26, 27). The only type II PRMTs identified to date are the Janus kinase-binding protein JBP1/PRMT5 (28) and PRMT7 (29, 30).

In this manuscript, we have reported the identification of PRMT6 as a protein methyltransferase able to efficiently methylate *in vitro* and *in vivo* HMGA1a. PRMT6 has been recently identified as a type I PRMT enzyme with an exclusive nuclear localization (31). PRMT6 *in vivo* cellular substrates are unknown. In fact, if we exclude its automethylation activity, the only substrate identified so far is the viral Tat human immunodeficiency virus (HIV) protein (32). Tat-modified Arg residues have not been identified; thus its substrate specificity remains unknown. We report that PRMT6 methylates HMGA1a at the level of Arg^{57} and Arg^{59} within the second AT-hook domain. This is a critical region for HMGA1a function, which has been shown to have the highest affinity for DNA binding and also to be involved in protein-protein interaction, thus implying an important role for arginine methylation in modulating HMGA functions. In addition, this study, identifying the residues modified by PRMT6, lays down the basis for future studies aimed at establishing a consensus for its substrate specificity.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids pARHMGA1a, pARHMGA1a-(1–80), pARHMGA1a-(1–52), pARHMGA1a-(35–107), pARHMGA1a-(46–107) expressing the wild-type and deletion mutants of human HMGA1a proteins were generated by PCR using the human HMGA1a cDNA as the template. The primers used for expression vector construction were as follows: A5–1, 5′-AGG AGA TAT ACA TAT GAG TGA GTC GAG CT-3′; A5–35, 5′-GCA AGC AGC ATA TGG TGA GTC CCG GGA CA; A5–46, 5′-TGG TAG GGC ATA TGA AGG AGC CCA GCG A-3′; A3–107, 5′-GCA GCC CGG ATC CTT ATC ACT GCT CCT CCT C-3′; A3–80, 5′-GGG TCT GCC GGA TCC TTA TCA TCC TGG AGT TGT-3′; and A3–52, 5′-GCC CCG AGG GGA TCC TTA TCA TGG CAC TTC GCT-3′. The PCR products for deletion constructs at the C terminus were obtained using primer A5–1 in combination with the different A3 primers, whereas products for deletion constructs at the N terminus were obtained using A5–35 and A5–46 in combination with A3–107. The PCR products were cloned between the NdeI and BamHI sites of the bacterial expression vector pAR3038 under the bacteriophage T7 promoter. The resulting clones were verified by sequencing. Plasmids pARHMGA1aR25A, pARHMGA1aR57A, pARHMGA1aR59A, pARHMGA1aR57A,R59A were obtained using the QuikChange site-directed mutagenesis kit by Stratagene. The mutagenesis was performed via standard Stratagene protocol using as template the pARHMGA1a plasmid, except for pARHMGA1aR57A,R59A in which the template used was pARHMGA1aR59A, and using the following primers: R5759A (forward), 5′-CAC CTA AGA GAC CTG CGG GCG CAC CAA AGG-3′; R5759A (reverse), 5′-CCT TTG GTG CGC CCG CAG GTC TCT TAG GTG-3′; R57A (forward), 5′-CAC CTA AGA GAC CTG CGG GCC GAC CAA AGG-3′; R57A (reverse), 5′-CCT TTG GTC GGC CCG CAG GTC TCT TAG GTG-3′; R59A (forward), 5′-GAG ACC TCG GGG CGC ACC AAA GGG AAG C-3′; R59A (reverse), 5′-GCT TCC CTT TGG TGC GCC CCG AGG TCT C-3′; R25A (forward), 5′-AGA AGC GGG GCG CGG GCA GGC CGC-3′; R25A (reverse), 5′-GCG GCC TGC CCG CGC CCC GCT TCT-3′. Plasmids pcDNA3HA-PRMT6 and pcDNA3HA-PRMT1, expressing proteins in-frame with the hemagglutinin (HA) epitope, were obtained by cloning the open reading frames of PRMT6-GFP and PRMT1-GFP (31) into the BamHI/EcoRI sites and the EcoRI site of the pcDNA3HA vector, respectively. Plasmid pcDNA3HA-HMGA1a was obtained via subcloning using BamHI/EcoRI restriction sites from pcDNAI-HMGA1a (a gift of Dr. Thanos, Alexander Fleming Biomedical Sciences Research Cener, Vari, Greece), whereas the vector pcDNA3HA-nucleophosmin (NPM) was obtained via PCR from the PINCO-HA-NPM retroviral vector (a gift of Dr. Pelicci European Institute of Oncology, Milan, Italy) using the following primers including the BamHI/NotI sites: NPM (forward), 5′-GGC AGG GAT CCA TGG AAG ATT CGA T-3′ and NPM (reverse), 5′-TTA AAG CGG CCG CTT AAA GAG ACT TCC-3′ in the corresponding sites of pcDNA3HA. The plasmids pGEX6P1-PABP1 and pGEX2T-GAR, expressing the poly(A)-binding protein 1 (PABP1) and a protein arginine methyltransferase substrate composed of the first 148 amino acids (glycine arginine-rich domain) of the human fibrillarin protein (both in fusion with the glutathione *S*-transferase (GST)) have been described previously (33, 34).

Recombinant Protein Expression and Purification—Recombinant HMGA proteins were expressed, extracted, purified, and analyzed by mass spectrometry as previously described (35). HMGA1a-(45–75) was a degradation product obtained during the preparation of HMGA1a-(45–106). GST fusion proteins were produced and purified as already described (31).

Mass Spectrometry Analyses—LC-MS analyses on PRMT6-methylated HMGA1a were carried out with an API 1 mass spectrometer (PerkinElmer SCIEX) as previously described (14). Mass values are reported as Da ± 1. To identify the methylation sites, 10 μg of PRMT6-methylated HMGA1a were digested with 0.5 μg of Endoproteinase Lys-C sequencing grade (Roche Applied Science) for 18 h at 37 °C. HMGA1a fragments were analyzed by LC-MS as previously described (14) using a Waters Symmetry C18 3.5 μm, 1.0 × 150-mm column. All the LC-MS analyses performed with the API 1 mass spectrometer were carried out splitting the flow outside the chromatographic column allowing in this way to both record mass spectra and collect protein/peptide fractions for subsequent analyses. Tandem mass spectrometry (MS/MS) measurements were performed on a Q-TOF Micro mass spectrometer (Micromass, UK) equipped with a Z-spray nanoflow electrospray ionization interface. Mass spectra of the peptide digests of HMGA1a were acquired using the nano-electrospray source operating at capillary, cone, and extractor voltages of 1400, 30, and 1 V, respectively (positive ion mode). For the MS analyses, samples were dissolved in a 1:1 solution of acetonitrile:water containing 1% formic acid. Nano-electrospray ionization capillaries were prepared in-house from borosilicate glass tubes of 1 mm outer diameter and 0.78 mm inner diameter (Harvard Apparatus, Holliston, MA) using a Flaming/Brown P-80 PC micropipette puller (Sutter Instruments, Hercules, CA) and gold coated using an Edwards S-150B sputter coater (Edwards High Vacuum, West Sussex, UK). MS/MS analyses of the peptide-(55–61) and its methylated forms were performed utilizing the same parameters of the MS instrument as above, using argon as the collision gas and a collision energy setting of 18 V. Instrument control and data acquisition and processing were achieved using the MassLynx software (Micromass,

UK). The relative ratio of the monomethylated peptides was calculated as the ratio between the relative peak intensity of the monomethylated peptide (1-Me) and the relative peak intensity of the unmethylated (0-Me) plus the monomethylated peptide (0-Me + 1-Me). Peptide relative intensity ratio = 1-Me/(0-Me + 1-Me).

Blot Overlay—Blot overlay experiments were performed essentially as previously described (8). Subconfluent HEK293 cells seeded on 100 mm-diameter Petri dishes were transfected with pcDNA3HA, pcDNA3HA-PRMT6, or pcDNA3HA-NPM by the conventional calcium phosphate procedure. Thirty-six hours later, the cells were washed with ice-cold phosphate-buffered saline and then harvested in 1 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0,1% Nonidet P-40) supplemented with protease inhibitors. Lysis was performed at 4 °C for 20 min. The lysates were then clarified by centrifugation and utilized as probes in the blot overlay experiments. Blot overlay membranes were incubated with an α-HA primary antibody (Santa Cruz Biotechnology) and bound primary antibodies visualized by enhanced chemiluminescence.

Co-immunoprecipitation—Subconfluent HEK293 cells were transfected with the indicated vector as described above and immunoprecipitation was performed as previously described (36).

In Vitro Methylation—*In vitro* methyltransferase assay, electrophoresis, and fluorography of methylation reactions were carried out as previously described (31).

In Vivo Methylation—Subconfluent HEK293 cells seeded on 100 mm diameter Petri dishes were transfected with the indicated vectors as described above. Protein synthesis inhibition and *in vivo* methylation were carried out as previously described (33). Cells were washed with ice-cold phosphate-buffered saline and then harvested and lysed in 1 ml of ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0,1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 5 mM NaF, 1 mM Na_3VO_4) supplemented with protease inhibitors (Sigma). Cell lysates were quantified using a standard Bradford method and 1 mg incubated with 8 mg of α-HA primary antibody (Sigma) prebound to 50 ml of protein A-Sepharose (Amersham Biosciences). The beads were then washed three times in ice-cold lysis buffer, and the bound proteins were solubilized by the addition of SDS sample buffer. Proteins were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Western blot analyses were performed by standard procedures with an anti-HA primary antibody (Sigma) and enhanced chemiluminescence visualization. Radioactivity ([^{35}S]methionine and methyl-^{3}H) was visualized by fluorography; membranes were soaked in NAMP100 Amplify (Amersham Biosciences), air dried, and exposed to films at −80 °C for two months.

RESULTS

HMGA1a Is a Substrate of PRMT6—HMGA1a protein is methylated at the level of several arginine residues (9, 11). Because methyltransferases responsible for this post-translational modification were completely unknown, we decided to screen arginine methyltransferases for their ability to methylate HMGA1a. To this aim, pure recombinant HMGA1a protein, together with its isoform HMGA1b and the highly related HMGA2, were incubated with the different recombinant PRMTs in the presence of ^{3}H-labeled *S*-adenosyl-L-methionine ([^{3}H]AdoMet) as a methyl donor. Methylated proteins were separated by SDS-PAGE and visualized by fluorography. All of the PRMTs (PRMT1, PRMT3, PRMT4, PRMT6, and PRMT7) that maintain their enzymatic activity as recombinant proteins were tested. Fig. 1 clearly shows that PRMT6 is the only arginine methyltransferase that is able to efficiently methylate HMGA1a and also the other HMGA proteins.

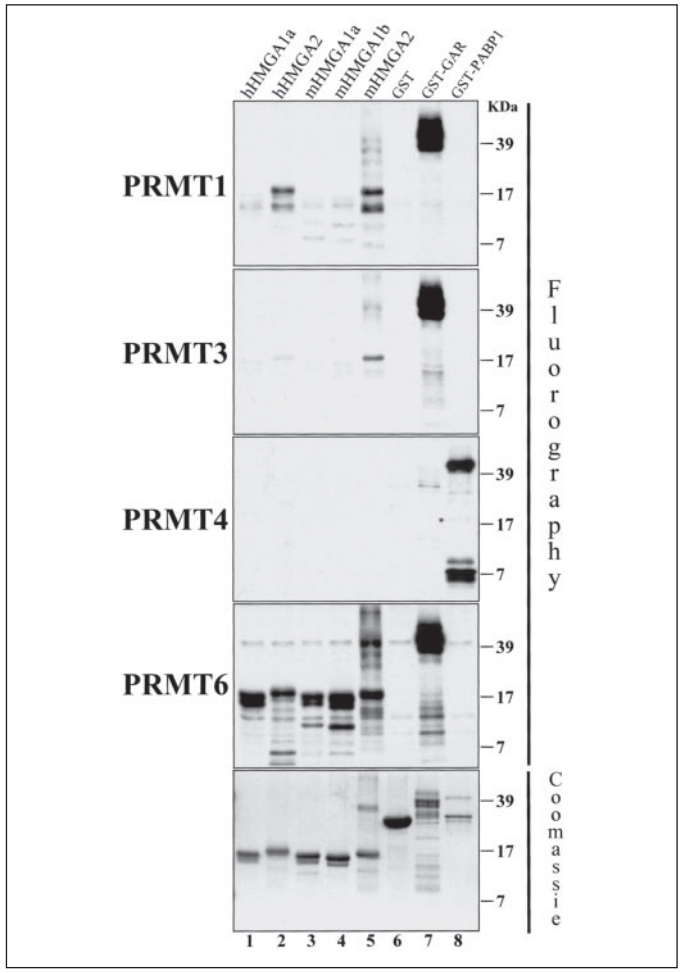


FIGURE 1. **HMGA proteins are *in vitro* methylated by PRMT6.** Comparable amounts of human (*h*) and murine (*m*) recombinant HMGA proteins were incubated with the indicated recombinant PRMTs in the presence of [^{3}H]AdoMet. Proteins were separated by SDS-PAGE and methylated proteins visualized by fluorography (exposure time, 24 h). For quantification purposes, the same amounts of recombinant proteins used in the methylation assay were separated by SDS-PAGE and stained with Coomassie Blue stain. GST-GAR and GST-PABP1 were used as positive controls, whereas GST is the negative control. Molecular mass markers are indicated on the *right*.

Interestingly, PRMT6 has an exclusive nuclear localization. Among the other methyltransferases tested, only PRMT1 and PRMT3 were able to weakly methylate HMGA2 (but not HMGA1a and HMGA1b). Recombinant GST-GAR was included as a positive control for PRMT1, PRMT3, and PRMT6, whereas GST-PABP1 is the positive control for PRMT4 (31). The newly described PRMT7 (29, 30) was tested at a later stage, but did not display any activity on HMGA proteins (data not shown).

PRMT6 Methylates Arg Residues within the Second AT-hook of HMGA1a—Because PRMTs are able to methylate short peptide sequences (33, 37, 38), to map the region of HMGA1a that is required for PRMT6 methylation, several HMGA1a deletion mutants were used. In addition, because methylation of Arg^{25} has been previously reported to occur at high levels in HMGA1a, point-mutated HMGA1aR25A was included. *In vitro* methylation reactions were performed as described above. Only the deletion of the second AT-hook region of HMGA1a (amino acids 52–79) is able to abolish methylation by PRMT6 (Fig. 2*A*, *lane 6*). All of the other deletion mutants that retain this *region* and also the point mutant R25A, which maps within the first AT-hook, are efficiently methylated by PRMT6 as the wild-type protein. As expected, PRMT1 did not display methylation activity on HMGA1a (Fig. 2*A*). The

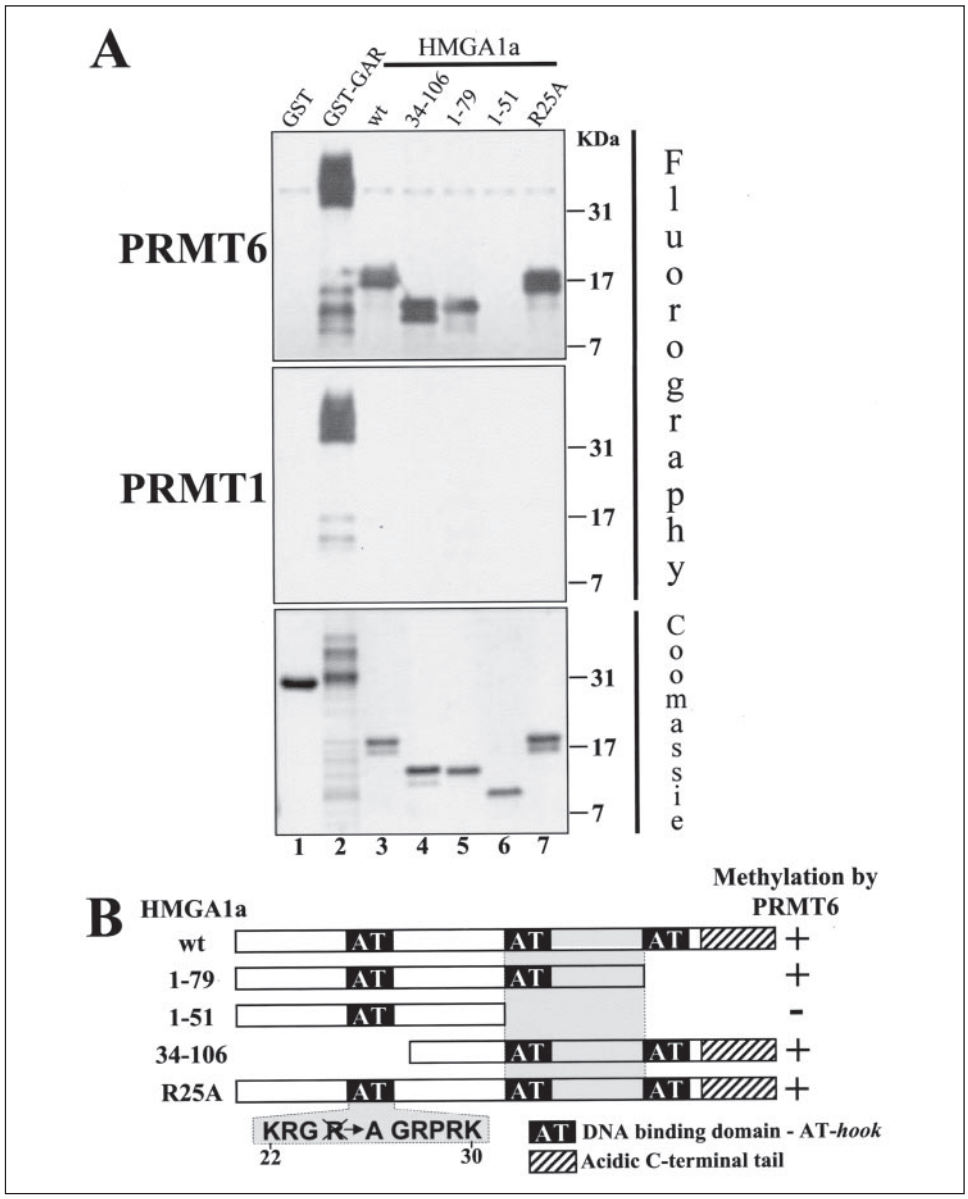


FIGURE 2. **Deletion analysis of HMGA1a defines the arginine-methylated region.** *A*, a series of recombinant HMGA1a deletion mutants and R25A point-mutated HMGA1a were incubated with PRMT6 and PRMT1 (negative control) in the presence of [^{3}H]AdoMet. Proteins were separated by SDS-PAGE and methylated proteins visualized by fluorography (exposure time, 24 h). For quantification purposes, the same amounts of recombinant proteins used in the methylation assay were separated by SDS-PAGE and stained with Coomassie Blue stain. GST-GAR and GST were used as the positive and negative control, respectively. Molecular mass markers are indicated on the *right*. *B*, schematic representation of the various HMGA1a forms used in the methylation assay. On the *right* is indicated their ability to be methylated by PRMT6. The *dotted box* over the five forms points out the region methylated by PRMT6. *wt*, wild type.

same experiment was conducted also on HMGA1b deletion mutants with essentially the same result (data not shown).

Arg^{57} and Arg^{59} Are Both Methylated by PRMT6—To establish that PRMT6 can methylate the second AT-hook of HMGA1a (in the context of the full-length protein), we subjected HMGA1a to an *in vitro* methylation assay with recombinant PRMT6. This methylated HMGA1a was then analyzed by mass spectrometry to identify the modified arginine residue(s). Because this region contains four Arg residues, to precisely map the one(s) modified by PRMT6, an *in vitro* methylation time course of recombinant HMGA1a was carried out at 0, 4, and 32 h, respectively. HMGA1a methylation was confirmed by LC-MS analyses as shown by the appearance in the reconstructed mass spectra of mono-, di-, and trimethylated forms of HMGA1a (Fig. 3*A*). Both the 4- and the 32-h methylated HMGA1a were high pressure liquid chromatography-purified and endoproteinase Lys-C-digested. The LC-MS-digested map is reported in Fig. 3*B*. In Fig. 3*C*, the relative ratio of monomethylated peptides is reported. The 4-h experiment gave only one methylated peptide-(55–61), whereas the 32-h experiment gave three methylated peptides (peptide-(23–30), -(55–61), and -(82–87)). The alignment of the sequence of these peptides (Fig. 3*B*, *right*) shows that they contain a similar arginine motif corresponding to the Arg-rich "core" domain of the three AT-hooks. These data indicate that, in agreement with the preceding *in vitro* radiolabeling experiments (Fig. 2), PRMT6 can methylate HMGA1a at multiple sites but that the preferred site maps to peptide-(55–61).

In Fig. 4*A*, a MS spectrum obtained with a Q-TOF mass spectrometer shows that the peptide-(55–61) (seen as triple-charged ion $[M + 3H]^{3+}$) can be either unmethylated (*m*/*z* 289.52), monomethylated (294.19), or dimethylated (298.86). By tandem mass spectrometry, we obtained typical b and y ions for each of the three forms (Fig. 4*B*). On this peptide, there are three potential methylation sites: Arg^{55}, Arg^{57}, and Arg^{59}. Because the b_1 (*m*/*z* 157.11) and the b_2 (*m*/*z* 254.15) ions are the same for all of the three forms (un-, mono-, and dimethylated) and no other signals are detected, we can conclude that Arg^{55} is never modified. Looking at the fragmentation ions obtained by the monomethylated form (*m*/*z* 294.19), Arg^{57} can be either unmethylated (b_3 ion, *m*/*z* 205.62) or monomethylated (b_3 ion, *m*/*z* 212.64). Because the precursor ion corresponds to the monomethylated peptide-(55–61), the implication is that when Arg^{57} is not modified, Arg^{59} must be methylated. This is confirmed from the y_4 ions (*m*/*z* 457.26 and

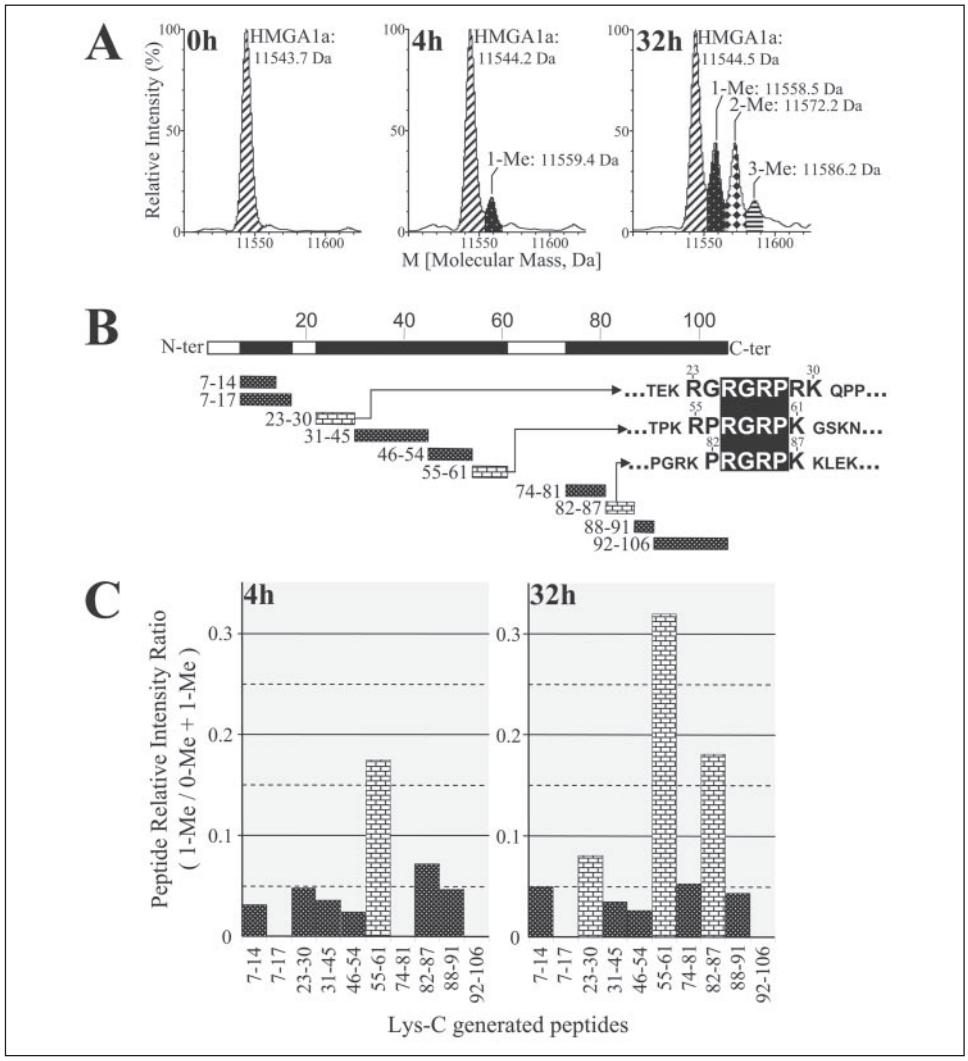


FIGURE 3. **The Arg-rich core motif of the AT-hooks of HMGA1a is methylated by PRMT6.** *A*, reconstructed mass spectra of recombinant HMGA1a *in vitro* methylated by PRMT6 for 0, 4, and 32 h, respectively. The various molecular mass values (*Da*) for each peak are indicated in the spectra. *B*, Lys-C digestion map of the 32-h methylated HMGA1a. The three peptides found methylated (peptide-(23–30), -(55–61), and -(82–87)) are evidenced, and their sequences are aligned on the *right* side. *C*, histogram graphs showing a comparison between the monomethylation degrees of the various peptides obtained after the 4- and 32-h *in vitro* methylation reactions and Lys-C digestions. The degree of monomethylation is calculated as a ratio between the intensity (relative intensity (%) in the *m/z* spectra) of the monomethylated peak and the sum of the intensities of the unmethylated (*0-Me*) and the monomethylated (*1-Me*) peaks. *2-Me*, dimethylated; *3-Me*, trimethylated.

471.27), which indicate that Arg^{59} can either be unmethylated or monomethylated. Fragmentation ions obtained from the dimethylated peptide (*m/z* 298.86) shows that Arg^{57} is found unmethylated (b_3 ion, *m/z* 205.62) or dimethylated (b_3 ion, *m/z* 219.65) but never monomethylated. Again, because Arg^{55} is not modified, when Arg^{57} is unmethylated, Arg^{59} must be dimethylated. Indeed the y_4 ions (*m/z* 457.24 and 485.30) obtained from the fragmentation of the dimethylated 55–61 form show that Arg^{59} can solely be unmethylated or dimethylated.

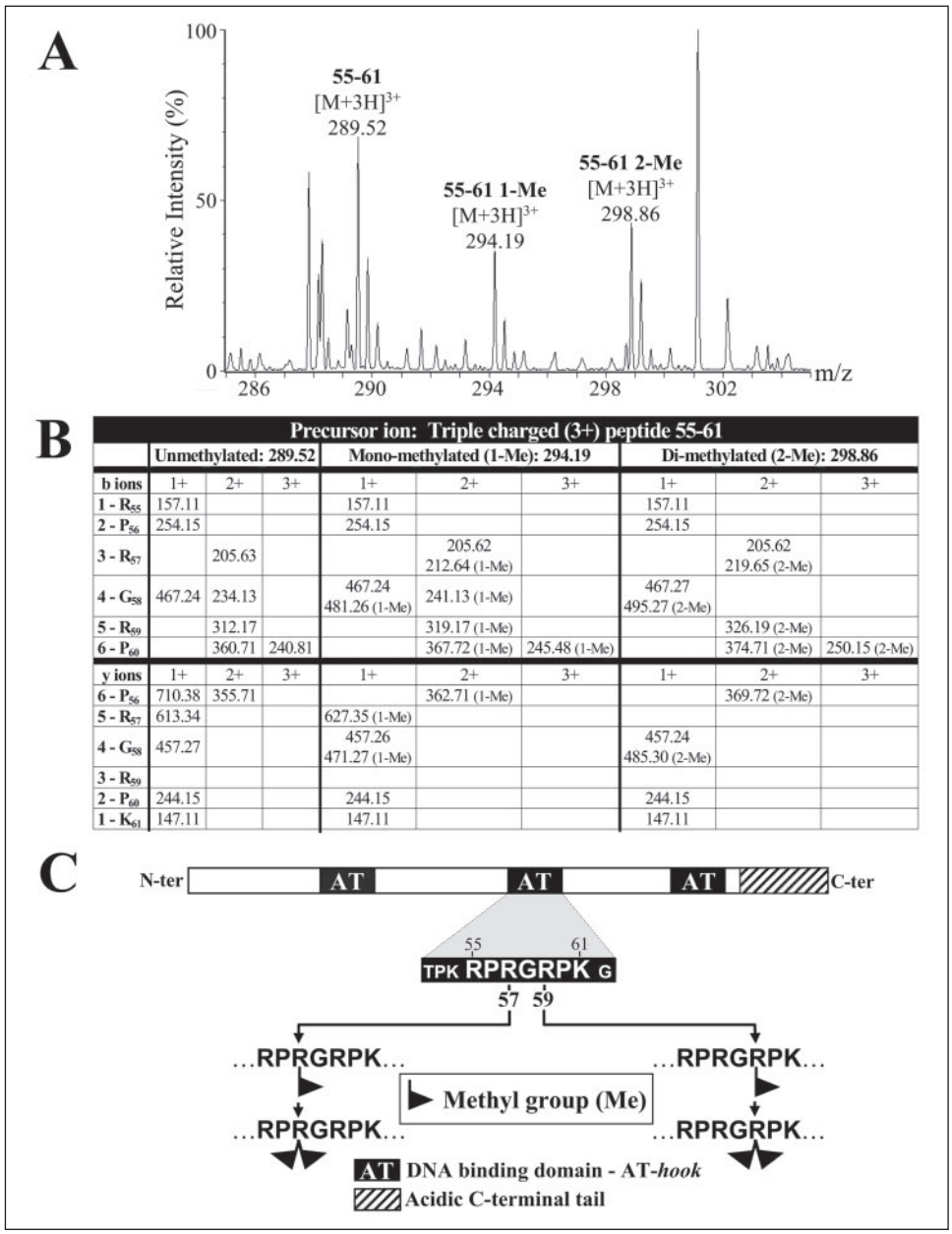
Therefore, MS/MS analysis indicates that both Arg^{57} and Arg^{59} can be methylated by PRMT6 but also that these two sites are mutually exclusive; once one of the two Arg is monomethylated, the addition of a second methyl group can only happen on the same residue. A scheme of the inferred methylation process is depicted in Fig. 4*C*.

MS/MS spectra of arginine-dimethylated peptides are often characterized by the presence of *m/z* signals arising from the loss of peculiar neutral fragments at the level of the methylated arginine (21). Depending on the symmetric or asymmetric nature of the dimethylation modification, it is possible to observe 70 or 45 Da neutral loss, respectively. The presence of a neutral loss of 45 Da, which corresponds to the loss of $[NH(CH_3)_2]$dimethylamine from the dimethylated peptide-(55–61) during the MS/MS fragmentation, clearly indicates that PRMT6 catalyzes the formation of asymmetric ω-N^G-N^G-dimethylarginine (data not shown).

To confirm that Arg^{57} and Arg^{59} are substrates of PRMT6, single point mutants HMGA1aR57A and HMGA1aR59A and the double mutant HMGA1aR57A,R59A were generated substituting arginine with alanine and tested in *in vitro* methylation assays using PRMT6. Fig. 5 clearly shows that the single point mutants are both methylated less efficiently than the wild type, whereas in the double mutant, the methylation is drastically reduced, thus confirming the identity of the modified residues. The faint residual radioactivity incorporation observable in the HMGA1aR57A, R59A mutant is likely due to methylation at the level of the first and third AT-hook. Indeed, these domains, as demonstrated by the mass spectrometry data shown in Fig. 4*C*, are less efficiently methylated by PRMT6.

HMGA1a Associates with and Is in Vivo Methylated by PRMT6—Certain PRMTs have been shown to interact with their substrates (39, 40). To demonstrate that HMGA1a can associate *in vitro* with PRMT6, a blot overlay was employed using wild-type HMGA1a along with several deletion mutants. As can be seen in Fig. 6*A*, HA-tagged PRMT6 expressed in HEK293 cells is able to associate *in vitro* with wild-type HMGA1a as well as with the other deletion mutants that retain the second AT-hook. Conversely, HMGA1a-(1–51), which has the second AT-hook removed, did not interact with PRMT6 (Fig. 6*A*, *lane 5*). HA-tagged NPM, a known interactor of HMGA1a (8), and the empty HA vector were used as positive and negative controls, respectively (Fig. 6*A*). The same region therefore that is methylated by PRMT6 is also responsible for the association with this enzyme.

To determine whether HMGA1a and PRMT6 interact *in vivo*, co-immunoprecipitation experiments were performed. HEK293 cells, which



B

Precursor ion: Triple charged (3+) peptide 55-61									
	Unmethylated: 289.52			Mono-methylated (1-Me): 294.19			Di-methylated (2-Me): 298.86		
b ions	1+	2+	3+	1+	2+	3+	1+	2+	3+
1 - R_{55}	157.11			157.11			157.11		
2 - P_{56}	254.15			254.15			254.15		
3 - R_{57}		205.63			205.62 212.64 (1-Me)			205.62 219.65 (2-Me)	
4 - G_{58}	467.24	234.13		467.24 481.26 (1-Me)	241.13 (1-Me)		467.27 495.27 (2-Me)		
5 - R_{59}		312.17			319.17 (1-Me)			326.19 (2-Me)	
6 - P_{60}		360.71	240.81		367.72 (1-Me)	245.48 (1-Me)		374.71 (2-Me)	250.15 (2-Me)
y ions	1+	2+	3+	1+	2+	3+	1+	2+	3+
6 - P_{56}	710.38	355.71			362.71 (1-Me)			369.72 (2-Me)	
5 - R_{57}	613.34			627.35 (1-Me)					
4 - G_{58}	457.27			457.26 471.27 (1-Me)			457.24 485.30 (2-Me)		
3 - R_{59}									
2 - P_{60}	244.15			244.15			244.15		
1 - K_{61}	147.11			147.11			147.11		

FIGURE 4. **Arg[57] and Arg[59] are mutually exclusively methylated by PRMT6.** *A*, mass spectrum showing the *m/z* signals of the three precursor ions, corresponding to the triple-charged peptide-(55–61) (unmethylated), -(55–61) (*1-Me*, monomethylated), and -(55–61) (*2-Me*, dimethylated) peptides selected for MS/MS analysis. *B*, table reporting the b and y series obtained in the MS/MS analyses. *C*, schematic representation of the HMGA1a domains and the mutually exclusive methylation on Arg[57] and Arg[59]. *N-ter*, N terminus; *C-ter*, C terminus.

express high levels of HMGA1a protein, were transfected with an HA-tagged PRMT6 expressing vector, cell lysates were immunoprecipitated with an α-HMGA1 antibody, and subsequently the immunocomplexes were visualized with an α-HA antibody. As shown in Fig. 6*C*, PRMT6 associates with HMGA1a (*lane 2*). Complex formation was not detected in the absence of PRMT6 overexpression (*lane 4*). As controls, cell lysates were tested for the expression of HA-tagged PRMT6 (Fig. 6*C*, *lanes 1* and *3*, *upper panel*) and endogenous HMGA1 (*lanes 1* and *3*, *lower panel*). Taken together, these results clearly demonstrate that PRMT6 and HMGA1a physically associate both *in vitro* and *in vivo*, providing further support that PRMT6 can methylate HMGA1a.

To determine whether HMGA1a is methylated *in vivo* by PRMT6, HEK293 cells were transiently transfected with HA-tagged forms of PRMT6 and HMGA1a, and methylation was assessed by metabolic labeling with L-[methyl-^{3}H]methionine as previously described (41). The *in vivo* methylation assay was performed in the presence of translation inhibitors to ensure that the labeling was not due to incorporation of methionine into newly synthesized proteins. HMGA1a was immunoprecipitated, resolved by SDS-PAGE, and visualized by fluorography. Fig. 7 shows that protein synthesis was inhibited (compare *lanes 1* and *2* with *lanes 4* and *5*, *lower panel*) and that both HA-tagged HMGA1a and PRMT6 were efficiently and specifically immunoprecipitated (*upper panel*). As can be seen in Fig. 7, HMGA1a is specifically methylated by PRMT6; in fact, a band of the correct size is detectable only in PRMT6-transfected cells (Fig. 7, *lane 11*, *lower panel*) and not in cells transfected with the empty vector. The band *below* 20 kDa, detected in *lanes 7* and *10*, which appears methylated, very likely corresponds to histones.

DISCUSSION

In this report, we have identified HMGA1a as an *in vivo* cellular substrate of PRMT6 and mapped the methylated residues. PRMT6 is a novel arginine methyltransferase that was recently discovered by data base screening (31). It appears to be unique among the other type I PRMT and, in fact, is the only one to display automethylating abilities. A

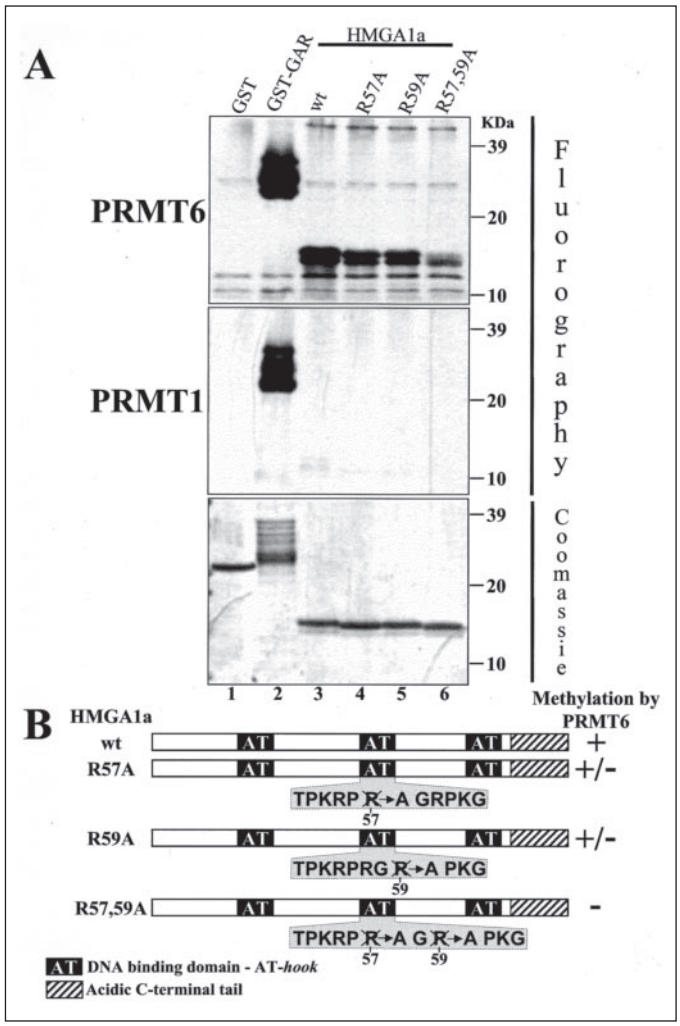


FIGURE 5. **The double point mutant HMGA1aR57A,R59A is not methylated by PRMT6.** *A*, wild-type HMGA1a, R57A, R59A, and R57A,R59A point-mutated forms were incubated with PRMT6 and PRMT1 (used as a negative control) in the presence of [^{3}H]AdoMet. Proteins were separated by SDS-PAGE and methylated proteins visualized by fluorography (exposure time, 24 h). For quantification purposes, the same amounts of recombinant proteins used in the methylation assay were separated by SDS-PAGE and stained with Coomassie Blue stain. GST-GAR and GST were used as the positive and negative control, respectively. Molecular mass markers are indicated on the *right*. *B*, schematic representation of the various HMGA1a mutants used in the methylation assay. On the *right* is indicated their ability to be methylated by PRMT6. *wt*, wild type.

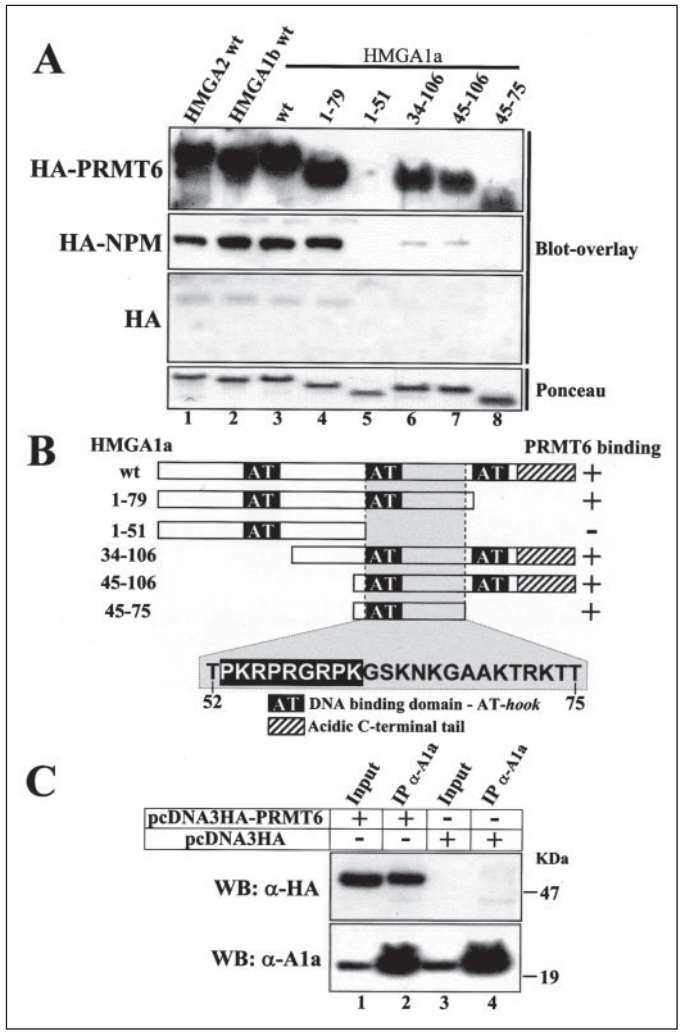


FIGURE 6. **HMGA1a and PRMT6 associate both *in vitro* and *in vivo*.** *A*, HMGA2, HMGA1b, HMGA1a, and a series of HMGA1a deletion mutants (fragments 1–79, 1–51, 34–106, 45–106, and 45–75) were SDS-PAGE-separated and transferred to polyvinylidene difluoride membranes. Blot overlay experiments were then performed, incubating the membranes with cell lysates obtained from HEK293 cells transfected with pcDNA3HA-PRMT6, pcDNA3HA-NPM, and pcDNA3HA vectors, respectively. HA-tagged proteins, bound to immobilized HMGA proteins, were detected by α-HA antibody. For quantification purposes, a Ponceau stained membrane is shown. *B*, schematic representation of the various HMGA1a forms used in the blot overlay assay. On the *right* is indicated their ability to bind to PRMT6. The *dotted box* over the six forms points out the protein-protein interaction region throughout which HMGA1a binds PRMT6. wt, wild type. *C*, lysates from HEK293 cells transfected with pcDNA3HA-PRMT6 (*lane 2*) or with the empty vector pcDNA3HA (*lane 4*) were immunoprecipitated with α-HMGA1a antibody. The immunoprecipitates (*IP*) were analyzed by Western blot (*WB*) with an α-HA antibody or an α-HMGA1a antibody. Total lysate used in the immunoprecipitation experiments (*Input*) was controlled for the amount of HA-PRMT6 and HMGA1a (*lanes 1* and *3*).

PRMT6 orthologue has been identified in fish, where expression is seen predominantly in the brain (42). Similar to PRMT4, PRMT6 displays an exclusive nuclear localization (when expressed as GFP fusion proteins) (31). Recently, the HIV transactivator protein Tat was identified as a substrate of PRMT6. Tat methylation, occurring in the Arg-rich transactivating domain, negatively regulates its transactivation activity and restricts HIV replication, thus demonstrating a role for PRMT6 in transcriptional regulation (32). Here we found that PRMT6 methylates HMGA1a principally in the second AT-hook, at Arg^{57} and Arg^{59} and to a lesser extent in the third AT-hook (at Arg^{83} and Arg^{85}, data not shown) and at a very low level in the first AT-hook. Interestingly, if we compare the sequence of the peptides found methylated in HMGA1a, we see that all of them share a core sequence constituted by Arg-Gly-Arg-Pro. The third AT-hook is more similar to the second, because there is a Pro and a Lys flanking the core sequence of both; therefore, the symmetrical sequence Pro-Arg-Gly-Arg-Pro seems to be the motif recognized in HMGA1a, even though addition flanking residues account for the preference for the second AT-hook. Of note, mass spectrometry data show that, within this sequence, only one Arg is found methylated (either mono- or dimethylated). Therefore, PRMT6 methylates Arg preceding a Gly independently on the orientation. This methylated sequence fits with the general consensus (F/G)GGRGG(G/F) suggested for the majority of type I PRMTs, with only the underlined Arg and Gly residues found in all methylated sites, and is most similar to the RGRG and PRG repeats methylated by PRMT1 (31). PRMT6 seems to be highly selective, having the ability to discriminate between subtle variations of the sequence surrounding the RG residues within HMGA1 AT-hook sequences. All three AT-hooks are highly conserved among the HMGA family, thus explaining why PRMT6 was able to methylate HMGA1b and the highly related HMGA2 in addition to HMGA1a. Furthermore, mass spectrometry data confirm the PRMT6 belonging to the type I

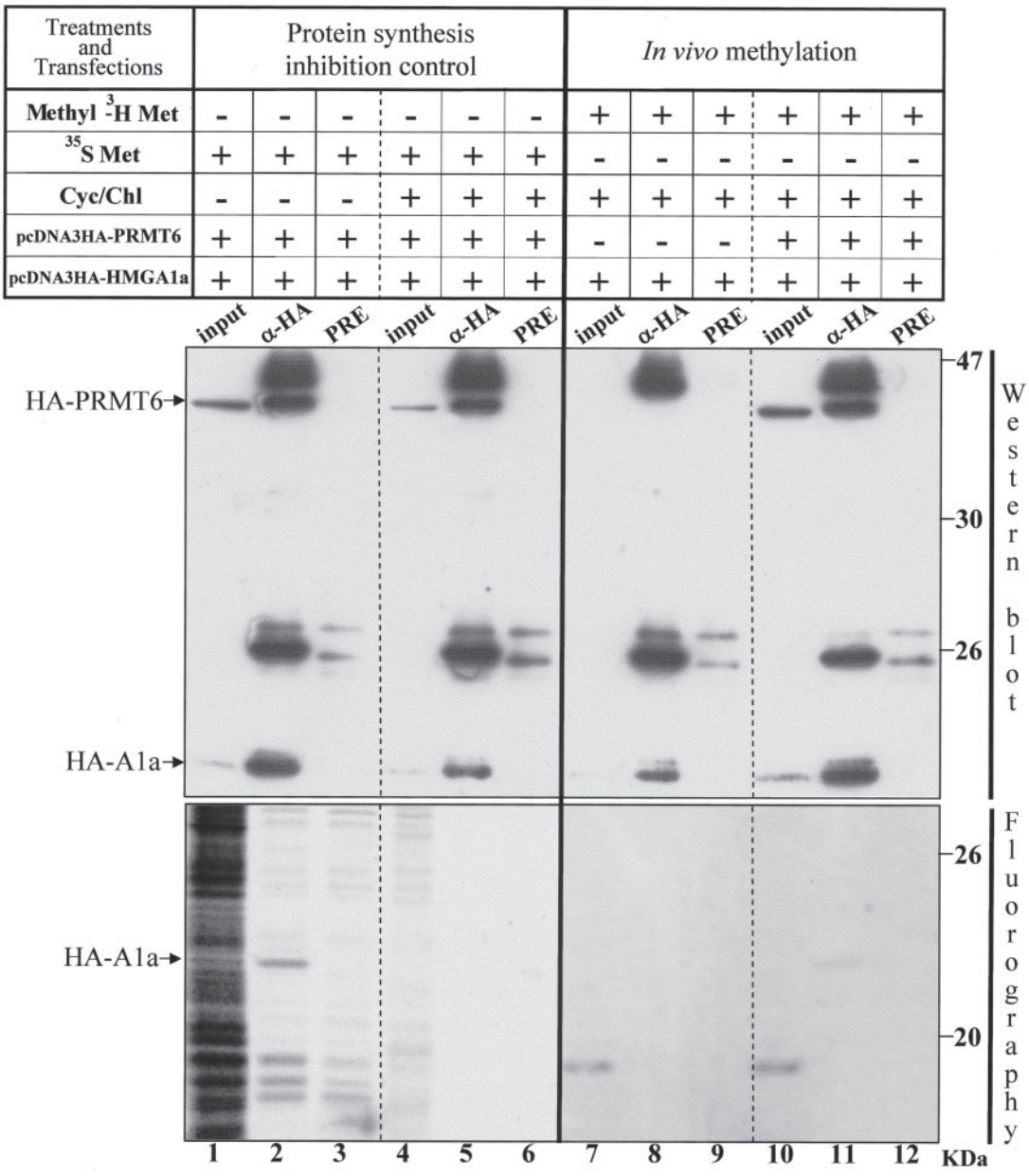


FIGURE 7. **HMGA1a is *in vivo* methylated by PRMT6.** HEK293 cells were transiently transfected to express the indicated proteins (HA-PRMT6 and HA-HMGA1a), treated with protein synthesis inhibitors cycloheximide and chloramphenical (*Cyc/Chl*), and methylated proteins were labeled *in vivo* (using methyl-^{3}H Met). Immunoprecipitations were carried out with α-HA antibody (*α-HA*) or with a pre-immune serum (*PRE*), and immunoprecipitated proteins were SDS-PAGE-analyzed, transferred onto nitrocellulose membranes, and visualized by Western blot using an α-HA antibody (*right*, *upper side*) and by fluorography (*right*, *lower side*) to detect ^{3}H-labeled proteins. Total lysates (*Input*) were analyzed in parallel to verify the expression of the HA-tagged proteins. In the *left* part of the figure, a parallel control experiment is shown. HEK293 cells, expressing both HA-PRMT6 and HA-HMGA1a, were incubated with [^{35}S]Met in the presence or absence of protein synthesis inhibitors to verify the protein synthesis inhibition. Bands detected in *lanes 2*, *3*, *5*, *6*, *8*, *9*, *11*, and *12* of the Western blot at ~26 kDa and just below 47 kDa correspond to the light and heavy chains, respectively, of the immunoglobulins used in the immunoprecipitation.

PRMT family, because it catalyzed the asymmetrical dimethylation of HMGA1a.

Arg^{25}, localized within the first AT-hook of HMGA1a, has been shown to be a major site of modification in tumor cells (11), but other Arg residues, in addition to those reported in this paper, have been found methylated *in vivo* (9, 10). In this study, we were unable to identify other PRMTs, except PRMT6, that modify HMGA1a protein. It is possible that a tissue-specific (or tumor-specific) factor could regulate the activity of a PRMT to facilitate HMGA1a methylation. Alternatively, there may be an HMGA code that requires an initiating PTM (such as phosphorylation or acetylation) that then facilitates subsequent arginine methylation.

All three AT-hooks of HMGA1a protein are involved in DNA interaction. All of them, in fact, are engaged at the same time when multiple stretches of AT bases are present in the DNA. In the absence of long AT run, it is the second AT-hook that has the highest affinity for DNA and that is engaged in DNA binding, either alone or in combination with the first or the second, depending on the DNA target (43, 44). NMR data obtained using a truncated HMGA1a form containing the second and third AT-hooks and a DNA target showed that the central Arg-Gly-Arg motif is engaged in interactions with both the DNA backbone and its bases establishing extensive hydrophobic and polar contacts (45). Here we report that the core of the second AT-hook can be subjected to PTM that can potentially modify its affinity for DNA.

As mentioned, HMGA proteins not only interact with DNA but also with different nuclear proteins, including transcription factors (6, 8). The region where most of the interactions have been mapped includes the second AT-hook (6, 8); the possibility exists therefore that this modification can alter protein-protein interaction as well.

PTMs mapping within this region (phosphorylation by p34/cdc2 at Thr^{52} and Thr^{77} and acetylation by p300/CBP and PCAF/GCN5 at Lys^{64} and Lys^{70}, respectively) have already been shown to alter HMGA1 functions (15, 17, 19, 44). A possibility exists that either methylation *per se* could alter HMGA1a protein-DNA and/or protein-protein interaction or could participate in the interplay of PTM as has been shown for the methylation of histones. In the case of histone, PTMs are mechanistically linked, and different combinations of acetylation, phosphorylation, and monoubiquitination regulate chromatin structure and transcription by stimulating or inhibiting the binding of specific proteins (46).

PTMs can have a profound impact on HMGA1 function affecting its biological effect. Indeed, it has been shown that acetylation of Lys^{59} in HMGA1b (which corresponds to Lys^{70} of the isoform HMGA1a) was required for HMGA1b-induced apoptosis in normal rat thyroid cells (47). Arginine methylation in HMGA1a has been found to correlate with apoptosis and tumor progression (10, 11). It is therefore possible that this PTM can play a direct role in modulating the HMGA1a function in these two processes.

Arginine methylation is an emerging regulator of protein function; however, the practical consequences of this modification and its mode of regulation remain largely unknown. The finding, reported in this paper that the recently described PRMT6 associates with and *in vivo* methylates the HMGA1a protein, suggests a possible pathway for this PRMT, placing it in the highly connected network of chromatin proteins involved both in differentiation and neoplastic transformation.

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