Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 336 (2005) 831–835
BBRC
www.elsevier.com/locate/ybbrc

Protein arginine methyltransferase 6 specifically methylates the nonhistone chromatin protein HMGA1a

Tina Branscombe Miranda [a], Kristofor J. Webb [a], Dale D. Edberg [b], Raymond Reeves [b], Steven Clarke [a,*]

[a] *Department of Chemistry and Biochemistry, Molecular Biology Institute, UCLA, Los Angeles, CA 90095-1560, USA*
[b] *The School of Molecular Biosciences, Washington State University, Pullman, Washington 99164, USA*

Received 15 August 2005
Available online 1 September 2005

Abstract

The HMGA family proteins HMGA1a and HMGA1b are nuclear nonhistone species implicated in a wide range of cellular processes including inducible gene transcription, modulation of chromosome structure through nucleosome and chromosome remodeling, and neoplastic transformation. HMGA proteins are highly modified, and changes in their phosphorylation states have been correlated with the phase of the cell cycle and changes in their transcriptional activity. HMGA1a is also methylated in the first DNA-binding AT-hook at Arg25 and other sites, although the enzyme or enzymes responsible have not been identified. We demonstrate here that a GST fusion of protein arginine methyltransferase 6 (PRMT6) specifically methylates full-length recombinant HMGA1a protein in vitro. Although GST fusions of PRMT1 and PRMT3 were also capable of methylating the full-length HMGA1a polypeptide, they recognize its proteolytic degradation products much better. GST fusions of PRMT4 or PRMT7 were unable to methylate the full-length protein or its degradation products. We conclude that PRMT6 is a good candidate for the endogenous enzyme responsible for HGMA1a methylation.

Keywords: Protein arginine methylation; PRMT6; HMGA1a

The mammalian high-mobility group (HMG) nonhistone chromatin proteins are abundant nuclear proteins functioning in multiple processes such as gene transcription and DNA replication, recombination, and repair [1]. They are divided into subfamilies based on the type of structurally distinct DNA-binding domain they possess. The HMGA (formerly HMG-I/Y) subfamily, containing HMGA1a, HMGA1b, and HMGA2, participates in specific protein–DNA and protein–protein interactions that induce structural changes in the chromosome and are involved in the formation of enhanceosomes in the promoter and enhancer regions of genes they regulate [2,3]. These proteins have a relative molecular mass of ~10,000 and share three highly conserved "AT-hook" DNA-binding domains [4]. The domains are comprised of a conserved Arg-Gly-Arg-Pro sequence flanked by positively charged amino acids [5]. The AT-hooks enable the HMGA proteins to bind to the minor groove of AT-rich DNA stretches, recognizing the local structure and not the nucleotide sequence [6]. The HMGA proteins are coded for by two genes. HMGA2 is coded on the *HMGA2* gene located on chromosome 12 [7], and the related splice variants HMGA1a and HMGA1b proteins are transcribed from the single *HMGA1* gene located on chromosome 6 [8]. The HMGA1a and HMGA1b proteins are identical in sequence with the exception of a deletion of an 11 amino acid region between the first and second AT-hook domain [9]. HMGA1 proteins behave as architectural transcription factors, inducing changes in chromatin structure by directly binding AT-rich regions and nucleosomes [6]. It has been shown that tumorigenic cells show increased levels of *HMGA1* gene products and the overexpression of HMGA1a or HMGA1b in rat 1a cells correlates to increased neoplastic transformation [10].

* Corresponding author. Fax: +1 310 825 1968.
E-mail address: clarke@mbi.ucla.edu (S. Clarke).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.08.179

The diversity of the functions HMGA1 proteins are involved in may be directed by a variety of posttranslational modifications including phosphorylation, acetylation, methylation, and ADP-ribosylation [11–14]. It has been found that phosphorylation and acetylation modulate the binding affinity of individual HMGA proteins to DNA and enhanceosomes [6]. An additional modification of HMGA1a is the methylation of arginine residues. Monomethylation of arginine 25 was detected in human leukemia, human prostate tumor, and rat thyroid transformed cells [13]. Later asymmetric and symmetric dimethylation of arginine 25 was detected in PC-3 human prostate cancer cells [15]. This methylation occurs in the Arg-Gly-Arg-Pro consensus sequence of the first AT-hook DNA-binding motifs. Edberg et al. [14,16] have also reported the presence of monomethyl- and dimethylarginine in HMGA1a and HMGA1b at multiple sites in proteins from cultured human breast cancer cells of differing metastatic potentials, including methylated peptides containing the region where Arg 25 is located. The variation in arginine methylation states seen in the HMGA proteins may be due to the cell types the HMGA proteins were isolated from and the degree of malignancy.

The methylation of arginine residues has recently come into light as an important posttranslational modification involved in the regulation of RNA processing, signal transduction, and DNA repair [17]. Two major types of protein arginine methyltransferases (PRMT) are known in mammalian cells. The type I enzymes catalyze the formation of ω-N^G-monomethylarginine and asymmetric ω-N^G, ω-N^G-dimethylarginine, whereas the type II enzymes catalyze the formation of ω-N^G-monomethylarginine and symmetric ω-N^G, ω-N^G-dimethylarginine [18]. A family of at least seven mammalian protein arginine methyltransferases have been described. PRMT1 [19], PRMT3 [20], PRMT4 [21], and PRMT6 [22] have been shown to be type I enzymes; PRMT5 [23] has been shown to be a type II enzyme. The initial characterization of PRMT7 suggests it might be a type II enzyme [24,25]. No activity has yet been associated with PRMT2 [26].

In this work, we screened all the known type I protein arginine methyltransferases for their ability to methylate HMGA1a in vitro. We provide evidence for a role of PRMT6 in this modification.

Experimental procedures

Construction and purification of GST-PRMT1, GST-PRMT3, GST-PRMT4, GST-PRMT6, and GST-GAR. Bacterial expression clones for GST-PRMT1 [19], GST-PRMT3 [20], GST-PRMT4 [21], GST-PRMT6 [22], GST-PRMT7 [24], and a GST fusion polypeptide containing the N-terminal portion of human fibrillarin (GST-GAR) [20] were described previously. GST-PRMT1, GST-PRMT3, GST-PRMT4, GST-PRMT7, and GST-GAR were overexpressed in *Escherichia coli* DH5α (Invitrogen), while GST-PRMT6 was overexpressed in *E. coli* BL21 DE3 (Invitrogen). The cells were induced with a final concentration of 0.4 mM isopropyl-β-D-thiogalactopyranoside for 4–5 h at 37 °C. Washed cells were resuspended in 2 ml phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4) per gram of cells (wet weight) with a final concentration of 100 μM phenylmethylsulfonyl fluoride and were subsequently lysed by six 20-s sonicator pulses (50% duty; setting 4) at 0 °C with a Sonifier cell disruptor W-350 (SmithKline Corp.). The resulting lysate was centrifuged for 50 min at 13,000*g* at 4 °C. The GST fusion protein was then batch-purified from extracts by binding to glutathione–Sepharose 4B beads (Amersham Biosciences) and washed in PBS as per the manufacturer's instructions in the presence of phenylmethylsulfonyl fluoride. The purified proteins were eluted from the beads with 30 mM glutathione, 50 mM Tris–HCl, pH 7.5, 120 mM NaCl, and 2% glycerol.

HMGA1a isolation and purification. Full-length recombinant human (rh) HMGA1a proteins were prepared from *E. coli* BL21 DE3 pLysS cells (Invitrogen) as previously described [27]. Briefly, acid soluble proteins were extracted with dilute (5%) trichloroacetic acid from cells induced to overexpress the rhHMGA1a protein. The rhHMGA1a proteins were then purified using a reverse-phase high-performance liquid chromatography (RP-HPLC) C4 Microsorb analytical column with a linear 12–25% acetonitrile, 0.2% trifluoroacetic acid gradient over 72 min. Further purification of the full-length rhHMGA1a was accomplished with a RP-HPLC C18 Dynamax analytical column utilizing a linear 0–23% acetonitrile, 0.2% trifluoroacetic acid gradient over 80 min. Purity of the isolated rhHMGA1a protein fractions was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), Western blotting, and verified by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry.

In vitro methylation of HMGA1a and GST-GAR. Either 2 μg of HMGA1a or 10 μg of GST-GAR was added to 1 μg of either GST-PRMT1, GST-PRMT3, GST-PRMT4, GST-PRMT6 or GST-PRMT7 and incubated with 1.8 μM *S*-adenosyl-L-[methyl-^{3}H]methionine (Amersham Pharma Biotech, 75 Ci/mmol, in dilute HCl/ethanol (9:1), pH 2–2.5) in 50 mM sodium phosphate buffer, pH 7.5, in a final volume of 30 μl. Reactions were stopped by adding 30 μl of 2× SDS gel sample buffer (180 mM Tris–HCl, pH 6.8, 4% SDS, 0.1% β-mercaptoethanol, 20% glycerol, and 0.002% bromphenol blue) and heating at 100 °C for 3 min. Samples were electrophoresed at 35 mA for 5 h using the buffer system described by Laemmli [28] on a gel prepared with 12.6% (w/v) acrylamide and 0.43% (w/v) *N*,*N*-methylenebisacrylamide (1.5 mm thick, 10.5 cm resolving gel, 2 cm stacking gel). Gels were stained with Coomassie brilliant blue R-250 for 30 min and destained in 10% (v/v) methanol, 5% (v/v) acetic acid overnight. For fluorography, gels were treated with EN^{3}HANCE (Perkin-Elmer Life Sciences) as per the manufacturer's instructions. Gels were dried at 70 °C in vacuo and exposed to Kodak X-Omat AR scientific imaging film at −80 °C for the times indicated in the figure legends.

Results and discussion

To search for protein arginine methyltransferases that might be responsible for the methylation of HMGA1a in vivo, we did a screen to see which enzymes could methylate recombinant human HMGA1a in vitro. GST fusions of PRMT1, PRMT3, PRMT4, PRMT6, and PRMT7 were purified and their activity towards GST-GAR was determined. GST-GAR is a fusion polypeptide containing the N-terminal portion of human fibrillarin and has been shown to be a good substrate for many of the enzymes [20,23]. Proteins were incubated in the presence of [^{3}H]AdoMet, electrophoresed on a SDS–polyacrylamide gel, and exposed to X-ray film as described in the Experimental procedures section. We found similar activities with PRMT1, PRMT3, and PRMT6, with GST-PRMT3 being the most active and GST-PRMT6 being less active (Fig. 1). As expected, no activity was found with PRMT4 and PRMT7 (data not shown) [22,24]. Interestingly, the three enzymes active on GST-GAR recognized primarily

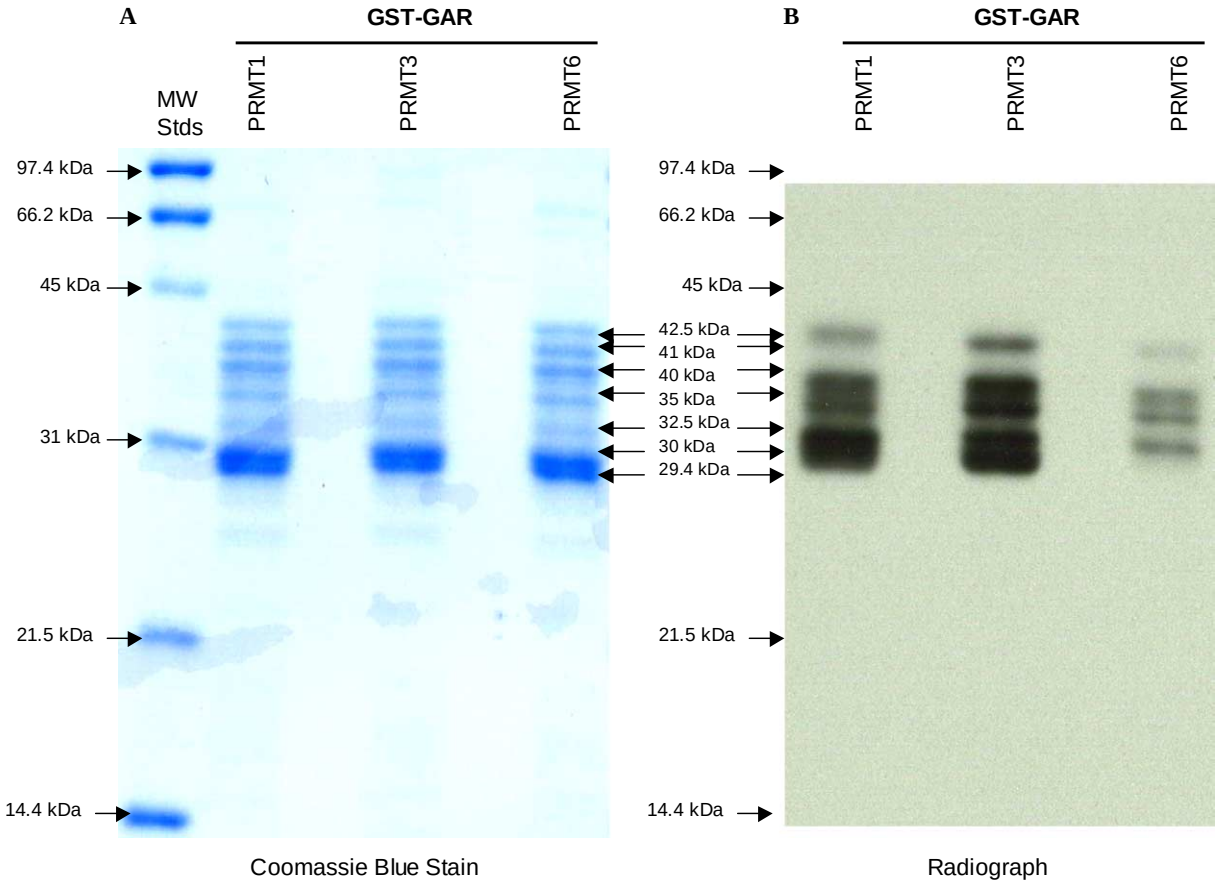

Fig. 1. Methylation of GST-GAR and its degradation products by protein arginine methyltransferase family members. Recombinant PRMT1, PRMT3, and PRMT6 GST fusion proteins were incubated with GST-GAR in the presence of [^{3}H]AdoMet as described under Experimental procedures. Reactions were directly loaded onto a 12.6% gel for SDS–polyacrylamide gel electrophoretic analysis. Gels were stained with Coomassie brilliant blue (A), treated with EN^{3}HANCE, dried, and exposed to film for 18 h (B). Molecular weight standards include bovine phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), chicken ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg lysozyme (14.4 kDa). The full-length GST-GAR migrates at 42.5 kDa, the more rapidly migrating species appearing to be proteolytic degradation products. The calculated sizes of GST-GAR polypeptides and radiolabeled species were found by interpolation of the standard polypeptide migrations.

its degradation products as has been seen previously [20,22]. In fact, PRMT1 and PRMT6 appear to prefer the degradation products over the full-length GST-GAR. PRMT1 and PRMT3 recognize the same group of polypeptides while PRMT6 did not methylate the 30 kDa degradation product (Fig. 1). The specificity for GST fusions of PRMT1, PRMT3, and PRMT6 is summarized in Fig. 2.

GST fusions of PRMT1, PRMT3, PRMT4, PRMT6, and PRMT7 were then incubated with HMGA1a in the presence of [^{3}H]AdoMet, and the products were separated on a SDS–polyacrylamide gel and analyzed as described above. GST-PRMT1 recognized the full-length HMGA1a poorly, and most of the methylation products were lower molecular weight degradation products. In contrast to the situation with GST-GAR, we do not detect significant amount of degradation products by Coomassie staining (Fig. 3). Thus, the methylation of these products indicates that they are recognized much better than the full-length polypeptide. GST-PRMT3 recognized the full length and the degradation products of HMGA1a. GST-PRMT3 has

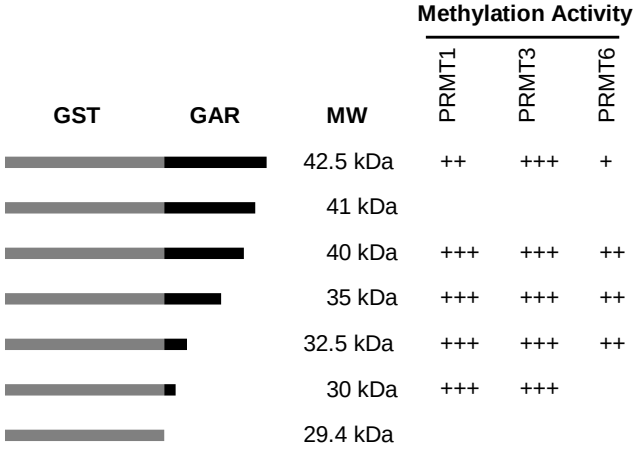

Fig. 2. Summary of the methylation activity of PRMT1, PRMT3, and PRMT6 on GST-GAR and its degradation products. At left, the polypeptides are diagramed, assuming that the GST domain is stable and that the lower molecular weight bands occur by proteolysis in the GAR region. Methylation selectivity of PRMT1, PRMT3, and PRMT6 to GST-GAR degradation products is indicated with (+) signs.

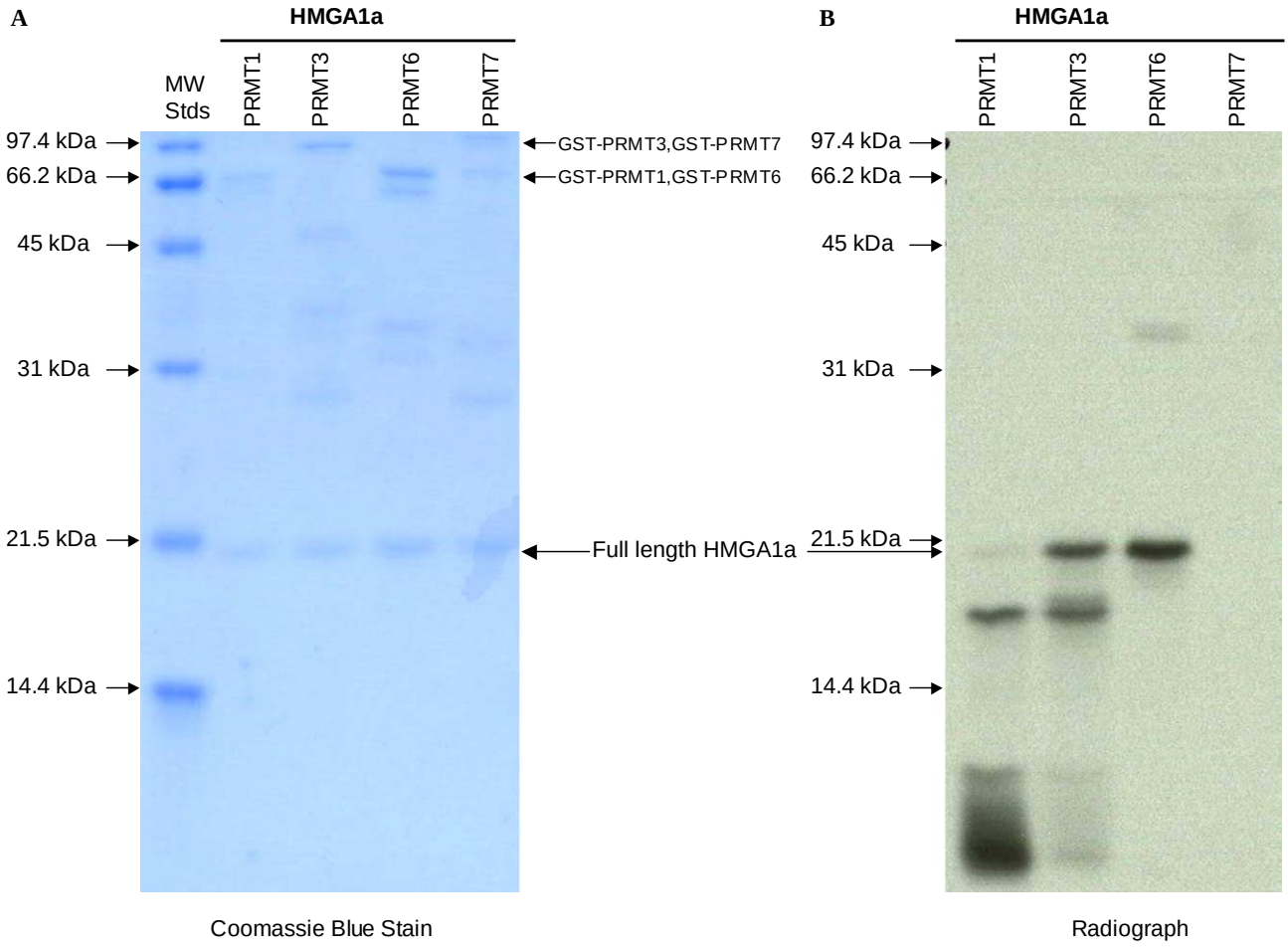

Fig. 3. PRMT6 specifically methylates full-length HMGA1a. Recombinant PRMT1, PRMT3, PRMT6, and PRMT7 were incubated with recombinant HMGA1a in the presence of [^{3}H]AdoMet as described under Experimental procedures. Reactions were directly loaded onto a 12.6% gel for SDS–polyacrylamide gel electrophoretic analysis. Gels were stained with Coomassie (A), treated with EN^{3}HANCE, dried, and exposed to film for 9 days (B). The position of the molecular weight standards is shown as indicated in Fig. 1; the positions of the Coomassie-stained bands of the GST protein arginine methyltransferase fusion proteins are indicated. The minor radiolabel species in the PRMT6 lane at 66 kDa and 33 and 34 kDa represent automethylated PRMT6 and its degradation products.

a similar activity to GST-PRMT1 methylating the lower molecular weight polypeptides, but in addition it recognizes the full-length peptide much better. No activity was seen with PRMT4 (data not shown) or PRMT7 (Fig. 3)

Interestingly, GST-PRMT6 was found to specifically methylate just the full-length HMGA1a and not the degradation products (Fig. 3). The ability of PRMT6 to selectively methylate the full-length HMGA1a differs from its looser selectivity with GST-GAR. PRMT1 and PRMT3 appear to be much less selective with regard to the full-length HMGA1a, methylating it and its degradation products, similar to their activity with GST-GAR. Why does PRMT6 only recognize full-length HMGA1a? One possibility is that PRMT6 recognizes some secondary or tertiary structure in HMGA1a that is absent in its proteolytic degradation products. This possibility may be unlikely due to HMGA1a having apparently little secondary structure when unbound in solution [29,30]. The other possibility is that the methylation site for PRMT6 is located near the N-terminus or the C-terminus and all of the degradation products lack these regions. For instance, if Arg 25 is the site of methylation, polypeptides truncated at the N-terminus by proteases would lack this residue.

HMGA1a is the third known specific substrate for PRMT6, along with the HIV tat protein and itself in an automethylation reaction [22,31]. Boulanger et al. [31] created peptides of regions of the HIV-I tat protein and found the peptide corresponding to residues 49–63 with the sequence RKKRRQRRRAPQDSQ was methylated in vitro, suggesting that one or more of the arginine residues in this region represent the modification site(s). This interpretation may be complicated if the enzyme specificity for the intact protein differs from that for the peptide. In HMGA1a the site of methylation at Arg 25 is EKRG**R**GRPR. There does not appear to be any sequence similarity here to the region methylated by PRMT6 in the tat peptide, nor is there any obvious similarity of the tat peptide sequence to those of the other methylation sites in HMGA1a identified by Edberg et al. [14]. Thus, it is not clear what determines substrate recognition for PRMT6 in the tat and HMGA1a substrates.

Up to this time two distinct classes of in vitro substrates for mammalian protein arginine methyltransferase had been described [17]. One large class involves proteins containing GGRGG-type sequences (such as those in GST-GAR) that are methylated by PRMT1 and PRMT3. A second much smaller class consists of proteins more or less specifically modified by PRMT4 [21,32]. PRMT6 was previously thought to behave like PRMT1 and PRMT3 in vitro because it methylates GST-GAR and the yeast protein Npl3 over the PRMT4 substrate PABP [22]. However, when RAT1 extracts were methylated by PRMT1, PRMT4, or PRMT6, PRMT6 showed a substrate specificity different than that of PRMT1 or PRMT4 [22]. Here we provide additional evidence that PRMT6 is more specific than PRMT1 or PRMT3 in its recognition of HMGA1a. This unique substrate specificity along with the fact that PRMT6 is localized to the nucleus [22] makes PRMT6 the most likely candidate for being the methyltransferase that can methylate HMGA1a in vivo.

Acknowledgments

This work was supported by NIH Grants GM026020 (S.C.) and GM071760 (R.R.).

References

[1] M. Bustin, Regulation of DNA-dependent activities by the functional motifs of the high-mobility-group chromosomal proteins, Mol. Cell. Biol. 19 (1999) 5237–5246.
[2] D. Thanos, T. Maniatis, Virus induction of human IFN beta gene expression requires the assembly of an enhanceosomes, Cell 83 (1995) 1091–1100.
[3] R. Reeves, Molecular biology of HMGA proteins: hubs of nuclear function, Gene 277 (2001) 63–81.
[4] M. Bustin, Revised nomenclature for high mobility group (HMG) chromosomal proteins, Trends Biochem. Sci. 26 (2001) 152–153.
[5] R. Reeves, M.S. Nissen, The AT-DNA-binding domain of mammalian high mobility group I chromosomal proteins, J. Biol. Chem. 265 (1990) 8573–8582.
[6] R. Reeves, L. Beckerbauer, HMGI/Y proteins: flexible regulators of transcription and chromatin structure, Biochim. Biophys. Acta 1519 (2001) 13–29.
[7] K. Chau, U.A. Patel, K.D. Lee, H.P. Lam, C. Crane-Robinson, The gene for the human architectural transcription factor HMGI-C consists of five exons each coding for a distinct functional element, Nucleic Acids Res. 23 (1995) 4262–4266.
[8] M. Friedmann, L.T. Holth, H.Y. Zoghbi, R. Reeves, Organization, inducible-expression and chromosome localization of the human HMG-I(Y) nonhistone protein gene, Nucleic Acids Res. 21 (1993) 4259–4267.
[9] K.R. Johnson, D.A. Lehn, R. Reeves, Alternative processing of mRNAs encoding mammalian chromosomal high-mobility-group proteins HMG-I and HMG-Y, Mol. Cell. Biol. 9 (1989) 2114–2123.
[10] L. Wood, J.F. Maher, T.E. Bunton, L.M.S. Resar, The oncogenic properties of the HMG-I gene family, Cancer Res. 60 (2000) 4256–4261.
[11] V. Giancotti, A. Bandiera, C. Sindici, L. Perissin, C. Crane-Robinson, Calcium-dependent ADP-ribosylation of high-mobility-group I (HMGI) proteins, Biochem. J. 317 (1996) 865–870.
[12] G.C. Banks, Y. Li, R. Reeves, Differential vivo modifications of the HMGI(Y) nonhistone chromatin proteins modulate nucleosome and DNA interactions, Biochemistry 39 (2000) 8333–8346.
[13] R. Sgarra, F. Diana, C. Bellarosa, V. Dekleva, A. Rustighi, M. Toller, G. Manfioletti, V. Giancotti, During apoptosis of tumor cells HMGA1a protein undergoes methylation: identification of the modification site by mass spectrometry, Biochemistry 42 (2003) 3575–3585.
[14] D. Edberg, J.N. Adkins, D.L. Springer, R. Reeves, Dynamic and differential in vivo modifications of the isoforms HMGA1a and HMGA1b chromatin proteins, J. Biol. Chem. 280 (2005) 8961–8973.
[15] Y. Zou, Y. Wang, Tandem mass spectrometry for the examination of the posttranslational modifications of high-mobility group A1 proteins: symmetric and asymmetric dimethylation of Arg25 in HMGA1a protein, Biochemistry 44 (2005) 6293–6301.
[16] D. Edberg, J.E. Bruce, W.F. Siems, R. Reeves, In vivo posttranslational modifications of the high mobility group A1a proteins in breast cancer cells of differing metastatic potential, Biochemistry 43 (2004) 11500–11515.
[17] M.T. Bedford, S. Richard, Arginine methylation: an emerging regulator of protein function, Mol. Cell 18 (2005) 263–272.
[18] J.D. Gary, S. Clarke, RNA and protein interactions modulated by protein arginine methylation, Prog. Nucleic Acid Res. 61 (1998) 65–131.
[19] W. Lin, J.D. Gary, M.C. Yang, S. Clarke, H.R. Herschman, The mammalian immediate-early TIS21 protein and the leukemia-associated BTG1 protein interact with a protein-arginine *N*-methyltransferase, J. Biol. Chem. 271 (1996) 15034–15044.
[20] J. Tang, J.D. Gary, S. Clarke, H.R. Herschman, PRMT 3, a type I protein arginine *N*-methyltransferase that differs from PRMT1 in its oligomerization, subcellular localization, substrate specificity, and regulation, J. Biol. Chem. 273 (1998) 16935–16945.
[21] D. Chen, H. Ma, H. Hong, S. Koh, S. Huang, B.T. Schurter, D.W. Aswad, M.R. Stallcup, Regulation of transcription by a protein methyltransferase, Science 284 (1999) 2174–2177.
[22] A. Frankel, N. Yadav, J. Lee, T.L. Branscombe, S. Clarke, M.T. Bedford, The novel human protein arginine *N*-methyltransferase PRMT6 is a nuclear enzyme displaying unique substrate specificity, J. Biol. Chem. 277 (2002) 3537–3543.
[23] T.L. Branscombe, A. Frankel, J. Lee, J.R. Cook, Z. Yang, S. Pestka, S. Clarke, PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins, J. Biol. Chem. 276 (2001) 32971–32976.
[24] T.B. Miranda, M. Miranda, A. Frankel, S. Clarke, PRMT7 is a member of the protein arginine methyltransferase family with a distinct substrate specificity, J. Biol. Chem. 279 (2004) 22902–22907.
[25] J. Lee, J.R. Cook, Z. Yang, O. Mirochnitchenko, S.I. Gunderson, A.M. Felix, N. Herth, R. Hoffmann, S. Pestka, PRMT7, a new protein arginine methyltransferase that synthesizes symmetric dimethylarginine, J. Biol. Chem. 280 (2005) 3656–3664.
[26] H.S. Scott, S.E. Antonarakis, M.D. Lalioti, C. Rossier, P.A. Silver, M.F. Henry, Identification and characterization of two putative human arginine methyltransferases (HRMT1L1 and HRMT1L2), Genomics 48 (1998) 330–340.
[27] R. Reeves, M. Nissen, Purification and assays for high mobility group HMG-I(Y) protein function, Methods Enzymol. 304 (1999) 155–188.
[28] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.
[29] J.R. Huth, C.A. Bewley, M.S. Nissen, J.N.S. Evens, R. Reeves, A.M. Gronenborn, M. Clore, The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif, Nat. Struct. Biol. 4 (1997) 657–665.
[30] A. Slama-Schwok, K. Zakrzewska, G. Léger, Y. Leroux, M. Takahashi, E. Käs, P. Debey, Structural changes induced by binding of the high-mobility group I protein to a mouse satellite DNA sequence, Biophys. J. 78 (2000) 2543–2559.
[31] M. Boulanger, C. Liang, R.S. Russell, R. Lin, M.T. Bedford, M.A. Wainberg, S. Richard, Methylation of tat by PRMT6 regulates human immunodeficiency virus type 1 gene expression, J. Virol. 79 (2005) 124–131.
[32] J. Lee, M. Bedford, PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays, EMBO Rep. 3 (2002) 268–273.