Arginine Methylation of the Histone H3 Tail Impedes Effector Binding*

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Histone tail post-translational modification results in changes in cellular processes, either by generating or blocking docking sites for histone code readers or by altering the higher order chromatin structure. H3K4me3 is known to mark the promoter regions of active transcription. Proteins bind H3K4 in a methyl-dependent manner and aid in the recruitment of histone-remodeling enzymes and transcriptional cofactors. The H3K4me3 binders harbor methyl-specific chromatin binding domains, including plant homeodomain, Chromo, and tudor domains. Structural analysis of the plant homeodomains present in effector proteins, as well as the WD40 repeats of the WDR5, reveals critical contacts between residues in these domains and H3R2. The intimate contact between H3R2 and these domain types leads to the hypothesis that methylation of this arginine residue antagonizes the binding of effector proteins to the N-terminal tail of H3. Here we show that H3 tail binding effector proteins are indeed sensitive to H3R2 methylation and that PRMT6, not CARM1/PRMT4, is the primary methyltransferase acting on this site. We have tested the expression and that PRMT6, not CARM1/PRMT4, is the primary binding effector proteins are indeed sensitive to H3R2 methylation has on H3 effector binding.

In vitro methylation experiments show that R2me2 and K4me3 are present in CARM1-null embryos. We also screened a panel of histone binding domains for sensitivity to H3R2 methylation. We found that a large number of H3 binding domains are sensitive to H3R2 methylation and add additional domains to the list of methyl-specific chromatin binders. In vitro methylation experiments show that R2me2 and K4me3 can exist on the same histone molecule. In addition, the transcriptional activity of select genes, known to be affected by the transcriptional regulatory complexes containing the H3R2me2a-sensitive effectors, is altered when PRMT6 levels are reduced or increased.

EXPERIMENTAL PROCEDURES

In Vitro Methylation Reactions—The GST-PRMT1, GST-CARM1, and GST-PRMT6 were expressed and purified as described previously (18). In vitro methylation reactions were

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performed in a final volume of 30 μl of phosphate-buffered saline (pH = 7.4). The reaction contained 0.5–1.0 μg of substrate and 1 μg of recombinant GST-PRMT. All methylation reactions were carried out in the presence of 0.42 μM [3H]S-adenosyl-[1-3H]methionine (79 Ci/mmol from a 7.5 μM stock solution; PerkinElmer Life Sciences). The reaction was incubated at 30 °C for 1 h and then subjected to fluorography by separation on SDS-PAGE, transferred to a polyvinylidene fluoride membrane, treated with En3Hance™ (PerkinElmer Life Sciences), and exposed to film for 1–3 days at −80 °C.

Acid Extraction of Histones—HEK293 and U2OS cells were grown to 80% confluency. Cells were suspended in reticulocyte standard buffer (10 mM Tris-HCl, pH 7.4; 10 mM NaCl; 3 mM MgCl₂) and then centrifuged. The pellet was resuspended in reticulocyte standard buffer plus 0.5% Nonidet P-40, placed on ice for 10 min, and then centrifuged again (2500 × g). Nuclei were resuspended in 5 mM MgCl₂, an equal volume of 0.8 M HCl was added, and histones were extracted for 20 min on ice. Histones (in supernatant) were precipitated with 50% (w/v) trichloroacetic acid and centrifuged at 8,000 × g. The pellet was washed twice with cold acetone and then resuspended in deionized water and 2 μl of 1.0 M Tris-HCl, pH 8.8.

Histological Analysis and Antibodies—E18.5 embryos with their abdomens perforated were fixed in formalin and embedded in paraffin wax. Embryos were sectioned at 3 μm and subjected to immunohistochemical localization of α-CARM1 (Upstate Biotechnology), αH3R2me2a (Abcam), and αH3R17me2a (Upstate Biotechnology). Staining was performed using the En-Vision system (Dako), and the counterstain was hematoxylin. The H3R2me1 antibody is from Abcam, and the PRMT6 antibody is from Bethyl Laboratories, Inc.

Peptide Pulldowns—Biotinylated histone tail peptides (15 μg) were immobilized on 8 μl of streptavidin beads (Pierce) in 500 μl of pulldown buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM dithiothreitol, and 0.1% Nonidet P-40 (v/v), 1 μM ZnSO₄) for 2 h at room temperature. Immobilized peptide bead complexes were washed three times with pulldown buffer. 1 μg of GST fusion protein and 400 μl of pulldown buffer were then added to beads and rocked overnight at 4 °C. The beads were then washed five times with pulldown buffer, boiled in protein loading buffer, fractionated by 10% SDS-polyacrylamide gel electrophoresis, and subjected to Western blot analysis using an anti-GST antibody.

Stable shRNA Line Generation—Plasmids designed to express shRNAs targeting nucleotides 995–1014 of human PRMT6 (GenBank™ accession number AY043278) were constructed by using pSUPER RNA interference system from Oligoengine (Seattle, WA) according to the manufacturer’s protocol. The insert target sequences are 5′-gatccccGCAAGACGCGACGGTTTCAattcagaagATGAAAGCTCGTGCTTGCTtttggaaaaa-3′ (forward) and agctttctaaaaGCAAAGACGCGAGCAGACTTTCTCTTtgaaaaa-3′. U2OS cells were then transfected with pSUPERRetro vector encoding the PRMT6-shRNA using Lipofectamine 2000 (Invitrogen). A polyclonal population was selected in 2 μg/ml puromycin, and ring cloning was performed. Expression of PRMT6 in each clone was analyzed by Western blots in triplicate.

RNA Isolation and Quantitative real-time-PCR—Cells at 80% confluency were trypsinized and collected. The pellet was washed in phosphate-buffered saline, and 10% was used to make a whole cell extract for Western analysis on PRMT6 protein levels. The remaining cells were spun down and then RNA-isolated following the Qiagen RNeasy mini prep manufacturer’s protocol. An on-column DNA digestion was performed in each RNA sample preparation. cDNA was prepared from total RNA using the Applied Biosystems high capacity cDNA archive kit following the manufacturer’s protocol. Real-time prevalidated gene primer sets were purchased from the Applied Biosystems “Assays-on-Demand” and analyzed with the Applied Biosystems 7900HT real-time PCR instrument using the TaqMan universal master mix. 18 S RNA was used as the internal control.

ChIP Analysis—U2OS cells at 80% confluency were transfected with 20 μg of 3×FLAG-ING2 vector (6). 24 h after transfection, ChIP analysis was performed following the Upstate Biotechnology ChIP assay kit protocol (catalog number 17-295). For the immunoprecipitation, 2 μg of the αFLAG antibody (Sigma, catalog number F-3165) was used for each condition and incubated with the cross-linked complexes overnight at 4 °C. PCR of the cyclin D1 promoter on the input and isolated DNA was performed using Advantage 2 polymerase (Clontech 639201). The cyclin D1 promoter primer sequences were as follows: forward, 5′-GATTTTCTTTTAAAACACGTGTTAC-3′, and reverse, 5′-CTTTGGTGACCATTTGAGACA-3′.

RESULTS AND DISCUSSION

PRMT6 Is the H3R2 Arginine Methyltransferase—CARM1 is reported to methylate H3R2, based on peptide mapping of in vitro methylated H3 (14). We decided to further study this using recombinant PRMTs and CARM1 knock-out mice. Based on the previous reports, we hypothesized that CARM1-null embryos would lose immune reactivity with an H3R2me2a-specific antibody. Surprisingly, immunostaining of wild-type and CARM1-null E18.5 embryos reveals a loss of H3R17 methylation, but not of H2R2 methylation, upon CARM1 loss (Fig. 1A). We then performed in vitro methylation experiments on calf thymus core histones using a set of recombinant PRMTs. Both CARM1 and PRMT6 robustly methylated H3 (Fig. 1B). PRMT1 methylates histone H4 and H2A. Next, calf thymus H3 was again methylated in vitro with this same set of PRMTs. The methylated H3 was then used for fluorography and Western analysis, using antibodies specific for histones methylated at different sites. The specificity of the antibodies was tested and only recognizes H3 when modified at the indicated site (data not shown). Surprisingly, although CARM1 dramatically increased the amount of R17 methylation, it did not increase the levels of arginine 2 methylation. PRMT6, and to a lesser degree PRMT1, catalyzed the methylation of H3R2 in vitro (Fig. 1C). Therefore we conclude that PRMT6 is the primary enzyme responsible for H3R2 methylation.

Knockdown of PRMT6 Decreases H3R2me2a, whereas Overexpression Increases H3R2me2a—To further validate the data obtained from the in vitro methylation experiments, we overexpressed PRMT6 in HEK293 cells (and HeLa cells, data not shown), which clearly led to an increase in H3R2me2a on bulk
FIGURE 1. PRMT6 methylates H3R2 in vitro. A, immunohistochemical analysis of E18.5 CARM1 wild-type (+/+) and knock-out (−/−) embryo brains. Paraffin-embedded sections were stained with α-CARM1, α-H3R17me2, and α-H3R2me2 antibodies. S = skin, N = neopallial cortex, I = intermediate zone, and V = ventricular zone. B, calf thymus core histones were methylated in vitro with recombinant PRMT1, CARM1, and PRMT6. A radioactive methyl donor is used to label reaction products, which were then subjected to a fluorograph to visualize methylation events. The same histone samples were also subjected to a fluorograph to visualize methylation events. The same histone samples were also used for Western blot analysis using antibodies specific for H3R2me1 and me2a and for H3R17me2a.

FIGURE 2. PRMT6 regulates H3R2 methylation levels in cells. A, PRMT6 was transiently overexpressed in HEK293 cells by transfection of a pcAGGS-PRMT6 vector (+/PRMT6). 24 h after transfection, the cells were harvested. Analysis of total cell lysate revealed PRMT6 levels. Core histones were isolated and analyzed for H3R2me2a levels. B, a stable PRMT6 knockdown U2OS cell line was generated using shRNA. Core histones were isolated from the mock stable line (Mock) and the PRMT6 knockdown line and analyzed by Western blot for H3R2me2a levels. Western analysis of total cell lysates confirms efficiency of the PRMT6 knockdown.

histones (Fig. 2A). If overexpression of PRMT6 results in increased H3R2me2a levels, one would expect that by knocking down endogenous PRMT6 levels, we would see a decrease in H3R2me2a methylation levels on bulk histones. Indeed, this is what was observed when we generated a stable PRMT6 knockdown cell line in U2OS cells (Fig. 2B). These data further support the finding that PRMT6 is the H3R2 methyltransferase.

H3 Binding Domains Are Sensitive to H3R2 Methylation—The published structures of the ING2 PHD finger, the WDR5 WD40 domain, and the JMJD2A tudor domains all show important contacts between domain residues and arginine 2 of H3. Thus, we were interested in looking at the effects of H3R2 methylation on the binding of these domain types. To address this, we synthesized biotinylated peptide and tested the ability of recombinant PHD, tudor, and WD40 domains to bind unmodified (H3K4me0), H3K4me, and the H3R2me2aK4me3 “dual” modified peptide using a peptide pulldown approach. The selected domains tested included the tudors of JMJD2A, the WD40 domains of WDR5, and several PHD domains that have been reported to bind H3K4me3 (ING2 and BPTF) and others tested here for the first time (PHF2, DATF1, and RAG2). Clearly, the majority of domains tested in this assay are sensitive to H3R2 methylation, as seen by reduced binding to the dual peptide as compared with the H3K4me3 peptide (Fig. 3A). Importantly, not all domains (RAG2-PHD) are sensitive, implying that certain H3-binding proteins are not regulated by this mechanism (19, 20). WDR5 binds the unmodified H3 N-terminal tail peptide well, regardless of H3K4 methylation. However, again, we see that H3R2 methylation reduces this binding.

PRMT6 Can Methylate H3 Peptides Regardless of Lysine 4 Methylation Levels—Up to this point, it is unknown whether the combination of H3K4me3 and H3R2me2 exists on the same histone molecule in vivo. To address this question, we performed an in vitro methylation experiment using histone peptides. Peptides corresponding to amino acids 1–18 of H3 containing varying degrees of methylation were subjected to in vitro methylation by PRMT6. Fluorography of the peptides revealed that PRMT6 has the ability to methylate all the H3 peptides regardless of the lysine 4 methylation status (Fig. 3B), although methylation of the H3K4me3 substrate is less robust than the other lysine-methylated peptides tested. Thus, PRMT6 can lay down the R2me2 mark, whereas H3K4 is methylated in vivo. The endogenous baseline levels of the dual mark and the stability of the dual mark are currently unknown. However, ChIP-on-chip analysis of these two marks indicate that they do not co-segregate and are in fact mutually exclusive (21). The ability of PRMT6 to in vitro methylate a peptide that is already methylated at H3K4 suggests that this dual modification can exist in vivo and raises the possibility that it is not very stable. Perhaps, after the dual mark is generated, efficient lysine demethylation causes the rapid loss of the H3K4me3 mark. We have not tested the ability of SET domain-containing proteins.
PRMT6 methylates H3R2

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PRMT6 methylates H3R2 to methylate an H3R2me2a peptide. A large number of enzymes have been reported to methylate the H3K4 site (22).

PRMT6 Activity Affects HOXA5 and Cyclin D1 Gene Expression—As H3R2 methylation reduces binding of WDR5 and ING2 chromatin binding domains to H3 N-terminal tail peptides, we hypothesized that the recruitment of these complexes to gene promoters may be hindered when PRMT6 is overexpressed, or conversely, overactive when PRMT6 is knocked down. We collected RNA from mock stably transfected U2OS cells, U2OS cells that were transiently transfected with a PRMT6 expression vector (+PRMT6), and shPRMT6 U2OS cells for real-time PCR analysis on HOXA5 and cyclin D1 genes. The expression of these genes is regulated by the WDR5-MLL and ING2-HDAC1-mSin3a protein complexes (6, 23). Specifically, the WDR5-MLL complex positively regulates HOXA5 transcription, whereas the ING2-HDAC1 complex negatively regulates cyclin D1 transcription after DNA damage. In agreement with our hypothesis, overexpression of PRMT6 decreased HOXA5 expression, whereas PRMT6 knockdown increased expression more than 2-fold (Fig. 3C). In addition, repression of cyclin D1 after doxorubicin treatment, by the ING2-HDAC1 complex, was very robust in the PRMT6 knockdown cells and weakened in the PRMT6-overexpressing cells (Fig. 3D). The robust repression of cyclin D1 after doxorubicin treatment in the shPRMT6 cells is likely due to the increased binding of the ING2 repressive complex; H3R2 methylation antagonizes ING2 binding, and PRMT6 knockdown reduces bulk H3R2 methylation levels.

Here we identified a number of H3-binding proteins sensitive to arginine 2 dimethylation (Fig. 3A). We also showed that the H3R2 can be methylated by PRMT6 when lysine 4 is previously methylated. One can imagine, perhaps during cellular differentiation, PRMT6 being recruited to active genes to start a shutdown procedure. H3R2 methylation by PRMT6 would knock off K4me3-binding proteins and their associated transcriptional coactivators. Following this event, H3K4 demethylases may be recruited to remove this activating mark to permanently silence the region. Supporting this hypothesis, Guccione et al. (21) show a counter-correlation between R2me2 and K4me3 on genomic regions bound by the transcription factor Myc. Their result implies that the dual mark is transient and/or present at very low amounts. H3K4 demethylase recruitment is one possible mechanism resulting in a transient dual R2K4 modified state on H3.

This study highlights the importance of a combinatorial histone code and will open the door to many future studies on the effects adjacent histone modifications have on each other and effector protein binding. Already described is the interplay
between H3K9 methylation and H3S10 phosphorylation. Namely, HP1 binding to H3K9me3 is antagonized by H3S10 phosphorylation during mitosis (24). We performed a limited screen for genes affected by PRMT6 activity and found that HOXA5 and cyclin D1 expression/repression is responsive to PRMT6 levels, presumably through methylation of H3R2me2.

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Addendum—While this manuscript was in preparation, two reports that also focus on H3R2 methylation appeared in press (16, 17). The first study addresses the role of this methyl mark in *Saccharomyces cerevisiae* (17), and the second addresses the role of this methyl mark in mammalian cells (16). Both studies highlight the role of H3R2me2a as a transcriptional repressive mark.

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