Histone H3 Lysine 9 Methyltransferase G9a Is a Transcriptional Coactivator for Nuclear Receptors**

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Methylation of Lys-9 of histone H3 has been associated with repression of transcription. G9a is a histone H3 Lys-9 methyltransferase localized in euchromatin and acts as a corepressor for specific transcription factors. Here we demonstrate that G9a also functions as a coactivator for nuclear receptors, cooperating synergistically with nuclear receptor coactivators glucocorticoid receptor interacting protein 1, coactivator-associated arginine methyltransferase 1 (CARM1), and p300 in transient transcription assays. This synergy depends strongly on the arginine-specific protein methyltransferase activity of CARM1 but does not absolutely require the enzymatic activity of G9a and is specific to CARM1 and G9a among various protein methyltransferases. Reduction of endogenous G9a diminished hormonal activation of an endogenous target gene by the androgen receptor, and G9a associated with regulatory regions of this same gene. G9a fused to Gal4 DNA binding domain can repress transcription in a lysine methyltransferase-dependent manner; however, the histone modifications associated with transcriptional activation can inhibit the methyltransferase activity of G9a. These findings suggest a link between histone arginine and lysine methylation and a mechanism for controlling whether G9a functions as a corepressor or coactivator.

Activation and repression of transcription involve the recruitment of many coregulator (coactivator or corepressor) proteins to the regulated gene promoter by sequence-specific DNA binding transcription factors (1, 2). These coregulator proteins contribute to transcriptional regulation by helping to remodel chromatin conformation in the promoter of the gene and by influencing the recruitment and activation of RNA polymerase II and its associated basal transcription factors. The mechanisms by which coregulators accomplish these tasks include protein-protein interactions, ATP-dependent alterations in conformations of chromatin, and catalysis of post-translational modifications of histones and other protein components of the transcription machinery.

Post-translational modifications of the N-terminal tails of histones include acetylation, phosphorylation, ubiquitylation, and arginine and lysine methylation. Individual histone modifications or sequential or concurrent combinations of these modifications may constitute a histone code, which is then recognized by effector proteins to bring about distinct changes in chromatin structure or other aspects of transcription.

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The abbreviations used are: NR, nuclear receptor; GRIP1, glucocorticoid receptor interacting protein 1; CARM1, coactivator-associated arginine methyltransferase 1; PRMT, protein arginine methyltransferase; CBP, cAMP-response element-binding protein; CREB, CREB-binding protein; AD, activation domain; AR, androgen receptor; ER, estrogen receptor; SET, Su(var), Enhancer of Zeste, Trithorax; AKR, ankyrin repeats; PSA, prostate-specific antigen; HA, hemagglutinin A; MMTV, mouse mammary tumor virus; DHT, dihydrotestosterone; RLU, relative light units; DBD, DNA binding domain; siRNA, small interfering RNA; GST, glutathione S-transferase.
and is responsible for mono- and dimethylation of Lys-9 of histone H3 in euchromatin (17, 18). Previous studies found that G9a functions as a corepressor which can be targeted to specific genes by associating with transcriptional repressors and corepressors such as CDP/cut, Blimp-1/PRDI-BF1, and REST/NRSF (19–21). Here we show, surprisingly, that G9a functions as a coactivator for NRs, collaborating synergistically with CARM1 and other NR coactivators. We also tested the role of the enzymatic activities of G9a and CARM1 in their synergistic coactivator function, and we investigated potential regulatory mechanisms for the histone lysine methyltransferase activity of G9a. Our results suggest that promoter context and/or regulatory environment control whether G9a functions as a corepressor or a coactivator.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Proteins with N-terminal hemagglutinin A or FLAG epitope tags were expressed in mammalian cells and in vitro from pSG5.HA or pSG5.FLAG, each of which has SV40 and T7 promoters (8, 22). Plasmids encoding the following proteins were previously constructed in pSG5.HA: GRIP1, CARM1, and CARM1(VLD) (8); GRIP1 ΔAD1 and GRIP1 ΔAD2 (23); GRIP1 AN (22); PRMT1 (9); GRIP1 N (5–765), GRIP1 M (730–1121), GRIP1.C (1122–1462), CARM1(E267Q), PRMT2, PRMT3, and RMT1 (13). A PCR-amplified EcoRI-XhoI insert encoding the murine long form of G9a (17) was cloned into the EcoRI-XhoI sites of pSG5.HA and pSG5.FLAG. PCR-amplified EcoRI-Sall inserts of G9a residues 1–333, G9a (17) was cloned into the EcoRI-XhoI sites of pSG5.HA or pSG5.FLAG. G9a H1166K mutant was generated with the QuikChange site-directed mutagenesis kit (BD Biosciences Clontech) for Gal4 DNA binding domain (DBD) fusions and into the EcoRI-XhoI sites of pSG5.HA or pSG5.FLAG. G9a H1166K mutant was generated with the QuikChange site-directed mutagenesis kit (Stratagene) using pSG5.HA-G9a as the template. The pm-G9a(ΔNHLC 1165–1168) plasmid encoding the mutant G9a fused to Gal4 DBD was described previously (17). This region of G9a is conserved among lysine methyltransferases and essential for methyltransferase activity (15, 17, 24).

**Cell Culture and Transfections**—CV-1 and Cos-7 (25) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO2. CV-1 cells were seeded into 12-well plates 18 h before transfection with a total of 2.5 or 5 μg of expression vector. Cell culture and transfections were performed as described previously (9). Coimmunoprecipitation assays were performed as described previously (13) using anti-FLAG antibody (M2, Sigma), anti-HA antibody (3F10, Roche Applied Science) or normal mouse or normal rat IgG for immunoprecipitations followed by either anti-HA antibody (3F10, Roche Applied Science) or by anti-FLAG antibody for immunoblotting. Further antibodies used for immunoblotting were anti-G9a (Sigma), anti-β-actin (Santa Cruz Biotechnology), and anti-PSA (DAKO Corp.).

**Small Interfering RNA Transfection and Quantitative Reverse Transcription-PCR**—LNCaP cells were cultured in RPMI 1640 supplemented with 5% charcoal-stripped fetal bovine serum for 2 days in 6-well plates. Cells were transfected with 90 nM siRNA using siLentFect (Bio-Rad) according to the manufacturer’s instructions. Starting 48 h after transfection, cells were treated with 100 nM DHT for 20 h. Total cellular RNA was prepared using Trizol (Invitrogen), and a two-step reverse transcription-PCR method was used to quantify specific mRNAs. Total RNA (400 ng) was subjected to reverse transcription (iScript cDNA synthesis kit, Bio-Rad) followed by quantitative PCR with Brilliant SYBR Green PCR master mix and the Mx3000P system (Stratagene). The primer sequences for quantitative PCR are as follows: hG9a, 5′-GAGGTGTTACTGCAATGATGCC-3′ (forward) and 5′-CAGACGCTTCTGCTCACGGGC-3′ (reverse); hβ-actin, 5′-ACCCCTACGAGGACAGCCGATCG-3′ (forward) and 5′-GTCCAGGAGTTCATCATCGAT-3′ (reverse); human PSA, 5′-TCAGACTTACCTACTGCTCA-3′ (forward) and 5′-AGGTCGTTGGCTGGAGTCATC-3′ (reverse).

siRNA sequences are as follows (Dharmacon): siG9a, 5′-UGAGAGAGGAUGAUUUUAAU-3′ (sense) and 5′-UAAGAAUAACUCCUCUCAU-3′ (antisense); siControl (negative control siRNA having no perfect matches to known human or mouse genes), 5′-UAAGGCUAUAGAGAUAUU-3′ (sense) and 5′-GUAUCCUCUCAUAGCCUUAGU-3′ (antisense).

**Chromatin Immunoprecipitation Assays**—Chromatin immunoprecipitation assays were performed essentially as described (26, 27) using LNCaP cells cultured as above in 150-mm dishes before treatment with 100 nM DHT for from 0 to 60 min. Cross-linked and sheared chromatin was immunoprecipitated with anti-AR (Santa Cruz Biotechnology), anti-G9a (Sigma), or normal rabbit IgG. Quantitative PCR amplifications were performed as described above using the following primers representing different portions of the human PSA gene: enhancer forward, 5′-GGGGTTTGTTGCCCCAGTGTGAG-3′; reverse, 5′-GGGGAGCTTTCTCCATGGTT-3′; enhancer-promoter forward, 5′-TAGAAACGCTGGAAGTAGCTG-3′; reverse, 5′-AACTTCAATGGATCCGTGTTG-3′; promoter forward, 5′-TCTAGTTTCTGCTCAGAG-3′; reverse, 5′-TTCGGTTTCTGCAATTAAGTG-3′; intron 1 forward, 5′-CCAAGGACCTTTCAATCG-3′; reverse, 5′-AGGGATGAGATTCTTCC-3′; 3′ UTR (untranslated region) forward, 5′-TACGGCCATGCTTGGACGAC-3′; reverse, 5′-TGGCTCACAGCCTTCTCTAG-3′.

**Methyltransferase Assays**—Bacterially produced GST fusion proteins of full-length CARM1 or G9a residues 730–1263 (mouse long form) were incubated with histone H3 tail peptides (amino acids 1–21, 22).
Upstate Biotechnology Inc.) containing various post-translational modifications in the presence of $^3$H-labeled S-adenosyl-l-methionine. Radioactive methylated products were analyzed by standard denaturing SDS gel electrophoresis using 15% (acrylamide/Bis, 29:1) gels and autofluorography.

RESULTS

We first tested whether the histone H3 Lys-9 methyltransferase G9a can enhance or inhibit transcriptional activation of transiently transfected reporter plasmids by steroid hormone receptors in CV-1 cells. We used previously established conditions that allow synergistic effects of multiple coactivators to be observed (13). Reporter gene expression mediated by hormone-activated AR and ERα (Fig. 1, A and B) was enhanced by GRIP1 and further enhanced by CARM1. G9a alone exhibited little coactivator activity, but it cooperated strongly with GRIP1; furthermore, the combination of G9a, GRIP1, and CARM1 was highly synergistic, producing an activity level up to 20-fold higher than that achieved with GRIP1 and CARM1. The synergy was entirely dependent on the steroid hormone (Fig. 1A) as well as GRIP1 (Fig. 1B). Similar results were obtained with glucocorticoid receptor (Supplemental Fig. S1) and thyroid hormone receptor β1 (data not shown). G9a cooperated synergistically with selective combinations of coactivators. In the presence of GRIP1, G9a was highly synergistic with CARM1, but not with p300; however, the addition of G9a to the combination of GRIP1, CARM1, and p300 produced a dramatic synergy (Fig. 1C). Thus, although G9a cooperated with GRIP1, CARM1, and p300, its coactivator function was more highly dependent on GRIP1 and CARM1.

To test the requirement for CARM1 enzymatic activity in its synergistic action with G9a, we used two mutants of CARM1 that lack enzymatic activity in vitro; mutation of VLD (amino acids 189–191) to AAA in the SAM (S-adenosyl-l-methionine) binding domain and the E267Q mutation in the arginine binding pocket. Both mutants maintain the ability to bind to AD2 of GRIP1 and are expressed at wild type levels (8, 13). The synergistic enhancement of AR function by GRIP1, wild type CARM1, and G9a was completely lost when either of the two CARM1 mutants was substituted for wild type CARM1 (Fig. 2A). The activity observed with the CARM1 (E267Q) mutation was equivalent to that observed with no CARM1, whereas the CARM1 (VLD) mutant displayed a dominant negative behavior. Thus, the enzymatic activity of CARM1 is required for the coactivator synergy between G9a and CARM1.

To test whether the histone lysine methyltransferase activity of G9a is required for its synergistic cooperation with CARM1, we used mutants that lack the enzymatic activity in vitro; that is, mutation H1166K in the catalytic site of the SET domain or deletion of the entire SET domain. The synergistic coactivator function observed with GRIP1, CARM1,
FIGURE 2. The methyltransferase activity of CARM1 but not that of G9a is required for coactivator synergy. A and B, CV-1 cells were transfected with MMTV-LUC reporter plasmid (125 ng) and with expression vectors encoding AR (10 ng), GRIP1 (50 ng), CARM1 wild type or mutant (200 ng), and G9a wild type or mutant (50, 100, 200, and 400 ng). Cells were treated with 20 nM DHT before luciferase assays. The schematic diagram shows full-length G9a and the residue numbering of fragments used in this study. E, polyglutamate; Cys, cysteine-rich region; ANK, six ankyrin repeats; Pre and Post, SET domains; SET, H3 Lys-9 methyltransferase domain; asterisk, location of H1166K mutation in the SET domain. C, Cos-7 cells were transfected with the G9a expression vectors used in panel B above. Whole-cell extracts were analyzed for G9a expression by immunoblotting with anti-HA antibody. D, CV-1 cells were transfected with GK1 reporter plasmid (250 ng) and expression vectors encoding Gal4 DBD (vector) or Gal4 DBD fused to various G9a fragments (250 ng each). After 48 h cell extracts were analyzed for luciferase activity. The inset graph shows the same results on a different scale without assay 3.
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and wild type G9a was reduced but not eliminated when G9a (H1166K) or G9a (ΔSET) was substituted for wild type G9a (Fig. 2B). In contrast, a G9a fragment consisting of the SET domain alone and missing the ankyrin repeats (ΔANK) was inactive as a coactivator. The G9a (H1166K) and G9a (ΔSET) mutants were less efficient than wild type G9a when lower levels of the plasmids were transfected but were almost equivalent to wild type G9a when higher levels of plasmid were transfected. Although the G9a mutants are expressed at approximately wild type levels when overexpressed in Cos-7 cells (Fig. 2C), we cannot rule out the possibility that modest reductions in their expression levels may account for the lower activities observed in Fig. 2B. Thus, the methyltransferase activity of G9a is not absolutely required for, but may contribute to, the synergistic coactivator function of G9a with CARM1.

We next tested whether any part of G9a, when brought to a promoter, could activate transcription. Fig. 2D shows the results of cotransfecting fragments of G9a fused to the Gal4 DBD with a Gal4-responsive reporter gene. The fragment containing G9a residues 72–333 contains an autonomous activation domain, whereas no other isolated fragment of the protein has such an activity. Interestingly, the first 71 residues of the protein appear to have a negative effect on this autonomous activity (compare assays 2 and 3, Fig. 2D).

To further define the specificity of the synergy between CARM1 and G9a, we tested various combinations of arginine and lysine methyltransferases in the transient transfection assays. The coactivator synergy between G9a and CARM1 was not observed when mammalian arginine methyltransferase PRMT1, PRMT2, or PRMT3 or yeast RMT1 was substituted for CARM1 (Fig. 3A). Each of these arginine methyltransferases exhibited similar coactivator activity when assayed with GRIP1 (13). PRMT1 and PRMT3 methylate histone H4 on Arg-3 among other substrates (4). Similarly, the mammalian histone H4 Lys-20 methyltransferase PR-SET7 could not be substituted for G9a (Fig. 3B), although both proteins can be expressed at high levels (Fig. 3C). Thus, among various histone methyltransferases, CARM1 and G9a have specific characteristics that allow them to function with each other as synergistic coactivators.

Because GRIP1 was important for the synergy between CARM1 and G9a, we explored physical interactions between G9a and specific GRIP1 domains. In co-immunoprecipitation experiments, G9a bound strongly to GRIP1.N (5–765) but not to the middle or C-terminal regions of GRIP1 (Fig. 4A). The interaction of G9a with GRIP1.N was apparently indirect or required post-translational modification, because no binding was observed between G9a translated in vitro and bacterially produced GST-GRIP1.N (Fig. 4B). However, G9a did bind weakly to the GRIP1 C-terminal region in vitro. The region of G9a involved in its association with GRIP1.N was further mapped using N-terminal truncations (Fig. 4, C–E). Although truncated G9a proteins containing the ankyrin repeats as well as the full-length G9a protein bound to GRIP1.N equally well, the SET domain alone bound only very weakly (Figs. 4, C and D), although it was expressed in equal or greater amounts as compared with the larger proteins (Fig. 4E). In addition, point mutation of the SET domain in full-length G9a to a catalytically inactive form did not inhibit association with GRIP1.N (Fig. 4C). The binding of G9a to GRIP1 (whether direct or indirect) suggests a possible mechanism for recruitment of G9a to the promoter by NRs.

In transient transfection assays GRIP1 mutants lacking the N-terminal AD3 domain (which associates with G9a and binds several other coactivators), the C-terminal AD2 domain (which binds CARM1), or the AD1 domain (which binds p300/CBP) all had a substantially reduced ability to support the G9a-CARM1 coactivator synergy (Supplemental Fig. S2). The mutant GRIP1 proteins are all expressed at similar levels (13, 23, 27). These results reinforce the key role of GRIP1 as a primary coactivator that binds directly to NRs and serves as a scaffold for recruiting p300, CARM1, G9a, and other secondary coactivators to contribute to transcriptional activation.

FIGURE 3. Specificity of coactivator synergy among protein arginine and lysine methyltransferases. A and B, CV-1 cells were transfected with MMTV-LUC reporter plasmid (125 ng) and with plasmids encoding AR (10 ng), GRIP1 (50 ng), CARM1, PRMT1, PRMT2, PRMT3, or RMT1 (200 ng), and HA-tagged G9a or PR-SET7 (50, 100, 200, and 400 ng). Cells were treated with 20 nM DHT, and luciferase assays were performed. C, Cos-7 cells were transfected with the G9a and PR-SET7 vectors used in panel B or mock-transfected. Whole-cell extracts were analyzed for protein expression by immunoblotting with anti-HA antibody.
We examined the effect of reducing endogenous G9a on androgen-dependent activation of the prostate-specific antigen (PSA) gene in LNCaP prostate cancer cells. In a typical experiment siRNA against G9a lowered endogenous levels of G9a mRNA by about 75%, compared with cells receiving no siRNA or a control siRNA (Fig. 5A, assays 2–4). The positions of coimmunoprecipitated GRIP1.N and GRIP1.C and of immunoglobulin heavy chains (lgG) are indicated. B, 35S-labeled G9a was incubated with GST, GST-GRIP1.N, GST-GRIP1.M, or GST-GRIP1.C immobilized on glutathione-agarose beads. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. C, Cos-7 cells were transfected as in panel A with FLAG-G9a full-length (FL), either wild type (WT) or H1166K mutant, N-terminal truncations (residues 464–1263, 685–1263 or 936–1263/ΔANK), or no G9a (none) and with HA-GRIP1.N. Extracts were processed as in panel A; coimmunoprecipitated GRIP1.N is indicated. D, Cos-7 cells were transfected with G9a N-terminal truncations and either with or without HA-GRIP1.N. Cell extracts were subjected to immunoprecipitation and immunoblotting as indicated. E, extracts from Cos-7 cells with transfected HA-GRIP1.N in panel D were subjected to immunoprecipitation and immunoblotting as indicated. Arrows in panels D and E indicate positions of G9a N-terminal-truncated proteins.

FIGURE 4. Binding of G9a to GRIP1. A, Cos-7 cells were transfected with expression vectors (2.5 μg of each) for FLAG-G9a and one of the following: HA-GRIP1.N, HA-GRIP1.M, or HA-GRIP1.C. Cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG antibody or normal IgG and then immunoblotted (W) with anti-HA antibody. The positions of coimmunoprecipitated GRIP1.N and GRIP1.C and of immunoglobulin heavy chains (lgG) are indicated. B, 35S-labeled G9a was incubated with GST, GST-GRIP1.N, GST-GRIP1.M, or GST-GRIP1.C immobilized on glutathione-agarose beads. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography. C, Cos-7 cells were transfected as in panel A with FLAG-G9a full-length (FL), either wild type (WT) or H1166K mutant, N-terminal truncations (residues 464–1263, 685–1263 or 936–1263/ΔANK), or no G9a (none) and with HA-GRIP1.N. Extracts were processed as in panel A; coimmunoprecipitated GRIP1.N is indicated. D, Cos-7 cells were transfected with G9a N-terminal truncations and either with or without HA-GRIP1.N. Cell extracts were subjected to immunoprecipitation and immunoblotting as indicated. E, extracts from Cos-7 cells with transfected HA-GRIP1.N in panel D were subjected to immunoprecipitation and immunoblotting as indicated. Arrows in panels D and E indicate positions of G9a N-terminal-truncated proteins.

We examined the effect of reducing endogenous G9a on androgen-dependent activation of the prostate-specific antigen (PSA) gene in LNCaP prostate cancer cells. In a typical experiment siRNA against G9a lowered endogenous levels of G9a mRNA by about 75%, compared with cells receiving no siRNA or a control siRNA (Fig. 5A, assays 2–4). The addition of the AR agonist DHT caused strong induction of PSA mRNA levels (Fig. 5B, assays 1–2). The siRNA against G9a lowered the hormone-induced level of PSA mRNA by about 50%, whereas control siRNA had no effect (assays 3–4). The G9a and PSA mRNA levels were normalized to β-actin mRNA levels, thus demonstrating that the effects of the G9a-directed siRNA were gene-specific. The siRNA against G9a also compromised the hormonal induction of PSA protein (Fig. 5C). Thus, although many different coactivators are involved in mediating transcriptional activation by NRs, endogenous G9a is necessary for efficient induction of the endogenous PSA gene in response to hormone. Similar results were obtained with induction of the endogenous pS2 gene by estradiol in MCF7 breast cancer cells (data not shown).

G9a has been associated with transcriptional repression both through
its ability to associate with repressive transcription factors (19–21) and through its ability to methylate H3 Lys-9 (17). By fusing various fragments of G9a to Gal4 DBD, we confirmed that the C-terminal SET domain, which contains the methyltransferase activity, contains the transcriptional repression activity of G9a (Fig. 6A). Mutations in the SET domain (H9004/NHLC or H1166K) that eliminate the methyltransferase activity also eliminated transcriptional repression by full-length G9a and its C-terminal fragments, showing that the methyltransferase activity is required for repression by G9a. The SET domain mutant G9a proteins are expressed at levels comparable with the corresponding wild type proteins. (Ref. 17 and Fig. 6B).

We have, thus, demonstrated that G9a can function as a coactivator or corepressor; this presumably depends on the context of transcription factors and other coregulators at the promoter. What specific factors direct G9a to function as a coactivator rather than a corepressor? Elimination of its methyltransferase activity or the presence of a poor substrate should prevent G9a from functioning as a corepressor and could, therefore, possibly allow G9a to function as a coactivator. We tested whether histone H3 modifications associated with active genes could restrict the ability of G9a to methylate Lys-9. Using histone H3 peptides (amino acids 1–21) as substrates for methylation by G9a in the presence of 3H-labeled S-adenosyl-L-methionine, we found that acetylation of Lys-9 and phosphorylation of Ser-10 each caused complete inhibition of G9a methyltransferase activity, whereas Lys-14 acetylation had no effect (Fig. 6C). In contrast, CARM1 was able to methylate all of these peptides, except for the Ser-10-phosphorylated peptide. Therefore, a combination of Lys-9 acetylation and Ser-10 phosphorylation (marks associated with transcriptional activation) would presumably cause a dramatic restriction of G9a ability to methylate Lys-9 and, thus, could help direct G9a to function as a coactivator rather than a corepressor.

Finally, because G9a can function as a coactivator, we sought to verify that G9a could be found associated with transcriptionally active genes. To determine whether G9a is recruited to NR-responsive genes, we performed chromatin immunoprecipitation assays. Using the androgen-responsive PSA gene, we found that G9a is associated most strongly with the upstream enhancer region but, notably, also with other regions including the transcribed portion of the gene (Fig. 7). Although the association of the androgen receptor with the enhancer region is highly dependent on hormone, the association of G9a appears largely consti-
tutive to the regions examined. Nevertheless, these results demonstrate that G9a is found associated with both the inactive and transcriptionally activated states of the PSA gene. These results are consistent with a role for G9a in transcriptional regulation.

**DISCUSSION**

Di- and trimethylation of Lys-9 of histone H3 is generally low in active genes and higher in inactive genes (28, 29). However, a recent study suggests that the pattern of Lys-9 methylation may be more complex and have different roles in the promoters versus the transcribed regions of genes. Di- and trimethylation of histone H3 Lys-9 was found along with the methyl(Lys-9)-histone H3-binding protein HP1, as a common feature in the transcribed regions of active genes (30). Moreover, their presence was dependent on active elongation by RNA polymerase II. Although further studies are needed (in light of these new findings) to examine H3 Lys-9 methylation patterns in more detail, this result suggests a role for histone H3 Lys-9 methylation and HP1 proteins in the transcription of active genes. The enzyme responsible for these modifications has not yet been identified, but our results strongly suggest G9a as a prime candidate for this function.

The extensive mechanisms for modulating chromatin structure and for recruiting and activating RNA polymerase II involve many different coregulators and complexes of coregulators, each of which appears to play a specific role in transcriptional activation (1, 2). We have shown that G9a, a protein thought to be central to a large number of gene repression events in euchromatin, is also involved in transcriptional activation by several members of the large family of nuclear receptors (Figs. 1 and 5 and data not shown). G9a acts in synergy with the p160 coactivator GRIP1, the protein arginine methyltransferase CARM1, and the histone acetyltransferase p300 (Figs. 1–3). The repressive activity of G9a depends on its lysine methyltransferase activity; however, G9a methyltransferase activity is inhibited by histone modifications associated with transcriptional activation (Fig. 6). We propose that this restriction of G9a methyltransferase activity could allow G9a to operate as a coactivator.

To function as a coactivator, G9a must have a mechanism for associating with the gene that will be activated and a mechanism for transmitting an activating signal to the chromatin or transcription machinery. Several characteristics of G9a suggest potential mechanisms of recruitment to genes targeted for activation. First, G9a is associated with euchromatin, placing it in the proper chromatin domain. Second, G9a can bind to the N-terminal tail of histone H3. Third, G9a can associate with GRIP1 (Fig. 4). In fact, the coactivator function of G9a is highly dependent on GRIP1 (Fig. 1) and various functional domains of GRIP1. Deletion of any of the three activation domains of GRIP1 (N-terminal AD3 or C-terminal AD1 or AD2) compromised the ability of G9a to function as a coactivator (Supplemental Fig. S2). These results reflect the role of GRIP1 as an NR binding coactivator that functions as a scaffold to recruit p300/CBP (through AD1), CARM1 (through AD2), and G9a as well as other coactivators that bind to the N-terminal AD3 of GRIP1.

Our results also suggest possible mechanisms by which G9a may transmit the activating signal downstream toward the transcription machinery. The N-terminal region of G9a contains an autonomous activation activity (Fig. 2D). The centrally located ankyrin repeats, 4 D. Y. Lee, J. P. Northrop, and M. R. Stallcup, unpublished results.
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which are known to function in protein-protein interaction in other proteins, associate with GRIP-N but could also make contact with other upstream or downstream components of the coactivator signaling pathway. Although the C-terminal SET domain was not absolutely required for the coactivator function of G9a, G9a mutants with methyltransferase-inactivating deletions or point mutations in this domain appeared to have reduced activity at lower levels of G9a expression, suggesting that G9a methyltransferase activity may contribute to coactivator function in some way (Fig. 2B).

In addition to G9a, at least two other lysine methyltransferases have been shown to work as coactivators for nuclear receptors. Riz1, which can dimethylate Lys-9 of histone H3, acts as a coactivator for the estrogen and progesterone receptors but not for several other NRs (31). NSD1, which can methylate both H3 Lys-36 and H4 Lys-20 in vitro (32), acts as a coactivator and corepressor for NRs (33). These methylation marks have been associated with transcriptional elongation and gene repression, respectively.

What factors regulate whether G9a acts as coactivator or corepressor? We propose that promoter context determines whether G9a functions as a coactivator or corepressor. G9a is recruited as a corepressor by several sequence-specific DNA binding repressor proteins. Similarly, protein-protein interactions between G9a and coactivators such as GRIP1 (Fig. 4) could be a factor that switches G9a from corepressor to coactivator. The functional switch then could be simply mediated by recruitment to either the potentially activated or repressed promoter. Alternatively, because our chromatin immunoprecipitation data indicate that G9a is present at enhancer and promoter regions in the absence or presence of gene activation, absolute recruitment may not be a functional switch.

Rather, the nature of the recruiting proteins, such as histone H3 versus GRIP1, may convert G9a from corepressor to coactivator. Dimethylation of H3 Lys-9 has been associated with repression of transcription in euchromatin, and the methyltransferase activity of G9a, located in the SET domain, is required for its coactivator function (19–21). Our results also indicate that tethering of G9a to a promoter can repress transcription and that its methyltransferase activity is necessary for this repression (Fig. 6A). Therefore, another possible contributing mechanism for switching G9a from corepressor to coactivator would be to inhibit its methyltransferase activity, at least when it is recruited to an active promoter region. Acetylation and methylation of Lys-9 are obviously mutually inhibitory, as confirmed by our results (Fig. 6C). Methylation of Lys-9 of histone H3 has previously been shown to interact with other histone modifications. Lys-9 methylation is mutually inhibitory with Lys-4 methylation (16), and these marks are inversely correlated with each other in inactive and active chromatin (28, 29). In addition, Lys-9 methylation by Suv39H1 inhibits Ser-10 phosphorylation (15), and our results show that Ser-10 phosphorylation also inhibits G9a-mediated methylation (C. P. Fig. 6C). Therefore, histone H3 tails containing several histone marks associated with active transcription serve as poor substrates for G9a.

The cooperative coactivator function between CARM1 and G9a is quite specific for CARM1 in that no other PRMT tested was able to cooperate with G9a (Fig. 3). Furthermore, the methyltransferase activity of CARM1 was essential for the synergistic cooperation between CARM1 and G9a (Fig. 2C). As a result the expression of G9a and the methyltransferase activity of G9a with Arg-17-methylated H3 peptides. Thus far we have been unable to show a significant difference between unmodified peptides and Arg-17-methylated peptides in these assays (data not shown). However, we cannot rule out direct effects of Arg-17 methylation that, when combined with one or more other activating marks such as Lys-9 acetylation, Ser-10 phosphorylation, or Lys-4 methylation, might have significant effects on G9a recruitment and/or methyltransferase activity. Although transcription activating histone modifications in the promoter region may prevent Lys-9 methylation in the promoter, they apparently do not inhibit Lys-9 di- and trimethylation in the transcribed region of active genes, since levels of Lys-9 methylation were recently reported to be higher in the bodies of active versus inactive genes (30).

In summary G9a is capable of functioning either as a coactivator or as a corepressor depending on the promoter context to which it is recruited. Our results suggest that some level of regulation of the methyltransferase activity of G9a is necessary for coactivator activity and that G9a reads and responds to posttranslational modifications of histone H3, consistent with the existence of a histone code.

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