Histone H3-Lysine 9 Methylation Is Associated with Aberrant Gene Silencing in Cancer Cells and Is Rapidly Reversed by 5-Aza-2′-deoxycytidine

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Abstract

Epigenetic modifications of cytosine residues in DNA and the amino termini of histone proteins have emerged as key mechanisms in chromatin remodeling, impacting both the transcriptional regulation and the establishment of chromosomal domains. Using the chromatin immunoprecipitation (ChIP) assay, we demonstrate that aberrantly silenced genes in cancer cells exhibit a heterochromatic structure that is characterized by histone H3 lysine 9 (H3-K9) hypermethylation and histone H3 lysine 4 (H3-K4) hypomethylation. This aberrant heterochromatin is incompatible with transcriptional initiation but does not inhibit elongation by RNA polymerase II. H3-K9 methylation may, therefore, play a role in the silencing of tumor-suppressor genes in cancer. Treatment with 5-aza-2′-deoxycytidine (5-Aza-CdR), previously known for its ability to inhibit cytosine methylation, induced a rapid and substantial remodeling of the heterochromatic domains of the p14ARF/p16INK4a locus in T24 bladder cancer cells, reducing levels of dimethylated H3-K9 and increasing levels of dimethylated H3-K4 at this locus. In addition, 5-Aza-CdR increased acetylation and H3-K4 methylation at the unmethylated p14 promoter, suggesting it can induce chromatin remodeling independently of its effects on cytosine methylation.

Introduction

Methylation of cytosine residues at CpG dinucleotides is an important biological phenomenon that has been linked to transcriptional repression as well as regulation of chromatin structure. Extensive data correlate cytosine methylation with gene silencing and heterochromatinization: cytosine hypermethylation in the promoters of genes such as p16 and p14 has been linked to transcriptional repression (1–4). Recent work has demonstrated the importance of posttranslational modification of histone proteins as another epigenetic mechanism in the organization of chromosomal domains and gene regulation (5, 6). Acetylation of lysine 9 and methylation of lysine 4 of H3 have been associated with an open chromatin configuration such as that found at transcriptionally active promoters. In contrast, methylation of lysine 9 of H3 is a marker of condensed, inactive chromatin of the sort associated with the inactive X-chromosome and pericentromeric heterochromatin (7–10).

There is growing evidence that an interplay exists between cytosine methylation and histone modification. For example, the methyl-CpG binding protein, MeCP2, has been found to be associated with HDAC activity, providing a pathway by which histone modification can be induced by DNA methylation changes (11, 12). It has also been shown that histone modification is crucial to the process of DNA methylation in some organisms; abrogation of H3-K9 methylation in Neurospora results in the loss of DNA methylation (13). These data suggest a functional linkage between DNA methylation and H3-K9 methylation in gene repression and the establishment of a heterochromatic state.

To better understand the relationship between cytosine methylation and histone modification in cancer-associated gene silencing, we have performed ChIP analyses of the patterns of H3-K4 methylation and H3-K9 methylation at the p14ARF/p16INK4a locus in a variety of normal and tumor cell lines, each of which display unique cytosine methylation patterns at the three CpG islands contained within the locus. We also treated the T24 bladder cancer cell line with the cytosine methylation inhibitor, 5-Aza-CdR, to determine whether or not modulation of DNA methylation could affect histone modification.

Materials and Methods

Tissue Culture. The four cell lines analyzed (LD419, J82, T24, and HCT15) were cultured and maintained as described previously (4).

5-Aza-CdR Treatment. Cells were plated at 1 × 10^6 cells/150-mm dish and treated the next day with freshly prepared 3 × 10⁻⁶ M 5-Aza-CdR (Sigma Chemical Co., St. Louis, MO). The medium was changed 24 h after treatment, and ChIPs were performed and DNA/RNA extracted on days 1, 2, and 3 after drug treatment from exponentially growing cultures. Two independent 5-Aza-CdR treatment experiments were performed.

ChIP Assays. ChIP assays were performed as previously described (4) with 10 µl of anti-MeCP2, 10 µl of anti-K9-dimethylated H3, 10 µl of anti-acetylated H3 (lysine 9 and lysine 14 residues), or 5 µl of anti-K4-dimethylated H3. All of the antibodies were obtained from Upstate Biotechnology, Lake Placid, NY.

PCR Analysis of Immunoprecipitated DNA. PCR amplification of immunoprecipitated DNA was performed as previously described (4).

RT-PCR Analysis of p14 and p16 Expression. RT-PCR and subsequent PCR reactions were carried out with 100 ng of the cDNA product, dNTPs (Roche Molecular Biochemicals) and TaqDNA polymerase (Sigma) as described previously (4). The p14 and p16 primers used have been described previously (4).

Western Blot Analysis. T24 cell pellets were lysed in 10 mM HEPES (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, and 1.5 mM phenylmethyl-
methylation (Fig. 1). ChIP analysis using antibodies against dimethylated histones demonstrated that the p16 promoter and shared exon 2 in LD419 cells displayed less H3-K4 methylation than predicted for CpG islands that have negligible cytosine or H3-K9 methylation. Furthermore, there appeared to be no significant difference in the levels of H3-K4 methylation at the p14 promoter of T24 and HCT15 cells, even though the CpG islands are differentially methylated in the two cell lines (Fig. 1B). This suggests that the positive correlation between H3-K4 methylation and euchromatin may not be absolute.

5-Aza-CdR Induces Rapid Reduction of MeCP2 and K9-methylated H3 and Enhancement of Acetylated H3 and K4-methylated H3 at Silenced Loci. Previous work has shown that the treatment of the T24 bladder cancer cell line with 5-Aza-CdR caused substantial demethylation of the p16 promoter and shared exon 2 and strong reactivation of p16 expression (15, 16), although no change was induced in the level of expression of the p14 gene, which is unmethylated in these cells (Fig. 2; Ref. 4). Reactivation of the p16 gene was preceded by a rapid chromatin remodeling of the promoter and shared exon 2 regions in which the heterochromatic imprint was reversed and replaced by a euchromatic one. The levels of MeCP2 and methylated H3-K9 at the p16 promoter and shared exon 2 decreased dramatically just 24 h after treatment with 5-Aza-CdR, reaching their lowest points by 48 h after drug treatment and were maintained at low levels after 72 h (Fig. 3, A and B). In contrast, H3 acetylation and H3-K4 methylation increased at the same two CpG islands compared with untreated T24 cells (day 0), reaching their highest levels by 48–72 h after drug treatment (Fig. 3, C and D).

ChIP analysis of all four chromatin markers was also performed on the p14 promoter. CpG island, which has little cytosine methylation (3) or H3-K9 methylation in T24 cells (Fig. 3, A and B). The p14 gene was expressed relatively strongly, and its levels did not change over the time course of 5-Aza-CdR treatment (Fig. 2). This promoter region was also occupied by high levels of acetylated H3 and H3-K4 methylation (relative to the p16 promoter and shared exon 2) that were substantially augmented after treatment with 5-Aza-CdR, with increases observed as early as 24 h after treatment (Fig. 3, C and D). Therefore, these results suggest that 5-Aza-CdR can induce chromatin remodeling independently of its effects on cytosine methylation.

5-Aza-CdR Induces High Levels of DNA Hemimethylation but Does Not Dramatically Inhibit Overall Histone Modification. MeCP2 is known to specifically bind to symmetrically methylated CpG sequences, and has a poor affinity for unmethylated and hemimethylated DNA (17). We determined the levels of hemimethylated, fully methylated, and unmethylated DNA at the p16 exon2 CpG island after treatment of T24 cells with 5-Aza-CdR using a novel assay that we recently developed (14). The levels of hemimethylated dramatically increased within the first 24 h of treatment, suggesting that the precipitous decrease of MeCP2-binding after 5-Aza-CdR treatment (Fig. 4A) may be attributable to its inability to bind to a hemimethylated DNA substrate. Whereas the levels of hemimethylated decreased at 48 h and 72 h after treatment, the levels of unmethylated DNA increased at this time, which may further prevent MeCP2 binding. Western blot analysis showed that the overall levels of K9-methylated H3, K4-methylated H3, and acetylated H3, however, did not dramatically change after 5-Aza-CdR treatment of T24 cells (Fig. 4B), suggesting that the chromatin alterations detected by ChIP analysis were region-specific rather than global.

Discussion

It has become increasingly evident that cytosine methylation and histone modification exhibit a complex interplay that can contribute to gene regulation as well as to the establishment and maintenance of chromosomal domains. Cytosine methylation and H3-K9 methylation...
have both been associated with repressive chromatin of the type observed in constitutive and developmentally regulated (facultative) heterochromatin (8, 9). Moreover, methylation of CpG residues in promoters has been implicated in the aberrant silencing of genes, possibly through the formation of an altered chromatin structure that is resistant to transcriptional initiation. Our study now demonstrates that H3-K9 methylation is a prevalent component of such aberrant heterochromatin as well. These aberrant heterochromatic domains coincide with CpG islands that are enriched for cytosine and H3-K9 methylation and depleted for H3 acetylation and H3-K4 methylation. The presence of such a domain at a promoter island is associated with a lack of transcriptional initiation (e.g., the p16 promoter in T24 cells), suggesting that H3-K9 methylation may be involved in the aberrant silencing of genes. However, the same type of heterochromatin does not resist transcriptional elongation when present downstream of an active promoter (e.g., the silenced p16 promoter and shared exon 2 in T24 downstream of the active p14 promoter). Therefore, such heterochromatin can be termed “permissive” because it is not wholly incompatible with transcriptional activity.

In addition, our study is consistent with previous work that has linked H3-K4 methylation with active euchromatin (5). However, the data show that the inverse correlation between H3-K4 methylation and heterochromatin may not be absolute, suggesting that H3-K4 methylation may be more of a permissive rather than an exclusive

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Fig. 1. Association of H3-K9 hypermethylation and H3-K4 hypomethylation with silenced loci in cancer cells. A, ethidium bromide staining of ChIP PCR products of K9-methylated H3 (top panel) (IP, immunoprecipitated DNA; NAC, no-antibody control; Input, sample representing amplification from a 1:100 dilution of total input chromatin from each ChIP experiment). Bottom panel, UV quantitation of ChIP PCR results. Each ChIP experiment was repeated two times to confirm reproducibility of results, and data shown are an average of the quantitated values derived from independent duplicate experiments. Triple circles beneath each column, cytosine methylation status of a CpG island in a particular cell line as described previously (4): black circles, methylated; white circles, unmethylated. B, results of ChIP PCR for K4-methylated H3.

Fig. 2. RT-PCR analysis of p16, p14, and GAPDH expression in T24 bladder cancer cells at various timepoints after treatment with 3 × 10⁻⁸ M 5-Aza-CdR. PCR products were probed with end-labeled oligonucleotides specific for each transcript, and the blots were exposed overnight.
marker of euchromatin. The lack of a complete correlation may also be explained by the limitations of our PCR-based assay, which does not allow precise quantitation. However, such a limitation does not influence the major findings of our study, and it should be noted that the data are internally consistent and reproducible (based on two independent experiments).

The mechanisms by which heterochromatic domains are established at CpG islands are poorly understood. However, data from this study, in conjunction with previous work from our laboratory, suggest that certain regions of a gene are more vulnerable than others to heterochromatinization and may serve as foci for the seeding of aberrant cytosine and H3-K9 methylation (18). It has been postulated that transcription through a gene region may facilitate de novo cytosine methylation, and we have previously demonstrated that downstream/exonic regions of a gene become aberrantly methylated more easily than promoter regions in normal and cancer cells (18, 19). The data in this study indicate that CpG islands are more heterochromatic when they are located downstream of an active promoter. For example, the p16 promoter and shared exon 2 of LD419 exhibit little cytosine or H3-K9 methylation, and yet both show relatively lower K9-acytlation and K4-methylation than would be predicted for CpG islands that are devoid of methylation (compared with the p14 promoter in LD419). Indeed, these two CpG islands displayed far less accessibility to endonuclease than did the equally unmethylated p14 promoter (4). The low level of H3 acetylation in downstream regions of a gene could predispose them to heterochromatic change by providing more sites for K9 histone-methyltransferase activity. Decreased levels of acetylation also imply higher occupancy by HDACs.

Fig. 3. 5-Aza-CdR causes rapid reduction in the binding of MeCP2 (A) and K9-dimethylated H3 (B) and enhancement of acetylated H3 (C) and K4-dimethylated H3 (D) at silenced loci in T24 cells. (ChIP PCRs and quantitation were as described for Fig. 1.)

Fig. 4. Effects of 5-Aza-CdR on modified histone and DNA hemimethylation levels. A, levels of hemimethylated (H), fully methylated (F), and unmethylated (U) DNA at the first HpaII site of p16 exon 2 after 5-Aza-CdR treatment. Values are expressed as relative percentages; error bars, the SD of three independent determinations. B, left panel, Western blot analysis of modified and total histone protein levels; right panel, quantitation by densitometry. Protein levels in 5-Aza-CdR treated cells were expressed relative to the protein levels in the untreated cells and normalized to H3. Error bars, the SD of the data.
which may recruit DNA-methyltransferase activity to a region as suggested by data demonstrating in vivo interactions between HDAC and DNMT1 (20–22). These data may explain why there is a greater prevalence of aberrant cytosine methylation and inactivation of p16 than there is of p14 in numerous tumor cell types (3).

The colocalization of aberrant cytosine and H3-K9 methylation to the heterochromatic microdomains of the p14/p16 locus in the various cancer cell lines raises another intriguing question: how do these two epigenetic marks interact to stably repress a gene, or, even more interesting, which epigenetic modification is dominant in abnormal gene silencing? It is entirely conceivable that cytosine methylation can facilitate H3-K9 methylation. For example, it is known that the DNA methylation signal is translated by methyl-CpG binding proteins, such as MeCP2 or MBD2; MeCP2 is able to recruit HDAC activity and induce deacetylation of K9 residues on H3, thus allowing methylation of K9 by histone methyltransferases (11, 12). The opposite pathway, in which H3-K9 methylation might regulate cytosine methylation, is supported by work done in Neurospora, in which it was shown that abrogation of H3-K9 methylation led to a loss of DNA methylation, as well as data from kinetic analyses of X inactivation demonstrating that H3-K9 methylation is the earliest epigenetic mark during inactivation, preceding even cytosine methylation (7, 13).

5-Aza-CdR treatment induced a precipitous remodeling of chromatin structure, drastically reducing MeCP2-binding and H3-K9 methylation while enhancing H3 acetylation and H3-K4 methylation. A recent study has also demonstrated a similar decrease in MeCP2 occupancy and increase in histone H3 acetylation at the promoter of the MDRI gene following 5-Aza-CdR treatment (23). These changes appeared to be regional, because no changes in the overall levels of modified histones were detected by Western blot analysis. Given that MeCP2 does not have a high affinity for hemimethylated DNA (17), the dramatic decrease in MeCP2-binding at methylated CpG islands might be explained by an increase in the levels of hemimethylated DNA after drug treatment. A substantial increase in hemimethylation immediately after 5-Aza-CdR treatment has been previously predicted because of the inhibition of maintenance DNA methylation machinery (16). As a result, DNA replication is not accompanied by DNA methylation. This work represents the first experimental evidence of 5-Aza-CdR-induced hemimethylation in the context of a hypermethylated CpG island. The presence of a hemimethylated DNA substrate may prevent MeCP2 binding at these sequences after drug treatment.

Furthermore, the inhibition of H3-K9 methylation by 5-Aza-CdR seemed to be more powerful than its inhibition of cytosine methylation. Previous work has shown that significant cytosine demethylation (an ~30% decrease) is achieved between 36 and 48 h after treatment with 5-Aza-CdR (16). The data from this study show that H3-K9 methylation levels precipitously decreased by almost two-thirds (a 66% reduction) a mere 24 h after treatment with 5-Aza-CdR.

Drug-induced remodeling of chromatin was also observed at the p14 promoter that was enriched for H3 acetylation and H3-K4 methylation and showed little, if any, MeCP2 binding or H3-K9 methylation before drug treatment. The levels of the two euchromatic markers were substantially increased after drug treatment, showing that the drug could induce chromatin remodeling in the absence of DNA methylation.

Taken together, these data suggest that 5-Aza-CdR is able to cause a regional remodeling of chromatin, by diminishing H3-K9 methylation and augmenting H3 acetylation and H3-K4 methylation, independently of its effects on DNA methylation or gene expression. Substantial enhancement of the two euchromatic markers at the p14 promoter was not accompanied by a concomitant increase in p14 expression, and chromatin remodeling preceded changes in expression of p16, suggesting that the observed changes in the levels of the chromatin markers after 5-Aza-CdR treatment are not secondary to transcriptional effects. Our data are supported by previous work showing that 5-Aza-CdR can induce hyperacetylation of pericentric heterochromatin (which is transcriptionally inert) in mice independently of its cytosine demethylation activity (24).

How does 5-Aza-CdR induce chromatin remodeling and modulate histone methylation? Drug-induced up-regulation of genes encoding chromatin-modifying proteins, such as SWI-SNF or HAT (histone acetyltransferase) enzymes, represent a possible mechanism by which 5-Aza-CdR could modulate histone methylation by inducing nucleosomal remodeling and turnover. Also, extensive data show that DNMT1 interacts with HDAC activity in complexes bound to DNA, suggesting that it can recruit histone modifiers to DNA (20–22). Conceivably, the trapping of DNA-methyltransferase by 5-Aza-CdR would prevent DNMT1 from targeting HDAC activity to certain regions of DNA, thereby affecting chromatin structure. Furthermore, it is still possible that histone methylation is dependent on cytosine methylation despite our observation that H3-K9 methylation diminished more rapidly than cytosine methylation after 5-Aza-CdR treatment. Methyl-CpG binding proteins, such as MeCP2 and MBD2, are known to interact with HDAC activity in vivo and may also interact with other unidentified chromatin modifiers (11, 12, 25). A loss of cytosine methylation because of sequestration of functional DNA-methyltransferase would eliminate binding sites for methyl-CpG binding proteins, subsequently decreasing recruitment of HDACs to DNA.

Acknowledgments

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References


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