Physical and Functional Interaction of DNA Methyltransferase 3A with Mbd3 and Brg1 in Mouse Lymphosarcoma Cells

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Abstract

Dnmt3a and Dnmt3b are de novo DNA methyltransferases that also act as transcriptional repressors independent of DNA methyltransferase activity. To elucidate the underlying mechanism of transcriptional repression, Dnmt3a was purified from mouse lymphosarcoma cells (P1798) by extensive fractionation on five different chromatographic matrices followed by glycerol density gradient centrifugation. Liquid chromatography electrospray tandem mass spectrometry analysis of Dnmt3a-associated polypeptides identified the methyl CpG binding protein Mbd3, histone deacetylase 1 (Hdac1), and components of Brg1 complex (Brg1, Baf155, and Baf57) in the purified preparation. Association of Dnmt3a with Mbd3 and Brg1 was confirmed by coimmunoprecipitation and coimmunolocalization studies. Glutathione S-transferase pulldown assay showed that the NH2-terminal ATRX homology domain of Dnmt3a interacts with the methyl CpG binding domain of Mbd3 and with both bromo and ATPase domains of Brg1. Chromatin immunoprecipitation assay revealed that all three proteins are associated with transcriptionally silent methylated metallothionein (MT-I) promoter in the mouse lymphosarcoma cells. To understand the functional significance of their association with the promoter, their role on the MT-I promoter activity was analyzed by transient transfection assay. The results showed that Mbd3 and Dnmt3a specifically inhibited the methylated promoter, and the catalytic activity of Dnmt3a was dispensable for the repression. In contrast, the wild-type but not the ATPase-inactive mutant of Brg1 suppressed MT-I promoter irrespective of its methylation status, implicating involvement of ATP-dependent chromatin remodeling in the process. Coexpression of two of the three interacting proteins at a time augmented their repressor function. This study shows physical and functional interaction of Dnmt3a with components of nucleosome remodeling machinery. (Cancer Res 2005; 65(23): 10891-900)

Introduction

Epigenetic modification of genome by DNA methylation plays an important role in the regulation of gene expression in animal cells. Recent studies have established the importance of DNA methylation in embryonic development (1, 2), genomic imprinting (3, 4), X chromosome inactivation (5), and repression of tumor suppressor genes in cancer (6, 7). In mammalian genome, the CpG islands located in the promoters of many genes are methylated, whereas the small stretches of CpG islands found in the housekeeping and tissue-specific genes remain essentially methylation-free (8). Transcriptional repression observed upon methylation of CpG base pairs in somatic cells or aberrant CpG methylation in cancer is mediated either by denying access of transcription factors to their cognate sequences on the promoter or by assembling repressor complex in the nearby promoter leading to condensed chromatin structure (8, 9).

Transfer of the methyl group from S-adenosyl methionine to the C-5 position of cytosine in DNA is catalyzed by three distinct DNA methyltransferases (Dnmt; i.e., Dnmt1, Dnmt3a, and Dnmt3b) in mammals (10). Dnmt1 is ubiquitously expressed, exhibits a preference for hemimethylated substrates, and therefore maintains the preexisting methylation pattern during replication (11). Dnmt3a and Dnmt3b are highly expressed in ES cells, early embryo, and developing germ cells (1) and function primarily as de novo methyltransferases, establishing DNA methylation pattern during embryogenesis and gametogenesis (2). Recent studies have shown that Dnmt3a is required for the establishment of methylation imprints in the oocytes (3). Dnmt3 family members share some structural similarity with Dnmt1 at the C-terminus catalytic region that is responsible for DNA methylation but exhibits no sequence similarity in the NH2-terminal regulatory domain (12). Apart from similarity to a cysteine-rich region with a plant homeodomain (PHD)–like motif present in the ATRX protein (13), the NH2-terminal noncatalytic domains of Dnmt3a and Dnmt3b do not share any sequence homology that accounts for their distinct biological functions.

In addition to their enzymatic function, all three Dnmts also exhibit transcriptional repressor activities (14–16). Dnmt3a and Dnmt3b suppress transcription in a methylation-independent manner through their PHD-like motif (16). Recent studies have suggested a direct interaction between Dnmts and histone deacetylases (Hdac; refs. 14, 17–19). Furthermore, the targeting of Dnmts to particular genomic regions might occur through protein-protein interactions. To enhance our understanding of how the methylation patterns are regulated in cells and how Dnmts function as transcriptional repressors, it is important to investigate the association of Dnmts in the native state with components of the chromatin-remodeling machinery. A direct approach to address this issue is to perform extensive biochemical fractionation of cell extracts and identify the polypeptides that coelute with Dnmts and then confirm their interactions by coimmunoprecipitation and coimmunolocalization. Towards this end, Robertson et al. did extensive fractionation of HeLa cell extracts and showed association of Dnmt1 with few components of DNA methylation machinery that includes Hdac1 (15). A recent study using similar
approach showed an association of Dnmt3b with components of the mitotic chromosome condensation machinery (20).

In an earlier study, we reported separation of Dnmt3a from Dnmt1 and Dnmt3b (18) by biochemical fractionation of mouse lymphosarcoma cell extracts. Here, we report extensive purification of Dnmt3a containing complex from lymphosarcoma S-100 extract and show its direct association in vivo with methyl CpG binding protein Mbd3 and the chromatin-remodeling protein Brg1, a member of the SWI/SNF-2 family. This study also shows that interaction among Dnmt3a, Mbd3, and Brg1 involves the PHD-like ATRX domain of Dnmt3a, the MBD of Mbd3, and the ATPase and bromo domains of Brg1. We further show the role of Dnmt3a, Mbd3, and Brg1 in modulating the metallothionein-I (MT-I) promoter activity.

Materials and Methods

Plasmid construction. All the mammalian expression vectors (i.e., wild type and deletion mutants of Dnmt3a, Brg1, and Mbd3) were amplified using Vent polymerase (New England Biolabs, Beverly, MA) from pcDnmt3a, pcBrg1 (21), and mouse lung cDNA library (Clontech, Palo Alto, CA), respectively. The details of plasmid construction and primers are provided in the Supporting Material.

Cell culture and transfection. HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum. The S-100 extract, mouse lymphosarcoma cell extracts, and HeLa cell extract (3 g protein from a 30-liter culture) was fractionated as detailed in (18). The recombinant Mbd3, Dnmt3a, and Brg1 were detected by Western blot analysis with the anti-flag antibody (M2, Sigma, St. Louis, MO).

Immunoprecipitation studies. Immunoprecipitation was done using standard methods, in which mouse lymphosarcoma cell extract was prepared in IPH buffer [50 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 0.1 mmol/L PMSF] in each case, 250 μL (500 μg protein) of cell extract were first precleared with protein A-agarose beads (Amersham) and subjected to immunoprecipitation using anti-Mbd3, anti-Dnmt3a, or anti-Brg1 antibodies. The beads were washed four times with IPH buffer; bound proteins were separated by SDS-PAGE and subjected to Western blot analysis with respective antibodies.

Glutathione S-transferase pulldown assay. The details of the assay are provided in the Supporting Material.

Immunofluorescence assay. HepG2 cells at 50% to 60% confluence were cultured directly on coverslips in a six-well plate. The transfection of these cells for immunofluorescence microscopy was done using Ca phosphate precipitation method as described (23). To show the coimmunolocalization of Dnmt3a/Brg1, Mbd3/Brg1, and Dnmt3a/Mbd3, HepG2 cells were cotransfected separately with FL-Dnmt3a (pcDNA 3.1)/FL-Brg1-3xFlag (CMV14), FL-Mbd3 (pcDNA3.1)/FL-Brg1/3xFlag (CMV14), and FL-Mbd3-3xFlag (CMV14) expression vectors, respectively. After 48 hours of transfection, cells were washed thrice with PBS, then fixed, and incubated with primary and secondary antibodies as described (19, 29). The cells were washed four times with IPH buffer; bound proteins were separated by SDS-PAGE and subjected to Western blot analysis with respective antibodies.

Chromatin immunoprecipitation. Chromatin immunoprecipitation was done as described (31).

RNA isolation, reverse transcription, and quantitative reverse transcription-PCR analysis. Total RNA treated with Turbo DNase I (Ambion, Austin, TX) was reverse transcribed with random hexamers and M-MulV reverse transcriptase from 3 μg of total RNA in 20 μL of total volume following manufacturer's protocol (Applied Biosystems, Foster City, CA). An aliquot of the cDNA (equivalent to 100 ng RNA for MT-I and 10 ng buffer B [20 mmol/L HEPES-KOH (pH 8), 0.1 mmol/L EDTA, 2 mmol/L [3H]-mercaptoethanol, 5 mmol/L MgCl2, 0.5 mmol/L PMSF, and 10% glycerol] supplemented with 100 mmol/L KCl and fractionated through a heparin Sepharose column. The bound fractions were eluted with 10-bed volume of linear KCl gradient (0.1-1.0 mol/L), and 2-mL fractions were collected. The fractions enriched in Dnmt3a (0.3-0.4 mol/L KCl) were pooled and fractionated through the subsequent columns as shown in Fig. L4.

Liquid chromatography electrospray tandem mass spectrometry analysis. The purified proteins from glycerol density gradient were subjected to SDS-PAGE on 4% to 12% gradient gel (Bio-Rad Laboratories, Hercules, CA), and the gel was used for mass spectrometric proteomic analyses. Gel slices were excised and digested in situ with trypsin, and peptides were analyzed by liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) using a CapLC system and a quadrupole time-of-flight mass spectrometer (QTOF2, Waters Corp., Milford, MA; refs. 26, 27). Protein identifications from MS/MS data used ProteinLynx Global Server (Waters) and Mascot (Matrix Science, Boston, MA) search engines and the Swiss-Protein and National Center for Biotechnology Information protein sequence databases (26, 27).
Results

Mbd3 and Brg1 copurify with Dnmt3a during extensive fractionation of mouse lymphosarcoma cell extract. DNA methyltransferases are unique proteins that participate in epigenetic regulation of gene expression by virtue of their catalytic activity and ability to act as transcriptional repressors. Following methylation of the promoter region by Dnmts, methylated cytosine residues recruit many repressors and corepressors that form a large repressor complex, preventing transcription from the initiation site (15, 32, 33). To identify the repressors present in the complex with Dnmt3a in vivo, we undertook extensive biochemical fractionation of S-100 extract from lymphosarcoma cells (P1798) that express all three Dnmts at significantly high levels (28). In an earlier study, we were able to separate Dnmt3a from Dnmt1 and Dnmt3b by fractionation through the initial Q-Sepharose column (18). The present investigation focused on the identification of polypeptides associated with Dnmt3a after extensive fractionation of the lymphosarcoma cell extract and the functional relevance of this association. Following fractionation through five different chromatographic columns and a final glycerol density gradient centrifugation (schematically shown in Fig. 1A), Dnmt3a coeluted with at least 12 polypeptides of distinct molecular sizes (Fig. 1B). During purification, Dnmt3a, Dnmt3b, and Dnmt1 were monitored by Western blotting using highly specific antibodies raised in our laboratory (18, 30). The identity of the major polypeptides copurified with Dnmt3a was determined by mass spectrometry (LC-MS/MS) using well-established mass spectrometric and bioinformatics methods (Fig. 1B). The most noteworthy polypeptides in the complex included Mbd3, a methyl CpG binding protein, Hdac1, and several components of chromatin-remodeling complex (i.e., Brg1, Baf155, and Baf57). Copurification of these components with Dnmt3a was further confirmed by Western blotting of the glycerol gradient fractions with respective antibodies (Fig. 1C). The peak fractions of all the proteins eluted in the same fraction (#9). Hdac1 was, however, separated into two peaks, and one of the Hdac1 peak fractions (#9) matched with those of Dnmt3a and other proteins (Fig. 1C). The fractions containing the maximum amount of Dnmt3a protein also exhibited maximal DNA methyltransferase activity (Fig. 1C). This data strongly suggests that the major chromatin-remodeling factors exist in a large complex in vivo in lymphosarcoma cells.

Figure 1. Profile of polypeptides copurified with Dnmt3a. A, fractionation scheme of Dnmt3a from mouse lymphosarcoma S-100 extract. B, identity of polypeptides copurified with Dnmt3a as determined by LC-MS/MS. The fraction of glycerol density gradient maximally enriched in Dnmt3a was separated on SDS-PAGE. Major polypeptides stained with Coomassie blue were subjected to MS. C, Western blot analysis of glycerol density gradient fractions with specific antibodies. Top, de novo DNA methyltransferase activity measured in glycerol density gradient fractions enriched in Dnmt3a. Bottom, location of Dnmt3a and other proteins of nucleosome remodeling complex. D, Dnmt3a coimmunoprecipitates with Brg1 and Mbd3. Mouse lymphosarcoma cell extracts (500 μg) were immunoprecipitated with anti-Dnmt3a, anti-Brg1, or anti-Mbd3 antibodies in TNN buffer. The corresponding preimmune serum served as the control. The immunoprecipitated polypeptides were analyzed by Western blotting sequentially with the antibodies against each protein after stripping in the buffer [100 mmol/L Tris-HCl (pH 8.8), 2%SDS, 100 mmol/L mercaptoethanol] at 55°C for 30 minutes.
Dnmt3a (Fig. 1D, lane 3). Similarly, anti-Mbd3 and anti-Brg1 antibodies were able to immunoprecipitate the respective proteins along with other two interacting proteins (lanes 4 and 5). None of these proteins were pulled down by preimmune serum, indicating specific interactions among the proteins.

ATRX domain of Dnmt3a, MBD of Mbd3, and ATPase and Bromo domains of Brg1 are involved in interaction among the three proteins. To identify the domain of each polypeptide that is involved in direct interaction among the three proteins, glutathione S-transferase (GST) pulldown assay was done. For this purpose, the Dnmt3a-GST/Mbd3-GST fusion protein coupled to reduced glutathione beads was used to pull down different deletion mutants of Mbd3-Flag/Dnmt3a-Flag expressed in HepG2 cells. The pulldown proteins were then analyzed by Western blot analysis with anti-Flag antibody. The expression of full-length Mbd3 (Mbd3-FL) and two of its deletion mutants (i.e., ΔMBD/Mbd3 [MBD-deleted Mbd3] and ΔC/Mbd3 [COOH-terminal domain-deleted Mbd3]; Fig. 2A) were comparable (Fig. 2A, lanes 1-3). GST-Dnmt3a was able to pull down Mbd3-FL and ΔC/Mbd3 (residues 1-214 amino acids) but not ΔMBD/Mbd3 (residues 80-285 amino acids; Fig. 2A, lanes 7-9). GST alone did not pull down Mbd3 from the same extracts (lanes 4-6). This data suggests that MBD of Mbd3 is necessary for its interaction with Dnmt3a. We also identified the domain of Dnmt3a that interacts with Mbd3 using full-length Dnmt3a (3a-FL) and different deletion mutants of Dnmt3a such as NH2-terminal deleted (ΔN-3a), catalytic domain deleted (ΔC-3a), or ATRX homology domain deleted (ΔATRX-3a) Dnmt3a (Fig. 2B). GST-Mbd3 efficiently pulled down full-length Dnmt3a as expected, and in addition, only the catalytic domain mutant ΔC-3a (residues 1-690) was pulled down under this condition (Fig. 2B, lanes 10 and 12). Although expressed at comparable levels (lanes 1-3), ΔN-3a (residues 589-908) and ΔATRX-3a (Δ455-588 amino acids) Dnmt3a were not pulled down by GST-Mbd3 (lanes 9 and 11). Because the ATRX homology domain present in ΔN-3a and ΔATRX is retained in ΔC-3a, it can be concluded that ATRX homology domain of Dnmt3a interacts with Mbd3. Because additional NH2-terminal residues in ΔN-3a mutant are deleted, we cannot completely rule out the possible role of the NH2-terminal region in this interaction. GST alone was not able to pull down Dnmt3a or its deletion mutants (lanes 5-8).

Next we sought to define the domain(s) of Brg1 required for its association with Dnmt3a and Mbd3. For this purpose, full-length Brg1 (Brg1-FL), NH2-terminal domain (N-term), ATPase domain (ATPase), or bromo domain (bromo) of Brg1 tagged to triple Flag epitope (Fig. 2C) was used. Both GST-Dnmt3a and GST-Mbd3 were able to pull down full-length Brg1 protein very effectively (Fig. 2C, lanes 9 and 13), whereas neither protein was pulled down by GST-Mbd3.
amplified from input DNA. The amount of promoter amplified was quantitated using ImageQuant software and expressed as ratio of promoter amplified from immunoprecipitated DNA to that digested. MT-I promoter was amplified from these DNAs with specific primers. Mbd3, or Brg1 antibodies (see Materials and Methods for details), and the precipitated DNA was divided into three aliquots that were digested with and Methods for details).

Mbd3 antibody was used to detect Mbd3. Colocalization of Dnmt3a and Brg1 when both proteins were overexpressed in HepG2 cells (Fig. 3) showed a high degree of colocalization with Mbd3, particularly in the DAPI-dense region when horizontally stained by DAPI (Fig. 3). Dnmt3a colocalized extensively with Mbd3 in HepG2 cells (Fig. 3A, a-d). Similarly, a significant population of Dnmt3a colocalized with Mbd3, particularly in the DAPI-dense region when overexpressed together (Fig. 3A, e-h). Cotransfection of Mbd3 and Brg1-Flag into HepG2 cells showed a high degree of colocalization of these two proteins in the DAPI-dense region (Fig. 3A, i-l). These results further reinforce our observation that Dnmt3a, Brg1, and Mbd3 interact with each other and exist as a large complex in vivo.

Dnmt3a, Mbd3, and Brg1 interact with each other and exist as a large complex in vivo. In vitro association of Dnmt3a with Mbd3 and Brg1 in the mouse lymphosarcoma cells (P1798) led us to explore the functional significance of their interaction in modulating gene expression. Because the MT-I gene is silent in P1798 cells due to extensive promoter methylation (25, 34), we investigated whether these three proteins involved in epigenetic regulation of genes associate with the promoter by chromatin immunoprecipitation assay. The formaldehyde cross-linked chromatin from P1798 cells was immunoprecipitated with anti-Dnmt3a, Mbd3, or Brg1 antibodies (see Materials and Methods for details), and the precipitated DNA was divided into three aliquots that were digested with MspI, HpaII, or mock digested. MT-I promoter was amplified from these DNAs with specific primers. D, quantitative analysis of the association of these proteins with MT-I promoter. The amount of promoter amplified was quantitated using ImageQuant software and expressed as ratio of promoter amplified from immunoprecipitated DNA to that amplified from input DNA.

Dnmt3a, Mbd3, and Brg1 colocalize at the subnuclear level. To further confirm the interaction among Dnmt3a, Mbd3, and Brg1, we examined their cellular localization by confocal microscopy after transient overexpression in HepG2 cells. Dnmt3a was present essentially in the heterochromatin region throughout the nuclei strongly stained by DAPI (Fig. 3). Dnmt3a colocalized extensively with Mbd3 both proteins were overexpressed in HepG2 cells (Fig. 3A, a-d). Similarly, a significant population of Dnmt3a colocalized with Mbd3, particularly in the DAPI-dense region when overexpressed together (Fig. 3A, e-h). Cotransfection of Mbd3 and Brg1-Flag into HepG2 cells showed a high degree of colocalization of these two proteins in the DAPI-dense region (Fig. 3A, i-l). These results further reinforce our observation that Dnmt3a, Brg1, and Mbd3 interact with each other and exist as a large complex in vivo.

by GST alone (Fig. 2C, lanes 5–8). It is, therefore, evident that the Brg1 protein directly interacts with Dnmt3a and Mbd3 in vitro. Assay with various deletion mutants of Brg1 showed that GST-Dnmt3a as well as GST-Mbd3 pulled down both ATPase and bromo domains of Brg1 (Fig. 2C, lanes 11 and 12 and 15 and 16, respectively) but not the NH2-terminal domain (Fig. 2C, lanes 10 and 14). All three Brg1 mutants were expressed at a comparable level (Fig. 2C, lanes 1–4). This data shows that the NH2-terminal domain of Brg1 is dispensable for its interaction with Dnmt3a or Mbd3.

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with the methylated MT-I promoter (Fig. 3C, lanes 10-12 and D). Similarly, significant association of Mbd3 with the methylated promoter was detected in these cells (Fig. 3C, lanes 16-18 and Fig. 3D). Association of Mbd3 with the methylated promoter is probably mediated through its interaction with Dnmt3a, as Mbd2 that can also recruit Mbd3 to methylated DNA, is expressed at a very low level in P1798 cell (28). Immunoprecipitation with Brg1 antibody showed strong association of Brg1 with the methylated MT-I promoter in P1798 cells (Fig. 3C, lanes 22-24 and Fig. 3D).

This data indicate that all three proteins are associated with the methylated MT-I promoter in vivo and are likely to play significant role in silencing the methylated gene in lymphosarcoma cells.

Dnmt3a, Mbd3, and Brg1 suppress the methylated MT-I promoter activity. The association of Dnmt3a, Mbd3, and Brg1 with the MT-I promoter in lymphosarcoma cells led us to explore further the role of these proteins in modulating MT-I expression. To address this issue, we studied the potential alteration in the MT-I promoter activity following ectopic expression of these proteins. The relatively high transfection efficiency of HepG2 cells prompted us to use these cells instead of P1798 cells that grow in suspension and are very difficult to transfect. Mouse MT-I promoter was cloned into promoterless pGL2-Basic vector (pMT-Luc; ref. 22) and methylated with M. HhaI (Fig. 4A). The cloned region of MT-I promoter in the pMT-Luc harbors four $Hh{a}_1$ sites, and methylation of those sites inhibited the constitutive (C) or Zn-induced (Zn) promoter activity by 65% compared with the unmethylated promoter (Fig. 4A), implicating that the inhibition (65%) of the methylated pMT-Luc is a direct effect of methylation on the promoter as methylation of the vector (pGL2-basic) had no significant effect on the luciferase activity under this experimental condition (data not shown).

To assess the role of Dnmt3a and its interacting proteins in regulating the methylated MT-I promoter, we cotransfected cells with respective expression vectors and methylated pMT-Luc. The internal control (pRL-tk) used to monitor transfection efficiency was not included in these assays, because CpG binding proteins and chromatin-remodeling factors affected its activity as also observed in another laboratory (35). Monitoring expression of the Flag-tagged Dnmt3a, Mbd3, or Brg1 by Western blot analysis ensured comparable transfection efficiency, and the experiments were done in triplicate and repeated at least three times. Variance among the experiments was insignificant. Transient overexpression of Dnmt3a in HepG2 cells inhibited both the constitutive (C) and Zn-induced (Zn) activity of the methylated MT-I by ~40% and 51%, respectively (Fig. 4B, compare lanes 1 and 2 with lanes 3 and 4). To ascertain if the catalytic activity of Dnmt3a is essential for its transcriptional repressor activity, a catalytically inactive, point mutant (C706S) of Dnmt3a (36) was coexpressed with methylated pMT-Luc into HepG2 cells. Both the wild type and C706S mutant exhibited comparable inhibitory effect on MT-I promoter activity (Fig. 4B, compare lanes 3 and 4 with lanes 5 and 6), suggesting that the catalytic activity of Dnmt3a is not essential for promoter suppression. Among different deletion mutants of Dnmt3a analyzed (Fig. 2B), ΔN-3a and ΔATRX had no effect on the MT promoter activity (Fig. 4B, compare lanes 1 and 2 with lanes 7 and 8 and 9 and 10), whereas ΔC-3a exhibited inhibitory activity like the C706S mutant (35% of basal activity and 45% of Zn-induced activity; Fig. 4B, compare lanes 5 and 6 with lanes 11 and 12). Western blot analysis showed expression of all the proteins (wild type and mutants) at comparable levels (Fig. 4B, bottom).

Similarly, ectopic expression of Mbd3 resulted in significant inhibition of both constitutive (C) and Zn-induced (Zn) methylated MT-I promoter activity (35-48%; Fig. 4C, compare lanes 1 and 2 with lanes 3 and 4). Overexpression of pΔC-Mbd3 but not pΔMBD-Mbd3 inhibited the methylated MT promoter activity of comparable level with that of the full-length Mbd3, suggesting the involvement of COOH-terminal domain in the repression process (Fig. 4C, compare lanes 3 and 4 with lanes 7 and 8). Neither Dnmt3a nor Mbd3 had any effect on mock-methylated MT-I promoter (data not shown). Western blot analysis revealed comparable expression of all the Mbd3 proteins (Fig. 4C, bottom).

Unlike Dnmt3a and Mbd3, Brg1 at a relatively low level inhibited basal activity (C) of both the mock-methylated (52%) and methylated (48%) MT-I promoter (Fig. 4D, compare lane 3 with lane 15 with 13). Similarly, the Zn-induced (Zn) activity of both mock-methylated and methylated promoter was strongly inhibited by Brg1 (unmethylated: 72%, lanes 2 and 4 and methylated: 52%, lanes 14 and 16). Because Brg1 requires DNA-dependent ATPase activity for chromatin remodeling, we investigated whether the ATPase activity of Brg1 is involved in suppression of the MT-I promoter. Indeed, the ATPase activity of Brg1 is essential for the impediment of MT-I promoter irrespective of the methylation status, because the catalytically inactive mutant (K798R) of Brg1 (37) had no effect (Fig. 4D, compare lanes 1 and 2 with lanes 5 and 6 and lanes 13 and 14 with lanes 17 and 18). The NH$_2$-terminal or the bromo domain of Brg1 alone could not impede the MT-I promoter activity, whereas ATPase domain alone inhibited both the basal and Zn-induced activity of the unmethylated (40% and 50%, respectively) and methylated promoter (30% and 34%, respectively). Western blot analysis showed that the expressions of the wild-type and mutant Brg1 were comparable (Fig. 4D, bottom). These data showed that all three proteins (i.e., Dnmt3a, Brg1, and Mbd3) play a significant role in silencing the MT-I promoter particularly when it is methylated in vivo.

To elucidate the functional significance of the observed physical interaction among Dnmt3a, Mbd3, and Brg1 on transcriptional regulation of the MT-I gene, we cotransfected HepG2 cells with methylated pMT-Luc and two of the three interacting proteins at a time. For this purpose, we first optimized the level of ectopic proteins to achieve minimal inhibition (30-50%) of the promoter activity with individual proteins. Figure 5A shows the effect of Dnmt3a and Mbd3 coexpression on MT-I promoter activity. As observed before, expression of Dnmt3a and Mbd3 individually resulted in ~30% inhibition of the constitutive MT-I promoter activity, and the Zn-induced activity was repressed by 47% and 28% respectively (Fig. 5A, lanes 1-6). However, overexpression of both proteins resulted in 64% inhibition of the constitutive activity and 71% inhibition of Zn-induced activity. This data shows that Dnmt3a and Mbd3 alone can suppress transcription from the methylated MT-I promoter, and overexpression of both resulted in additive effect. Similar results were observed when Dnmt3a and Brg1 were coexpressed (Fig. 5B). Because the three polypeptides BAF-55, BAF-57, and BAF-155 were associated with Dnmt3a by virtue of their presence in the Brg1 complex, the present study focused on the core subunit of this complex. When expressed alone, Dnmt3a and Brg1 resulted in 30% and 51% inhibition of the basal MT-I promoter activity and 45% and 56% inhibition of Zn-induced activity, respectively (Fig. 5B, lanes 1-6). Coexpression of both the proteins resulted in ~86% to 88% inhibition of both constitutive and Zn-induced activity of the MT-I promoter. Finally, we studied the
effect of ectopic expression of Mbd3 and Brg1 separately and in combination on the MT promoter activity (Fig. 5C). Expression of Mbd3 or Brg1 alone led to 32% and 51% inhibition of constitutive MT-I promoter activity and 36% and 59% inhibition of the Zn-induced activity, respectively (Fig. 5C, lanes 1-6). Overexpression of both the proteins resulted in 92% inhibition of the constitutive activity and 94% inhibition of the Zn-induced activity (Fig. 5C, compare lanes 1 and 2 with lanes 7 and 8), which indicates suppressive effect of one protein is potentiated in presence of the other. Demonstration of functional cooperation among the three proteins is shown in Figure 4A. Western blot analysis of the overexpressing proteins (bottom). C, MBD of Mbd3 is required for suppressing methylated MT-I promoter activity. HepG2 cells were cotransfected with the mammalian expression vector (Vector), full-length Mbd3 (Mbd3-FL), MBD-deleted Mbd3 (∆MBD), and COOH-terminal domain–deleted (∆C) mutants (Fig. 2A) along with M. HhaI methylated MT-Luc. Zn treatment and luciferase assay was done as described for (A). Western blot analysis of the overexpressing proteins (bottom). D, the wild type but not the ATPase domain mutant of Brg1 inhibits MT-I promoter activity irrespective of its methylation status. HepG2 cells were transfected with wild-type Brg1 (WT), catalytic site mutant (Mut), different domains of Brg1 (Fig. 2C), or empty vector (Vec) along with mock-methylated or M. HhaI–methylated pMT-I-Luc (see Materials and Methods for additional details). Luciferase activity in each sample (in arbitrary units) normalized to same amount of protein in each extract was compared and represented as fold alteration of the promoter activity upon overexpression of different Brg1 mutants. Western blot analysis of the overexpressing proteins (bottom).

Figure 4. Modulation of MT-I promoter activity by Dnmt3a, Mbd3, and Brg1 in transient transfection assay. A, schematic diagram showing HhaI sites on mouse MT-I promoter. Methylation of these sites by M. HhaI methylase represses the constitutive (C) or Zn-induced (Zn) promoter activity. HepG2 cells were cotransfected with mock or methylated pMT-Luc along with pRL-tk. After 24 hours, cells were split into two and were either left untreated (−) 12 hours later or treated with 100 μmol/L zinc sulfate (+) for an additional 6 hours. The luciferase activity was measured in whole cell extract using Dual Luciferase assay kit (Promega, Madison, WI). B, Dnmt3a suppresses methylated MT-I promoter activity. HepG2 cells were cotransfected with the mammalian expression vector (Vector), wild-type Dnmt3a (WT-3a), catalytic site mutant (mut-3a), and different deletion mutants (Fig. 2B) of the protein along with M. HhaI methylated pMT-Luc. Zn treatment and luciferase assay was done as described for (A). Western blot analysis of the overexpressing proteins (bottom). C, MBD of Mbd3 is required for suppressing methylated MT-I promoter activity. HepG2 cells were cotransfected with the mammalian expression vector (Vector), full-length Mbd3 (Mbd3-FL), MBD-deleted Mbd3 (∆MBD), and COOH-terminal domain–deleted (∆C) mutants (Fig. 2A) along with M. HhaI methylated pMT-Luc. Zn treatment and luciferase assay was done as described for (A). Western blot analysis of the overexpressing proteins (bottom). D, the wild type but not the ATPase domain mutant of Brg1 inhibits MT-I promoter activity irrespective of its methylation status. HepG2 cells were transfected with wild-type Brg1 (WT), catalytic site mutant (Mut), different domains of Brg1 (Fig. 2C), or empty vector (Vec) along with mock-methylated or M. HhaI–methylated pMT-I-Luc (see Materials and Methods for additional details). Luciferase activity in each sample (in arbitrary units) normalized to same amount of protein in each extract was compared and represented as fold alteration of the promoter activity upon overexpression of different Brg1 mutants. Western blot analysis of the overexpressing proteins (bottom).
proteins in transient transfection assay is technically challenging and has not been successfully executed. Nevertheless, this series of experiments show functional significance of the observed biochemical interaction of Dnmt3a with Mbd3 and Brg1.

Ectopic expression of Brg1 in Brg1-null cells inhibits MT gene expression. To further our understanding of the role of Brg1 in regulating MT gene expression, we chose SW13 cells, an adrenal carcinoma cell line that do not express Brg1 (38). Level of MT-I gene expression was analyzed by real-time PCR in SW-13 cells and compared with the cells that are ectopically expressing Brg1 using primers common to all isoforms of MT-I. Expression of MT-I was inhibited (44%) in Brg1-expressing cells compared with the vector-transfected controls (Fig. 6A).

We next analyzed the MT promoter activity in the Brg1-null background by transient transfection assay. SW-13 cells were cotransfected with either unmethylated or methylated pMT-Luc and increasing amount of Brg1 expression vector. A dose-dependent inhibition of both unmethylated (38-54%) and methylated (36-61%) MT-I promoter activity was observed when Brg1 was overexpressed (Fig. 6B). This data validates the previous observation where ectopic expression of Brg1 inhibited both methylated and unmethylated MT-I promoter activity in HepG2 cells (Fig. 4D).

Discussion

The mechanisms underlying DNA methylation and subsequent transcriptional repression have been the subject of intense research in recent years. The mechanism of selective recruitment of DNA methyltransferases to specific sites on the chromatin still remains to be elucidated. The present study was undertaken to explore the repressor function of Dnmt3a and its association with other proteins in vivo that might augment its repressor activity as well as facilitate its targeting to distinct gene promoters. We

Figure 5. Coexpression of Dnmt3a-associated proteins potentiates the repressive activity of Dnmt3a on methylated MT-I promoter. A, repressive effect of Mbd3 and Dnmt3a is additive when coexpressed. HepG2 cells were transfected with Dnmt3a and Mbd3 expression vectors alone or in combination, along with methylated pMT-Luc. After 24 hours, cells were split into two, and 12 hours later, these cells were either left untreated (-) or treated with 100 μmol/L zinc sulfate (+) for an additional 6 hours. The luciferase activity was measured in whole cell extract using Dual Luciferase assay kit (Promega). Luciferase activity in each sample (arbitrary units) was normalized to same amount of protein in each extract was compared. B, coexpression of Brg1 and Dnmt3a potentiates their repressive effect. Dnmt3a and Brg1 expression vectors were transfected alone or in combination into HepG2 cells. The cells were treated with Zn and luciferase activity measured as described (A). C, Brg1 and Mbd3 coexpression increases efficiency of silencing of methylated MT-I promoter. Mbd3 and Brg1 expression vectors were transfected alone or in combination into HepG2 cells along with methylated pMT-Luc. The cells were treated with Zn, and luciferase activity measured as described in (A).
interaction of Dnmt3a with Mbd3 and Brg1

Mbd3 recruits Hdac complex as well as Dnmt1 to establish transcriptionally repressed chromatin (43). The present data suggests that Mbd3 can also be recruited to the target site by virtue of its interaction with Dnmt3a.

An important aspect of this study is the demonstration that Brg1 and Mbd3 could inhibit the cotransfected methylated MT-I promoter, and that the DNA-dependent ATPase activity of Brg1 is essential for repression of the MT-I promoter. This is particularly relevant in view of our previous reports that MT-I is methylated and silenced in some tumors (22, 44). It is striking that Dnmt3a could suppress the methylated MT-I promoter activity independent of its the catalytic domain. Dnmt3a is indeed recruited by sequence-specific transcription factor RP58 to suppress its target genes by hypoacetylation of their promoters (17). Similarly, a recent study in our laboratory showed that the noncatalytic domain Dnmt3b plays a key role in the differentiation of PC12 cells to neuronal cells (19).

In an earlier study using a partially purified preparation, we showed that a histone H3 K9-methyl transferase and Hdac1 also associate with Dnmt3a in P1798 cells (18). LC-MS/MS analysis of the highly purified Dnmt3a fraction did not identify any histone methyltransferase. Most probably, the amount of HMTase is very low or it is a novel HMTase. On the contrary, Hdac1 remained associated with Dnmt3a even after extensive biochemical fractionation of the cell extracts. This is consistent with the association of Hdac1 with purified Dnmt1 (15) and Dnmt3b (39). Transient transfection assay also suggests that inhibition of the methylated MT-I promoter activity by Dnmt3a is mediated through interaction with Hdac, as the suppression can be partially recovered by the Hdac inhibitor, trichostatin A.² It is evident that at least one of the HdaCs forms part of the repressor complex with all three DNA methyltransferases that control the epigenetic silencing of genes. It has recently been reported that both in vivo and in vitro sumoylation of Dnmt3a regulate its interaction with Hdac1/Hdac2 (45). At this juncture, we cannot rule out the involvement of posttranslational modifications in interaction of Dnmt3a with Mbd3 or other members of the chromatin-remodeling complex.

Recent studies have identified new protein partners for the Dnmts, which reveal novel mechanisms for gene silencing. In addition to DNA methylation and histone modifications, chromatin remodeling also plays a critical role in the epigenetic regulation of genes. A complex picture is emerging as to how the chromatin-modifying factors facilitate the gene silencing by DNA methylation. The potential role of chromatin-remodeling factors in altered methylation at rDNA repeats has been reported (46). The present study has shown direct interaction between Dnmt3a and the ATP-dependent chromatin-remodeling factor Brg1. In this context, it is noteworthy that association of another chromatin-remodeling enzyme, SNF2h, with Dnmt3b has been recently shown (20).

Brg1 is known to be the central subunit of the SWI/SNF complex that plays a crucial role in transactivation (37, 47). Recent studies showed transcriptional repressor activity of the SWI/SNF complex (48, 49). Furthermore, the interaction of Brg1 (the ATPase subunit of SWI/SNF complex) with other proteins causes transcriptional repression in mammalian cells. Brg1 exhibits its repressor activity, whereas it is present in a multiprotein supercomplex (50). This finding is consistent with the association of Brg1 with the methylated MT-I promoter in lymphosarcoma cells, impediment of both basal and zinc-induced MT-I promoter activity following overexpression of the wild-type Brg1 in HepG2 cells and the role of ATPase domain of Brg1 in this process. To gain mechanistic insight into the role of Dnmt3a, Mbd3 and Brg1 in regulation of MT-I gene expression, it is necessary to study chromatin remodeling and transcriptional activities using in vitro assembled nucleosomal template in presence and absence of these factors. Nevertheless, the present study has clearly showed the physical and functional interaction between the factors involved in different steps of the epigenetic machinery.

² S. Majumder, unpublished data.

Figure 6. Ectopic expression of Brg1 suppresses the MT-I promoter activity in Brg1 null (SW13) cells. A, MT-I expression is suppressed after ectopic expression of mBrg1 in SW13 cells. cDNAs prepared from SW13 cells after 36 hours of transfection with pBJ5 or pBJ5-Brg1 (2 μg) were subjected to real-time PCR with primers specific for human MT-I or 18S rRNA. Copy number for each mRNA was calculated based on a standard curve generated from 10-fold serial PCR with primers specific for human MT-I or 18S rRNA. Copy number for each mRNA was compared. Western blot analysis of the overexpressing Brg1 protein (bottom).
Acknowledgments

Received 4/27/2005; revised 9/2/2005; accepted 9/14/2005.

Grant support. NIH/USPHS grants ES10874, CA86978, and CA86978. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Steve Goff (Department of Biochemistry and Molecular Biophysics, Columbia University, New York, NY) for providing a wild-type and mutant BrGlu expression vectors; Dr. En Li (Novartis Institutes for Biomedical Research, Cambridge, MA) for providing Dnmt3a cDNA; Dr. Said Sif (Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, OH) for providing antibodies against BrGlu, BrD, BrH3, and H3K14 acetylated and Dr. Taeam Motiwala (Department of Molecular and Cellular Biochemistry, Ohio State University, Columbus, OH) for critically reviewing the article.

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