Abstract

Methyl-CpG binding domain (MBD) proteins have been shown to couple DNA methylation to transcriptional repression. This biological property suggests a role for MBD proteins in the silencing of tumor suppressor genes that are hypermethylated at their promoter CpG islands in cancer cells. Despite the demonstration of the presence of MBDs in the methylated promoter of several genes, we still ignore how general and specific is this association. Here, we investigate the profile of MBD occupancy in a large panel of tumor suppressor gene promoters and cancer cell lines. Our study shows that most hypermethylated promoters are occupied by MBD proteins, whereas unmethylated promoters are generally devoid of MBDs, with the exception of MBDA. Treatment of cancer cells with the demethylating agent 5-aza-2’-deoxycytidine results in CpG island hypomethylation, MBD release, and gene reexpression, reinforcing the notion that association of MBDs with methylated promoters in the methylated promoter of p16INK4a in colon cancer cells. Despite the selectivity of MBDs for methylated DNA and their potential involvement in gene repression, as methylated CpG islands are an attractive substrate on which to exert their function. A major breakthrough in this field came when a mechanism link between MBD proteins and histone deacetylation–mediated transcriptional repression was found by showing that these proteins are able to associate histone deacetylase (3–15).

Introduction

Ablant hypermethylation of promoter CpG islands and the resulting transcriptional silencing is nowadays a widely accepted mechanism of inactivation of tumor suppressor genes in cancer that actively contributes to tumorigenesis (1–4). One of the key achievements in cancer epigenetics has been the recognition of profiles of aberrant CpG hypermethylation that are specific to the tumor type (5, 6). The existence of these profiles provides a powerful set of markers for outlining the disruption of critical pathways in tumorigenesis and for deriving sensitive molecular detection strategies for virtually every human tumor type. The systematic study of DNA methylation patterns in human cancer cell lines (7, 8) has shown that these are appropriate models for this type of study as they show methylation patterns that resemble their corresponding tumor types (7, 8).

As a further step in the characterization of epigenetic inactivation of tumor suppressor genes in cancer, it is of inherent interest to characterize the pattern of nuclear factors involved in translating the information encoded by DNA methylation to transcriptional silencing. Methyl-CpG binding proteins constitute a key element in this scenario. The founding member of this family of proteins, MeCP2, was discovered and characterized in the early 1990s (9). The remaining members [methyl-CpG binding domain (MBD) 1, MBD2, MBD3 and MBD4] were identified from database searches done with the minimum portion able to recognize a single methylated CpG site, the MBD (10). Current evidence indicates that, in mammals, only MeCP2, MBD1, and MBD2 are bona fide methylated DNA-binding proteins. Despite the lack of methyl-CpG binding activity, mammalian MBD3 (10, 11), which is an integral subunit of the Mi-2/NuRD complex, can be recruited to methylated DNA through interaction of the complex with MBD2 (12).

Early reports of MBD proteins had already suggested their potential involvement in gene repression, as methylated CpG islands are an attractive substrate on which to exert their function. A major breakthrough in this field came when a mechanistic link between MBD proteins and histone deacetylation–mediated transcriptional repression was found by showing that these proteins are able to associate histone deacetylase (13–15).

The selectivity of MBDs for methylated DNA and their transcriptional repression properties suggest that they could exert their function in methylated promoters. The presence of MBD proteins in the methylated promoter of a gene in cancer was first shown in 2000, when Magdinier and Wolffe (16) identified MBD2 in the methylated promoter of p16INK4a in colon cancer cells. Subsequent reports have shown MBDs to be present in other methylated promoters (17–21) and it is currently thought that MBD proteins may possess some specificity in their association with methylated promoters. However, this issue remains as an open question in the field.

In this report, we address the question of MBD occupancy in aberrantly methylated promoters in the context of cancer cells. We have comprehensively analyzed a panel of transformed cells corresponding to different tumor types and selected tumor suppressor genes that are known to undergo CpG island promoter hypermethylation. Our results prove that MBDs are a common feature of all methylated genes in cancer with a profile of MBD distribution and occupancy that exhibits gene promoter– and cell type–dependent specificity.
Materials and Methods

Human cancer cell lines. The 10 human cancer cell lines used in this study were obtained from the American Type Culture Collection (Rockland, MD) and the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Six tumor types were represented in this set of tumor cell lines: breast (MCF7 and MDA-MB-231), colon (HCT15, LoVo, and SW48), lymphoma (Raji and U937), leukemia (HL60), lung (H1299), and cervix (HeLa). Cell lines were maintained in the appropriate medium and treated with 5-aza-2′-deoxycytidine at a concentration of 1 μmol/L for 3 days to achieve demethylation.

Western blot analysis. To check the levels of MBP proteins in the cells used for the study, cells were lysed with RSB buffer [10 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, 3 mmol/L MgCl2] in the presence of 1% NP-40 (17). Raw nuclei were then washed with RSB buffer and resuspended in SDS-PAGE loading buffer. Equal amounts of proteins (~5 μg) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were then hybridized with antibodies against MeCP2, MBD1, MBD2, and MBD3 (Abcam, Cambridge, United Kingdom). The proteins were detected with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Analysis of CpG island methylation status. DNA methylation patterns in the CpG islands were determined by bisulfite-mediated conversion of unmethylated, but not methylated, cytosines to uracil as described previously (6, 8). We established methylation status by PCR analysis of bisulfite-modified genomic DNA using two procedures. First, all genes studied were analyzed by bisulfite genomic sequencing of their corresponding CpG islands. Both strands were sequenced. The second analysis used methylation-specific PCR (MSP) for all genes (6). Placental corresponding CpG islands were determined by bisulfite-mediated conversion of the CpG islands were used for the study, cells were lysed with RSB buffer [10 mmol/L Tris (pH 7.5), 10 mmol/L NaCl, 3 mmol/L MgCl2] in the presence of 1% NP-40 (17). Raw nuclei were then washed with RSB buffer and resuspended in SDS-PAGE loading buffer. Equal amounts of proteins (~5 μg) were separated on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were then hybridized with antibodies against MeCP2, MBD1, MBD2, and MBD3 (Abcam, Cambridge, United Kingdom). The proteins were detected with the Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Results and Discussion

MBDs are differentially expressed in human cancer cell lines. Potential differences in the expression levels of different MBPs for each cell line and cell type may be key to understand MBP distribution in promoters. We observed a wide range of levels of expression of MBP proteins among different human cancer cell lines (Fig. 1). Only MBD3 expression seemed to be roughly similar in different cell lines, whereas MeCP2, MBD1, and MBD2 exhibited a wide range of expression.

MeCP2 was strongly expressed in H1299 (lung), SW48 and HCT15 (colon), HL60 (leukemia), U937 (lymphoma), and MCF7 (breast) cells. In contrast, MDA-MD-231 (breast) and Raji cells (lymphoma) had very low levels of MeCP2 expression. For MBD2, Raji and MDA-MB-231 had the highest levels of expression. Interestingly, these two cell lines exhibited the lowest levels of MeCP2 expression, whereas SW48 and U937 weakly expressed MBD2 but strongly expressed MeCP2. We may speculate that this apparent complementarity of patterns of MBP expression is functionally significant. This possibility would be supported if the same promoter had a differential distribution of associated MBPs that correlated with the availability or expression levels of MeCP2 and MBD2 in different cell lines. This particular issue is discussed below.

For MBD1, the three studied colon cancer cell lines (HCT15, LoVo, and SW48) showed the highest levels of expression. The MBD1 antibody, raised against a common peptide to all five described MBD1 isoforms (17), shows that only two forms are substantially expressed among cell lines. Some of the cell lines expressed only one of the MBD1 isoforms, whereas others expressed both detectable isoforms (Fig. 1). For instance, the two breast cancer cell lines, MCF7 and MDA-MB-231, had very different patterns of MBD1 isoform expression (Fig. 1).

Statistical analysis. Data were statistically analyzed by the χ2 test and calculation of Kendall’s τ correlation coefficients. Statistical differences between percentages were examined with Fisher’s exact test. Because ~40 different statistical tests were done in this work, we chose a Bonferroni-adjusted α level of 0.001 (calculated as 0.05/40) as the threshold of significance. For all analyses, the null hypothesis tested was that MBPs exhibited no preference for binding to a particular site (methylated/ nonmethylated) and that all of them had the same probability of binding a particular promoter. To identify statistical differences for the levels of MBP binding among several cell lines or among several genes, we used the Kruskal-Wallis nonparametric test.

Figure 1. Expression of MBPs in human cancer cell lines tested by Western blot. The 10 cell lines were analyzed with anti-MeCP2, MBD1, MBD2, and MBD3 antisera. Both MBD1 and MBD3 show two previously described isoforms. Nucleolin was used as a loading control.
Finally, as mentioned above, MBD3 was expressed at similar levels in all the cell lines studied, and only MDA-MB-231 had low levels of expression. The similarity of expression levels of MBD3 among cell lines is consistent with the fact that this protein is an integral subunit of a general complex involved in many regulatory processes (15). However, it is interesting to note that the two MBD3 isoforms detected had distinct proportions in different cells (Fig. 1).

A MBD occupancy profile in hypermethylated promoter CpG islands of tumor suppressor genes in human cancer cell lines. We next investigated the CpG island promoter methylation profile and MBD occupancy in a large panel of promoters (n = 22) in the 10 described cell lines. We selected promoters of different tumor suppressor genes for which CpG island promoter hypermethylation has been described previously in human cancer (1–8). A total of 22 genes, including well-characterized tumor suppressor genes (p16INK4a, p15INK4b, p14ARF, and p73), DNA repair genes (hMLH1, GSTP1, and MGMT), and genes related to metastasis and invasion (CDH1, CDH13, TIMP2, and TIMP3), apoptosis (DAPK1 and TMS1), cell signaling and mitosis (SYK, SOCS1, CHFR, and RASSFIA), and hormone receptors (PR, ERα, RARB2, and CRBB1) were studied. All of these genes possess a CpG island in their 5' region that is unmethylated in corresponding normal tissues (1–8). Three sets of primers were designed for every promoter. Two of the primer sets were designed for bisulfite genomic sequencing and MSP (unmethylated and methylated pairs), whereas the third was intended for ChIP assays. The three sets of primers were designed to amplify the same DNA sequence. Examples of bisulfite genomic sequencing are shown in Fig. 2A. In addition, primers for RT-PCR were designed for all the above genes. Examples of RT-PCR results are shown in Fig. 2B. Therefore, for each sample, DNA and RNA were isolated in parallel with formaldehyde fixation. In all cases, presence of CpG island hypermethylation was associated with transcriptional silencing. Illustrative examples are provided in Fig. 2A and B and the data are summarized in Supplementary Table S1. ChIP assays were done in triplicate and four antibodies (anti-MeCP2, anti-MBD1, anti-MBD2, and anti-MBD3) were included in all cases. Figure 2C shows the ChIP results of representative genes and cell lines, where specific profiles of MBD distribution are observed. The overall features of the promoter CpG islands bound to each MBD are summarized in Supplementary Table S2.

The combination of ChIP experiments for MBDs, CpG island methylation status, gene loci, and tissue type originated a characteristic profile of MBD occupancy in human cancer depicted in Fig. 3. An initial conclusion that can be drawn from these data is that there is a significant association between MBDs and hypermethylated CpG islands. This result is consistent with the in vitro properties of MBDs (20) and previous results obtained on a smaller scale (16–21).

The MBD occupancy data can be rationalized in terms of number and type of MBDs that occupy methylated and unmethylated promoters (Fig. 4). The methylation analysis of 10 cell lines for 22 promoter sequences yielded 136 methylated loci and 84 unmethylated loci. In this context, over 89% (121 of 136) of the methylated promoters were occupied by MBD proteins, whereas only 28% (24 of 84) of the unmethylated genes showed an association of any MBD to the promoters (Fig. 4A). This difference was statistically significant (P < 0.001, Fisher’s exact test). Considering each of the MBDs separately, we found that the 136 methylated loci accumulated 189 MBDs, whereas the 84 unmethylated sites accumulated only 26 (Fig. 4B). These values reflect a preference for MBDs to be recruited by methylated sites (P < 0.001, χ² test) and a clear correlation between gene hypermethylation and MBD recruitment (Kendall’s τ correlation coefficient r = 0.549; P < 0.001).

This close association of MBD presence and methylated CpG islands is enhanced if we exclude MBD1. In fact, most of these occupied unmethylated promoters are associated with MBD1 (23 of 26, 88%; Fig. 4C), which possesses DNA binding domains outside the MBD (10). Excluding MBD1, only ~5% of the unmethylated sites contain any MBD proteins, in contrast to the 89% of methylated genes that contain any MBD protein. This difference is statistically significant (P < 0.001, Fisher’s exact test). Another interesting issue is the presence of 11% of methylated CpG islands not occupied by any MBD. One of the most likely explanations is

Figure 2. Analysis of CpG island promoter methylation, gene expression, and MBD occupancy. A, illustrative examples of bisulfite genomic sequencing. Unmethylated Cs become Ts on bisulfite modification, whereas methylated Cs are retained as Cs. The promoter CpG islands of TIMP2 and p14 are unmethylated in U937 and Raji, respectively. The promoter CpG islands of CDH13 and p16 are methylated in U937 and Raji cells, respectively. B, illustrative examples of gene expression by semiquantitative RT-PCR. For U937 cells, the methylated genes CDH13 and PR are not expressed, whereas the unmethylated TIMP2 is expressed. For Raji cells, the methylated genes DAPK1 and RARB2 are silenced, whereas the unmethylated p14 is expressed. C, illustrative examples of ChIP analysis for MBDs for two different promoters (p16INK4a and CDH13) among different cell lines (left) and for two cell lines (Raji and U937) among different gene promoters (right). Left, CpG island methylation status. Red square, methylated; green square, unmethylated. Input lane is followed by the “bound” fractions of the no antibody (NAb) negative control and the samples corresponding to the four MBD antibodies.
the existence of other putative transcriptional repressors with affinity for methylated CpGs that we have not included in the study, such as new members of the MBD family (22), the thymine DNA glycosylase MBD4 (23), and the methyl-DNA binding protein Kaiso (24) and Kaiso-like proteins, such as ZBTB4 and ZBTB38 (25).

In summary, the analysis of MBD distributions show that MBDs are preferentially bound to hypermethylated promoter CpG islands, whereas unmethylated promoter CpG islands are devoid of MBDs or are occasionally occupied by MBD1.

The binding occupancy of MBDs in different promoters.

Having established the close association between MBD presence and hypermethylated CpG islands, two further analyses can be developed: one can study at the distribution of MBDs in a chosen promoter in all the different cell lines or one can focus attention on a cell line, analyzing the distribution of MBDs across all promoters.

The first analysis will address whether there is specificity in MBD occupancy for a particular promoter and, if so, whether it is constant regardless of the cell type. The second analysis would help us to understand whether a particular cell line has a preference for using a particular MBD protein.

To address the first aspect, we analyzed MBD distribution for each particular promoter CpG island. We observed that, for MeCP2 and MBD2, the profile of MBD binding was significantly promoter specific among the 22 genes analyzed ($P = 0.011$, Kruskal-Wallis nonparametric test). This was not the case for MBD1 and MBD3. For instance, a gene like CDH13 is methylated across the entire panel of cell lines, except for the cervical cancer cell line HeLa. MeCP2 was present in all but one of the methylated cases (8 of 9). However, not all promoters reflect this specificity for MBD occupancy. For example, the PR and ER1 promoters show heterogeneity in the MBD distribution and none of the MBDs preferentially occupies these promoters. It is remarkable that genes that are methylated in a greater number of cell lines seem to exhibit more specificity in recruiting a particular MBD than those with less tendency to become methylated. For instance, CDH13 is methylated in 90% (9 of 10) of the cell lines and MeCP2 is present in 8 of these 9 cases (Fig. 3).

In the second analyses, we investigated the MBD distribution in each cell line for the entire panel of CpG islands. We observed that human cancer cell lines tended to use a particular MBD. Using the statistical test of Kruskal-Wallis, a different usage among the 10 cell lines was shown for MeCP2 ($P = 0.026$), MBD1 ($P < 0.001$), and MBD3 ($P = 0.020$) but not for MBD2 ($P > 0.05$). For example, U937 and SW48 had a preference for using MeCP2, whereas H1299 and HL60 “preferred” MBD1 (Fig. 3). An easy explanation would be that these data may reflect the expression levels of different MBDs. We therefore decided to compare these results with the expression data analyzed by Western blot (Fig. 1). However, in general, the
strong expression of a particular MBD in a cell line was not necessarily associated with a preferential use in promoters (Fig. 3). Thus, MBD recruitment to target sequences could be achieved by specific cell type nuclear factors that interact with MBD-containing repressor complexes.

Our two combined analyses, promoter wise and cell type wise, suggest that MBD distribution in hypermethylated promoter CpG islands of tumor suppressor genes in cancer cells exhibits a characteristic profile. These findings should stimulate lines of research directed to identify putative sequence-dependent and transcription factor-mediated mechanisms for MBD recruitment to these regulatory regions and to obtain a more comprehensive understanding of the role of MBDS in human tumorigenesis.

**References**

A Profile of Methyl-CpG Binding Domain Protein Occupancy of Hypermethylated Promoter CpG Islands of Tumor Suppressor Genes in Human Cancer

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