Methyl-CpG Binding Domain Proteins and Their Involvement in the Regulation of the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 Gene Promoters

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Abstract
Promoter hypermethylation is responsible for the restricted expression of the tumor-associated MAGE antigens. In order to elucidate the mechanism underlying methylation-dependent repression, we examined the involvement of methyl-CpG binding proteins, MBD1, MBD2a, and MeCP2, in silencing of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 genes. Electroforetic mobility shift assays displayed binding of MBD1 to the methylated and unmethylated MAGE-A promoters. Using chromatin immunoprecipitation assays, in vivo binding of MBD1 and MeCP2 to the promoters could be observed in MCF-7 and T47D cells. Transient transfection assays of MCF-7 cells were done with the transcriptional repression domains (TRD) of MBD1, MBD2a, and MeCP2, and MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters. Whereas the TRD of MBD1 and MeCP2 repressed the MAGE-A promoters, the TRD of MBD2 had no inhibiting effect on the promoter activity. Furthermore, cotransfections of Mbd1-deficient mouse fibroblasts and MCF-7 cells with MBD2a, MeCP2, and the MBD1 splice variants, 1v1 and 1v3, showed that strong methylation-dependent repression of the MAGE-A promoters could not be further down-regulated by these proteins. However, the two MBD1 splice variants, 1v1 and 1v3, were able to repress the basal activity of unmethylated MAGE-A promoters. Additional cotransfection experiments with both isoforms of MBD1 and the transcription factor Ets-1 showed that Ets-1 could not abrogate the MBD1-mediated suppression. In contrast with the repressive effect mediated by MBD1, MBD2a was found to up-regulate the basal activity of the promoters.

In conclusion, these data show, for the first time, the involvement of methyl-CpG binding domain proteins in the regulation of the MAGE-A genes. (Mol Cancer Res 2007;5(7):749–59)

Introduction
The MAGE-A gene family is located on chromosome Xq28 (1) and is comprised of 12 members (MAGE-A1–12). They encode tumor-associated antigens which are recognized by CTLs in conjunction with MHC class I molecules of various haplotypes on the tumor cell surface (2). Their expression is restricted to different histologic tumors types including melanoma, lung, mamma, bladder, and stomach carcinoma (3-8). In normal adult tissues, they are silent, except for testicular germ cells (spermatogonia and primary spermatocytes) and placenta (9). Hypermethylation of CpG-rich MAGE-A promoters causes gene silencing and prevents Ets transcription factors from accessing their binding sites (1, 10, 11), which are responsible for the high transcriptional activation of the MAGE-A1 gene (12).

Alterations in DNA methylation occur during the pathogenesis of human tumors, and a global DNA hypomethylation of the genome, including the tumor-associated MAGE-A genes, has been observed in various carcinomas. Recent clinical studies showed that immunotherapy against MAGE-A antigens caused a significant tumor regression in patients with various tumor entities, pointing out that vaccination with a MAGE-A peptide could be a promising target for the treatment of patients with tumors (13-16). Nevertheless, heterogeneous intratumor expression of the MAGE-A genes may hamper the effectiveness of immunotherapy and urgently requires a detailed examination of the regulation of these genes in tumors. The mechanism leading to partial DNA hypomethylation of the genes during carcinogenesis remains poorly understood. In our previous article, we addressed this issue and found that besides DNA methylation, histone deacetylation, which leads to a compact and transcriptional inactive chromatin structure (17), is also involved in the partial repression of MAGE-A genes in tumor cells and may impede their activation (18). Whether methyl-CpG binding proteins, which recruit histone deacetylases to methylated DNA, contribute to the silencing of MAGE-A genes has not yet been clarified.

To date, five methyl-CpG binding proteins have been identified: MBD1, MBD2, MBD3, MBD4, and MeCP2. All of these proteins are involved in the transcriptional repression
of methylated DNA (19, 20). The methyl-CpG binding domain protein 2 (MeCP2) can interact with both the corepressor Sin3 and histone deacetylases (21-23). It can also repress the transcription of distinct promoters independently of histone deacetylases, probably through direct interaction with basal transcription factors (24). MeCP2-deficient mice show neuronal defects, which likely lead to their short survival time of 2 to 3 months (25). The other members of the family (MBD1, MBD2, and MBD3) also associate with histone deacetylases. MBD1 is alternatively spliced to produce five protein isoforms (PCM1, MBD1v1, MBD1v2, MBD1v3, and MBD1v4) which differ in the number of cysteine-rich (CXXC) domains and the carboxyl-terminal sequence. All five MBD1 isoforms repress transcription from methylated promoters and the variants with three CXXC domains additionally repress unmethylated promoters (26, 27). Two isoforms of MBD2 are known: MBD2a and MBD2b. The shorter form, MBD2b, starting at the second methionine therefore lacks the NH2-terminal sequence of MBD2a (28). Recent reports described MBD2a as both an activator and a repressor of transcription (29-31). Mbd1- and Mbd2-deficient mice are viable and fertile. However, the activator and a repressor of transcription (29-31). Mbd1- and Mbd2-deficient mice are viable and fertile. However, the methylation status of the MAGE-A1 promoter when nuclear extract from MCF-7 cells was used (Fig. 1B, lanes 2-4). Binding was competed by the addition of an excess of cognate, unlabeled oligonucleotides which were either unmethylated or methylated indicating that the complexes were specific (Fig. 1B and C, lanes 5-10). Furthermore, the complexes competed by SssI-methylated oligonucleotides had a weaker intensity (Fig. 1B and C, lanes 8-10) than those competed by unmethylated oligonucleotides (Fig. 1B and C, lanes 5-7), suggesting that the binding affinity of nuclear extracts was higher to methylated than unmethylated DNA.

To characterize the nature of the complexes, we did supershift experiments using antibodies that specifically react with different methyl-CpG binding domain proteins. When added to the EMSA reaction, an antibody specific for MBD1 produced a supershift (SS, Fig. 2), whereas antibodies specific for MBD2a and MeCP2 had no effect on the binding reaction (data not shown). Our findings show that MBD1 had a binding affinity to both methylated and unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters (Fig. 2). Figure 2A shows such a supershift caused by MBD1 using increasing amounts of nuclear extract from MCF-7 (lanes 4 and 5), MDA-MB-231 (lanes 8 and 9), and T47D (lanes 12 and 13) breast cancer cells and the unmethylated MAGE-A1 probe. As shown in Fig. 2B and C, an antibody specific for MBD1 generated supershifted complexes containing nuclear extracts derived from MCF-7 (B) or T47D cells (C) and methylated or unmethylated MAGE-A2, MAGE-A3, and MAGE-A12 probes (lanes 5-7).

Results

In vitro Binding of MBD1 to the 5′-Regions of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 Genes

In our previous studies, we reported the association of DNA methylation and histone deacetylation in silencing of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 genes (18). Because methyl-CpG binding proteins can recruit histone deacetylases to methylated DNA, we investigated whether these proteins are able to bind to hypermethylated MAGE-A promoters. For these analyses, we applied two techniques: the in vitro electrophoretic mobility shift assay (EMSA) and in vivo chromatin immunoprecipitation (ChIP) assay.

EMSA was done using the regions extending from −77 to −36 of MAGE-A1, −158 to −83 of MAGE-A2, −160 to −102 of MAGE-A3, and −109 to −63 of MAGE-A12 genes (Fig. 1A). We chose these sequences because they contained Ets binding motifs which have been shown to be important for the MAGE-A1 promoter activity (10). There were four, six, two, and five CpG dinucleotides in the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoter fragments, respectively (Fig. 1A). In vitro DNA methylation of the fragments was carried out by DNA methylases SssI and HpaII, which methylate each cytosine residue of the CpG dinucleotides and the second cytosine of the sequence CCGG, respectively. For EMSA, we used nuclear extracts derived from three breast cancer cells (MCF-7, T47D, and MDA-MB-231) as well as the epithelioid cervical carcinoma cell line HeLa. Nuclear extracts from HeLa cells were used because methyl-CpG binding proteins were recently found to be components of HeLa cells and served as control for the binding analyses (30, 34). When the unmethylated, HpaII- and SssI-methylated probes were incubated with various nuclear extracts, prominent complexes were formed as detected by EMSA. Figure 1B and C show representative examples of such binding reactions between nuclear extracts derived from MCF-7 or T47D breast cancer cells and the unmethylated or methylated MAGE-A1 (Fig. 1B, lanes 2-4) and MAGE-A3 sequences (Fig. 1C, lanes 2-4). A striking increase in the formation of complexes paralleled with the methylation status of the MAGE-A1 promoter when nuclear extract from MCF-7 cells was used (Fig. 1B, lanes 2-4). Binding was competed by the addition of an excess of cognate, unlabeled oligonucleotides which were either unmethylated or methylated indicating that the complexes were specific (Fig. 1B and C, lanes 5-10). Furthermore, the complexes competed by SssI-methylated oligonucleotides had a weaker intensity (Fig. 1B and C, lanes 8-10) than those competed by unmethylated oligonucleotides (Fig. 1B and C, lanes 5-7), suggesting that the binding affinity of nuclear extracts was higher to methylated than unmethylated DNA.

As EMSA revealed in vitro binding, specifically of MBD1 to methylated and unmethylated MAGE-A promoters, we did ChIP assays to monitor in vivo binding of methyl-CpG binding proteins to the MAGE-A promoters in MCF-7 and T47D cells. Following cross-linking of nuclear proteins to DNA with formaldehyde and immunoprecipitation of the protein-DNA complexes with the antibodies specific for MBD1, MBD2, or MeCP2, the immunoprecipitated DNA fragments were amplified by PCR using gene-specific primers and visualized by gel
Our recent studies on various cell lines indicated that promoter hypermethylation and histone deacetylation are associated with silencing of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters than MBD1; possibly caused by the higher precipitation efficiency of the MeCP2-specific antibody (Fig. 3A).

To verify the data of the standard PCR, we additionally carried out a quantitative real-time PCR with primers specific for the MAGE-A1 sequence and the RPLP0 (ribosomal protein, large protein) housekeeping gene. The primer pairs were chosen due to the stringent conditions of the real-time PCR. A low amount of immunoprecipitated RPLP0 DNA could be observed in the bar chart of Fig. 3B based on the characteristics of a housekeeping gene, which is constitutively expressed, whereas its promoter region is unmethylated. In MCF-7 cells which do not express MAGE-A1 (18), the yield of MAGE-A1 DNA immunoprecipitated with antibodies against MBD1 and MeCP2 was 2- and 2.5-fold higher, respectively, than the yield of DNA immunoprecipitated with the antibody against MB2 or IgG, which served as a negative control (Fig. 3B). When DNA originated from T47D cells, there was no difference in the yield of MAGE-A1 DNA precipitated with antibodies against MBD1, MBD2, and MeCP2, in comparison to the DNA precipitated with IgG (Fig. 3B), indicating that these cells expressed MAGE-A1 (18). Taken together, these findings show that MBD1, as well as MeCP2, interact in vivo with all four MAGE-A promoter sequences in MCF-7 cells.

Transcriptional Repression of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 Genes by the Transcriptional Repression Domains of MBD1 and MeCP2

To functionally test the effect of the methyl-CpG binding proteins on MAGE-A promoters, we constructed a series of reporter plasmids in which we inserted sequences derived from the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters immediately downstream of the Gal4 DNA-binding sites. The expression plasmids contained the transcriptional repression domains (TRD) of MBD1, MBD2, and MeCP2 linked to the heterologous Gal4 DNA-binding domain (28). Both reporter and expression plasmids were transiently cotransfected with a reference plasmid into MCF-7 cells; cell lysates were then assayed for luciferase activity 48 h posttransfection. Immunoblot analysis using antibodies specific for MBD1, MBD2, and MeCP2 documented efficient expression of the fusion proteins in these cells (data not shown). Targeting of the TRD of MBD1 to Gal4 DNA-binding sites upstream of the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters produced a 7-, 25-, 25-, and 8-fold decrease in promoter-driven luciferase activity, respectively, when compared with the basal activity of Gal4-MAGE-A constructs (Fig. 4). Although the Gal4-linked TRD of MeCP2 had a weaker effect, it was able to repress the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 transcription by 2-, 4-, 5- and 2-fold, respectively (Fig. 4). These data show that the TRDs of MBD1, and to a lesser extent, MeCP2, but not that of MBD2, are able to inhibit each of the tested MAGE-A promoter activities.

Transcriptional Repression of Unmethylated MAGE-A Genes by the Splice Variants of MBD1, and Activation by MBD2a

Our recent studies on various cell lines indicated that promoter hypermethylation and histone deacetylation are associated with silencing of MAGE-A1, MAGE-A2, MAGE-A3,
and MAGE-A12 genes (18). Furthermore, our present findings suggest that MBD1 and MeCP2 may be able to bind to and repress MAGE-A promoters (Figs. 1-4). To more precisely define the role of the methyl-CpG binding proteins in MAGE-A gene repression, we transiently cotransfected full-length MBD1, MBD2a, and MeCP2 together with methylated and unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 reporter constructs into MCF-7 cells, and assessed their influence on transcription in response to methylation by SssI and HpaII. We expanded our transfection assay with embryonic fibroblast cells from a Mbd1-knockout mouse3 to exclude interference from endogenous MBD1.

The Mbd1-knockout mouse was analyzed by PCR using genomic DNA from tail biopsies and a primer set gene-specific for the wild-type and knockout allele, as illustrated in the schematic view of Fig. 5A. The primers binding at −695 and −266 bp relative to the start site of MBD1 (ATG) produced a PCR product of 430 bp in size when genomic DNA from wild-type mice were amplified (Fig. 5B, wt lane). No PCR product of 430 bp was generated when DNA originated from homozygous Mbd1-knockout mice (Fig. 5B, Mbd1−/− lane). The primer pair at −695 bp and +1383 bp amplified a PCR product of 2078 bp in size when DNA was derived from wild-type mice (data not shown), and a 520-bp-long product when deleted DNA from Mbd1-knockout mice was used (Fig. 5B, Mbd1−/− lane). Consequently, amplification of DNA from heterozygous mice formed three PCR products at 430, 520, and 2078 bp (Fig. 5B, Mbd1+/− lane).

The genetic characterization of embryonic mouse fibroblasts from the Mbd1-knockout mouse was carried out by amplification of their genomic DNA using the same primer set. Whereas amplification with the primers specific for the wild-type allele did not lead to a PCR product of 430 bp in size (Fig. 5C, wt lane), amplification with the primers specific for the Mbd1 sequence with the deleted exon resulted in a 520-bp-long PCR product (Fig. 5C, Mbd1−/− lane). For cotransfection of MCF-7 cells and Mbd1-deficient embryonic fibroblast cells, we used two isoforms and a mutant form of MBD1. Whereas MBD1v1

![Figure 2. EMSA supershift experiments with an antibody against MBD1 (Ab).](image-url)

A. EMSA was done with unmethylated MAGE-A1 promoter fragments and different amounts of nuclear extracts (NE) derived from MCF-7 (lanes 2-5), MDA-MB-231 (lanes 6-9), and T47D (lanes 10-13) cell lines. B and C. EMSA was done with unmethylated (lanes 2 and 5; U), HpaII- (lanes 3 and 6; H), and SssI-methylated (lanes 4 and 7; S) fragments of MAGE-A2, MAGE-A3, and MAGE-A12 promoters and nuclear extracts derived from MCF-7 (B) and T47D (C) cell lines as indicated above the polyacrylamide gels. SS, supershifted bands of MAGE-A1/MBD1, MAGE-A2/MBD1, MAGE-A3/MBD1, and MAGE-A12/MBD1 complexes (A, lanes 4, 5, 8, 9, 12, and 13; B and C, lanes 5-7). The positions of the MAGE-A/NE complexes (C) and free DNA are indicated.
contains two CXXC domains, MBD1v3 harbors three CXXC domains. The mutant form of MBD1 is functionally inactivated by a 126-bp deletion in the methyl-CpG binding domain and a mutation in the TRD (amino acids 537-556), and served as a negative control. Evaluation of promoter-driven luciferase activities showed that DNA methylation of the reporter plasmids by SssI caused a nearly complete loss of MAGE-A promoter activity in mouse Mbd1<sup>−/−</sup> fibroblasts (Fig. 5D). Similar results were obtained when transfection assays were done with SssI- and HpaII-methylated constructs in MCF-7 cells, as described in our recent article (18). Due to the strong methylation-dependent repression, cotransfected methyl-CpG binding proteins had no further inhibitory effect on methylated MAGE-A constructs in Mbd1-deficient mouse cells (Fig. 5D) and MCF-7 cells (data not shown). In contrast, the transcriptional activity of the unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters in Mbd1-deficient mouse cells was efficiently reduced 3.5-, 2.5-, 3-, and 8.5-fold by isoform MBD1v1 and 1.5-, 3-, 2-, and 1.5-fold by isoform MBD1v3, respectively (Fig. 5D). In MCF-7 cells, MBD1v1 repressed the basal activity of unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters by 8-, 6-, 3-, and 17-fold, respectively, whereas MBD1v3 did not affect the promoters (Fig. 6). MBD1v1 with its three CXXC domains usually affected the repression more strongly than MBD1v3 with its two CXXC domains. The mutant version of MBD1 failed to repress the reporter plasmids indicating the specificity of repression by MBD1v1 and MBD1v3 (Figs. 5D and 6).

**FIGURE 3.** MBD1 and MeCP2 bind to MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters in vivo. **A.** PCR amplifications of the immunoprecipitated DNA derived from MCF-7 cells were carried out with primer sets specific for MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters. The intensity of the PCR products derived from the MBD1-, MBD2-, and MeCP2–immunoprecipitated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 DNA are shown relative to the intensity of the DNA immunoprecipitated with the antibody IgG. The amplification products with and without an asterisk belong to two independent experiments. Both amplifications should therefore be considered independently. Amplification of sonicated genomic DNA (gDNA) with the gene-specific primers prior immunoprecipitation served as positive control. Lane M, contains marker DNA. **B.** Quantitative real-time PCR was done on a realplex<sup>4</sup> system with primer sets specific for the MAGE-A1 and RPLP0 promoters and immunoprecipitated DNA derived from MCF-7 and T47D cells. Columns, amounts of IgG-, MBD1-, MBD2-, and MeCP2–immunoprecipitated DNA.
activity of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 transcription, we transiently transfected MCF-7 cells and embryonic mouse fibroblasts with an expression plasmid encoding Ets-1. Ets-1 could stimulate the promoter activity of MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 in Mbd1-deficient fibroblasts and MCF-7 cells 2-, 4-, 2-, and 2-fold (Fig. 5D) and 2-, 4-, 6-, and 3-fold (Fig. 7), respectively. We conclude that Ets is not only a transactivator for MAGE-A1 but for the other tested promoters as well.

By additional cotransfection experiments, we examined the influence of Ets-1 on MBD1-mediated repression and MBD2a-mediated activation. As shown in Figs. 5D and 7, Ets-1 could neither alleviate the suppression (Fig. 5D) nor affect the activation (Fig. 7) of the promoters by MBD1 and MBD2a, respectively.

Discussion

The current study shows for the first time the different regulations for unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoters by methyl-CpG binding proteins. Thus far, these epigenetic factors have been found to be mainly associated with methylated promoters of tumor suppressor genes (32-36).

Our transfection assays of MCF-7 cells and embryonic mouse Mbd1<sup>−/−</sup> fibroblasts show that promoter hypermethylation seems to be sufficient for silencing of the MAGE-A genes. However, considering that mice deficient of the different methyl-CpG binding proteins and the resulting phenotypes (25, 32, 33), it could be possible that other methyl-CpG binding proteins, like MeCP2, could compensate for the loss of MBD1 in the repression of methylated MAGE-A promoters. Moreover, our binding analyses of EMSA show that MBD1 bound to methylated as well as to unmethylated MAGE-A promoters, and sustains the transfection data that binding of MBD1 to unmethylated DNA may lead to repression of the promoters. The ability of MBD1 to repress unmethylated promoters in vitro is supported by a recently published study showing methylation-independent repression (35). Repression of unmethylated genes depends on the third CXXC domain, and repression of methylated genes requires the methyl-CpG binding domain (26, 36). The isoform MBD1v1 contains the additional third CXXC domain and could therefore repress promoters regardless of their methylation status. However, MBD1v3 lacks the third CXXC domain. Although the repression by MBD1v3 was weaker than the suppression by MBD1v1 and only observed in Mbd1-deficient mouse fibroblasts, our findings indicate that the two other CXXC domains may also contribute to the repression of unmethylated promoters, however, with a weaker affinity. The repressive effect on the examined MAGE-A promoters by MBD1v3 could also be ascribed to the fact that endogenous DNA methylases 3a and 3b in the transfected cells might methylate de novo the introduced reporter plasmids containing each of the MAGE-A promoters (37). Furthermore, the specific repression of MAGE-A promoters by MBD1v1 and MBD1v3 was confirmed by the use of mutated MBDB1 in our overexpression studies. MBD1mut, which lacks the methyl-CpG binding domain and harbors a nonfunctional TRD, congersously showed no effect on MAGE-A gene expression.

The MAGE-A promoters contain putative Ets motifs and the transcription factor Ets has been shown to be responsible for the high transcriptional activation of MAGE-A1 (10). Our results show that transfection of expression plasmid encoding Ets-1...
resulted in the activation of all tested MAGE-A promoters. It is noteworthy that cotransfection of MBD1v1 or MBD1v3 together with Ets-1 did not lead to an abrogation of MBD1-mediated repression. Concerning the potential role of MBD1 as a repressor of unmethylated MAGE-A promoters and the mechanisms underlying the transcriptional repression, it is of considerable interest to investigate the relation between MBD1 and Ets-1. It is unclear whether, in the cotransfection assay using both MBD1 and Ets-1, the MBD1-mediated repression depends on basal or Ets-1–mediated activity and whether MBD1 is able to prevent the binding of Ets-1 to its motif.

Although EMSA and ChIP assays show that MBD2a lacked binding activity with any of the examined MAGE-A promoters, transfected MBD2a could stimulate the luciferase activity of unmethylated reporter plasmids containing MAGE-A promoter fragments. The stimulatory effect of MBD2a on unmethylated promoters was recently reported for cyclic AMP–responsive genes (29). The absence of binding activity of MBD2a to these

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**FIGURE 5.** The splice variants MBD1v1 and MBD1v3 repress basal transcription of unmethylated MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 genes in Mbd1-knockout embryonic mouse fibroblasts. **A.** Schematic view of the MBD1 gene with the start site (ATG) and the deleted region from −365 to +1194 bp. Primers used for the characterization of the wild-type and Mbd1-knockout mice (arrows) lead to PCR products of 430 and 520 bp, respectively. **B.** Characterization of the Mbd1-knockout mouse was done by PCR using genomic DNA from tail biopsies of homozygous (Mbd1+/−), heterozygous (Mbd1+/+), and wild-type mice and gene-specific primers in **A.** **C.** Genetic characterization of embryonic mouse fibroblasts from the Mbd1-deficient mouse was done by PCR with genomic DNA from these cells and the same primer set in **A.** **D.** Embryonic fibroblasts were transfected with unmethylated (unmeth.) and methylated (methyl.) reporter plasmids containing fragments of MAGE-A1, MAGE-A2, MAGE-A3, or MAGE-A12 promoters and expression plasmids encoding MBD1mut, MBD1v1, MBD1v3, or the transcription factor Ets-1. The signals derived from the reference plasmid pCMV LacZ were used to normalize the variability in transfection efficiency. The activities are shown relative to the activity of the reporter construct containing the MAGE-A1 sequence which was arbitrarily set to 100%.

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genes and their activation by MBD2a were explained by its interaction with the RNA helicase A and by a hypothetical model illustrating MBD2a as a factor in the transcriptional coactivator complex which is associated with the RNA polymerase II (29). Whether MBD2a forms a link with one of the components of the basal transcription machinery or even interacts with Ets-1 will require further investigation. Moreover, we cannot completely exclude that MBD2a may promote transcription mediated by other transcription factors, like Sp1 which is also involved in MAGE-A1 promoter activation (10).

Our ChIP assays show that both MBD1 and MeCP2 were able to bind to each of the four examined MAGE-A promoters in MCF-7 cells. As we previously reported, MAGE-A promoters are heavily methylated in this cell line (18). The cells have been shown to possess a high maintenance of DNA methyltransferase activity (38). In contrast with our ChIP results, the in vitro data obtained by EMSA show the binding activity of MBD1 to the MAGE-A promoter fragments, but no binding of MeCP2. The reason for this discrepancy seems to be the use of short DNA fragments extending not more than 80 bp 5′ from the start site of the promoters in the EMSA assays. For in vitro studies, longer DNA sequences were amplified in the PCR reaction following the ChIP assays. These findings suggest that MBD1 does not bind to the same site of the promoter as MeCP2 does. Apparently, MBD1 and MeCP2 have different binding preferences to the MAGE-A promoters.

In conclusion, our results refer to substantial aspects of the MAGE-A gene repression and reveal why promoter demethylation must not result in the activation of the MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 genes. It is noteworthy that we could show for the first time that binding of MBD1 to the unmethylated MAGE-A promoters lead to gene repression which could not be abrogated by the transcription factor Ets-1. Studies are under way to investigate the interplay between MBD1 and Ets-1 at unmethylated MAGE-A promoters.

Materials and Methods

Cell Cultures

The human cell lines HeLa derived from an epithelioid cervical carcinoma and the three breast cancer cell lines, MCF-7, MDA-MB-231, and T47D, as well as the primary cell
culture of embryonic fibroblast cells from a Mbd1-deficient mouse were maintained in DMEM (Invitrogen), supplemented with 10% FCS, and 2 mmol/L of L-glutamine (Invitrogen).

Preparation of Primary Mouse Embryonic Fibroblasts from Mbd1-Knockout Mice

For the construction of the Mbd1-deficient mouse, the second exon of Mbd1 containing the first ATG of the translation start site was deleted. A second ATG is located in the third exon in another reading frame, and is directly followed by a stop codon. By introducing the deletion of the second exon, no functional Mbd1 protein can be translated. The generated Mbd1-knockout mice are, however, viable and fertile. The embryonic fibroblasts were isolated from these mice at embryonic day 13. Embryos were dissected, and heart and organs were removed. The embryonic tissues were washed with PBS and mechanically digested following incubation for 2 h on ice with trypsin/EDTA (Invitrogen). The genotypes of the Mbd1-knockout fibroblasts and primary embryonic fibroblasts were verified by PCR using the following forward primer and two reverse primers specific for MBD1: 5'-CCT CGA GAC GGG AGA AAT AGT TAG A-3' (−695 to −671), 5'-CCC GGT GTT CTA GCT GTA TTT ATG G-3' (−290 to −266), and 5'-GAC TAA GAG CCA ATG GCT TTA GAG G-3' (+1359 to +1383). The primer position was relative to the first ATG in exon 2.

Preparation of Nuclear Extracts and EMSA

Nuclear extracts were isolated from three breast cancer cell lines (MCF-7, MDA-MB-231, and T47D) and HeLa cells derived from an epithelioid cervical carcinoma with 300 μL of a buffer containing 10 mmol/L of HEPES (pH 7.9), 10 mmol/L of KCl, 0.1 mmol/L of EDTA, 0.1 mmol/L of EGTA, 1 mmol/L of DTT, and 0.5 mmol/L of phenylmethylsulfonyl fluoride. The suspension was incubated for 15 min on ice and 20 μL of 10% Nonidet P40 was added and subsequently centrifuged for 60 s at 10,000 × g. The nuclear pellet obtained was resuspended in 100 μL of a buffer containing 20 mmol/L of HEPES (pH 7.9), 25% glycerol, 0.4 mmol/L of NaCl, 1 mmol/L of EDTA, 1 mmol/L of EGTA, 1 mmol/L of DTT, and 0.5 mmol/L of phenylmethylsulfonyl fluoride, and incubated for 20 min at 4°C. After centrifugation for 5 min, 10,000 × g at 4°C, the supernatant, corresponding to the nuclear extract, was collected.

For EMSA, we incubated 10 fmol of 32P-end-labeled oligonucleotides with 2 or 4 μg of nuclear extract, 1 μg poly(dI/ dC), and 0.4 μg salmon sperm DNA in 15 μL of a buffer containing 4% Ficoll, 1 mmol/L of EDTA, 1 mmol/L of DTT, 20 mmol/L of HEPES (pH 7.9), and 50 mmol/L of KCl. After 15 min of incubation at room temperature, the binding reaction was separated on a native 4% polyacrylamide gel (29:1 cross-linked) at 10 V/cm for 2 to 3 h at room temperature. Competition experiments were done by mixing 1,500 fmol of unlabeled competitor DNA to the binding reaction before adding the nuclear extract. For supershift assay experiments, 3 μL of the polyclonal antibodies recognizing a peptide of MBD1 (436-455 amino acids; Abcam), MBD2/3 (Upstate; 152-262 amino acids), and McCP2 (Upstate; 465478 amino acids) were added to the nuclear extract and incubated at room temperature for 10 min. The following oligonucleotides were synthesized for EMSA: MAGE-1 (−36 to −77 relative to the start site), 5'-TCC CGC CAG GAA ACA TTC GGG TGC CCG GAT GTG ACG CCA CTG-3'; MAGE-A2 (−83 to −157), 5'-ATC CCC ATG CCC GGA CAA TCC TGC ACC CTT GCT GTG AAC CCA GGG AAG TCA CGG GGC CCG ATG TGA CGC CAC-3'; MAGE-A3 (−102 to −160), 5'-ACC CCC ATC CGA TCC CTA AGG CAG AAT CCA GTC CCA CCC CTG CCC GGA ACC AAC CCA GG-3'; and MAGE-A12 (−47 to −93), 5'-AAG TCA CGG GCC CCG ATG TGA CGC TGA CTT GGG CTT AGG TC-3'. The putative Ets binding sites are indicated in boldface. All oligonucleotides were purified using a nucleotide removal kit (Qiagen).

ChIP Assays

MCF-7 and T47D cells at 80% cell confluency were fixed with 20 mL of 1% formaldehyde in minimal medium for 10 min at room temperature. The DNA/protein cross-linking reaction was stopped by adding a glycine stop-fix solution. The cells were washed with ice-cold PBS, scraped and pelleted by centrifugation for 10 min, 720 × g at 4°C. Cells were lysed with a hypotonic lysis buffer, and the nuclei were pelleted by centrifugation for 10 min, 2,400 × g at 4°C. The nuclei pellet was sheared in 1 mL of shearing buffer by sonication at 25% power for 4 min on ice (Sonicator UP50H; Dr. Hielscher GmbH) to chromatin fragment lengths of 200 to 1,000 bp. The stop-fix solutions, the hypotonic lysis buffer, and the shearing buffer were obtained from the ChIP-IT kit (Active Motif). The extract was precleared with protein G beads and an aliquot of the precleared supernatant was used as a positive control (gDNA). Aliquots (170 μL) of the supernatant were immunoprecipitated overnight using 4 μg of the antibodies specific for IgG (Active Motif), MBD1 (Abcam), MBD2a (Abcam), or McCP2 (Abcam) at 4°C. The DNA/protein/antibody complexes were incubated with the blocked protein G beads for 2 h at 4°C. After washing the beads, the immunoprecipitated DNA was eluted from the beads with 100 μL elution buffer containing 1% SDS and 50 mmol/L of NaHCO3 for 15 min and protein-DNA crosslinks were reversed with 200 mmol/L of NaCl by incubation at 65°C for 4 h. Digestion of the proteins was done with 0.1 mmol/L of EDTA, 20 mmol/L of Tris-HCl (pH 6.8) and 2 μL of Proteinase K solution (Active Motif) for 2 h at 42°C. The DNA purified by mini-columns (Active Motif) was amplified by standard PCR or quantitative real-time PCR.

For standard PCR, the following primers were used: MAGE-A1, 5'-TGT CCC GCC GCG AAA CAT-3' and 5'-GGG GCT CTC TAT TTG GAG-3' (PCR product of 198 bp); MAGE-A2, 5'-AAA CAG CCA GGA GTG ACG AAG A-3' and 5'-TTG GGG GAT GGG ATT GGT GAG GGT-3' (881 bp); MAGE-A3, 5'-TCA CCC AGA CCA CAC TT-3' and 5'-GCC GTC GCT GTG TCA G-3' (430 bp); MAGE-A12, 5'-GGT AAA CTT AGG CAA TAA TGT CAC CC-3' and 5'-CAG CAG CAG CCG CGC TG-3' (540 bp). The reaction was done in a final volume of 25 μL containing 5 μL of purified ChIP DNA, PCR buffer (Qiagen), 5 mmol/L of each deoxynucleotide triphosphate (Roche), 10 pmol of each primer set, and 2.5 units of Taq polymerase (Qiagen). Template DNA
was amplified in 35 cycles. After agarose gel electrophoresis, the PCR products were quantified densitometrically using the software NIH Image 6.2f (NIH).

Quantitative real-time PCR was done on a realplex4 system (Mastereceller ep gradient S; Eppendorf AG). PCR reactions contained 8 µL of purified imunoprecipitated DNA, 5 pmol of each primer set, 10 µL of DNA QuantitTeck SYBR Green PCR kit (Qiagen), and water to a final volume of 20 µL. The immunoprecipitated DNA was amplified during 45 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 15 s. The following primers were used: MAGE-A1, 5'-GTT CCC GCC AGG AAA CAT C-3' and 5'-GAA CTC TAC GCC GTC CCT CAG-3'; RPLP0 (housekeeping gene), 5'-TTA GTC GTT TGA GCT CGC CAG-3' and 5'-TCT TGA GCT GCT GCC ACC TG-3'. After amplification, melting curve analysis was used to determine the specificity of the PCR products. For quantification, a serial dilution of genomic DNA was used in each run. Each sample was thermocycled in triplicate and all experiments were repeated at least thrice.

RT-PCR

For cloning of the different MBD1 isoforms and MBD2a, total RNA was prepared from HeLa cells using the RNeasy RNA-Isolation Kit (Qiagen) and were done according to the manufacturer's description. cDNA synthesis was carried out using the SuperScript First-Strand System and priming with the oligonucleotides dT (Invitrogen). PCR amplification of cDNA was done with primers specific for MBD1: 5'-ATG GCT GAG GAC TGG CTG CAC CCG-3' and 5'-TCT AGT ATG CTG TAG AAG CCT CCA GTC TAC TGC-3' and for MBD1mut: 5'-GAC AGG ATG CAG AAA GTT GAG CTG-3' and 5'-TCT AGT CTG TAG AAG CCT CCA GTC TAC TGC-3', and for MBD2a: 5'-ATG CGC GCG CAC CCG GGG-3' and 5'-TTA GCC TTC ATC TTC ACT-3'. The reaction was done in a final volume of 20 µL containing PCR Buffer (Qiagen), 200 µmol/L of each deoxynucleotide triphosphate (Roche Applied Science), 0.5 µmol/L of each primer, and 2.5 units of Pfu Turbo hot start polymerase (Stratagene). Template DNA was amplified in 35 cycles.

Construction of Plasmids

Reporter plasmids were constructed by cloning the MAGE-A1 (−82/+116), MAGE-A2 (−549/+332), MAGE-A3 (−418/+12), and MAGE-A12 (−425/+115) promoter fragments into the BglII and HindIII sites of the pGL2-Luciferase reporter plasmid (Promega). Five Gal4 DNA-binding motifs derived from the plasmid pFR-Luc (Stratagene) were inserted into the MluI and XhoI sites upstream of the promoter fragments of the pGL2-Luciferase reporter plasmids. Expression plasmids were constructed by cloning different isoforms of MBD1 (MBD1v1 and MBD1v3) and mutated MBD1 (MBD1mut) into the EcoRI and XhoI sites of the pcDNA3.1(+) vector. MBD1mut has a 126-bp-long deletion of the methyl-CpG binding domain and a frame-shift mutation at position 1608 to 1668 bp. The expression plasmids containing MBD2a were inserted into the BamHI and XhoI sites of the pcDNA3.1(+) vector. All clones were verified by restriction digestion and DNA sequencing (MWG-Biotech AG). The expression plasmids containing sequences encoding the Gal4 DNA-binding domain and the TRD of MeCP2 (amino acids 196-486, MeCP2-TRD), MBD1 (383-605, MBD1-TRD), or MBD2 (45-262, MBD2-TRD), full length MeCP2, and the transcription factor Ets-1 have been previously described (28, 42).

In vitro Methylation of Plasmid DNA

Twenty micrograms of the reporter plasmid constructs containing MAGE-A1, MAGE-A2, MAGE-A3, and MAGE-A12 promoter fragments were methylated by SssI or HpaII methylase (New England Biolabs) for 4 h. Efficient methylation of plasmid DNA was confirmed by its resistance to digestion with the methylation-sensitive restriction enzyme HpaII (New England Biolabs). A control digestion was done using the isoschizomer MspI (New England Biolabs).

Transfection and Luciferase Reporter Assay

The MCF-7 cell line was transiently transfected with 0.5 µg of reporter plasmids and expression plasmids using the FuGENE 6 Reagent (Roche Applied Science). The mouse embryonic fibroblasts were transfected with 0.4 µg of the same plasmids, however, the FuGENE HD Reagent was used. For efficiency control of transfection assays, 0.2 µg of a CMV-β-galactosidase vector was additionally transfected as an internal control. Following 48 h of incubation, the transfected cells were lysed using the Luciferase Reporter Gene Assay Lysis Buffer (Roche Applied Science). Promoter-driven luciferase activity was measured by a 20/20n Luminometer (Turner Biosystems) and normalized by β-galactosidase activity (Galacto-Light; Applied Biosystems). Each transfection experiment was carried out in triplicate wells and repeated at least thrice.

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