Methyl-CpG-binding Protein, MBD2*

Transcriptional Repression by Interacting with a Methyl-CpG-binding Protein, MBD2*

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MBD2, a methyl-CpG-binding protein, is a component of the MeCP1 histone deacetylase (HDAC) complex and plays a critical role in DNA methylation-mediated transcriptional repression. To understand the molecular basis of the methylation-associated repression, we attempted to identify MBD2-interacting proteins by a yeast two-hybrid system. Using MBD2 as bait, we isolated a novel zinc finger protein, referred to as MIZF. A direct interaction between MBD2 and MIZF was confirmed by in vitro binding assays and immunoprecipitation experiments. Four of seven zinc fingers present in the C-terminal region of MIZF are required for binding with MBD2. The MIZF mRNA is expressed in all human tissues and cell lines examined. The subcellular localization of MIZF is distinct from that of MBD2, although both proteins co-localize in some areas of the nuclei; MIZF localizes diffusely in the nucleoplasmic region, whereas MBD2 preferentially localizes in major satellites. A reporter assay demonstrated that MIZF significantly abrogates transcriptional activities. This repression is attenuated by an HDAC inhibitor, trichostatin A, and is completely dependent on the interaction with MBD2. These results suggest that MIZF is abundantly present in cells and functions as a negative regulator of transcription by binding to MBD2 and recruiting HDAC-containing complexes.

Methylation of CpG dinucleotides is the major epigenetic modification in higher eukaryotic genomes. This reaction is mediated by DNA methyltransferases (1, 2), and the biological importance of the CpG methylation is directly demonstrated by the fact that mice lacking the gene of a DNA methyltransferase exhibit a defect in embryogenesis at midgestation (3). CpG methylation also plays important roles in a wide range of biological steps, including tissue-specific gene expression, X-chromosome inactivation, and genomic imprinting (4–8). MBD2 can also interact with the NuRD complex (20). To understand the precise mechanisms for the recruitment of the two HDAC complexes by MBD2 and for MBD2-dependent transcriptional repression, further identification and characterization of novel proteins associated with MBD2 is required. In the present study, we performed a yeast two-hybrid screening using mouse MBD2 as bait to search for MBD2-interacting molecules and found a novel zinc finger protein. This MBD2-binding zinc finger (MIZF) protein represses transcription by associating with MBD2 and a histone deacetylase complex. The results suggest that MIZF, in concert with MBD2, recruits HDAC complexes, which in turn results in transcriptional repression.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screening and Isolation of the Full-length cDNA—The full-length mouse MBD2b cDNA was cloned into the pGBT9, a GALA DNA-binding domain (GAL4-DBD) vector (CLONTECH, Palo Alto, CA), and transfected into yeast PJ69–4A strain. An expression library consisting of human fetal brain cDNA (CLONTECH) was then introduced into the yeast and screened by growth on plates lacking Ade, His, Leu, and Trp, but containing 2 mM glutathione S-transferase; NuRD, nucleosome remodeling histone deacetylase; GST, glutathione S-transferase; TSA, trichostatin A.

† The abbreviations used are: MeCP, methyl-CpG-binding protein; MBD, methyl-CpG binding domain; HDAC, histone deacetylase; GST, glutathione S-transferase; NuRD, nucleosome remodeling histone deacetylase; GFP, green fluorescent protein; PAGE, polyacrylamide gel electrophoresis; TSA, trichostatin A.
MIZF, a Novel MBD2-interacting Zinc Finger Protein

FIG. 1. Primary structure of MIZF. A, amino acid sequence of human MIZF. The predicted zinc finger domains are shaded, and the acidic region is underlined. The portion of MIZF recovered in a yeast two-hybrid screen starts at amino acid residue 197. Numbers are shown for the amino acid sequence. B, alignment of the amino acid sequences of the zinc finger domains. MIZF possesses seven C2H2 zinc fingers (ZF1–ZF7) conforming to the C2H2 consensus, C_{X_{4-5}}C_{X_{2-3}}(F/C)_{X_{3}}(F/L)x{X_{4-5}}H, in which X represents any amino acid.

3-aminoaziridine as described (25). The colonies were tested for β-galactosidase activity, and positive clones were analyzed for the insertion sequences. To isolate the full-length cDNA, 5′-rapid amplification of cDNA ends combined with nested polymerase chain reaction was performed using a human fetal brain cDNA library (Marathon-Ready cDNA, CLONTECH) as a template.

Plasmid Construction—For the expression of a green fluorescent protein (GFP) fused to MBD2, the MBD2b cDNA was subcloned into the appropriate sites of pEGFP-C1 (CLONTECH) to obtain pGFP-MBD2. Epitope-tagged derivatives of MBD2 and MIZF, containing amino-terminal FLAG and carboxy-terminal Myc tags, respectively, were generated using a pcDNA3 expression vector (Invitrogen, San Diego, CA). For epitope-tagged derivatives of MBD2b were generated by inserting the cDNA fragments into pGEX-4T-1 (Amersham Pharmacia Biotech) (GST-MBD2 and GST-MIZF). Deletion mutants of MIZF, various deletion mutants of MIZF, and MBD2 were synthesized using the indicated pGBKT7 plasmids as templates in a TNT coupled transcription-translation system (Promega). The λ system was rehydrated with a digoxigenin-labeled MIZF cDNA probe (up panel) or a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe (lower panel). Size markers and the corresponding mRNA are indicated on the left and right sides, respectively.

In Vitro Binding Assay—GST fusion proteins were expressed in Escherichia coli BL21 (DE3) and purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech). 5′S-Methionine-labeled full-length MIZF, various deletion mutants of MIZF, and MBD2 were synthesized using the indicated pGBKT7 plasmids as templates in a TNT T7 quick coupled transcription-translation system (Promega). The labeled, in vitro-translated proteins were incubated with various GST fusion proteins immobilized on glutathione-Sepharose beads for 4 h at 4 °C in buffer containing 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, 0.2% Nonidet P-40, 1 mM dithiothreitol, and 1 μg/ml leupeptin. After washing with the same buffer, the bound proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Subcellular Localization—COS-7 cells growing on glass coverslips were transfected with pGFP-MBD2 or pMIZF-Myc using Effectene Transfection Reagent (Qiagen, Hilden, Germany) as described elsewhere (28). The cells were fixed with 3.7% formaldehyde in phosphate-buffered saline and permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 5 min. For observation of Myc-tagged MIZF cells were incubated with anti-Myc antibody (9E10) and stained with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (BIO-SOURCE, Camarillo, CA). The cell preparation was observed using a confocal laser-scanning microscope.

Immunoprecipitation and Western Blot Analysis—Transfected 293
FIG. 3. Association of MIZF with MBD2. A, binding of MIZF to GST-MBD2. [35S]Methionine-labeled MIZF produced by an in vitro translation system was incubated with an equal amount of GST (lane 2) or GST-MBD2 (lane 3), which was immobilized on glutathione-Sepharose beads. Bound and input (lane 1) proteins were separated on SDS-PAGE and visualized by fluorography. B, binding of MBD2 to GST-MIZF. [35S]Methionine-labeled MBD2 was incubated with GST (lane 2) or GST-MIZF (lane 3). Bound and input (lane 1) proteins were separated on SDS-PAGE and visualized by fluorography. C, 293 cells were transfected with pMIZF-Myc in combination with control vector alone or pGFF-MBD2 (indicated by the plus and minus at the top of each lane). Total lysates were immunoprecipitated (IP) with anti-GFP antibody, and the resultant immunoprecipitates and lysates were analyzed by Western blotting (WB) with anti-Myc antibody to detect MIZF (upper two panels). The same lysates were immunoprecipitated with anti-Myc antibody, and the resultant immunoprecipitates and lysates were analyzed by Western blotting with anti-GFP antibody to detect MBD2 (lower two panels). Molecular mass markers and the corresponding proteins are indicated on the left and right sides, respectively.

Identification of MIZF—When a total of 5 × 10^6 clones were tested in a yeast two-hybrid screening system with MBD2 cDNA, ~300 cDNA clones were obtained as first candidates exhibiting Hist (+) and Ade (+) properties. Most of these were, however, eliminated by a second screening for their ability to activate the β-galactosidase gene. One clone showed strong β-galactosidase activity and was chosen for further analysis. To obtain the full-length sequence of the clone, 5'-rapid amplification of cDNA ends combined with polymerase chain reaction was performed using a human fetal cDNA library. Finally, we obtained a cDNA comprising 2285 base pairs including an open reading frame encoding a polypeptide of 517 amino acids with a predicted molecular mass of 59.7 kDa. BLAST search revealed that the cDNA is identical to human cDNA NC1:4917 (accession number BC001945). This cDNA encodes a novel protein with homology to zinc finger proteins. Therefore, we name this protein MIZF (MBD2-interacting zinc finger protein). As shown in Fig. 1, the MIZF protein contains seven zinc finger domains similar to the C2H2 zinc finger motif (30) and a stretch of negatively charged amino acids residues from position 49 to 63.

RESULTS

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Tissue and Subcellular Expression of MIZF—Expression of
MIZF was examined by Northern blot analysis with RNA samples from various human tissues and cell lines. The expression level of MIZF was detectable as a predominant single signal in all tissues and cell lines examined (Fig. 2, A and B). The apparent size of the MIZF transcript is ~2.3 kilobase, consistent with the size of the cDNA clone. Among tissues examined, the highest level of MIZF was detected in the brain, heart, skeletal muscle, and kidney, with moderate levels of the transcript seen in the colon, thymus, spleen, liver, small intestine, placenta, and lung (Fig. 2A). The MIZF transcript was detected in all cell lines examined at relatively constant levels (Fig. 2B).

We examined the subcellular localization of MIZF and compared it with that of MBD2. When COS-7 cells were transiently transfected with pMIZF-Myc, MIZF localized exclusively to the cell nucleus except the nucleolus (Fig. 2C, a). The nuclear localization of MIZF proteins was confirmed by difference interference contrast image (Fig. 2C, b). Consistent with the previous observations on MBD2, GFP-MBD2 displays a diffuse nucleoplasmic staining pattern with prominent nuclear dots that are known to be highly methylated regions of the genome (Fig. 2C, c and d) (15). These results indicate that MIZF is a nuclear protein with subcellular localization distinct from that of MBD2.

Association of MIZF with MBD2—The association of the MIZF protein with MBD2 was confirmed by in vitro binding assays. The MIZF protein was produced by an in vitro translation system and tested for binding to GST-MBD2, and vice versa. As shown in Fig. 3A, MIZF bound to GST-MBD2 but not to GST alone. Similarly, the in vitro translated MBD2 protein bound to GST-MIZF but not to GST alone (Fig. 3B). This association was further confirmed in 293 cells subjected to cotransfection with pMIZF-Myc and pGFP-MBD2. When GFP-MBD2 was immunoprecipitated with anti-GFP antibody, MIZF-Myc (68 kDa) was present in the resultant immunoprecipitates (Fig. 3C). Similarly, GFP-MBD2 was detected in immunoprecipitates with anti-Myc antibody. These results demonstrate that MIZF associates specifically with MBD2 in vitro, as well as inside the cell.

To determine the region of MIZF responsible for binding to MBD2, several deletion mutants were examined (Fig. 4A). As shown in Fig. 4B, C125–517 and C201–517 could bind to MBD2 (lanes 10–15). The removal of an additional 228 amino acids (C429–517) resulted in a complete loss of the binding activity to MBD2 (lanes 16–18), indicating that the N-terminal ~200 amino acids are dispensable for the interaction with MBD2. In fact, a mutant N1–201 failed to bind to MBD2 (lanes 4–6). Deletion of the C terminus (N1–429) did not affect the binding ability, suggesting that the C-terminal 89 amino acids are also dispensable for the interaction with MBD2 (lanes 7–9). These results indicate that the region comprising amino acids 201–429 of the MIZF protein, containing four zinc finger domains (ZF4–ZF7) (Fig. 1B), is required for its association with MBD2.

A series of MBD2 deletion mutants was also examined to map the region responsible for the interaction with MIZF (Fig. 5A). MIZF could bind to the MBD2 mutant C53–262, which lacks the 52 amino acids at the N terminus (Fig. 5B, lane 7), but not to mutants N1–52, C154–262, and C212–262, indicating that the N-terminal 52 amino acids are not responsible for the binding to MIZF (lanes 4, 8, and 9). In addition, MIZF could bind to deletion mutants N1–154 and N1–212, indicating that the C-terminal 108 amino acids are dispensable for the interaction with MIZF (lanes 5 and 6). Thus, the region from amino acid 53 to 154 of MBD2 is necessary for the association with MIZF.

Involvement of MIZF in Transcriptional Repression—The effect of MIZF on transcription was examined using a reporter assay with a DNA polymerase β promoter and GAL4-binding sites (Fig. 6A). When the full-length MIZF cDNA fused with GAL4-DDB was transfected into cells, transcription of the reporter gene was significantly inhibited by MIZF in a dose-dependent manner (Fig. 6B). Similar results were obtained using an MIZF mutant, C201–517, which possesses binding activity to MBD2. These results suggest that MIZF functions as a negative regulator for transcription and that MIZF-mediated transcriptional repression is dependent on the binding to MBD2, and, in turn, the association with an HDAC complex. In contrast, another MIZF mutant, N1–201, which does not bind to MBD2, did not repress the transcription (Fig. 6B). In addition, the results shown in Fig. 6B demonstrate that transcriptional repression by MIZF can be substantially relieved by TSA, an inhibitor of histone deacetylases. To examine whether MIZF interacts with endogenous HDAC1, an immunoprecipitation experiment followed by Western blotting analysis was performed on lysates from 293 cells transiently expressing MIZF-Myc. As expected, HDAC1 was detected in the immunoprecipitates containing MIZF. MIZF-Myc was specifically coimmunoprecipitated with HDAC1 by an anti-HDAC1 antibody but not by normal rabbit serum (Fig. 6C).

Enhancement of the MBD2-mediated Repression by MIZF—To confirm whether MIZF can modulate MBD2-mediated repression, a luciferase reporter assay was performed in the presence or absence of MIZF. MIZF enhanced the MBD2-mediated repression in a dose-dependent manner (Fig. 7A). Basal transcription of GAL4-DDB alone was not affected by MIZF. To investigate the involvement of HDAC in this effect of MIZF, cells expressing FLAG-MBD2 and MIZF-Myc were analyzed by an immunoprecipitation assay. As shown in Fig. 7, B and C, the expression of MIZF produced significant increases in
DISCUSSION

A close relationship between methylation-dependent repression and histone deacetylation has been demonstrated. The transcriptional effects of CpG methylation are mediated by MBD-containing proteins. Among MBD proteins, MeCP2 is known to bind to methylated CpG pairs and functions as a transcriptional repressor by recruiting Sin3A and HDACs (16–18). MBD2, another methyl-CpG binding protein, has also been shown to be a component of the MeCP1-HDAC complex that includes RbAp46/48 and Sin3A (24). In addition, MBD2 interacts with the NuRD complex (21). These findings suggest that MBD2 is likely to function as a molecular link between methyl-CpG and HDACs. To understand the mechanisms underlying the MBD2-dependent transcriptional repression and recruitment of HDACs, we identify MBD2-interacting molecules using a yeast two-hybrid system. When a human fetal brain cDNA library was screened using mouse MBD2 as bait, a novel zinc finger protein, MIZF, was specifically isolated. This MIZF protein binds to MBD2 and represses the promoter-driven transcription activity. Like the MBD2 protein, the MIZF protein is expressed in a wide variety of tissues and cells. These results suggest that MIZF is expressed abundantly in cells and functions as a negative regulator for transcription by binding to MBD2 and to HDAC systems.

We demonstrated that the C-terminal region of MIZF (amino acids 201–517) is required for the association with MBD2 (Fig. 4) and for the TSA-sensitive repression (Fig. 6). The N-terminal region of MIZF (amino acids 1–201), which lacks MBD2-binding activity, does not repress transcription (Figs. 4 and 6). These results suggest that MIZF-derived repression is totally dependent on binding to MBD2 and to HDAC systems. Indeed, MIZF was coimmunoprecipitated with endogenous HDAC1 (Fig. 6). It has become clear that several transcriptional repressors, such as YY1, Bcl-6, and Rb family proteins, are able to associate directly with HDAC1 or HDAC2 (31–33). The minimal structures required for the association with HDAC are thus far identified as the LXXC-like motif in Rb family proteins, a 30-amino acid glycine-rich region in YY1, and the POZ domain in Bcl-6. However, MIZF does not possess regions homologous to these motifs. In addition, we could not detect the interaction with any
MIZF is a unique, C2H2-type zinc finger protein involved in methylation-dependent transcriptional repression. The C2H2 zinc finger structure was initially identified in DNA-binding molecules such as the transcription factors TFIIB and Kruppel, and at present a number of transcription factors are known to utilize C2H2 zinc fingers as DNA-binding domains (35). Most of these proteins contain multiple fingers, which are required for the recognition of a specific range of DNA sequences. Concerning an analogy with these proteins, it is possible that MIZF is a DNA-binding protein, and our preliminary results reveal that MIZF binds to some DNA sequences (data not shown). Thus, it is probable that MIZF functions as a repressor, in terms of its suppressive effect on transcription and DNA binding activity. As shown in Fig. 2C, we demonstrate here that MIZF and MBD2 are expressed quite abundantly and ubiquitously in cells and tissues, suggesting their important functions in cell. Although these two proteins co-localize in some areas in nuclei, their main localizations are quite different; MIZF localizes diffusely in the nucleoplasmic region, whereas MBD2 preferentially localizes in the major satellite, which is known to be a highly methylated region of the genome. Thus, it is possible that MIZF functions as a repressor in the regulation of the transcription of specific genes. Identification of the DNA sequences that interact with MIZF is necessary to understand this issue.

It has become clear that zinc fingers are also involved in protein-protein interactions. Homodimerization of the multifinger protein Ikaros is mediated by its two C-terminal fingers (36). Likewise, metastasis-associated protein 2, a subunit of the NuRD complex, which contains a C4-type zinc finger, also modulates HDAC activity by interacting with MBD3 (20). MIZF contains seven zinc fingers (ZF1–ZF7), and four fingers in the C-terminal region are required for the interaction with MBD2. Our preliminary study demonstrates that MIZF also associates with MBD3 in a manner similar to its binding with MBD2 (data not shown). MBD3 is a component of the NuRD complex (34). Thus, it is possible that MIZF recruits the NuRD complex through its interaction with MBD3. The analysis of MIZF-interacting proteins may provide a clue to understanding the mechanism of transcriptional repression induced by MIZF.

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