SWI/SNF Chromatin-Remodeling Factors Induce Changes in DNA Methylation to Promote Transcriptional Activation

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Abstract

Brahma (Brg1) and brahma-related gene-1 (Brg1) are mammalian homologues of SWI/SNF chromatin-remodeling factor subunits that can regulate both transcriptional activation and repression. Both Brg1 and Brm are mutated or deleted in numerous cancer cell lines, leading to the altered expression of genes that influence cell proliferation and metastasis. Here, we find that the promoters of two such genes, CD44 and E-cadherin, are hypermethylated in cells that have lost Brg1 or Brm. In two carcinoma cell lines that lack functional Brg1 and Brm, CD44 and E-cadherin expression are induced by the demethylating agent 5-aza-2′-deoxycytidine. Transfection with either Brg1 or Brm also induces CD44 and E-cadherin transcription and protein expression in these cells, as well as loss of methylation at sequences in the promoters of both genes. Chromatin immunoprecipitation assays show that Brg1 and Brm associate with these regions of the CD44 and E-cadherin promoters, suggesting that SWI/SNF protein complexes may directly influence the loss of DNA methylation. In vivo, Brm-deficient mice also show methylation and silencing of the CD44 promoter. Collectively, these data implicate loss of SWI/SNF-mediated transcriptional activation as a novel mechanism to increase DNA methylation in cancer cells and provide insight into the mechanisms underlying aberrant gene induction and repression during tumor progression. (Cancer Res 2005; 65(9): 3542-7)

Introduction

Mammalian SWI/SNF chromatin-remodeling complexes, which use energy from ATP hydrolysis to disrupt histone-DNA interactions, contain one of two catalytic ATPase subunits, called Brm (for brahma; also called SNF2h) and Brg1 (for brahma-related gene-1; also called SNF2j). Both Brg1 and Brm have been implicated in transcriptional activation and repression (1). Mutations or deletions of these and related genes lead to altered gene expression in cancer cell lines through largely unknown mechanisms (2). Several SWI/SNF-related factors have been implicated in transcriptional silencing through DNA methylation (3–5). Indeed, DNA methyltransferase 3B (DNMT3B) interacts with the ATP-dependent chromatin-remodeling enzyme SNF2H (6). It was unclear, however, if transcriptional activation conversely involved loss of DNA methylation. We previously found that cell lines and tissues lacking functional Brg1 or Brm do not express the CD44 transmembrane glycoprotein (7, 8), a cell adhesion protein whose loss in some cell types has been implicated in tumor progression (9). When these cells were transfected with Brg1 or Brm, endogenous CD44 transcription was induced (7, 8). Interestingly, CD44 transcription is silenced in a number of cancer cell lines by hypermethylation of CpG islands within the CD44 promoter that normally remain unmethylated at all times (10–14). Here, we tested if transcriptional activation by Brg1 and Brm involves loss of methylation of CpG islands in promoter sequences of both the CD44 gene and another gene whose transcription is often silenced by DNA methylation, E-cadherin (15). We find, for the first time, that Brg1- and Brm-mediated transcriptional activation involves direct interactions with promoter sequences of affected genes combined with the loss of DNA methylation.

Materials and Methods

Plasmids. The pCG-BRM, pBrg4-BRG1, dBRG1, and dBRM constructs were described previously (7, 8). Plasmids were transfected using either calcium phosphate or FuGene (Roche, Nutley, NJ) according to the manufacturer’s instructions. Cells were analyzed 48 hours following transfection.

Cells. The SW13, C33A, HeLa, and Saos-2 cell lines, and the BOS-1 clone of NIH 3T3 cells, carrying a tetracycline-inducible dominant-negative Brg1 construct, were all grown as previously described (7).

Immunoblotting. Cell lysates were prepared and analyzed by immunoblotting as previously described (7). Mouse anti-Brm (1:250, clone 24) and mouse-anti–E-cadherin (1:1,000) were obtained from BD Transduction Laboratories (Lexington, KY); goat-anti-Brm (1:250, N-19), mouse-anti-Brg1 (1:10,000, G-7), rabbit anti-Brg1 (1:1,000, H-88), and goat anti-actin (1:1,000, I-19) were from Santa Cruz Biotechnology (Santa Cruz, CA); the mouse anti-human CD44 (1:50, Hermes-3) was from American Type Culture Collection (Manassas, VA); mouse anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1:4,000) was from Ambion (Austin, TX); horseradish peroxidase–conjugated secondary antibodies were from Bio-Rad (Richmond, CA) and were used at 1:1,000 (for anti-rabbit antibodies) or 1:2,000 (for anti-goat and anti-mouse antibodies).

In vitro methylation and luciferase assays. A 0.5-kb CD44 promoter fragment was cloned from rat genomic DNA then subcloned into the PGL2 luciferase vector (Promega, Madison, WI). Plasmid DNA was methylated by incubating 1 μg of DNA with 2.5 units of SspI methylase (New England Biolabs, Beverly, MA) and 160 μmol/L S-adenosylmethionine for 2 hours at 37°C followed by a 20-minute incubation at 65°C. As a control, DNA was incubated in the presence of buffer alone. DNA was precipitated and suspended in 10 μL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA) and cotransfected into cells with a CMV-β-galactosidase expression

Note: A recently published report by Harkrishnan et al. (Nat Genet 2005;37:254–64) shows that Brm can interact with the methyl-CpG-binding protein MeCP2 and that Brm complexes are recruited to methylated DNA. It is therefore possible that both SWI/SNF interactions with methylated DNA can mediate both gene induction and repression.

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vector. After 48 hours, cells were washed with PBS, harvested, and analyzed for β-galactosidase and luciferase activity using a Luciferase Kit (Promega).

**PCR analysis of methylated DNA.** Genomic DNA from cells and tissues was extracted, precipitated, and suspended in TE (16). For each sample, 250 ng of genomic DNA were incubated with HpaII, MspI, or HhaI (New England Biolabs) in the recommended buffer or with buffer alone in a total volume of 20 μL for 2 hours at 37°C. Samples were incubated for 20 minutes at 65°C. DNA was precipitated, suspended in 10 μl water, and used for PCR in a final volume of 20 μL. Amplifications were carried out using a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany) using the Advantage-GC genomic PCR Kit (Clontech, Palo Alto, CA) or PureTag Ready-to-Go PCR beads (Amersham, Arlington Heights, IL). For DNA from human cell lines, the forward CD44 primer was 5′-GGATGGGCGCGGATGAGAT-3′ and the reverse primer was 5′-TCCGCTGGCGATAGGCTG-3′; for E-cadherin, 5′-GAGCTCTGCGAAGGACCT-3′ and 5′-GAGCTCTGAACT-CCAGCC-3′; for GAPDH, 5′-GCCGTCCTCTCCACAGC-3′ and 5′-GCCGTCCTCTCCACAGC-3′. For amplification of mouse CD44, 5′-ATAA GTCCAGCTTCCTCAGC-3′ and 5′-AGAAGGTGTGGGCAGAAGAAAA-3′; for mouse GAPDH, 5′-GGAATGGATGGGTGGATTGT-3′ and 5′-GAACTCAGCCAAGTGTAAAAGCC-3′; for mouse β-actin, 5′-GAAATGTG-3′ and 5′-AGTTATYGG-3′; for CD44, 5′-ATAA GTCCAGCTTCCTCAGC-3′ and 5′-AGAAGGTGTGGGCAGAAGAAAA-3′; for GAPDH, 5′-GCCGTCCTCTCCACAGC-3′ and 5′-GCCGTCCTCTCCACAGC-3′. DNA was denatured by 4 minutes of incubation at 94°C and amplified using the following conditions: denaturation at 94°C for 40 seconds; annealing at 64.9°C (for human CD44), 66.0°C (for mouse CD44), or 60.3°C (for E-cadherin and GAPDH) for 2 minutes; and extension at 72°C for 2 minutes for 40 cycles. Amplification was followed by 10 minutes of incubation at 72°C. Five microliters of the PCR products were then separated in a 0.8% agarose gel. The PCR products were visualized by ethidium bromide staining.

Alterations in DNA methylation were confirmed by sequencing DNA (from at least 10 distinct clones) following treatment with sodium bisulfite as previously described (17). For the first CD44 PCR reaction, the forward primer was 5′-TTTTGGTTTATGGCTTATAG-3′ and the reverse primer was 5′-AAATACGAGGCAAGCCCTAAT-3′ with an annealing temperature of 64°C. For the second reaction, we used 5′-GAGA AATTTAGGGGAAAGG-3′ and 5′-TCCACCTAAACTAAACTC-3′ with an annealing temperature of 66.5°C. For mouse CD44, the forward primer was 5′-GAATACTGCTTCTCATTGG-3′ and the reverse primer was 5′-GAATACTGCTTCTCATTGG-3′ with an annealing temperature of 64°C. For the second CD44 PCR reaction, the forward primer was 5′-GAGA AATTTAGGGGAAAGG-3′ and the reverse primer was 5′-TCCACCTAAACTAAACTC-3′ with an annealing temperature of 66.5°C. For mouse GAPDH, 5′-GGAATGGATGGGTGGATTGT-3′ and 5′-GAACTCAGCCAAGTGTAAAAGCC-3′; for mouse β-actin, 5′-GAAATGTG-3′ and 5′-AGTTATYGG-3′; for mouse CD44, 5′-ATAA GTCCAGCTTCCTCAGC-3′ and 5′-AGAAGGTGTGGGCAGAAGAAAA-3′; for mouse GAPDH, 5′-GCCGTCCTCTCCACAGC-3′ and 5′-GCCGTCCTCTCCACAGC-3′. The annealing temperatures for these reactions were 56°C and 58°C, respectively. For E-cadherin, the forward primer in the first reaction was 5′-AAATACGAGGCAAGCCCTAAT-3′ with an annealing temperature of 50°C. For the second PCR, 5′-GAGA AATTTAGGGGAAAGG-3′ and 5′-TCCACCTAAACTAAACTC-3′ with a 60.3°C annealing temperature. PCR products were purified and subcloned into pGEM-T easy vector (Promega).

**Reverse transcription-PCR.** RNA was isolated from cells using Trizol (Invitrogen, San Diego, CA) and reverse transcribed using a Thermoscript reverse transcription-PCR (RT-PCR) kit (Invitrogen) according to the manufacturer’s instructions. CD44 and E-cadherin sequences were amplified using the following primers for human CD44, forward primer 5′-AGAAGGTGTGGCGAGGAGG-3′ and reverse primer 5′-CACTGCTTCCTCCTCG-3′; for human E-cadherin, 5′-GCACGAGGCACTC-3′ and 5′-AATGGCCAGGAATTGCAACTC-3′; for GAPDH, 5′-ACCACGATTCGCTACCTC-3′ and 5′-TCCACACCTGTGCAGTA-3′. PCR reactions were run with Platinum Taq polymerase (Invitrogen) at 94°C for 2 minutes, 58°C for 40 seconds, and 72°C for 1 minute, with 28 cycles followed by 5 minutes at 72°C.

**Chromatin immunoprecipitation assays.** Chromatin immunoprecipitation (ChIP) assays were done using the protocol described in a ChIP Assay Kit from Upstate Cell Signaling Solutions (Lake Placid, NY). Immunoprecipitations were done using 5 μg of either the mouse anti-BrG1 (G-7), goat anti-BrM (N-19), or rabbit anti-HDAC1 (H-51) antibodies (Santa Cruz Biotechnology). The primers used to amplify E-cadherin and GAPDH are the same as those described above for the analysis of methylated DNA. To analyze the human CD44 promoter sequences that interacted with BrG1 and BrM, we used either the primers used above or the forward primer 5′-CCTCTCCACCTCCTCCACCTCC-3′ and the reverse primer used above. The PCR conditions were similar to those described above with an annealing temperature of 68°C.

**Results and Discussion.** We first examined the possibility that the CD44 promoter is hypermethylated in cell lines lacking functional BrG1 or BrM and which do not express CD44. The human cervical carcinoma cell line C33A and the human adenocarcinoma cell line SW13, which lack functional BrG1 and/or BrM, do not express CD44 (Fig. 1A; note that C33A cells express very low levels of BrM that can be detected following longer exposure; e.g., see Fig. 2A) compared with BrG1- and BrM-positive cell lines that express CD44, including the osteogenic sarcoma cell line Saos2 (data not shown) and the
mammary carcinoma cell line, MCF-7 (Fig. 1B). However, after ≥2 days in the presence of 50 μmol/L 5-aza-2′-deoxycytidine (5-aza), which causes genomic hypomethylation by interfering with the activities of DNA methyltransferase 1 (DNMT1), CD44 expression was induced in the C33A and SW13 cell lines but was not further induced in cell lines that expressed Brg1 or Brm (Fig. 1A and B). 5-Aza did not affect the expression of Brg1 or Brm (Fig. 1A and B).

To confirm that hypermethylation inhibits CD44 transcription in the cell lines used in these studies, we examined whether methylating a stretch of the CD44 promoter was sufficient to inhibit transcriptional activation. A fragment of the CD44 promoter cloned into a luciferase reporter construct was treated with the SssI CpG methylase and transfected into SW13 (Fig. 1C), Saos2 and C33A (data not shown) cells. High levels of luciferase activity were observed in all three cell lines following transfection with unmethylated constructs, indicating that Brg1 and Brm function are not required for the transcriptional activation of promoter sequences in plasmid DNA (Fig. 1C). Luciferase activity was abolished, however, in cells transfected with the methylated construct (Fig. 1C), confirming that CD44 transcription is silenced by methylation of promoter sequences.

We next tested the possibility that other genes might also be transcriptionally silenced by DNA methylation in cells lacking Brg1 or Brm. E-cadherin transcription is inhibited in numerous cancer cell lines, including C33A cells (18), and in tumor tissues by hypermethylation (15). We therefore tested whether E-cadherin expression was altered in C33A and SW13 cells by treatment with 5-aza, as above. C33A cells expressed no detectable E-cadherin, whereas SW13 cells expressed only low levels of the protein as determined by Western blotting (Fig. 1D). As with CD44, however, E-cadherin expression was induced in both cell lines following treatment with 5-aza (Fig. 1D). In contrast, 5-aza did not influence the expression of E-cadherin in SW13 cells, which express moderate levels of E-cadherin (Fig. 1B).

Consistent with our previous findings (7, 8), CD44 protein expression (Fig. 2A, top) and transcription (Fig. 2A, bottom) was induced in both SW13 and C33A cells following transfection with either Brg1 or Brm. Mutants of either protein that lack the ATPase domain failed to induce CD44 expression, indicating that ATPase activity is required for transcriptional activation (Fig. 2A). Similarly, we found here that Brg1 and Brm both induced E-cadherin expression in these cells in an ATPase-dependent manner (Fig. 2B).

Collectively, these data indicate that the CD44 and E-cadherin genes are both hypermethylated in cells that lack functional Brg1 and Brm and suggest that transcriptional activation by Brm or Brg1 may involve loss of DNA methylation. To test this hypothesis, C33A and SW13 cells were transfected with Brg1, Brm, or vector alone, or, as a control for demethylation, treated with 5-aza as above. Genomic DNA was isolated and incubated with HpaII or MspI, restriction enzymes that are sensitive to cytosine methylation but which have distinct sensitivities for different methylated sequences (19, 20), and amplified by PCR using E-cadherin (Fig. 3A) or CD44 (Fig. 3C) promoter-specific primers. HpaII has weak nicking activity in the unmethylated strand of the hemimethylated sequence m5CCGG/CCGG, whereas MspI is sensitive to m5CCGG sequences (19). In the case of the E-cadherin promoter, DNA from untreated cells could not be digested by either HpaII or MspI (Fig. 3A). Both enzymes, however, at least partially digested E-cadherin promoter DNA following transfection with Brg1 (Fig. 3A). Interestingly, in SW13 cells, which constitutively express low levels of E-cadherin (Fig. 1D), we did not observe a similar change in methylation following transfection with Brm, with only slight increases in HpaII digestion (Fig. 3A, top). In contrast, both enzymes could digest E-cadherin promoter DNA in C33A cells transfected with Brm (Fig. 3A, bottom) suggesting that Brg1 and Brm may differ in their effects on DNA methylation in different cell lines. To verify that Brg1 and Brm induce specific changes in the methylation of the E-cadherin promoter, we used sodium bisulfite sequencing of the same promoter region amplified in the PCR assays described above. We consistently (>80% of clones sequenced) observed changes in three regions of a 5′ untranslated region of E-cadherin encompassing nucleotides 863 to 1138 (Genbank accession no. L34545) and containing CpG islands at positions 887, 901, and 920 following transfection with brg1 or brm in both C33A and SW13 cells (Fig. 3F). The cytosines at 901 and 920 had lost methylation in all of the clones sequenced.

CD44 promoter sequences from cells transfected with empty vector were always at least partially digested by HpaII but not by
Both HpaII and MspI could completely digest CD44 promoter sequences, however, if cells had been treated with 5-aza or were transfected with either Brg1 or Brm (Fig. 3C), suggesting that at least a small number of 5mC CGG sequences in the CD44 promoter may become demethylated in the presence of these SWI/SNF family members. These changes were verified by bisulfite sequencing as above, which revealed that the cytosines at positions −167 and −151 had become unmethylated in 100% of the clones analyzed following transfection with Brg1 or Brm. E. DNA methylation analysis of a 380-bp stretch of the 5′ untranslated region of the GAPDH promoter, showing that neither Brg1 nor Brm influence the methylation of this gene. F. Results from bisulfite sequencing of genomic DNA isolated from tetracycline-inducible NIH 3T3 cells carrying an expression construct for dnBrg1. After four passages in the presence of tetracycline (TET), dnBrg1 expression was repressed and the CD44 promoter was methylated in some clones at −742 but not at −722. In the absence of tetracycline for the same number of passages, there was no significant change in methylation at −742, but 20% of clones demonstrated methylation at −722.
expression as assessed by immunohistochemistry (8). We therefore isolated genomic DNA from the brains of brm-null mice and wild-type littermates and assayed for changes in methylation in the CD44 promoter. For the mouse promoter sequence, we used the *Hha*I methylation-sensitive restriction enzyme, which efficiently digested this CD44 promoter DNA from wild-type mice but only partially digested DNA from *brm*-null mice, indicating that this sequence becomes methylated in the absence of Brm (Fig. 3C, bottom).

We previously found that overexpression of the Brg1 mutant lacking the ATPase domain at least partially inhibited CD44 expression in NIH 3T3 cells (7). Because we found that transcriptional activation of CD44 involves loss of promoter DNA methylation, we tested whether this mutant Brg1 inhibited CD44 transcription through a mechanism that involved increased DNA methylation. NIH 3T3 cells induced to express the mutant Brg1 protein (dnBrg1) showed reductions (20-40% by Western blotting and immunocytochemistry compared with uninduced cells) in CD44 protein expression, but we did not detect any alterations in DNA methylation either by PCR using *Hha*I digestion as above or by sodium bisulfite sequencing (data not shown). However, if cells were grown for four passages with constant induction of dnBrg1, CD44 expression was even more reduced compared with controls (50-70%), and we found increased incidence of DNA methylation in some but not all CpG islands in the CD44 promoter (Fig. 3F). These data suggest that although Brg1 and Brm can induce transcription via a mechanism that includes loss of DNA methylation, reducing the activity of Brg1, and possibly Brm, is not sufficient to immediately induce DNA hypermethylation. However, loss of Brg1 or Brm may promote conditions that lead to hypermethylation and transcriptional silencing following multiple rounds of cell division.

To determine if SWI/SNF complexes containing Brg1 or Brm interact directly with the CD44 and E-cadherin promoters, we did ChIP assays using Brg1 and Brm antibodies and amplified DNA sequences using primers within the same regions where we observed Brg1- or Brm-dependent loss of DNA methylation. As shown in Fig. 4A, both factors interacted with the CD44 promoter in Saos2 cells, which constitutively express CD44 and express wild-type Brg1 and Brm. Similar results were obtained using MCF-7 and HeLa cells (data not shown). In SW13 cells, only HDAC1 interacted with the CD44 promoter (Fig. 4A, top). When SW13 cells were transfected with Brg1, however, Brg1 did interact with CD44 promoter sequences (Fig. 4A, middle). Brg1 and Brm similarly interacted with the E-cadherin promoter in MCF-7 cells (data not shown) and in SW13 cells transfected with either factor (Fig. 4B, top and middle) but not with the GAPDH promoter, whose expression is not regulated by Brg1 or Brm (Fig. 4C). The dnBrg1 and dnBrm proteins also interacted with the CD44 and E-cadherin promoters (Fig. 4A and B, bottom), further confirming that the ATPase chromatin remodeling activities of Brg1 and Brm are required for transcriptional activation of CD44 and E-cadherin but not for recruitment to DNA.

**Figure 4.** Brg1 and Brm interact with the CD44 and E-cadherin promoters. ChIP assays for interactions between Brg1 and Brm with the CD44 (A), E-cadherin (B), and GAPDH (C) promoters. Using primers encompassing the same regions of the promoters analyzed in Fig. 3, cross-linked DNA was isolated from cells and immunoprecipitated with Brg1, Brm or HDAC1 (A, as a positive control) antibodies. A, ChIP assays were performed using Saos2 cells (top), which express endogenous Brg1 and Brm, and SW13 cells that lack both proteins. A nonspecific band (*) was amplified in some experiments. Middle, ChIP assays were repeated in SW13 cells transfected with Brg1 to confirm that Brg1 interacts with the CD44 promoter in these cells. Bottom, SW13 cells were transfected with wild-type Brg1 or dnBrm, showing that the mutant proteins, lacking an ATPase domain, still interact with the CD44 promoter. B, as above, untransfected SW13 cells (top) and cells transfected with Brg1 (middle left), Brm (middle right), or dnBrg1 (bottom) were analyzed by ChIP using primers that amplify the E-cadherin promoter. C, ChIP assay of Saos2 cells showing that neither Brg1 nor Brm interact with the 5′ untranslated region of GAPDH.
Brg1 and Brm could influence the methylation of the E-cadherin and CD44 promoters through either a direct or indirect mechanism. It is possible, for example, that either factor could promote the activities of other genes that in turn alter DNA methylation. However, given our finding that Brg1 and Brm both interact with the CD44 and E-cadherin promoter sequences within the regions of CpG islands that become unmethylated 48 hours following transfection with either factor, we believe it is more likely that SWI/SNF factors can promote the transcription of some genes by either blocking the ability of methyltransferases, like DNMT1, to methylate promoter sequences or by recruiting a demethylase to promoter sequences.

No matter what the mechanism may be, the controlled recruitment and activation of methyltransferases or demethylases to DNA through the activities of chromatin remodeling factors provides a powerful means of regulating tissue-specific gene expression both during development and in disease. This model explains how changes in the transcriptional activation of numerous genes may be altered in cancer cells with Brg1 or Brm mutations, and opens a new avenue of research into the link between DNA methylation and chromatin remodeling activities.

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References
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