The BRG-1 Subunit of the SWI/SNF Complex Regulates CD44 Expression

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Matthew W. Strobeck§§, Marc F. DeCristofaro$§§, Fatima Banine¶, Bernard E. Weissman‡‡, Larry S. Sherman*, and Erik S. Knudsen‡

From the *Department of Cell Biology, University of Cincinnati College of Medicine, Vontz Center for Molecular Studies, Cincinnati, Ohio 45267-0521, the ‡Department of Pathology and Laboratory Medicine, University of North Carolina Chapel Hill, North Carolina 27599, and **The Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina 27599

Aberrant regulation of CD44, a transmembrane glycoprotein, has been implicated in the growth and metastasis of numerous tumors. Although both CD44 overexpression and loss have been implicated in tumor progression, the mechanism of CD44 down-regulation in these tumor types is not known. By immunoblot and reverse transcription-polymerase chain reaction analysis we determined that a cervical carcinoma cell line, C33A, lacks CD44 expression. To determine how CD44 is down-regulated in C33A cells, we utilized cell fusions of C33A cells with a CD44-expressing cell line (SAOS-2). We found that SAOS-2 fusion restored CD44 expression in C33A cells, suggesting that a trans-acting factor present in SAOS-2 cells promotes CD44 production. C33A cells are BRG-1-deficient, and we found that CD44 was absent in another BRG-1-deficient tumor cell line, indicating that loss of BRG-1 may be a general mechanism by which cells lose CD44. Reintroduction of BRG-1 into these cells restored CD44 expression. Furthermore, disruption of BRG-1 function through the use of dominant-negative BRG-1 demonstrated the requirement of BRG-1 in CD44 regulation. Finally, we show that Cyclin E overexpression resulted in the attenuation of CD44 stimulation, which is consistent with previous observations that Cyclin E can abrogate BRG-1 action. Taken together, these results suggest that BRG-1 is a critical regulator of CD44 expression, thus implicating SWI/SNF components in the regulation of cellular adhesion and metastasis.

The CD44 family of transmembrane glycoproteins has been implicated in cell-cell and cell-matrix adhesion, cell growth, and metastasis (1–3). A number of different CD44 proteins are produced through alternative RNA splicing, and these proteins are extensively modified. Many tumors express higher than normal levels of total CD44 protein as well as splice variants that do not occur in normal cells (1, 3, 4). How CD44 expression is regulated in normal cells and in tumors is poorly understood.

A role for CD44 in tumor progression has been documented in numerous clinical and experimental studies (1, 2). Ectopic expression of some forms of CD44 can enhance metastasis and tumor growth both in vitro and in vivo (5–7). It is believed that CD44 expression in some tumors increases as the tumor becomes more proliferative and invasive (1). These findings suggest that CD44 might be regulated by environmental or genetic factors that have been shown to contribute to cancer progression. The expression of activated oncogenes like v-Ras, v-Src, and v-Fos, which promote transformation and invasion, have been reported to induce CD44 expression (8–10). In addition, the epidermal growth factor receptor has also been shown to stimulate CD44 (10, 11).

In contrast to studies that correlate CD44 overexpression with cancer progression, a significant number of reports also indicate that loss of CD44 expression can contribute to tumorigenesis (12). Specifically, it has been shown that loss of CD44 in cervical carcinomas, neuroblastomas, prostate carcinomas, melanomas, and small cell lung carcinomas correlates with increased aggressiveness of these tumors (13–17). It has been hypothesized that loss of CD44 may facilitate the abrogation of epithelial-mesenchymal interactions in some tumors, leading to a more metastatic phenotype (4, 12). Unlike positive regulators of CD44, no activity has been identified that leads to loss of CD44 expression.

In the present study, we have identified the Brahma-related gene, BRG-1, as a critical regulator of CD44 expression. BRG-1 is a component of the SWI/SNF complex that regulates gene transcription through ATPase-dependent remodeling of chromatin (18–21). BRG-1 has been shown to inhibit the transcription of a subset of genes involved in cancer such as Cyclin A and c-fos (22–25). In addition, BRG-1 can facilitate estrogen receptor, glucocorticoid receptor, c-Myc, and BRCA1-mediated transcription (26–30).

We demonstrate that loss of BRG-1 correlates with a lack of CD44 expression in several cell lines. Reintroduction of BRG-1 restored expression of CD44 in BRG-1-deficient cells, suggesting that BRG-1 regulates basal CD44 levels. Moreover, we show that functional BRG-1 is required for stimulating CD44, because dominant-negative BRG-1 inhibits CD44 production. In addition, we show that Cyclin E can antagonize BRG-1 regulation of CD44. As such, this report provides the first
evidence that the SWI/SNF complex regulates genes involved in cell adhesion and cancer metastasis and suggests that BRG-1 loss in human tumors contributes to the formation of a metastatic phenotype.

MATERIALS AND METHODS

Cells, Cell fusions, Plasmids, and Transfection—C33A, SAOS-2, MCF-7, U87, PC3, U2OS, RD, and SW13 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin-streptomycin, and 2 mM l-glutamine at 37 °C in 5% CO2. 3T3 cells (B50-1) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated calf serum (Calbiochem), 100 units/ml penicillin-streptomycin, 2 mM l-glutamine, 350 units of Hygromycin B, and 0.12 mg/ml Geneticin either in the presence or absence of 2 μg/ml tetracycline (28). Plasmids were transfected using the calcium phosphate method (31). Antibiotic-resistant C33A and SAOS-2 cells were generated as previously described (32). The following plasmids: CMV-Neo, pBluescript-Puro, Cyclin E, pSM-Neo-B, and BRG-1 have been previously described (33–36). The cell fusions were generated as previously described (32).

Immunoblotting—Approximately 1 × 106 C33A and SW13 cells were plated in 10-cm dishes 24 h before transfection. C33A and SW13 cells were cotransfected with effectors and the puromycin-selectable plasmid (pBabe-Puro), and transfected cells were subsequently selected with 2.5 μg/ml puromycin (Sigma) for 48–72 h and then harvested for immunoblot analysis. To isolate BRG-1, total protein was isolated from subconfluent cultures using an 8 M urea extraction (8 M urea, 0.1 M NaH2PO4, 10 mM Tris, pH 8). For immunoblotting, cells were trypsinized and subsequently washed with PBS. C33A and SW13 cell pellets were resuspended in radioimmune precipitation buffer supplemented with the following protease inhibitor mixture (10 mg/ml, 1,10-phenanthroline, 10 mg/ml aprotinin, 10 mg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) and incubated for 15 min on ice. The lysates were brieﬂy sonicated and then centrifuged for 10 min at 20,000 × g at 4 °C. The lysates were then separated via SDS-polyacrylamide gel electrophoresis and then transferred onto Immobilon-P membranes (Millipore). The membranes were then incubated with either of the following antibodies: anti-CD44 (clone 1H2; Santa Cruz Biotechnology), anti-Cyclin E (clone C19, Santa Cruz), anti-actin (Sigma A-2066), anti-p53 (Dr. K. Fukasawa, University of Cincinnati, College of Medicine, Cincinnati, OH), anti-pRB (Dr. J. Wang, University of California at San Diego, 851 polyclonal antibody), and anti-BRG-1 (Dr. Weidong Wang, National Institutes of Health, Baltimore, MD). The blots were then incubated with either horseradish peroxidase-conjugated anti-rabbit or anti-mouse for 1 h at room temperature. The antibody-antigen complex was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The levels of Cdk4 and Cdk4 were quantitated using Metamorph software (Universal Imaging Corp.) for two independent PCR reactions.

Immunohistochemistry—Cells were fixed in 3.7% formaldehyde for 15 min and then permeabilized (PBS, 0.3% Triton X-100, 5 mg/ml bovine serum albumin) for 5 min staining or directly blocked with 5% normal goat serum for CD44 staining. The p53 antibody (1:100 dilution, 211 polyclonal antibody), and anti-p53 (Dr. K. Fukasawa, University of Cincinnati, College of Medicine, Cincinnati, OH) were utilized. The antibody-antigen complex was detected by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech). The levels of Cdk4 and Cdk4a were quantitated using Metamorph software (Universal Imaging Corp.) for two independent PCR reactions.

RESULTS

CD44 Expression Is Down-regulated in the C33A Cervical Carcinoma Cell Line—CD44 is lost soon in cervical carcinoma, and this loss correlates with increased metastatic potential (13, 14). We identified a human cervical carcinoma cell line, C33A, which demonstrates loss of CD44 expression as determined by immunoblot analysis with a CD44-specific antibody (Fig. 1A, lane 1). By contrast, the osteogenic sarcoma cell line, SAOS-2, readily expresses detectable CD44 (Fig. 1A, lane 2). The level of CD44 RNA was normalized to that of GAPDH. The results are representative of two independent RT-PCR experiments.

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Factors Expressed by SAOS-2 Cells Act in-trans to Restore CD44 Signaling in C33A Cells—To examine the underlying differences, we utilized C33A-SAOS-2 (C-S) cell fusion lines that have been previously characterized (32). Four independent C-S fusion clones (#1–4) exhibited robust expression of CD44 comparable to that observed in parental SAOS-2 cells (Fig. 2A, lanes 1–6). Immunohistochemical analysis confirmed that CD44 expression was restored and appropriately localized in the cell fusions (Fig. 2B, right panel). As a control, p53 staining confirmed that

![FIG. 1. C33A cells have reduced CD44 expression. A, C33A (lane 1) and SAOS-2 (lane 2) cells were harvested and lysed, and equal total protein was resolved by SDS-PAGE and immunoblotted for CD44 and Cdk4. B, C33A cells were harvested as in A, total RNA was prepared, and RT-PCR was performed utilizing 1 μg of total RNA as described under "Materials and Methods." As a control, PCR analysis of isolated RNA was carried out from C33A (lane 1) and SAOS-2 (lane 2). The level of CD44 RNA was normalized to that of GAPDH. The results are representative of two independent RT-PCR experiments.

![FIG. 2. CD44 expression is restored in the C33A-SOAS-2 cell fusion. A, immunohistochemistry for CD44 was performed on C33A and SAOS-2 cells (A, lane 1) and on the C33A-SOAS-2 cell fusion (A, lanes 2–6). B, immunohistochemistry for CD44 was performed on C33A and SAOS-2 cells (B, lane 1) and on the C33A-SOAS-2 cell fusion (B, lanes 2–6). The results are representative of two independent cell fusion experiments.]
the fusion expressed p53 protein (contributed from C33A nuclei), because SAOS-2 are p53-negative (Fig. 2B, left and middle panel). These findings suggest that C33A cells lack a factor(s) required for CD44 signaling, which is restored after nuclear fusion with SAOS-2.

**Loss of BRG-1 Correlates with Reduced CD44 Expression—** A known difference between SAOS-2 cells and C33A cells is that SAOS-2 cells express BRG-1, a component of the SWI/SNF complex involved in both gene regulation and chromatin remodeling, whereas C33A lack BRG-1 (36). To examine whether loss of BRG-1 correlated with lack of CD44 expression, we analyzed several tumor lines that were either positive or negative for BRG-1 expression. Immunoblot analysis revealed that those tumor lines that were positive for BRG-1 were also positive for CD44 expression (Fig. 3A, upper and middle panel, lanes 2, 3, 5, 6, and 7). However, the SW13 adenocarcinoma cell line, which is BRG-1-negative, was deficient in CD44 protein expression, similar to that observed in C33A cells (Fig. 3A, upper and middle panel, lanes 1 and 4) (36). As a control, the detection of actin shows the relative amount of protein loaded per lane (Fig. 3A, lower panel, lanes 1–7). These data demonstrate that loss of BRG-1 correlates with a lack of CD44 protein, suggesting that BRG-1 is required for CD44 expression.

**BRG-1 Restores CD44 Expression in C33A and SW13 Cells—** To determine whether BRG-1 is sufficient to induce CD44 expression, BRG-1 was restored in BRG-1-negative (C33A and SW13) cells. C33A and SW13 cells were cotransfected with vector, a phosphorylation site mutant of RB (PSM-RB) as controls, or BRG-1 along with a puromycin resistance plasmid (pBabe-Puro). After selecting the transfected cells with puromycin, cells were harvested and subjected to immunoblot analysis. In C33A and SW13 cells, transfection of either vector or PSM-RB did not alter CD44 levels. However, ectopic expression of BRG-1 caused a significant increase in CD44 protein levels (Fig. 4A, upper panel, lanes 1–6). Immunoblotting for BRG-1 and PSM-RB confirmed their expression in C33A and SW13 cells, whereas Cdk4 levels indicate that equal total protein was loaded per lane (Fig. 4A, middle and lower panels, lanes 1–6). Because RB has been shown to recruit BRG-1 for transcriptional repression, we also examined whether expression of PSM-RB could alter BRG-1 stimulation of CD44. To test this, we cotransfected PSM-RB with BRG-1 into C33A cells and found that PSM-RB did not alter the ability of BRG-1 to modulate CD44 production (data not shown).

Because BRG-1 is known to mediate its function through transcriptional regulation, we investigated whether BRG-1 stimulates CD44 mRNA levels (37). C33A cells were transfected with either vector or PSM-RB along with pBabe-Puro and selected in puromycin. RT-PCR analysis revealed that the level of CD44 mRNA quantitated from vector-transfected cells was equal to that of PSM-RB-transfected cells after normalization to GAPDH (Fig. 4B, upper and lower panels, lanes 4, 5, 8, and 9). In contrast, C33A cells transfected with BRG-1 showed an ~40-fold induction in CD44 mRNA when compared with that of vector- or PSM-RB-transfected cells and normalized against GAPDH (Fig. 4B, upper and lower panels, lanes 4–9). PCR analysis of the RNA prior to reverse transcribing revealed no amplification, thus eliminating the possibility of DNA contamination (Fig. 4B, upper and lower panels, lanes 1–3). The effect of ectopic BRG-1 expression on the CD44 promoter was assessed by reporter analysis in transient transfection assays. Ectopic expression of BRG-1 did not stimulate CD44 reporter activity, and high basal activity of the reporter was observed in cells either expressing or deficient in BRG-1 (data not shown). This finding is consistent with the idea that BRG-1 stimulates transcription through the remodeling of chromatin, because we show that BRG-1 clearly stimulates the expression of endogenous CD44 RNA and protein.

**Functional BRG-1 Is Required for Stimulating CD44 Production—** Because the data presented imply that BRG-1 may have a role in maintaining the production of CD44, we investigated whether BRG-1 is required for this process. To test this we utilized an NIH3T3 cell line, called B05-1, that inducibly expresses dominant-negative BRG-1 (dnBRG-1) (28). The mutant BRG-1 protein that is induced in B05-1 cells contains a mutation in the ATP-binding site that renders it catalytically inactive, and in the presence of tetracycline, B05-1 cells fail to demonstrate detectable levels of dnBRG-1 (Fig. 5A, lanes 1 and 2).
However, after incubation in the absence of tetracycline for 72 h, a strong induction of dnBRG-1 was observed. Immunostaining of the BO5-1 cells grown in the absence of tetracycline revealed that expression of dnBRG-1 results in the reduction of CD44 expression when compared with uninduced BO5-1 cells (Fig. 5B). Microscopic analysis revealed that expression of dnBRG-1 significantly inhibits CD44 staining in 90% of induced cells when compared with BO5-1 cells grown in the presence of tetracycline (Fig. 5C). These data imply that functional BRG-1 is required for stimulating CD44 expression.

**DISCUSSION**

Aberrant expression of CD44 is associated with advanced tumor progression and poor clinical outcome (1–3). However, little is known about what factors are required for maintaining CD44 expression. Here we show that BRG-1 plays a critical role in regulating CD44 transcription. Reintroduction of BRG-1 restored CD44 expression in BRG-1-negative (C33A and SW13) cells, whereas expression of dominant-negative BRG-1 in NIH3T3 cells inhibited CD44 production. Furthermore, ectopic expression of Cyclin E inhibited the ability of BRG-1 to stimulate CD44, consistent with the notion that at least some components of the cell cycle machinery could also mediate CD44 expression. These results indicate that BRG-1 is required for regulating CD44 expression, suggesting that loss of BRG-1 may contribute to the invasive and metastatic potential of certain cancers.

BRG-1 plays a critical role in transcriptional regulation and chromatin remodeling and can both stimulate and repress the
transcription of specific genes (22, 26–30, 32). BRG-1 is an ATP-dependent chromatin remodeler that regulates transcription through its ability to modify nucleosome structure (37). Activation of CD44 expression is apparently dependent on chromatin remodeling, because the dominant-negative BRG-1 used in this study specifically disrupts the ATPase activity of BRG-1. In the case of CD44, BRG-1 likely converts the CD44 promoter from a transcriptionally inactive state to an active one. The finding that transiently transfected CD44 reporter constructs maintained high basal activity in the absence of BRG-1 suggests that the plasmid-borne CD44 promoter construct already exists in a transcriptionally active state, thus masking any BRG-1 stimulatory effect at the level of chromatin (data not shown).

The loss of CD44 expression by some tumors correlates with increased metastatic potential (1). The mechanism of CD44 loss in these tumors is largely unknown; however, in prostate carcinoma, CD44 expression is repressed through the methylation of the CD44 promoter (40). Interest-

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