EVI1 Promotes Cell Proliferation by Interacting with BRG1 and Blocking the Repression of BRG1 on E2F1 Activity*  

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Yiqing Chi, Vitalyi Senyuk, Soumen Chakraborty, and Giuseppina Nucifora‡

From the Department of Pathology and The Cancer Center, University of Illinois, Chicago, Illinois 60607

EVI1 is a complex protein required for embryogenesis and inappropriately expressed in many types of human myeloid leukemia. Earlier we showed that the forced expression of EVI1 in murine hematopoietic precursor cells leads to their abnormal differentiation and increased proliferation. In this report, we show that EVI1 physically interacts with BRG1 and its functional homolog BRM in mammalian cells. We found that the C terminus of EVI1 interacts strongly with BRG1 and that the central and C-terminal regions of BRG1 are involved in EVI1-BRG1 interaction. Using reporter gene assays, we demonstrate that EVI1 activates the E2F1 promoter in NIH3T3 cells but not in BRG1-negative SW13 cells. Ectopic expression of BRG1 is able to repress the E2F1 promoter in vector-transfected SW13 cells but not in EVI1-transfected SW13 cells. Finally, we show that EVI1 up-regulates cell proliferation in BRG1-positive 32Dc13 cells but not in BRG1-negative SW13 cells. Taken together, these data support the hypothesis that the interaction with BRG1 is important for up-regulation of cell growth by EVI1.

EVI1 is an evolutionarily conserved DNA-binding protein that belongs to the Kruppel family of proteins and is characterized by two domains of seven and three repeats of the Cys2-His2-type zinc finger motif (1, 2). EVI1 was first identified in the mouse as the integration site of an activating ecotropic retrovirus leading to myeloid tumors in susceptible strains of mice (1, 3). EVI1 is not detected in normal hematopoietic organs including bone marrow, and the inappropriately expression of EVI1 is often triggered by chromosomal rearrangements that disrupt the 3q26 chromosomal region where this gene is located (4, 5). The most frequent rearrangements associated with deregulation of EVI1 expression are the t(3;3)(q21;q26) and inv(3)(q21q26) observed in very aggressive acute myeloid leukemia and myelodysplastic syndrome (6, 7). Chronic myelogenous leukemia patients frequently display EVI1 up-regulation although their chromosomes appear normal by conventional cytogenetics, indicating that the inappropriately activation of EVI1 occurs also by unknown mechanisms. The forced expression of EVI1 in cell lines and in murine embryonic stem cells reduces the cellular response to growth inhibition by transforming growth factor-β (8–10) and affects the normal hematopoietic differentiation of murine bone marrow precursors (6) and embryonic stem cells (9). In addition, the inappropriate expression of EVI1 in cell lines leads to up-regulation of cell replication (9, 11). The mechanisms by which EVI1 disrupts normal cell replication and hematopoietic differentiation are not known. Using reporter gene assays, several investigators showed that EVI1 is a transcription repressor (12, 13). EVI1 interacts with corepressors including the C-terminal-binding protein 1 and histone deacetylases, and with co-activators, including the cAMP-responsive-binding protein and the p300/cAMP-responsive-binding-protein-associated factor (11).

BRG1 and BRM are mammalian orthologs of Saccharomyces cerevisiae SWI2/SNF2 and have ATP-dependent helicase activity that is necessary for the chromatin remodeling function of SWI/SNF complexes (14–19). The human SWI/SNF is a complex of about 2 MDA that contains 10–15 subunits. In addition to either BRG1 or BRM, which are the core members of the complex, the mammalian SWI/SNF complex contains other components like BAF170, BAF155, BAF60, BAF53, and IN11 (19–21). The BRG1/BRM family members do not bind to specific DNA sequences but are recruited to promoters by DNA-binding proteins to alter the structure of chromatin. This process utilizes the energy of ATP hydrolysis to remodel the chromatin structure and to make specific DNA sequences more accessible (gene activation) or less accessible (gene repression) to the basal transcription machinery (22, 23). BRG1 and BRM are implicated in targeted gene regulation and are involved in varieties of critical aspects of cellular growth and genomic stability. They have been associated with gene activation by nuclear hormone receptors (15, 24–26) and BRCA1 (27) and are required for regulation of genes involved in cell cycle control such as E2F (28) and c-Myc (29). BRG1 and its family members have also been identified in T-cell development (30, 31) and in activation of erythroid (32, 33), myeloid (34), and muscle-specific (35) gene regulation. In addition, BRG1 and BRM have been linked to host immune defense system by activating the master regulators of major histocompatibility complex class I and II expression (36, 37).

In this study, we investigated whether the ability of EVI1 to up-regulate the cell cycle requires the association with BRG1. We show that the C-terminal region of EVI1 and the central and C-terminal regions of BRG1 are involved in EVI1-BRG1 interaction. This interaction is biologically significant, and by reporter gene assay we show that BRG1 is required for the activation of the E2F1 promoter by EVI1. These data suggest that the interaction of these proteins could explain the mechanism of cell cycle up-regulation by EVI1, and this model is confirmed by in vivo results showing that EVI1 up-regulates the cell cycle in BRG1-positive but not in BRG1-negative cells.

EXPERIMENTAL PROCEDURES

DNA Constructs—FLAG-tagged full-length EVI1 and deletion mutants of EVI1 were described previously (11). To clone the deletion

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‡ Scholar of the Leukemia and Lymphoma Society. To whom correspondence should be addressed: Dept. of Pathology and The Cancer Center, Molecular Biology Research Bldg., M/C 737, University of Illinois at Chicago, 900 S. Ashland Ave., Chicago, IL 60607. Tel.: 312-413-4686; Fax: 312-413-0548; E-mail: nucifora@uic.edu.
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**Fig. 1.** EVI1 interacts with BRG1 and BRM in vivo. A, top, 293T cells were cotransfected with BRG1 and FLAG-EVI1 (lanes 1 and 3) or with BRG1 and empty vector (lanes 2 and 4). Forty-eight h after transfection, whole cell lysates were collected and incubated with agarose-conjugated anti-FLAG antibody (lanes 1 and 2) or analyzed directly by Western blot (lanes 3 and 4). The proteins were separated by electrophoresis, transferred to a PVDF membrane, and probed with anti-BRG1 antibody. A band corresponding to BRG1 is observed with lysates of cells cotransfected with FLAG-EVI1 (lane 1), whereas no band is observed with lysates of cells transfected with the empty vector (negative control; lane 2). Equivalent amount of BRG1 is expressed in the cell lysates (lanes 3 and 4). Bottom, the membrane was stripped and hybridized to anti-BRG1 antibody to visualize the level of FLAG-EVI1 in the IP reaction (lane 1) and in the cell lysate (lane 3). B, top, 293T cells were transfected with FLAG-EVI1 (lanes 1 and 2) or empty vector (lanes 2 and 4). Forty-eight h after transfection, whole cell lysates were prepared and incubated with anti-BRG1 antibody and protein A agarose beads (lanes 1 and 2) or analyzed directly by Western blot (lanes 3 and 4). The proteins were separated by electrophoresis, transferred to a PVDF membrane, and probed with anti-FLAG antibody. EVI1 was immunoprecipitated with endogenous BRG1 (lane 1) but not observed in transfection with the empty vector (lane 2). Bottom, the membrane was stripped and hybridized to anti-BRG1 antibody to visualize the level of endogenous BRG1 in the IP reaction (lanes 1 and 2) and in the cell lysates (lanes 3 and 4). C, top, 293T cells were cotransfected with HA-BRM and FLAG-EVI1 (lanes 1 and 2) or empty vector (lanes 2 and 4). Forty-eight h after transfection, whole cell lysates were prepared and incubated with agarose-conjugated anti-FLAG antibody (lanes 1 and 2) or analyzed directly by Western blot (lanes 3 and 4). The proteins were separated by electrophoresis, transferred to a PVDF membrane, and probed with anti-HA antibody. HA-BRM was immunoprecipitated with FLAG-EVI1 antibody (lane 1) but not observed with the negative control (empty vector; lane 2). Equivalent amount of HA-BRM is expressed in the cell lysates (lanes 3 and 4). Bottom, the membrane was stripped and reprobed with anti-FLAG antibody to visualize the amount of FLAG-EVI1 in the IP-reaction (lane 1) and in the cell lysate (lane 3).

**Fig. 2.** The C terminus of EVI1 interacts with BRG1. A, diagram of the EVI1 constructs used in this study. Gray boxes indicate the position of the zinc finger domains. The full-length EVI1 contains 1051 amino acids. The deletion mutants and their size are indicated in the remaining lines. B, 293T cells were cotransfected with BRG1 and full-length EVI1 (FLAG-EVI1–1051) (lane 1) or deletion mutants of EVI1 including FLAG-EVI1–653 (lane 2), FLAG-EVI1–514 (lane 3), FLAG-EVI1–283 (lane 4), or empty vector (lane 5). Lane 6 refers to mock transfected cells. Top, 48 h after transfection, the cells were lysed and incubated with agarose-conjugated anti-FLAG antibody. The proteins were separated by electrophoresis, transferred to a PVDF membrane, and probed with anti-BRG1 antibody. BRG1 was efficiently immunoprecipitated (IP) when cotransfected with full-length EVI1 (lane 1) or less efficiently with the deletion mutants FLAG-EVI1–653 (lane 2) and FLAG-EVI1–514 (lane 3). No BRG1 band was detected with the deletion mutant FLAG-EVI1–283 (lane 4), the empty vector (lane 5), and with mock transfected lysates (lane 6). Middle, the membrane was stripped and reprobed with anti-FLAG antibody to determine the expression of EVI1 and the deletion mutants. Bottom, the cell lysates were directly analyzed by Western blot (WB) and probed with anti-BRG1 antibody.

The first fragment to generate BRG1 (1–1088) and BRG1 (382–1088), respectively. To construct HA1-tagged BRG1 mutants, a modified pCMV-myc-nuc (Invitrogen) containing the HA epitope was used (38). The different fragments of BRG1 were cloned in-frame in the NotI site of pCMV-myc-nuc-HA to generate HA-BRG1 (382–1088), HA-BRG1 (1088–1598), and HA-BRG1 (382–917). All cloning junctions were verified by DNA sequencing.

**Cell Culture**—The adherent cell lines 293T and NIH3T3 were cultured in Dulbecco’s modified minimum essential medium (Invitrogen) supplemented with 10% newborn calf serum. The BRG1-negative SW13 cells were maintained in Dulbecco’s modified minimum essential medium with 10% fetal bovine serum. The suspension cell line 32Dcl3 was maintained in RPMI 1640 medium supplemented with 10% calf serum and 10% WEHI-3B conditioned medium as a source of interleukin 3.

**DNA Transfection**—DNA transfection of adherent cells was performed by calcium phosphate precipitation method (39). Ten μg of each plasmid was used for each 10-cm plate, unless indicated otherwise.
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FIG. 3. The central and C-terminal regions of BRG1 are required for efficient interaction with EVI1. A, schematic diagram of the full-length BRG1 (top line) and BRG1 deletion mutants (lines 2–7). The full-length BRG1 contains 1648 amino acids. The gray box represents the ATP helicase domain (774–1237). Other domains are indicated on the top of the diagram. The boundaries of all the deletions are indicated by the amino acid numbers. The BRG1 deletion mutants BRG1 (1–382), BRG1 (1–1088), and BRG1 (3382–1088) (lines 2–4) are detected with anti-BRG1 antibody. The BRG1 deletion mutants HA-BRG1 (382–1088), HA-BRG1 (1088–1598), and HA-BRG1 (382–917) (lines 5–7) are detected with anti-HA antibody. B, 293T cells were cotransfected with BRG1 deletion mutants (as noted on the left side of the figure) and FLAG-EVI1 (lanes 1 and 3) or empty vector (lanes 2 and 4). Forty-eight h after transfection, cell lysates were prepared, and the proteins were immunoprecipitated with agarose-conjugated anti-FLAG antibody (lanes 1 and 2) or were analyzed directly by Western blot (lanes 3 and 4). After protein transfer, the PVDF membranes were probed with anti-BRG1 antibody (left panel) or with anti-HA antibody (right panel). The levels of expression of all the deletion mutants are also shown (lane 3 and 4).

Stably transfected 32Dcl3 cell clones were obtained by retroviral infection as described previously (11). To generate EVI1-positive or vector (control) SW13 stable transfectants, the cells were transfected with pCMV-myc-nuc-HA-EVI1 or the empty vector by calcium phosphate precipitation method. Twenty-four h later, the culture medium was replaced with selection medium containing G418 (400 μg/ml) (BioWhittaker). After 7 days, single colonies were isolated and expanded. The expression of full-length EVI1 was confirmed by reverse transcriptase-PCR analysis.

Co-immunoprecipitation and Western Blot—Transiently transfected 293T cells expressing the SV40 large T antigen were used for the assays. Forty-eight h after transfection, the cells were harvested, and immunoprecipitations were carried out as described (11). The proteins were separated by electrophoresis in a 7% SDS-polyacrylamide gel. After transfer to polyvinylidene difluoride (PVDF) membrane, the protein bands were visualized with the following commercially available antibodies: rabbit polyclonal BRG1 antibody (H-88) (Santa Cruz Biotechnology, Inc.), monoclonal HA antibody (Roche Applied Science), and mouse monoclonal FLAG antibody (M2) (Sigma). Reporter Gene Assay—Dual-Luciferase Reporter Assay System (Promega) was used according to the manufacturer’s instructions. The reporter construct E2F1-Luc that expresses firefly luciferase under the control of E2F1 promoter was a gift of Dr. Raychaudhuri. NIH3T3 or SW13 cells plated in 6-well plates were transfected with 3 μg of the reporter construct E2F1-Luc and 3 μg of pRL-TK (Promega), which expresses Renilla luciferase as internal control. Forty-eight h after transfection, the cells were lysed with three cycles of freezing and thawing. The total cellular extracts were centrifuged, and the activity of the enzymes in the supernatant was measured using Luminometer TD-20/20 (Turner Designs Instrument). All assays were performed in triplicates, and each experiment was repeated at least twice.

Flow Cytometry—One million stably transfected exponentially growing EVI1–32Dcl3 and EVI1-SW13 cells were collected, washed with phosphate-buffered saline, and fixed in cold 70% ethanol for 1 h. Fixed cells were washed and incubated with 20 μg/ml propidium iodide (Sigma) containing 100 μg/ml RNase A (Sigma) at 37 °C for 1 h. The analysis of cell cycle profile was performed with a fluorescence-activated cell sorter using a Cell Quest package (BD Biosciences).

RESULTS

EVI1 Interacts with BRG1 and BRM in Vivo—Although the human BRG1 and BRM complexes have been often associated with transcription activation (22, 40), each complex is also implicated in transcription repression (41). BRG1 and BRM complexes that contain components of the co-repressor SIN3 complex have been purified from HeLa cell nuclear extracts (42). SIN3 is involved in the repression of transcription by a variety of transcription factors. It has also been reported that BRG1 associates with a co-repressor multiprotein complex, N-CoR1, which possesses histone deacetylase activity (43). BRG1 also interacts with the mammalian heterochromatin protein 1-α and enhances heterochromatin protein 1-mediated transcription repression (44). Finally, combined studies from other investigators indicate that BRG1 interacts with the retinoblastoma tumor suppressor protein (RB) and enhances RB-mediated transcription repression of key cell cycle targets, including E2F and cyclin A (28, 45).

We first tested the interaction between EVI1 and BRG1 using co-immunoprecipitation analysis. After transient transfection, the proteins were incubated with agarose-conjugated anti-FLAG antibody and were separated by electrophoresis. BRG1 was visualized by anti-BRG1 antibody. The results clearly show that BRG1 is immunoprecipitated with EVI1 in cells cotransfected with BRG1 and EVI1 (Fig. 1A, top panel, lane 1) but not in cells cotransfected with BRG1 and the empty vector (Fig. 1A, top panel, lane 2). In both cases, BRG1 was expressed in the cell lysates at level equivalent to EVI1 (Fig. 1A, top panel, lanes 3 and 4). The expression of the transfected FLAG-EVI1 was confirmed after stripping the membrane and hybridization to anti-FLAG antibody (Fig. 1A, bottom panel, lanes 1 and 3). The interaction between EVI1 and BRG1 was further confirmed by the fact that EVI1 is immunoprecipitated by endogenous BRG1 in cells transfected with FLAG-EVI1 (Fig. 1B, top panel, lane 1) but not in cells transfected with the empty vector (Fig. 1B, top panel, lane 2). The amount of endogenous BRG1 in the IP reaction is shown in Fig. 1B, bottom panel, lanes 1 and 2. The amount of endogenous BRG1 in the cell lysates is shown in Fig. 1B, bottom panel, lanes 3 and 4.

Because the mammalian SWI/SNF complexes contain either BRG1 or the highly related BRM as ATPase subunit, we tested the interaction between FLAG-EVI1 and HA-BRM. Anti-HA antibody was used to visualize HA-BRM. As with BRG1, we
found that FLAG-EVI1 interacts with HA-BRM (Fig. 1C, top panel, lane 1). The interaction was not observed with the empty vector (Fig. 1C, top panel, lane 2). The expression of HA-BRM (Fig. 1C, top panel, lanes 3 and 4) and EVI1 (Fig. 1C, bottom panel) is also shown.

To identify the region of EVI1 that interacts with BRG1, the FLAG-tagged full-length EVI1 and a series of deletion mutants (Fig. 2A, lanes 1–4) were cotransfected with BRG1 in 293T cells. The full-length EVI1 interacts strongly with BRG1 (Fig. 2B, top panel, lane 1). The deletion mutants FLAG-EVI1–653 (lane 2) and FLAG-EVI1–514 (lane 3) interact weakly. The shortest deletion mutant FLAG-EVI1–283 (lane 4) and the empty vector (lane 5) do not interact with BRG1. The expression of EVI1 and its deletion mutants was confirmed after the membrane was stripped and reprobed with anti-FLAG antibody (Fig. 2B, middle panel). The amount of BRG1 input in different reactions is about the same (Fig. 2B, bottom panel). The result obtained with mock-transfected 293T cells is also shown (lane 6). The faint band in the bottom panel identifies the endogenous BRG1 present in 293T cells.

The Central and C-terminal Regions of BRG1 Are Required for Efficient Interaction with EVI1—A schematic representation of BRG1 and its domains is shown in Fig. 3, line 1. To map the region of BRG1 responsible for mediating the interaction with EVI1, we generated a series of BRG1 deletion mutants and tested their ability to interact with full-length FLAG-EVI1. The first group of deletion mutants (Fig. 3A, lines 2–4) contains the intact N-terminal region of BRG1 (1–382 amino acids) and can be detected by anti-BRG1 antibody (raised against amino acids 209–296 of BRG1). The second group of deletion mutants lacking the N terminus (Fig. 3A, lines 5–7) was cloned in pCMV-myc-nuc-HA vector to provide a nuclear localization signal and the HA epitope, and anti-HA antibody was used for their identification. The BRG1 deletion mutants were separately cotransfected with either full-length FLAG-EVI1 (Fig. 3B, lanes 1 and 3) or the empty vector (Fig. 3B, lanes 2 and 4). FLAG-EVI1 failed to co-immunoprecipitate BRG1 (1–382), which contains only the N terminus of BRG1 (Fig. 3B, left top panel, lane 1). However, the other deletion mutants, which contain either the central region or the C terminus of BRG1, are able to interact with FLAG-EVI1 but not with the empty vector (Fig. 3B, left middle and bottom panels and right panel, lane 1). Lanes 3 and 4 in Fig. 3B show the protein expression in the cell lysates.

EVI1 Co-operates with BRG1 in the Activation of the E2F1 Promoter—Previous studies (28, 45) demonstrated that BRG1 interacts with the tumor suppressor protein RB and enhances RB-mediated transcription repression of key cell cycle targets, including E2F and cyclin A. RB is the master regulator of cell cycle, and the repression of E2F-mediated transcription is thought to underlie RB-dependent cell-cycle arrest (46). E2F controls the expression of numerous genes directly involved in cell cycle progression or in metabolic processes coupled to DNA replication (47, 48).

To evaluate the potential significance of the EVI1-BRG1 interaction in the regulation of cell cycle, we used the E2F1 promoter linked to the luciferase reporter gene in reporter gene assays. We first assessed the ability of EVI1 to modulate the E2F1 promoter activity in NIH3T3 cells. NIH3T3 cells were cotransfected with FLAG-EVI1 or the empty vector, together with E2F1-Luc reporter plasmid and the internal control pRL-TK. In NIH3T3 cells, full-length FLAG-EVI1 activates E2F1 promoter activity about 3-fold compared with the empty vector (Fig. 4A).

To investigate the potential role of BRG1 in EVI1’s activation of the promoter, we utilized SW13 cells, a BRG1- and BRM-deficient cell line derived from human small-cell carcinoma (49, 50). In contrast to what we found in NIH3T3 cells, in the absence of BRG1 and BRM, there is no difference between the
response of the E2F1 promoter in presence or absence of added EVI1 (Fig. 4B, white bars). However, when a functional BRG1 was introduced into the SW13 cells by cotransfection, BRG1 was able to suppress E2F1 promoter activity with the empty vector but not with FLAG-EVI1 (Fig. 4B, gray bars), confirming the results obtained with BRG1-positive NIH3T3.

EVI1 Up-regulates Cell Proliferation in BRG1-positive 32Dcl3 Cells but Not in BRG1-negative SW13 Cells—We reported earlier that the forced expression of EVI1 in 32Dcl3 and embryonic stem cell lines leads to a significant up-regulation of cell replication (9–11). To assess the functional importance of BRG1 in EVI1-dependent up-regulation of cell proliferation, we carried out cell cycle analyses of SW13 and 32Dcl3 cell lines that stably express EVI1. As controls, we used the same cell lines stably transfected with the empty vector. We found that the forced expression of EVI1 in the BRG1-positive 32Dcl3 cells reduced the number of cells in G1 by over 20% (from 61% in vector-32Dcl3 cells to 40% in EVI1–32Dcl3 cells) and increased the number of cells in G2/M by about 12% (from 9.6% in vector-32Dcl3 cells to 21% in EVI1–32Dcl3 cells) (Fig. 5A). In contrast, there was no significant difference in the fluorescence-activated cell sorter profile of BRG1-negative SW13 cells that expressed or did not express EVI1 (Fig. 5B).

DISCUSSION

A large number of genes contain consensus sequences for E2F binding in their promoters. Some of these genes encode well studied cell cycle regulators such as cyclin E, cyclin A, cdc2, cdk2, and even E2F1 and E2F2 themselves (51). Others encode enzymes that are important for DNA synthesis, such as DNA polymerase α (52), thymidine kinase (53, 54), and dihydrofolate reductase (55). E2F regulates gene expression in different ways depending on promoter contexts. It acts as a transcription activator for the genes involved in DNA synthesis and thus promotes DNA synthesis (56, 57). In contrast, for cell cycle regulators E2F mediates a transcription repression by association with RB family proteins and assembly of a repressor complex (51). RB contains distinct surfaces that enable the interaction with RB family proteins and assembly of a repressor complex (51). RB includes the E7 motif. It is therefore possible that the association of BRG1 controls E2F promoter by interaction with RB.

Co-immunoprecipitation analyses (not shown) suggest that BRG1 and RB associate to enhance E2F1 functions and support the results of our mapping analysis with RB. BRG1 and RB contain the LXCXE motif in their E7 homology domain at their C-terminal regions. This motif is common to other RB-interacting proteins, such as human papillomavirus E7, simian virus 40 large T antigen, and adenoavirus E1A (61), and it was shown that the deletion of the E7 homology region from BRG1 and BRM completely eliminates its ability to bind to RB (28). The results of our mapping analysis indicate that the region of BRG1 that associates with EVI1 includes the E7 motif. It is therefore possible that the association with EVI1 could block the interaction between BRG1 and RB. These data provide insight on a novel pathway that could lead to a better understanding of the mechanism of up-regulation of cell proliferation by EVI1.

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