Recruitment of SWI/SNF complex is required for transcriptional activation of SLC11A1 gene during macrophage differentiation of HL-60 cells

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The solute carrier family 11 member 1 (SLC11A1) gene is strictly regulated and exclusively expressed in myeloid lineage cells. However, little is known about the transcriptional regulation of the SLC11A1 gene during myeloid development. In the current study, we used HL-60 cells as a model to investigate the regulatory elements/factors involved in the transactivation of the SLC11A1 gene during phorbol 12-myristate 13-acetate (PMA)–induced macrophage differentiation of HL-60 cells. Promoter deletion analysis shows that a seven-base AP-1-like element (TGACTCT) is critical for the SLC11A1 promoter’s responsiveness to PMA. Stimulation by PMA induces the binding of ATF-3 and the recruitment of two components of the SWI/SNF complex, BRG1 and β-actin, to this element in an ATF-3-dependant manner. RNAi-mediated depletion of ATF-3 or BRG1 markedly decreases SLC11A1 gene expression and its promoter activity induced by PMA. Luciferase reporter experiments demonstrate that ATF-3 cooperates with BRG1 and β-actin to activate the SLC11A1 promoter. Furthermore, we showed that PMA can induce the proximal (GT/AC)n repeat sequence to convert to Z-DNA structure in the SLC11A1 gene promoter, and depletion of BRG1 results in a significant decrease of Z-DNA formation. Our results demonstrate that recruitment of the SWI/SNF complex initiates Z-DNA formation and subsequently helps to transactivate the SLC11A1 gene.

The solute carrier family 11 member 1 (SLC11A1) gene, also known as Ity/Lsh/BCG or natural resistance-associated macrophage 1 (NRAMP1) gene, is associated with host resistance to infection. In mice, mutations in the Slc11a1 gene, whether naturally occurring or experimentally induced, cause susceptibility to infection with unrelated intracellular pathogens such as Salmonella, Leishmania and Mycobacteria (1-5). The multiple pleiotropic effects of the SLC11A1 gene on macrophage activation, including regulation of the chemokine KC and cytokines (e.g., TNFα), as well as induction of nitric oxide (NO) release, MHC class II molecule expression and oxidative burst (6,7), displays its potential importance in autoimmune and infectious diseases. In humans, polymorphic variants of the SLC11A1 gene are associated with susceptibility to infectious diseases such as tuberculosis, leprosy, and HIV infection, as well as autoimmune diseases such as rheumatoid arthritis, sarcoidosis, diabetes, and Crohn’s disease (6,8). Furthermore, the polymorphisms of the SLC11A1 gene have been linked with esophageal cancer risk (9).

In humans, the SLC11A1 gene is located on chromosome 2q35 and has 15 exons spanning...
about 14 kb. The gene encodes a transmembrane protein exclusively expressed in the myeloid lineage: monocytes, macrophages, polymorphonuclear neutrophils and dendritic cells (10,11). SLC11A1 gene expression is strictly regulated during myeloid development and SLC11A1 protein expression parallels with its mRNA level (12), suggesting that SLC11A1 expression may be controlled primarily at the level of transcription. The human promyelocytic leukemia cell line HL-60 has been shown to be a useful model to study the regulation of SLC11A1 gene expression. SLC11A1 gene expression is undetectable in HL-60 cells; however, it can be strongly induced both at mRNA and protein levels in these cells when differentiated towards either the monocyte/macrophage pathway or the granulocyte pathway (10,13). Previous studies have shown that PMA induces the transcriptional activation of the SLC11A1 gene, and its mRNA stability is mediated by HuR-AU-rich element interaction in HL-60 cells (13). However, the molecular mechanism of transcriptional activation of the SLC11A1 gene during the macrophage-like differentiation remains largely unknown. Identification of the specific determinants controlling SLC11A1 expression in response to PMA treatment demonstrated in this study sheds light on the regulatory cis-acting elements and trans-acting factors involved during myeloid differentiation and immune responses. This will further improve our understanding of the possible influence of SLC11A1 promoter gene polymorphisms in human susceptibility to diseases.

Chromatin remodeling is involved in the regulation of gene transcription, including pre-initiation complex formation, transcriptional initiation, and elongation (14-18). Currently, several ATP-dependent chromatin remodeling complexes have been characterized by the identity of their central catalytic subunit, including the SWI/SNF complex (19). The SWI/SNF chromatin remodeling complex can alter chromatin structure by either shifting nucleosomes along the DNA or twisting DNA to modulate the nucleosome structure in an ATP-dependent manner (20), thereby yielding a permissive or non-permissive state. In yeasts, SWI/SNF complexes regulate hundreds of genes involved in a wide variety of cellular functions through strictly controlled targeting mechanisms (21,22) and can both promote and suppress gene expression (23,24). There is a growing body of evidence demonstrating the roles of the SWI/SNF complex in cell differentiation (25-27), proliferation (28-30), neural development (31), hematopoietic development (32-34), as well as in malignant processes (35). The human SWI/SNF complex includes a heterogeneous mixture of proteins, where most purified complexes contain BRG1 (or hBRM) as the central ATPase subunit, as well as BRG1 (or hBRM)-associated factors (BAFs) such as BAF47/INI1 and actin (36,37). Biochemical analysis demonstrates that β-actin is directly bound to BRG1 in the SWI/SNF complex. The association of β-actin with BRG1 is so tight that it is impossible to break this interaction without denaturing BRG1 (37). To date, all studies have shown that SWI/SNF complexes are recruited to target genes via association with transcription factors such as c-Myc (38) and C/EBPβ (39), or nuclear receptors (16) such as glucocorticoid receptor (40,41) and estrogen receptor (42,43).

PMA-induced chromatin remodeling is critical for the transcriptional regulation of MMP-9 gene transcription. Upon PMA stimulation, transcription factors, the SWI/SNF chromatin-remodeling complex and the coactivators CBP/p300, as well as coactivator-associated arginine methyltransferase I, are recruited to the MMP-9 promoter in a stepwise manner. This ordered recruitment results in a relaxed or "open" chromatin structure and allows for the binding of pol II to the promoter to initiate transcription (44). Transcriptional
activation of the HIV-1 promoter in human cells in response to PMA involves the recruitment of the SWI/SNF chromatin remodeling complex (45) and cellular proteins with histone acetyltransferase activity (46). Our previous study demonstrated that during PMA-induced differentiation of HL-60 cells towards macrophages, nuclear β-actin and RNA pol II are recruited to the SLC11A1 promoter and are involved in the transcriptional activation of the SLC11A1 gene (47). However, the mechanism by which β-actin contributes to transcriptional activation of the SLC11A1 gene remains unknown. β-actin is a component of SWI/SNF complex and has been shown to be involved in chromatin remodeling, suggesting a possible functional relationship between the β-actin-containing SWI/SNF complex and chromatin remodeling in the transcriptional activation of the SLC11A1 gene during the differentiation of HL-60 cells towards macrophages.

In the current study, to understand how the SLC11A1 gene is transactivated in response to PMA treatment, we used promoter deletion analysis to define a seven-base AP-1-like element in the SLC11A1 promoter which is critical for responsiveness to PMA. We found that the SWI/SNF complex is recruited to this element via interaction with transcriptional factor ATF-3, and is required for PMA–induced activation of human SLC11A1 gene transcription. The SLC11A1 gene promoter contains a (GT/AC)n repeat sequence that favors left-handed Z-DNA formation. Our results also demonstrate that PMA can induce the Z-DNA formation in the SLC11A1 gene promoter, and that BRG1 is essential during this process. Our results suggest that recruitment of the SWI/SNF complex initiates Z-DNA formation and subsequently helps to transactivate the SLC11A1 gene.

**Experimental Procedures**

*Cells and culture*— HL-60 cells were purchased from the American Type Culture Collection, and maintained in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-Glutamine and 1% penicillin/streptomycin at 37°C in a 5% CO₂ incubator.

*Plasmid constructs*— pREP4-luc was constructed as described previously (23). For construction of SLC11A1 promoter -1625Luc, human genomic DNA (Roche, Laval, QC) was first amplified by PCR using the primers: 5’-ACTCTTGTCAACAAAGCTGAAGTGC-3’ and 5’-TCTTTGTTCTCAAGTCTCC ACCA-3’. The PCR product was then amplified again using a forward primer 5’-GAGCTAGCATGTTAAC CAGGATGGTCTCG -3’, bearing a NheI restriction site (underlined) and a reverse primer 5’- CAAGCTTATGCGCTCTGCTTACAT CA-3’ bearing a HindIII restriction site (underlined). The final PCR product (spanning nucleotides -1625 to +19 of the SLC11A1 promoter, numbers relative to the major transcription start site) was gel-purified and inserted into the NheI-HindIII sites of the pREP4-luc. The construct was sequenced to ensure accurate replication and used as a template for several other constructs: -938Luc, -550Luc, -395Luc, -264Luc and -161Luc using a constant reverse primer as the same as above bearing the HindIII restriction site and the following forward primers, respectively: 5’-GAGCTAGCATGAGCAAGACGCCATCTCA -3’; 5’-GAGCTAGCATGATCTGGTGACAAAT CTCAAGTG-3’; 5’-GAGCTAGCATGAGGAAAAGTCAAT GAGACTCGCATTTAGG-3’; 5’-GAGCTAGCT GTGGTCATGGGTTATGGAC-3’; 5’-GAGCTA GCATGTGTTGTTGGGCACAG-3’. The two deletion mutants [-550(ΔAP-1)Luc and -395(ΔAP-1)Luc], lacking the AP-1-like element TGACTCT, were constructed using QuickChange II XL site-directed mutagenesis kit. Primers used for deletion were 5’-AAAGAGATAAAGAAAGACCGTGTGTGTACGTGTG GT-3’ and 5’-CACACACGTACACACACACG
To create a pCG-β-actin expression plasmid, human β-actin was amplified by PCR using a pAcGFP1-Actin vector (BD Biosciences-Clontech, Mountain View, CA) as a template, and subcloned into XbaI/BamHI sites of the pCG plasmid. Plasmid pCG-ATF-3 was generously provided by Dr. Hai T. (Ohio state university, Columbus, OH). Two expression plasmids encoding human BRG1 (pBJ5 BRG1) and the ATPase-defective variant of BRG1 (pBJ5 BRG1 DN, K798R mutant) were obtained from AddGene (Cambridge, MA, USA).

**Expression and purification of ZaaFOK**

The fusion protein His-ZaaFOK was expressed and purified as previously described (48). Briefly, *Escherichia coli* BL21 were transformed with the plasmid pET-His-ZaaFOK (kindly provided by Dr. Zhao K., Laboratory of Molecular Immunology, NIH, Bethesda, MD), grown in LB medium (containing 60 µg/ml ampicillin) until OD₅₉₅ ≅ 0.45, and then induced with 1 mM IPTG (final concentration) for 3 hrs. After induction, cell pellets were washed with and then resuspended in lysis buffer (0.3M NaCl, 0.1mM EDTA and 0.5% NP-40 in 1×PBS). Following sonication, the lysate was centrifuged and the supernatant was incubated in Ni-NTA agarose beads (Amersham Biosciences, Baie d’Urfé, QC) for 30 min at 4°C. The beads were washed with wash buffer (lysosome buffer plus 15 mM imidazole) three times, and the recombinant proteins were then eluted from the beads with elution buffer (20 mM Tris, pH8.0, 10% Glycerol, 0.4 M imidazole, 1mM EDTA, 0.1 M NaCl and 0.1% NP-40). A protease inhibitor cocktail was added to the lysis buffer and elution buffer (Roche, Laval, QC) just before use.

**Western blot analysis**

After appropriate treatments, cells were collected and total cellular extracts or nuclear fractions were prepared. Western blot analysis was then performed as described previously(13). A mouse monoclonal antibody against β-actin and a rabbit polyclonal antibody against Brm were purchased from Sigma (Saint Louis, MO) and Abcam (Cambridge, MA), respectively. Rabbit polyclonal antibodies against BRG1 (sc10768x), ATF-3 (sc188x), c-fos (sc-253x), c-jun (sc-1694x), Jun B (sc-8051x) and Jun D (sc-74x) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

**DNA affinity pull-down assays**

DNA affinity pull-down assays were performed as described previously(45). Two hundred micrograms of Dynabeads M280 streptavidin (Dynal; Invitrogen) was prepared, concentrated and resuspended in 20 μl of buffer T (10mM Tris[pH 7.5], 1 mM EDTA, 1 M NaCl), which included 10 pmol of biotinylated AP-1 Wt or AP-1 mutant probe (Figure 2A). The mixture was gently agitated for 1 hr at room temperature (RT), and the beads were then washed 4 times in buffer T to remove unbound probes. Bead-coupled probes were equilibrated in buffer R (10 mM Tris[pH 7.5], 1mM MgCl₂, 0.1% NP-40, 1mM EDTA, 10 mM DTT, 5% glycerol, 60 mM KCl, 12 mM HEPES[pH 7.9], 0.03% BSA) for 30min, centrifuged and resuspended in buffer R containing 200 μg of nuclear extract and 40 ng/μl of poly(dG-dC) (120 μl final volume), and agitated for 30 min at RT. Following the binding reaction, the beads were washed three times using buffer R containing 10 ng/μl of poly(dG-dC). The bound proteins were eluted by boiling them in SDS sample buffer, and the presence of AP-1 family transcription factors, BRG1 and β-actin was detected by Western blot analysis.

**Chromatin Immunoprecipitation (ChIP) assay, Re-ChIP and ChIP-qPCR**

The ChIP assay was performed using a chromatin immunoprecipitation assay kit (Upstate, Lake Placid, NY) according to the manufacturer’s instructions. Briefly, cells were fixed, lysed and sonicated to yield short DNA fragments. The DNA/protein complexes were immunoprecipitated with either non-specific IgG
or the indicated specific antibodies. Immunoprecipitates were washed and eluted, and the cross-links were reversed. The precipitated DNA fragments were purified. The 5′-promoter region spanning the AP-1 like-element (nucleotide position -389 to -190) were amplified by PCR using a pair of primers: 5′-TGAAGACTCGCATTAGGCCAACGA-3′, and 5′- TGTGCCTCCAAAGTTAGCTCTGAT-3′. For Re-ChIP assays, after immunoprecipitation with the first antibody, the primary immunocomplex was eluted by 10 mM dithiothreitol with agitation at 37°C for 30 min. The eluate was diluted 50 times with buffer (20mM Tris-HCl, p 8.1, 150 mM NaCl, 2Mm EDTA, and 1% Triton X-100) and was immunoprecipitated using the second antibodies. For ChIP-qPCR assays, the precipitated DNA fragments were purified, and quantified with the Quant-iT™ dsDNA Assay Kit (Molecular Probes, Eugene, Oregon). They were then amplified by real-time qPCR using the same primers as for regular ChIP.

Quantitative real-time PCR- Total RNA was extracted from cells using TRIzol Reagent (Invitrogen, Burlington, ON, Canada) according to the manufacturer’s instructions. One µg of total RNA was reverse-transcribed with the QuantiTect reverse transcription kit (Qiagen, Mississauga, ON, Canada). An equal amount of cDNAs or purified DNA fragments from ChIP was then amplified by real-time PCR using the Stratagene Mx-4000 and the Brilliant®SYBR Green QPCR Master Mix. Gene expression was normalized to a house-keeping gene (GAPDH) and the relative expression values between the samples were calculated based on the threshold cycle (C_T) value using the 2^-ΔΔCT method(49). The following forward and reverse SLC11A1-specific primers were used for cDNA amplification: 5′-AGCGGACATCGAGAAG CCAACAT-3′ and 5′-CTGCCCAGAAG GACAGC CATGACAAA-3′, respectively.

Small RNA interference experiment- HL-60 cells were transiently transfected with control, ATF-3 or BRG1 siRNA using the Cell Line Nucleofector® kit V (Amaxa, Gaithersburg, MD) according to the manufacturer’s instructions. Briefly, 2×10^6 log-growth cells were suspended in 100 µl of Cell Line Nucleofector® Solution V and mixed with 100 pmol of control siRNA, ATF-3 siRNA or BRG1siRNA (1µM at final concentration). The siRNA duplexes used in the experiment are as follows: the previously described siATF-3(50), 5′-GCACCUCUGCCACCGGAUGdTdT-3′ and 5′-CAUCCGGUGGCGAGGGUGCdTdT-3′ (synthesized by Dharmacon, Lafayette, CO); the siRNAs against BRG1 and control siRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Transfection was performed with a Nucleofector® II device using the program T-019. Transfected cells were cultured for 6 hrs at 37 °C in 5% CO₂ and were then further treated with PMA (10 ng/ml) for 48 hrs or left untreated before cell harvest.

Immunoprecipitation assays- The immunoprecipitation assays were prepared as described previously (51). For IP, protein A-Sepharose beads (Sigma) were coated with the appropriate antibodies against ATF-3 (Santa Cruz), BRG1 (Santa Cruz) or nonspecific IgG as a control. Antibody-coated beads were then added to a mixture of 100µl (200µg) nuclear lysate and 400 µl NT2 buffer (50mM Tris-HCl[pH7.4], 150mM NaCl, 1mM MgCl₂, and 0.05% NP-40), and the mixture was rotated gently at 4°C for 2 hrs. After washing four times with NT2 buffer, the precipitated proteins were eluted by adding 100 µl 2×SDS sample buffer and heating at 95°C for 10 min. Samples were resolved on a 4-12% Bis-Tris gel (Invitrogen, Burlington, ON) and electrophoretically transferred to PVDF membranes (Millipore, Bedford, MA). The blots were blocked with 5% milk in TBS-T (Tris-buffered saline/0.1% Tween 20) for 2 hrs and were then incubated with primary antibodies recognizing ATF-3 or
BRG1 for 1 hr (both at RT). After washing 5 times with TBS-T (5 min. each), blots were incubated with a 1:5000 dilution of Protein A-HRP (Amersham) for another 1 hr at RT. The blots were washed the same way as above and developed with ECL detection reagents (Amersham).

**Luciferase activity** - To detect the effect of knockdown of ATF-3 or BRG1 on the transcriptional activity, the HL-60 cells were co-transfected with luciferase reporter constructs and control, ATF-3 or BRG1 siRNA. To detect the overexpression of ATF-3, BRG1 or β-actin on the transcriptional activity, plasmids expressing BRG1, BRG1DN mutant, ATF-3 or their different combinations, as well as luciferase reporter constructs, were co-transfected into HL-60 cells. Transfections were performed using the Cell Line Nucleofector™ kit V (Amaza, Gaithersburg, MD) according to the manufacturer’s instructions. As needed, empty plasmids pCG and pBJ5 were added to adjust the total amount of transfected DNA. Six hrs after transfection, cells were treated with PMA (10 ng/ml) for 48 hrs or left untreated; following this the cells were harvested. Luciferase reporter assays were performed using the Dual-Luciferase® Reporter Assay System (Promega, Madison, WI) and the luminescence was measured with a Turner Designs model TD-20/20 luminometer. Firefly luciferase activity was normalized to Renilla luciferase activity.

**Detection of Z-DNA structure** - Detection of Z-DNA structure was performed as described previously (48,52). HL-60 cells were transfected with empty vector pREP4-Luc, reporter construct -395 Luc or siRNA specific to BRG1. Six hrs after transfection, transfected or untransfected HL-60 cells were treated with PMA for 48 hrs and the cells were then cross-linked with 1% formaldehyde for 15 minutes. Following cross-linking, the cells were washed once with 1M glycine and once with 1×PBS, and then permeabilized with 0.5% Triton-X 100 in 1×PBS for 3 minutes. The cells were digested with 5ng/µl ZaaKOF in 100 µl of l × NEB4 buffer (20 mM Tris-acetate [pH 7.0], 10mM magnesium acetate, 50 mM potassium acetate and 1 mM DTT) for 30 min at 37°C. The digestion was stopped by the addition of 100 µl of 1mg/ml proteinase K and 1% SDS in TE buffer, and was maintained at 55°C for 2 hrs. After reverse cross-linking at 65°C for 6 hrs, the DNA was purified, digested completely with Neol and analyzed by linker ligation-mediated PCR (LM-PCR). Primers used in LM-PCR are as follows: universal linker primers (WL1: 5’-GCGGTGACCCGGGAGATCTGAATTC-3’ and WL2: 5’-GAATTCAGATC-3’), and primers specific to the luciferase cDNA in the reporter construct (reverse PCR primer: 5’-TATGACATTGCTTCCACGC-3’ and reverse labeling primer: 5’-GGTTCCATCTTCCACGGATAGA A-3’) or to the endogenous SLC11A1 promoter (forward PCR primer:5’-CATGATCTGGTGCAATCTCAAGTG-3’ and forward labeling primer: 5’-CATGTCCCTTCTGCAGTGCCT-3’).

**Statistical analysis** - All data are presented as means ± standard errors (SE) of three or four experiments. Analysis was performed using unpaired student’s t test. P<0.05 was considered significant.

**Results**

**Identification of the PMA responsive element** - We have previously demonstrated that PMA can activate the human SLC11A1 gene transcription (13). To identify the cis-acting elements responsive to PMA stimulation, we generated a series of deletion constructs (Figure 1A) and evaluated them in transient transfection assays. The luciferase reporters -1625Luc, -938Luc, -550Luc, -395Luc, -264Luc and -161Luc represent their 5’-ends corresponding to nucleotide position -1625, -938, -550, -395, -264 and -161 from the transcription start site,
respectively. As shown in Figure 1B, PMA stimulated luciferase activity driven by the SLC11A1 gene promoter. The promoter activities of -1625Luc, -938Luc, -550Luc and -395Luc were markedly increased following PMA treatment, whereas -264Luc and -161 Luc were not affected, indicating that the sequence between nucleotide -395 and -264 is required for PMA response. Inspection of the sequence between nucleotides -395 to -264 revealed the presence of an AP-1-like element (TGACTCT). To test if the AP-1-like element is necessary for induction of SLC11A1 gene promoter activity in response to PMA, this element was deleted in the luciferase reporters -550 Luc and -395 Luc. As shown in Figure 1B, in PMA-treated HL-60 cells, the luciferase activity driven by these two mutant reporter constructs was significantly lower than the activity driven by the corresponding wild-type reporters, revealing that the 7-base element is essential for induction of luciferase activity by PMA. Our results demonstrate that the AP-1-like element within the proximal region of the SLC11A1 gene promoter is necessary for PMA-induced transcriptional activation of this gene.

**Binding of ATF-3/Jun B to the AP-1-like element is inducible by PMA**- The AP-1-like element is also known as ATF-3 binding site, which has been shown to be bound by ATF and AP-1 transcription factor families. To identify the proteins that interact with the AP-1-like element, DNA affinity pull-down assays were performed using antibodies against ATF-3 and members of the AP-1 transcription factor family, such as c-Jun and Jun B, as indicated. The wild-type and mutant probes used in this assay are shown in Figure 2A. Western blot analysis (Figure 2B, left panel) clearly showed that ATF-3, c-fos, c-Jun, Jun B and Jun D are all present in the nuclear extract, of which ATF-3, c-Jun and Jun B expression is PMA inducible. As shown in Figure 2B (middle panel), c-fos, c-Jun, and Jun D show little or no affinity for the AP-1-like element, irrespective of PMA treatment. Interestingly, binding of ATF-3 and Jun B to this element increased in response to PMA treatment. None of these transcription factors is bound to the mutant probe. In order to know if ATF-3 and Jun B are associated with the AP-1-like element within the promoter of the SLC11A1 gene in living cells, we performed ChIP assays using antibodies to ATF-3 and Jun B. An antibody against Jun D or nonspecific rabbit IgG was used as a control. A pair of primers spanning a 200-bp DNA fragment (-389/-190) of the proximal region encompassing the AP-1-like element was used for PCR. As shown in Figure 2C (left panel), binding of ATF3 and Jun B to the region spanning the AP-1-like element was obviously increased in response to PMA treatment in living cells. However, neither ATF-3 nor Jun B was associated with the far upstream region of the SLC11A1 gene promoter in living cells (Figure 2C, right panel). The results demonstrate that binding of ATF-3/Jun B to the AP-1-like element at SLC11A1 promoter is PMA-inducible.

**BRG1 and β-actin are recruited to the AP-1-like element in response to PMA**- Given that ATF3 can recruit BRG1 to a gene promoter (e.g. human immunodeficiency virus type 1 promoter) (45) and that β-actin is involved in the transactivation of the SLC11A1 gene in response to PMA (47), we wanted to investigate whether the two components of SWI/SNF complex, β-actin and BRG1, are recruited to the AP-1-like element during the differentiation of HL-60 cells towards macrophages. HL-60 cells were treated with PMA (10ng/ml) for 24 and 48 hrs, and DNA affinity pull-down assays were performed using anti-BRG1 or anti-β-actin antibodies. As shown in Figure 3A, the recruitment of BRG1 and β-actin to the wild-type probe was markedly enhanced in response to PMA; however, neither BRG1 nor β-actin binds to the mutant probe even in response to PMA treatment. The negative control, Jun D, was not associated with
either wild-type or mutant probes. To investigate whether BRG1 and β-actin are associated with the proximal region of the SLC11A1 gene in living cells, we performed ChIP assays using antibodies to BRG1, β-actin or nonspecific IgG as a control. As shown in Figure 3B, both the antibodies recognizing BRG1 and β-actin precipitated the same promoter fragment as ATF-3 and Jun B antibodies did (Figure 2C), while the nonspecific rabbit or mouse IgG failed to do so. Our results suggest that β-actin and BRG1 may be recruited to the AP-1-like element via their interaction with transcription factor ATF-3.

**BRG1 and β-actin are recruited to the AP-1-like element of the SLC11A1 gene promoter in an ATF-3-dependent manner.** To determine whether ATF-3 actively recruits BRG1 and β-actin to the AP-1-like element, we transfected ATF-3 specific siRNA or control siRNA into HL-60 cells and treated the cells with PMA for 48 hrs. The effect of ATF-3 knockdown on the binding of BRG1 and β-actin to the AP-1-like element of the SLC11A1 gene promoter was then analyzed by using a DNA affinity pull-down assay. As shown in Figure 4A, transfection of specific ATF-3 siRNAs into HL-60 cells led to a substantial down-regulation of ATF-3 protein levels (approximately 63% decrease), without any effects on BRG1 and β-actin protein expression. The protein expression of Braham (Brm), an alternative ATPase subunit of the SWI/SNF complex, was also analyzed and its expression level was not affected by ATF-3 knockdown. Transfection using a control siRNA had no effect on either ATF-3, BRG1, β-actin or Brm protein levels. Interestingly, binding of either BRG1 or β-actin to the AP-1-like element in the SLC11A1 gene promoter was significantly diminished when using nuclear extract from ATF-3 siRNA-transfected cells (Figure 4B). The Brm did not bind to the AP-1-like element, suggesting that BRG1 but not Brm is specifically recruited to this element (Figure 4B). To further test the role of ATF-3 in mediating BRG1 and β-actin to bind to the SLC11A1 promoter in living cells, a ChIP-qPCR assay was performed using HL-60 cells transfected with ATF-3 siRNA. As shown in Figure 4C, transfection with ATF-3 siRNA significantly decreased the amount of BRG1 (left panel) and β-actin (right panel) bound to the SLC11A1 promoter. These results suggest that the ATF-3 transcription factor is essential for efficient binding of BRG1 and β-actin to the AP-1-like element in the SLC11A1 promoter.

**PMA induces complex formation and the binding of ATF-3, BRG1 and β-actin to the SLC11A1 gene promoter in HL-60 cells.** Since knockdown of ATF-3 diminished BRG1 and β-actin binding to the SLC11A1 gene promoter, we postulated that BRG1 and β-actin are recruited to the SLC11A1 promoter by forming a complex with ATF-3. To test this hypothesis, we performed co-immunoprecipitation experiments using nuclear extracts from HL-60 cells treated with PMA for 24 and 48 hrs, or which were left untreated. As shown in Figure 5A (upper panel), PMA induced the complex formation among ATF-3, BRG1 and β-actin. The BRG1 and β-actin were found in the complex with ATF-3 but not with the IgG control. The presence of ATF-3, BRG1 and β-actin in the nuclei was also detected by Western blot analysis. As shown in Figure 5A, bottom panel, the nuclear protein levels of ATF-3 and β-actin were significantly increased after 24 and 48 hrs of treatment with PMA while the protein level of Brg-1 did not change significantly. These co-immunoprecipitation assays were repeated in a reciprocal fashion using an anti-BRG1 antibody. Inducible association of ATF-3, β-actin and BRG1 was again detected by immunoblotting of these complexes with anti-ATF-3 or anti-β-actin antibodies (Figure 5B). We next determined by Re-ChIP analyses whether BRG1 and β-actin are associated with the ATF-3-containing SLC11A1 promoter fragment in living cells. Briefly,
chromatin fragments from cross-linked HL-60 cells were first immunoprecipitated using antibodies against ATF-3. The resulting immunocomplex was then eluted and subjected to immunoprecipitation using antibodies against BRG1 or β-actin. PCR analysis was then used to determine whether the promoter fragment was present in the final precipitate. As shown in Figure 5C, we found that the promoter fragment present in the first immunocomplex generated using anti-ATF-3 antibodies was pulled down again by either anti-BRG1 or anti-β-actin antibodies in PMA-treated cells. These results indicate an association among ATF-3, BRG1 and β-actin at the proximal promoter region containing the AP-1-like element in differentiating HL-60 cells.

**ATF-3 and BRG1 are required for PMA-induced expression of the SLC11A1 gene**

To further confirm the importance of ATF-3 and BRG1 in SLC11A1 gene expression, we used RNA interference to knockdown endogenous ATF-3 or BRG1. As shown in Figure 6A, transfection of HL-60 cells with specific ATF-3 or BRG1 siRNAs led to a significant down-regulation of ATF-3 and BRG1 protein levels, respectively, with no effect on β-tubulin expression. Transfection using a control siRNA had no effect on ATF-3, BRG1 and β-tubulin protein levels. As expected, SLC11A1 gene expression was significantly inhibited in ATF-3 or BRG1 knockdown cells. The levels of SLC11A1 protein and mRNA were reduced by 48% and 56%, respectively in ATF-3 knockdown cells, while in BRG1 knockdown cells they were reduced by 57% and 64% (Figures 6A and B). To further study the influence of ATF-3 and BRG1 on SLC11A1 promoter activity, we compared the SLC11A1 promoter-driven luciferase activity between HL-60 cells transfected with siRNA specific for ATF-3 or BRG1 and those with control siRNA. As shown in Figure 6C, ATF-3 or BRG1 knockdown had no significant effect on SLC11A1 promoter-driven transactivation of luciferase activity in untreated HL-60 cells; however, in PMA-treated cells, ATF-3 or BRG1 knockdown significantly decreased the luciferase activity driven by the SLC11A1 promoter. Our results demonstrate that ATF-3 and BRG1 are required for PMA-induced SLC11A1 gene transactivation.

**ATF-3 cooperates with the SWI/SNF complex to activate the SLC11A1 promoter**

Since the recruitment of BRG1 and β-actin to the SLC11A1 gene promoter is ATF-3–dependent and PMA inducible (Figures 2 and 3), we investigated the cooperation between ATF-3 and the SWI/SNF complex in transactivation of SLC11A1 gene in response to PMA stimulation. The expression plasmids for BRG1, a dominant negative mutant of BRG1, ATF-3, or different combinations of these plasmids were transiently co-transfected with luciferase reporter -395 Luc into HL-60 cells. As shown in Figure 7A, transfection of BRG1 alone increases PMA-induced transcriptional activity in a dose-dependent manner. Overexpression of dominant negative BRG1 inhibits the transcriptional activity, demonstrating that BRG1 activity is required for efficient transactivation of the SLC11A1 gene promoter. Co-transfection of ATF-3 and BRG1 caused an additive, but not synergistic, effect on the PMA-induced transcriptional activity. In order to further evaluate the cooperative effect of ATF-3 and the SWI/SNF complex, we performed luciferase reporter assays using the expression plasmids at lower concentrations. As shown in Figure 7B, transfection of expression plasmids for BRG1, ATF-3 or β-actin individually at lower concentrations (0.5 μg, 0.5 μg and 0.6 μg per transfection, respectively) with PMA treatment resulted in only a slight increase in transcriptional activity compared with PMA treatment alone. When BRG1 was used in combination with ATF-3, a clear additive effect on activation of the SLC11A1 gene promoter...
was seen. A similar result was also observed when β-actin was co-transfected with ATF-3. Not surprisingly, the highest induction of the transcriptional activity was obtained when BRG1, ATF-3 and β-actin were concomitantly transfected into HL-60 cells. In conclusion, these results reveal a cooperation between ATF-3 and SWI/SNF complex during the PMA-induced transcriptional activation of the SLC11A1 gene, and that ATPase activity of BRG1 is necessary for this process.

**BRG1 mediates Z-DNA-formation at the SLC11A1 gene promoter in response to PMA treatment**—BRG1-mediated transcriptional activation of the SLC11A1 gene and the recruitment of BRG1 to the SLC11A1 gene promoter strongly suggest that chromatin remodelling occurs at the promoter in response to PMA treatment. Through analysis of the SLC11A1 promoter, a Z-DNA forming dinucleotide repeat, t(gt)ac(gt)ac(gt)g, was identified immediately downstream of the AP-1-like element in the promoter (spanning nucleotides -273 to -317). This stretch of GT repeats is shown to have Z-DNA-forming propensity in vitro and in vivo (53). To confirm the Z-DNA formation in response to PMA stimulation, HL-60 cells were transfected with the reporter construct -395 Luc or the empty vector pREP4-Luc and the Z-DNA formation in the SLC11A1 promoter was detected by a combination of in vivo cross-linking and ZaaFOK digestion. ZaaFOK is a fusion protein which can specifically bind Z-DNA structure and make double-stranded cleavages within or around the Z-DNA region to which it is bound. The generation of cleavage sites was then detected by LM-PCR. As shown in Figure 8A, PMA treatment resulted in several new ZaaFOK cleavage sites within or near the GT/AC repeat sequence in the wild-type SLC11A1 gene promoter (compare lanes 3 and 4), indicating the formation of Z-DNA structure. However, no significant Z-DNA structure was detected in the empty vector (lanes 1 and 2) even in the presence of PMA. These results demonstrate that PMA can induce Z-DNA formation in the SLC11A1 promoter. As shown in Figure 8B, the formation of Z-DNA in the endogenous SLC11A1 promoter in response to PMA treatment was also detected. Similarly, several new cleavage products were generated when HL-60 cells were treated with PMA compared to the results from untreated HL-60 cells.

BRG1 assistance in Z-DNA formation is often needed to form open-chromatin structures in gene regulatory regions (23,52). Thus, we hypothesized that PMA-induced recruitment of the SWI/SNF complex to the AP-1-like element promotes the conversion of the (GT/AC)n repeat sequence to Z-DNA structure. To confirm this, HL-60 cells were transfected with BRG1 siRNA, and the effects of BRG1 depletion on Z-DNA formation were then analyzed. As expected, knockdown of BRG1 significantly reduced the intensity of bands (Figure 8C). Knockdown of BRG1 by RNA interference was analyzed using Western blot analysis. As shown in Figure 8D, BRG1 protein expression was significantly reduced in specific siRNA-transfected cells but not in control siRNA-transfected cells. As a control, Brm protein expression was not affected. Taken together, our results demonstrate that during the PMA-induced transcriptional activation of the SLC11A1 gene, recruitment of the SWI/SNF complex by ATF-3 to the SLC11A1 promoter is required for the (GT/AC)n repeat sequence to be converted to Z-DNA conformation.

**Discussion**

The *SLC11A1* gene expression is strictly regulated during myeloid differentiation. HL-60 cells have been shown to be a useful model to study the regulation of *SLC11A1* gene expression during experimentally induced granulocytic, monocytic or macrophage-like differentiation (10,13,54). Our previous study
demonstrated that HuR binds to an AU-rich element (ARE) present in the 3'UTR of SLC11A1 mRNA and significantly increases SLC11A1 mRNA stability and protein expression (13). Richer et al. demonstrated that transcription factors Sp1 and C/EBP are recruited to two cis-acting elements in the SLC11A1 promoter region and regulate its transcriptional activity during the monocytic differentiation of HL-60 cells by vitamin D(55). However, the role of the chromatin remodeling complex on the activation of SLC11A1 has not been determined. The present study provides evidence that during PMA-induced differentiation of HL-60 cells towards the macrophage pathway, two components of the SWI/SNF complex, BRG1 and β-actin, are recruited to the SLC11A1 gene promoter via interaction with transcription factor ATF-3, and activate gene transcription.

Role of ATF-3 in recruitment of the SWI/SNF complex to the SLC11A1 gene promoter- Using a series of deletion constructs, a promoter region, located between -395 and -264, was found to be necessary for PMA-induced transcriptional activation of the SLC11A1 gene. Sequence analysis of this promoter fragment revealed that it contains an AP-1-like element (TGACTCT). AP-1-like elements are highly homologous to the consensus AP-1 binding site (TGAC/GTCA) and are involved in transcriptional regulation of some genes via interaction with the AP-1 and ATF/CREB family factors or their dimmers (56). ATF3 is a member of the ATF/CREB transcription factor family and has at least 5 isoforms derived from alternative splicing events (57,58). The ATF-3 isoforms can form a homodimer or it can heterodimerize with other transcription factors such as Jun-B, c-Jun, ATF-2 and Smad3. In human umbilical vein endothelial cells (HUVECs) and cardiac myocytes, over-expression of ATF3 down-regulated p53 expression (59,60). The regulation occurred at the transcription level, through binding of ATF3 to the PF-1 sequence "TGACTCT", an AP-1-like site in the promoter of p53 (59,60). In this study, we observed that PMA induces ATF-3 to bind to the AP-1-like element within the SLC11A1 gene promoter. We also found that ATF-3 is required for recruitment of BRG1 and β-actin, two components of the SWI/SNF complex, to the SLC11A1 gene promoter, and for activating the transcription of the SLC11A1 gene. Therefore, ATF-3 may function either as a transcriptional activator or repressor according to its partner, target gene and cellular context.

Role of BRG1 and β-actin in the chromatin remodeling complex- Previous studies have shown that β-actin participates in chromatin remodeling as a component of the human SWI/SNF complex during gene activation (37,61,62). It has been proposed that β-actin and actin-related proteins are required for the maximum ATPase activity of SWI/SNF (61,63) and for the stable association of the chromatin remodeling complex with chromatin (64). Recently, it has been demonstrated that β-actin binds to the SLC11A1 promoter, and is involved in transcriptional activation of the SLC11A1 gene during the PMA-induced macrophage-like differentiation of HL-60 cells (47). In this study, we have established that β-actin is recruited to the AP-1-like element together with BRG1, suggesting that it is involved in chromatin remodeling during the transactivation of the SLC11A1 gene in differentiating HL-60 cells.

Modulation of chromatin structure by ATP-dependent remodeling SWI/SNF complexes has been implicated in cell differentiation, proliferation and transcriptional control of tissue-specific and inducible genes (25,26,28,29,32,65,66). The functional selectivity of the SWI/SNF complex at specific genes is attributable to its recruitment to the target genes through interaction with sequence-specific transcriptional activators or repressors (67,68). BRG1, one of the ATPase
subunits of the SWI/SNF chromatin remodeling complex, plays a critical role in SWI/SNF-mediated transcriptional regulation (69). The essential role of BRG1 in hematopoietic development has previously been established (33,34). A more recent study has demonstrated that Brg1 and INI1, two core subunits of the hSWI/SNF complex, associate with the acute myeloid leukemia 1(AML1/ RUNX1) transcription factor, and are recruited to RUNX1 target gene promoters to control hematopoietic-specific gene expression(32). Studies with ATPase-deficient BRG1 have demonstrated that the ATPase activity of SWI/SNF is required for expression of tissue-specific genes in muscle (27,70), adipose (71) and mammary epithelial cells (72), as well as for myeloid differentiation to granulocytes (33). Chromatin immunoprecipitation analysis revealed that NF-E2-related factor (Nrf2) recruits BRG1 to the regulatory region of its target gene HO-1, and the chromatin-remodeling activity of BRG1 is specifically required for HO-1 induction in response to oxidative stress(73). Similarly, the present study shows that BRG1 is recruited to the AP-1-like element in the SLC11A1 gene promoter in an ATF-3-dependent manner. BRG1 knockdown markedly decreased the PMA-induced expression of the SLC11A1 gene, as well as the transcriptional activity of its promoter. Furthermore, overexpression of BRG1 mutant also leads to a reduction in the PMA-induced transcriptional activity of the SLC11A1 gene promoter. Our results demonstrate that the ATPase activity of SWI/SNF is necessary for transcriptional activation of the SLC11A1 gene during macrophage differentiation. 

Role of Z-DNA formation in transcriptional activity- DNA is capable of adopting different conformational states besides the canonical Watson-Crick B-DNA. One of the best characterized alternative DNA conformations is left-handed Z-DNA, which can be formed by stretches of dinucleotide repeats such as (CG)n, (TG)n or (CA/TG)n (74,75). In the promoter region of SLC11A1, nine different alleles (alleles 1-9) with polymorphisms containing a functional Z-DNA forming repeat (GT/AC)n have been reported in different populations worldwide. Bayele HK et al. (53) demonstrated that a polymorphism of (GT/AC)n dinucleotides is associated with the transcriptional activity of the SLC11A1 promoter, and that the (GT/AC)n repeat has a propensity to form Z-DNA in vitro and in vivo. In this study, we confirm that the (GT/AC)n repeat (t(gt)5ac(gt)5ac(gt)9g) can be converted into Z-DNA structure in the SLC11A1 promoter in response to PMA stimulation, and we demonstrate that recruitment of the chromatin remodeling factor BRG1 to the SLC11A1 promoter is essential for Z-DNA formation. Taken together, these data suggest that a polymorphism in the (GT/AC)n repeat sequence may affect Z-DNA formation; consequently, it would affect the transcription and expression of the SLC11A1 gene.

In summary, we found that chromatin remodeling is involved in the transcriptional regulation of SLC11A1 gene expression during the macrophage-like differentiation of HL-60 cells. We demonstrated that a proximal (GT/AC)n repeat sequence is converted into Z-DNA conformation by the cooperation between transcription factor ATF-3 and SWI/SNF chromatin remodeling complex. Our findings provide new insights into the mechanism of transcriptional regulation of the SLC11A1 gene during myeloid development.
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**FOOTNOTES**

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The abbreviations used are: SLC11A1, solute carrier family 11 member 1; PMA, phorbol 12-myristate 13-acetate; BRG1, brahma-related gene 1; ChIP, Chromatin Immunoprecipitation; siRNA, small interference RNA; LM-PCR, linker ligation-mediated PCR


**FIGURE LEGENDS**

**Fig. 1.** The AP-1-like element is required for SLC11A1 transcriptional activation by PMA. A, schematic representation of the SLC11A1 reporter constructs. The AP-1-like element (also known as ATF-3 binding site) is shown. B, HL-60 cells were transfected with the indicated SLC11A1 promoter-reporter constructs. Six hours after transfection, cells were incubated in the presence or absence of 10 ng/ml PMA for 48 hrs before harvesting and were analysed for luciferase activity. The data shown (mean±SE) are the averages of at least three independent experiments performed in triplicate. Compared with corresponding wild-type promoter, *P*<0.001

**Fig. 2.** PMA induces the binding of ATF-3/Jun B to the AP-1 like element. A, nucleotide sequences of the two probes (AP-1 Wt and AP-1 mutant) used for isolation of the proteins that bind to the AP-1-like element. B, HL-60 cells were left untreated or treated with 10 ng/ml PMA for 24 and 48 hrs, and nuclear extracts (NE) were then prepared. Proteins that bind to the AP-1-like element at each time point were isolated from the nuclear extracts using the DNA affinity pull-down assay. They were then subjected to Western blot analysis with antibodies raised against different AP-1/ATF family members. Unpurified total nuclear extracts were also subjected to Western blot analysis and probed with the same antibodies. C, HL-60 cells were grown in the presence or absence of PMA for 24 and 48 hrs. Cells were then cross-linked and subjected to sonication, and the chromatin fragments were immunoprecipitated (ChIP) with the antibodies against ATF-3, Jun B or nonspecific rabbit IgG (rIgG) as a control. Immunoprecipitated DNA and input genomic DNA were amplified using primers targeting the core region (-389/-190, left panel) or the far upstream region (-3260/-3083, right panel) of the SLC11A1 promoter. PCR products were analyzed using 2.0 % agarose gels and stained with ethidium bromide.

**Fig. 3.** BRG1 and β-actin are recruited to the AP-1-like site in response to PMA. A, nuclear extracts were prepared from untreated HL-60 cells or cells treated with 10 ng/ml PMA for 24 and 48 hrs. Proteins that bind to the wild type (middle panel) or mutant (right panel) AP-1-like binding site were isolated from the nuclear extracts by using the DNA affinity pull-down assay, and resolved on a 4-12% Bis-Tris gel. The presence of BRG1 and β-actin was detected by Western blot analysis. Jun D was used as a negative control. The expression of BRG1 and β-actin in total nuclear extracts was also detected (left panel). B, ChIP assay was performed as described in Figure 2C, except that BRG1 and β-actin antibodies were used for immunoprecipitation. Nonspecific rabbit IgG (rIgG) and mouse IgG (mIgG) were used as controls.

**Fig. 4.** ATF-3 mediates the recruitment of BRG1 and β-actin to the SLC11A1 promoter. HL-60 cells were transfected alone with Mock (transfection reagent only), control siRNA (CTR) or siRNA specific for ATF-3. Six hrs after transfection, HL-60 cells were treated with PMA (10 ng/ml) for 48 hrs. A, total cell extracts were prepared from transfected HL-60 cells and protein expression was analyzed by Western blotting using antibodies specific to ATF-3, BRG1, β-actin and Brm. B, nuclear extracts were prepared and the binding of ATF-3, BRG1, β-actin and Brm to the AP-1-like element was analyzed by using the DNA affinity pull-down assay together with Western blot analysis. The probe used in the assay is AP-1 Wt. C, Cells were cross-linked and sonicated and ChIP assay was performed as described in Figure 3B. The precipitated DNAs and input DNA were quantified and then
amplified using real-time qPCR. The occupancy level of BRG1 or β-actin at the SLC11A1 promoter is represented as the ratio of signal from IP samples versus that of the input minus background of IgG control. The relative occupancy level of BRG1 or β-actin in mock-treated HL-60 cells is set as 100%. Data presented as mean ± SE (n=3).

**Fig. 5.** ATF-3 forms a complex with BRG1 and β-actin in response to PMA. A, nuclear extracts prepared from HL-60 cells left untreated or treated with PMA (10 ng/ml) for 24 and 48 hrs were subjected to immunoprecipitation (IP) with an ATF-3 antibody or nonspecific rabbit IgG (rIgG) as a negative control. The bound proteins were eluted by boiling in SDS sample buffer, separated on a NuPAGE 4-12% Bis-Tris gel and analyzed by Western blotting with antibodies against BRG1, β-actin and ATF-3 (upper panel). Unimmunoprecipitated total nuclear extracts were also subjected to Western blot analysis using the same antibodies (bottom panel). B, co-immunoprecipitation experiments and Western blot analyses were performed as in (A), except using BRG1 antibody for IP. The precipitated proteins were probed with antibodies against ATF-3, β-actin and BRG1. C, ChIP was first carried out using the ATF-3 antibody, and the immunocomplex was eluted using 10 mM dithiothreitol. The aliquots of the diluted elution were immunoprecipitated with antibodies against BRG1, β-actin or non-specific rIgG and mIgG used as negative controls. The precipitated DNA fragments were amplified by PCR using the primers specific to the SLC11A1 promoter region containing the AP-1-like element. The illustrated results are representative of three independent experiments.

**Fig. 6.** RNA interference-mediated depletion of BRG1 and ATF-3 reduces PMA-induced SLC11A1 expression. SLC11A1 gene expression is inhibited in BRG1 and ATF-3 knockdown cells. HL-60 cells were transfected with mock, siRNA control, siRNA specific for ATF-3 (siATF-3) or siRNA specific for BRG1 (siBRG1). Six hours after transfection, HL-60 cells were left untreated or treated with PMA for 64 hrs. A, total cell extracts were prepared from PMA-treated HL-60 cells and Western blot analysis was performed to detect the protein expression levels of ATF-3, BRG1, SLC11A1 and β-tubulin. The expression of β-tubulin is shown as a loading control (left panel). Protein expression was also quantified (right panel). Results represent the mean ± SE for three independent experiments. Compared with corresponding protein level in mock-treated cells,*P<0.01, **P<0.001. B, total RNA was isolated from PMA-treated HL-60 cells using TRIzol reagent. SLC11A1 mRNA levels were analyzed using real-time RT-QPCR. The relative SLC11A1 mRNA level is represented as a percentage of the SLC11A1 mRNA level in mock-treated HL-60 cells. Data are expressed as mean±SE of four independent experiments. Compared with the SLC11A1 mRNA level in mock-treated HL-60 cells, *P<0.001. C, effects of ATF-3 and BRG1 knockdown on the activation of the SLC11A1 promoter. The luciferase reporter vector pREP4-SLC11A1 (-395)-Luc (-395Luc) was co-transfected into HL-60 cells respectively with mock, siRNA control, siRNA specific for ATF-3 (siATF-3) or siRNA specific for BRG1 (siBRG1). The pRL-CMV reporter was also transfected as an internal control. Six hrs post-transfection, transfected cells were cultured for 48 hrs with or without 10 ng/ml of PMA, and the luciferase activity was analyzed by the dual-luciferase reporter assay system. Relative luciferase activity is expressed as a fold of the luciferase activity of HL-60 cells transfected with mock and treated with PMA. The data shown (mean±SE) are the averages of three independent experiments performed in triplicate. *P<0.001, when compared with the group of cells transfected with mock and treated with PMA.
**Fig. 7.** ATF-3, BRG1 and β-actin cooperate to activate the SLC11A1 promoter. A, HL-60 cells were transiently transfected with luciferase reporter construct -395Luc alone or in combination with different amounts of expression plasmids for BRG1, BRG1 mutant and ATF-3 as indicated. B, HL-60 cells were transiently transfected with luciferase reporter construct -395 Luc alone or in combination with expression plasmids for BRG1, β-actin and ATF-3 as indicated. Six hrs after transfection, the cells were treated with or without PMA (10 ng/ml) for another 48 hrs and subjected to luciferase assays. Results of the luciferase assays are expressed as relative luciferase activity (fold change), as compared with luciferase activity of HL-60 cells transfected with -395 Luc alone. The data shown (mean±SE) are the averages of three independent experiments performed in triplicate.

**Fig. 8.** Recruitment of BRG1 to the AP-1-like element is required for PMA-induced Z-DNA formation at the SLC11A1 promoter. A, Control reporter plasmid pREP4-Luc or luciferase reporter plasmid -395Luc were individually transfected into HL-60 cells. Six hours after transfection, cells were left untreated or treated with 10 ng/ml PMA for 48 hrs. Following cross-linking with formaldehyde, the cells were permeabilized and treated with ZaaFOK. The cleavage sites in the SLC11A1 promoter in the reporter constructs were detected by LM-PCR using primers specific to the luciferase cDNA in the constructs. The GT/AC repeat region is indicated on the right. B, HL-60 cells were cultured in the presence or absence of 10ng/ml PMA for 48 hrs. The cells were then cross-linked, permeabilized and treated with ZaaFOK. The DNA was purified and digested completely with NcoI, which recognizes a site downstream of the (GT/AC)n repeat sequence in the endogenous SLC11A1 promoter. The cleavage sites were detected by LM-PCR using SLC11A1 promoter-specific primers upstream of the repeat sequence. The GT/AC repeat region is indicated on the right. C, HL-60 cells were transfected with control siRNA or BRG1-specific siRNA. Six hours after transfection, cells were treated with 10 ng/ml PMA for 48 hrs. The Z-DNA structure was detected as described in B. D, The knockdown of BRG1 by RNA interference experiments in C was analyzed by Western blot analysis. Expression of Brm protein was also detected as a control. The result shown is representative of three independent experiments.
Figure 1

A

-1625
-938
-550
-550(ΔAP-1)
-395
-395(ΔAP-1)
-264
-161

AP-1 like element
Luc
Luc
Luc
Luc
Luc
Luc
Luc

B

Relative luciferase activity

-1625 -938 -550 (ΔAP-1) -550 (ΔAP-1) -395 (ΔAP-1) -395 (ΔAP-1) -264 -161

pREP4-SLC11A1-luc

- PMA
+ PMA

20
Figure 2

A  

AP-1wt  5’-biotin-TAAGAAAGACCTGACTCTGTGTGTGTAC-3’

AP-1mut  5’-biotin-TAAGAAAGACCACACACTGTGTGTGTAC-3’

B  

| PMA (h) | Total NE | DNA affinity pull-down | AP-1 wt | AP-1 mut |
|---------|----------|------------------------|---------|----------|
|         | - 24 48  |                        |         |          |
| ATF-3   | ![ATF-3 Image](image1.png) | ![ATF-3 Image](image2.png) | ![ATF-3 Image](image3.png) | ![ATF-3 Image](image4.png) |
| c-fos   | ![c-fos Image](image1.png) | ![c-fos Image](image2.png) | ![c-fos Image](image3.png) | ![c-fos Image](image4.png) |
| c-jun   | ![c-jun Image](image1.png) | ![c-jun Image](image2.png) | ![c-jun Image](image3.png) | ![c-jun Image](image4.png) |
| Jun B   | ![Jun B Image](image1.png) | ![Jun B Image](image2.png) | ![Jun B Image](image3.png) | ![Jun B Image](image4.png) |
| Jun D   | ![Jun D Image](image1.png) | ![Jun D Image](image2.png) | ![Jun D Image](image3.png) | ![Jun D Image](image4.png) |

C  

| PMA(h) | - 24 48 | - 24 48 | - 24 48 |
|--------|---------|---------|---------|
| ATF-3  | ![ATF-3 Image](image1.png) | ![ATF-3 Image](image2.png) | ![ATF-3 Image](image3.png) |
| Jun B  | ![Jun B Image](image1.png) | ![Jun B Image](image2.png) | ![Jun B Image](image3.png) |
| Jun D  | ![Jun D Image](image1.png) | ![Jun D Image](image2.png) | ![Jun D Image](image3.png) |
| Input  | ![Input Image](image1.png) | ![Input Image](image2.png) | ![Input Image](image3.png) |
### Figure 3

#### A

| PMA(h) | Total NE | DNA affinity pull-down |
|--------|----------|------------------------|
|        |          | AP-1Wt | AP-1 mutant |
| -      | 24       | 48     | -          | 24 | 48 |
| BRG1   | ![Image](image1) | ![Image](image2) | ![Image](image3) |
| β-actin | ![Image](image4) | ![Image](image5) | ![Image](image6) |
| Jun D  | ![Image](image7) | ![Image](image8) | ![Image](image9) |

#### B

| PMA(h) | 24 | 48 | - | 24 | 48 |
|--------|----|----|---|----|----|
| BRG1   | ![Image](image10) | ![Image](image11) | -3260/-3083 |
| -389/-190 | ![Image](image12) | ![Image](image13) |
| β-actin | ![Image](image14) | ![Image](image15) | -3260/-3083 |
| -389/-190 | ![Image](image16) | ![Image](image17) |
| rIgG  | ![Image](image18) | ![Image](image19) | -3260/-3083 |
| -389/-190 | ![Image](image20) | ![Image](image21) |
| mIgG  | ![Image](image22) | ![Image](image23) | -3260/-3083 |
| -389/-190 | ![Image](image24) | ![Image](image25) |
| Input | ![Image](image26) | ![Image](image27) | -3260/-3083 |
| -389/-190 | ![Image](image28) | ![Image](image29) |
Figure 4

A

| siRNA       | Mock | CTR | ATF-3 |
|-------------|------|-----|-------|
| ATF-3       |      |     |       |
| BRG1        |      |     |       |
| β-actin     |      |     |       |
| Brm         |      |     |       |

B

| siRNA       | Mock | CTR | ATF-3 |
|-------------|------|-----|-------|
| ATF-3       |      |     |       |
| BRG1        |      |     |       |
| β-actin     |      |     |       |
| Brm         |      |     |       |

C

![Bar chart](chart.png)

- Relative BRG1 occupancy level (%)
- Relative actin occupancy level (%)
**Figure 5**

**A**

| PMA (h) | IP: anti-ATF-3 | IP: rIgG |
|---------|----------------|----------|
| -       | –              | –        |
| 24      | –              | –        |
| 48      | –              | –        |

**B**

| PMA     | IP: anti-BRG1 |
|---------|---------------|
| –       | –             |
| 24      | –             |
| 48      | –             |

**C**

1st IP: mIgG rIgG

| ATF-3  | BRG1 β-actin |
|--------|--------------|
| PMA 48h|              |

2nd IP: anti-ATF-3

| PMA (-) |
|---------|
|         |
**Figure 6**

(A) Western blot analysis of protein levels in mock-treated cells transfected with siRNA for ATF-3, BRG1, and SLC11A1. 

(B) Relative mRNA expression of SLC11A1 in cells transfected with siRNA for ATF-3, BRG1, and SLC11A1. 

(C) Relative luciferase activity in cells treated with PMA or mock treatment with or without siRNA for ATF-3, BRG1, and SLC11A1.
Figure 7

A

B

Relative luciferase activity

BRG1 (µg)  -  - 0.5 1.0 1.5 2.0 2.0 2.0 -

ATF-3 (µg)  -  -  - 1.0 - - 1.0 -

BRG1(K798R) (µg)  - - - - - 1.0 3.0 2.0 -

pREP4-SLC11A1 (-395)-Luc

Relative luciferase activity

BRG1 (µg)  - - 0.5 1.0 0.5 - - - 0.5

β-actin (µg)  - - - - - 0.6 1.1 0.6 1.1

ATF-3 (µg)  - 0.5 - - 0.5 - - 0.5 0.5

pREP4-SLC11A1 (-395)-Luc

- PMA

+ PMA
Figure 8

A

| PMA | PREP4-Luc | .395 Luc |
|-----|-----------|----------|
|     |          |          |

GT/AC repeat

B

| PMA |          |
|-----|----------|
|     |          |

GT/AC repeat

C

| siRNA | BRG1 | CTR |
|-------|------|-----|
|       |      |     |

GT/AC repeat

D

| siRNA | Mock | CTR | BRG1 |
|-------|------|-----|------|
|       |      |     |      |
Recruitment of SWI/SNF complex is required for transcriptional activation of SLC11A1 gene during macrophage differentiation of HL-60 cells
Yong Zhong Xu, Thusanth Thuraisingam, Rafael Marino and Danuta Radzioch

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