Recruitment of the SWI/SNF protein Brg1 by a multiprotein complex effects transcriptional repression in murine erythroid progenitors

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SWI/SNF complexes are involved in both activation and repression of transcription. While one of two homologous ATPases, Brg1 [Brm (Brahma)-related gene 1] or Brm, is required for their chromatin remodelling function, less is known about how these complexes are recruited to DNA. We recently established that a DNA-binding complex containing TAL1/SCL, E47, GATA-1, LMO2 and Ldb1 stimulates P4.2 (protein 4.2) transcription in erythroid progenitors via two E box–GATA elements in the gene's proximal promoter. We show here that the SWI/SNF protein Brg1 is also associated with this complex and that both the E box and GATA DNA-binding sites in these elements are required for Brg1 recruitment. Further, Brg1 occupancy of the P4.2 promoter decreased with terminal erythroid differentiation in association with increased P4.2 transcription, while enforced expression of Brg1 in murine erythroleukaemia cells reduced P4.2 gene ex-

pression. Overexpression of Brg1 was associated with increased occupancy of the P4.2 promoter by the nuclear co-repressor mSin3A and HDAC2 (histone deacetylase 2) and with reduced histone H3 and H4 acetylation. Finally, a specific HDAC inhibitor attenuated Brg1-directed repression of P4.2 promoter activity in transfected cells. These results provide insight into the mechanism by which SWI/SNF proteins are recruited to promoters and suggest that transcription of P4.2, and most likely other genes, is actively repressed until the terminal differentiation of erythroid progenitors.

Key words: Brahma-related gene 1 (Brg1), co-repressor, erythroid progenitor, histone deacetylase, SWI/SNF, transcriptional repression.

INTRODUCTION

Development is a highly regulated process involving the ordered activation and repression of specific sets of genes. Since the eukaryotic genome is compacted into the complex structure known as chromatin, accessibility to DNA is an important issue for the factors that regulate transcription [1]. As a consequence, two classes of multiprotein complexes, chromatin-remodelling and histone-modifying, are essential for establishing the patterns of gene expression that ultimately mediate cell differentiation [2].

SWI/SNF complexes use energy from ATP hydrolysis to remodel nucleosomes [3]. While the yeast and *Drosophila* complexes possess a single ATPase, Swi2/Snf2 and Brm (Brahma) respectively, mammalian SWI/SNF complexes contain either of two proteins with similar ATPase activity, Brm and Brg1 (Brm-related gene 1). It is likely that these proteins also have distinct biochemical and functional properties [4,5]. As evidence, *Brm*-mutant mice develop normally [6], while embryos lacking an intact *Brg1* gene die at implantation [5].

SWI/SNF complexes can either promote or inhibit transcription [7–9]. While nucleosome remodelling is clearly important to their actions in transcriptional activation [10–12], the mechanism through which they repress transcription is less well understood [7,8]. The presence of transcriptional co-repressors and HDACs (histone deacetylases) in purified Brg1-containing complexes and the recruitment of these proteins to the promoters of Brg1-regulated genes imply that histone deacetylation is involved [8,13–15].

The recent discovery of tissue- and cell-type-specific forms of SWI/SNF complexes suggests that their component proteins could have specialized functions, especially in differentiation [16–18]. For example, a mutation in the zebrafish *Brg1* gene was shown to block retinal cell differentiation [19], while dysfunction of the Brg1-based WINAC chromatin remodelling complex is the cause of Williams syndrome, a hereditary disorder characterized by multiple defects in organogenesis [20]. In addition, overexpression studies demonstrated the requirement for Brg1 or Brm in MyoD-mediated myogenesis, C/EBP α (CCAAT/enhancer-binding protein α) activation of myeloid gene expression, and adipogenesis [21–23], while deletion of the *Brg1* gene in T-lymphocytes showed that it has multiple roles in thymocyte development [24,25]. Finally, loss-of-function mutations in *Brg1* and *Snf5/Ini1*, another core protein of SWI/SNF complexes, have been observed in malignancies [5,26,27].

A number of studies suggest a role for Brg1 in regulating gene expression in erythroid progenitors. Multiple Brg1-containing SWI/SNF complexes have been described in erythroid cells [16,18,28], and Brg1 has been found to interact with the β -globin proximal promoter, both *in vitro* and *in vivo* [16,29], and to act as a co-activator for EKLF (erythroid Krüppel-like factor) in β -globin transcription [4,16,30,31]. While Brg1 promoted the expression of β -globin and other markers of erythroid differentiation in *in vitro* transcription studies [4,16,30,31], Groudine and co-workers [32] suggested that it could also repress β -globin transcription via the gene's LCR (locus control region). Thus the exact role of Brg1 in erythroid gene expression (as transcriptional activator, inhibitor, or both) is unclear.

Abbreviations used: Brm, Brahma; Brg1, Brm-related gene 1; ChIP, chromatin immunoprecipitation; DTT, dithiothreitol; EKLF, erythroid Krüppel-like factor; EMSA, electrophoretic mobility-shift assay; FVA cell, Friend virus cell; GPA, glycophorin A; HDAC, histone deacetylase; HS2, hypersensitivity site 2; LCR, locus control region; MEL cell, murine erythroleukaemia cell; NP40, Nonidet P40; P4.2, protein 4.2; TSA, trichostatin A; UTR, untranslated region.

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We and others have characterized a DNA-binding complex in erythroid cells containing the basic helix–loop–helix transcription factors TAL1/SCL and E47, zinc finger transcription factor GATA-1, LIM domain protein LMO2 and LIM domain-binding protein Ldb1 [33,34] and showed that it positively regulates gene expression and terminal differentiation [34]. While our studies established that this ternary complex stimulates P4.2 (protein 4.2) expression, we also observed that it was present on this promoter in advance of significant gene transcription [34]. In the present study, we demonstrate that a previously unrecognized component(s) of the complex can inhibit its transactivating activity. We show that the SWI/SNF protein Brg1 is recruited to the P4.2 promoter by this E box–GATA-binding complex and demonstrate that enforced expression of Brg1 inhibits endogenous P4.2 gene expression in association with the nuclear co-repressor mSin3A and HDAC2. Our results are consistent with a model of active repression of P4.2 transcription by Brg1 and its mediation by histone deacetylation.

MATERIALS AND METHODS

Plasmid constructs

The pGL2-P4.2p1700-Luc reporter plasmid and pEFIREs-P expression vector have been described in [34]. Plasmids pCI-Ce-Brg1 and pCI-Ce-Brg1^{K798R} were generously provided by Dr Anthony Imbalzano (University of Massachusetts Medical School, Worcester, MA, U.S.A.). pEFIREs-Brg1 and pEFIREs-Brg1^{K798R} were constructed by transferring SalI fragments containing a FLAG-tagged wild-type human Brg1 cDNA and a K798R mutant cDNA from pCI-Ce-Brg1 and pCI-Ce-Brg1^{K798R} respectively into the SalI site of pEFIREs-P. The nucleotide sequence of both inserts was confirmed by DNA sequencing and the correct size of the expressed proteins was verified by Western-blot analysis using antibodies to the FLAG epitope and Brg1.

Cell culture and transient transfections

MEL (murine erythroleukaemia) cells (line F4-12B2) were cultured and transfected as described previously [34]. Briefly, 125 ng of the pGL2-P4.2p1700-Luc reporter, 7.5 ng of a *Renilla* luciferase expression vector as transfection control, and the indicated amount (55, 110, 220, 440 or 880 ng) of pEFIREs-Brg1 or pEFIREs-Brg1^{K798R} were transfected with DMRIE-C reagent (Invitrogen, Carlsbad, CA, U.S.A.) into MEL cells grown in 24-well plates. As needed, plasmid pCMV4 was added to adjust the total mass of transfected DNA to 1.0 µg. All extracts were prepared 48 h after transfection, luciferase activities were determined with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, U.S.A.), and reporter activities were normalized to *Renilla* luciferase activities. Each transfection was done in triplicate and repeated at least three times.

Preparation of stably transduced cells

pEFIREs-Brg1 or empty vector were introduced into MEL cells with DMRIE-C reagent as described above. Cells were selected with 0.5 µg/ml puromycin beginning 48 h after transfection. The puromycin concentration was increased to 10 µg/ml 5 days later and maintained.

EMSA (electrophoretic mobility-shift assay)

Nuclear extracts from MEL cells and splenic proerythroblasts from mice infected with the anaemia-inducing strain of FVA (Friend virus; anaemia-inducing strain) cells were prepared as

described in [34]. The details of EMSA of DNA-binding activity have also been described [34]. Normal rabbit immunoglobulin and antibodies to Tal1 and GATA-1 were used as before [34]. Goat polyclonal antibody to Brg1 (sc-12520X) and mouse monoclonal (sc-17828X) and goat polyclonal (sc-6450) antibody to Brm used in supershift analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Rabbit antibody to goat immunoglobulin and a soluble engineered form of Protein G were purchased from Sigma (St. Louis, MO, U.S.A.).

DNA affinity capture analysis of DNA-binding complexes

Streptavidin-conjugated beads (M-280 Dynabeads, Dynal, Oslo, Norway) were washed three times with BW buffer {TE buffer [10 mM Tris/HCl (pH 8.0)/1 mM EDTA] containing 1 M NaCl} before use. A biotinylated, double-stranded oligonucleotide (25 pmol) corresponding to the E1G1 element in the P4.2 promoter and the previously described E box, GATA, and combination E box–GATA mutants [34] were incubated with washed beads in 200 µl of BW buffer at room temperature for 20 min. DNA-loaded beads were washed twice with BW buffer and incubated with BW buffer containing 1% BSA at 4°C overnight to block non-specific protein binding. Nuclear extract (200 µg) from undifferentiated MEL cells was then added to DNA-bound beads in binding buffer [20 mM Hepes, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM DTT (dithiothreitol) and 25% (v/v) glycerol] and incubated at room temperature for 30 min. Finally, beads were washed three times with 500 µl of binding buffer containing 0.025% NP40 (Nonidet P40). DNA-associated proteins were released by boiling in Laemmli sample buffer (Sigma), and eluates were subjected to Western-blot analysis.

Western-blot and immunoprecipitation analyses

Western-blot analysis was carried out as previously described [35]. For immunoprecipitation analysis, nuclei from undifferentiated MEL cells were isolated as described in [35] and extracted on ice with lysis buffer (6 mM Na₂HPO₄, 4 mM NaH₂PO₄, 150 mM NaCl, 2 mM EDTA, 1 mM DTT, 1% NP40 and 25% glycerol). Nuclear extracts were then incubated with goat polyclonal antibody to Brg1 or normal goat IgG. Immunoprecipitated proteins captured by Protein G–agarose were washed three times with 10 mM Tris/HCl (pH 8.0), 150 mM NaCl and 0.1% NP40 and assayed by Western-blot analysis. In addition to the antibodies above, normal goat IgG (sc-2028) and rabbit polyclonal antibodies to mSin3A (sc-994X) and HDAC2 (sc-7899) were purchased from Santa Cruz Biotechnology. Rabbit polyclonal antibody to the FLAG epitope (F7425) was from Sigma.

ChIP (chromatin immunoprecipitation) analysis

ChIP analysis was performed with commercial reagents (Upstate Biotechnology, Charlottesville, VA, U.S.A.) as described previously [34]. Immunoprecipitated DNA was analysed by standard PCR using HotStarTaq DNA polymerase (Qiagen, Valencia, CA, U.S.A.) or by real-time PCR using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, U.S.A.). For quantitative ChIP analysis, the amount of immunoprecipitated DNA was determined from a standard curve generated by serial dilution of input DNA. Factor occupancy was quantified by subtracting the values obtained with normal rabbit IgG from those for the indicated antibodies. Normal rabbit IgG (sc-2027) and polyclonal antibodies to Brg1 (sc-10768X), mSin3A (sc-994X), and HDAC2 (sc-7899) obtained from Santa Cruz Biotechnology and to acetylated histone H3 (06-599) and histone H4 (06-866) obtained from Upstate Biotechnology were used in this analysis. The sequences of the primers

used in PCR were: GCAGTTCATCTCCAAAGAGC and CGAA-CCCAACTCTGAACCTC for the P4.2 promoter and TCTTT-CCCTGGTGGCTATTG and AGAGTACCCCGAAAACACC for P4.2 3'-UTR (3'-untranslated region).

RNA expression analysis

Total RNA was prepared from MEL cell transductants using an RNeasy Midi kit (Qiagen). Total RNA (1 µg) was then used for cDNA synthesis with the iScript cDNA Synthesis kit (Bio-Rad), followed by real-time PCR using iQ SYBR Green Supermix (Bio-Rad). The relative amount of cDNA was determined from a standard curve generated by serial dilution of cDNA prepared from vector-transduced MEL cells, and the level of P4.2 RNA was normalized to that of *S16*. The sequences of the primers used in PCR were: TCCCAAACAACCTCAACCGTC and TGGTATGAAACATCTGAACACCCC for P4.2 and TCG-GGAAAGATGAAGTCGGAG and GGTCGGATACACTGTGC-TATTCTCG for *S16*. Northern-blot analysis was carried out as previously described [34].

Autoradiographic analysis

Band intensities on photographic film were quantified with NIH Image software (version 1.5).

Statistical analysis

The significance of differences in means was evaluated with a two-tailed Student's *t* test.

RESULTS

Brg1 is recruited to the P4.2 promoter by a multiprotein DNA-binding complex

We have reported that a DNA-binding complex containing TAL1, E47, GATA-1, LMO2 and Ldb1 associates with two E box–GATA elements in the P4.2 promoter and promotes transcription of this gene [35]. Expression of all five nuclear proteins was required for maximal transcription, and mutation in either the E box or GATA site led to loss of at least 75 % of P4.2 promoter activity in differentiating MEL cells. Although the above-mentioned work established that the complex contributed positively to P4.2 transcription, it was unclear at the time why it was assembled on the promoter in less differentiated cells in advance of significant gene expression [35]. One explanation could be that a unique surface of the complex forms with differentiation and recruits a transcriptional co-activator. Alternatively, an unrecognized component(s) of the complex could inhibit its transactivating activity in undifferentiated cells. Given genetic data showing *Drosophila* Swi/Snf proteins Brm and OSA/BAF250 repress the transactivating functions of an E box–GATA-binding complex involved in proneural patterning [36,37], we investigated whether Brg1 or Brm altered the function of the P4.2 promoter-binding complex.

To determine, first, whether either of these SWI/SNF proteins were associated with this DNA-binding complex in erythroid cells, antibody supershift analysis was carried out. As described previously [34], incubation of MEL cell nuclear extracts with a ³²P-labelled E box–GATA probe from the P4.2 promoter led to the formation of two E box–GATA DNA-binding complexes with identical protein composition. This was confirmed using antibodies to two of its components, Tal1 and GATA-1 (Figure 1A, arrowheads) [33,34]. In addition, the two complexes were supershifted and ablated respectively by goat polyclonal (Figure 1A)

Figure 1 Identification of Brg1 in a Tal1- and GATA-1-containing DNA-binding complex

³²P-labelled E box–GATA double-stranded oligonucleotide was incubated with 5 µg of nuclear extract from undifferentiated MEL cells (A) or FVA cells (B). Where indicated, normal IgG and antibodies to Tal1, GATA-1, Brm (mouse monoclonal) and Brg1 were added in supershift analysis. Ternary complexes are marked with arrowheads, while a complex containing only GATA-1 is marked with filled circles. Supershifted complexes were further retarded by addition of Protein G or antibody to goat IgG.

and rabbit polyclonal antibody (results not shown) to Brg1 while being unaffected by antibody to Brm or normal IgG. These complexes were further retarded by addition of an IgG-binding agent to the Brg1 antibody, either anti-IgG or an engineered form of Protein G. In contrast, the mobility of a less retarded complex containing GATA-1 only (Figures 1A and 1B, filled circles) was not affected. To extend these findings to a more physiological setting, nuclear extracts from splenic proerythroblasts of mice infected with the anaemia-inducing strain of Friend virus were also used in EMSA. As shown in Figure 1(B), pre-incubation of antibody to Brg1 but not Brm or normal IgG with FVA cell extracts retarded the migration of the two E box–GATA DNA-binding complexes. As companion studies showed the presence of both *Brm* RNA (results not shown) and protein (see Supplemental data at <http://www.BiochemJ.org/bj/399/bj3990297add.htm>; Figure 1) in MEL cells, these results demonstrate the specificity in SWI/SNF protein interaction with the E box–GATA-binding complex. In summary, EMSA with erythroid extracts from two cell systems and polyclonal antibodies from two species demonstrated that Brg1 contributes to the P4.2 promoter E Box–GATA DNA-binding complex together with the five proteins described originally to comprise the complex.

To confirm Brg1 recruitment to the E box–GATA element by an independent method, DNA affinity capture analysis was also carried out. To this end, oligonucleotide-linked magnetic beads were incubated with MEL cell nuclear extracts and DNA-bound proteins identified by Western-blot analysis. As shown in Figure 2, Brg1 was captured by a double-stranded oligonucleotide corresponding to an E box–GATA element from the P4.2 promoter but not by oligonucleotides containing a mutation in the E box (E box mutant), GATA site (GATA mutant), or both (double mutant). Identical results were obtained with oligonucleotide-linked agarose beads (results not shown). Thus two different

Figure 2 DNA affinity capture analysis of Brg1 association with an E box-GATA sequence element from the P4.2 promoter

Nuclear extract (200 μ g) from undifferentiated MEL cells was incubated with a biotinylated, double-stranded E box-GATA oligonucleotide or oligonucleotides containing mutations in the E box (E box mutant), GATA site (GATA mutant), or E box and GATA sites (double mutant). DNA-associated proteins were captured with streptavidin-conjugated beads, the beads were washed extensively, and bound proteins were identified by Western-blot analysis. Brg1 protein is marked by an arrowhead. A cross-reacting protein is marked with a filled circle.

approaches confirmed the requirement for both the E box and GATA sites in this bipartite sequence-element for Brg1 binding. As all five of its original components and the integrity of both DNA-binding sites are required for formation of the complex [33,34], we conclude that Brg1 is recruited to the P4.2 promoter by the fully assembled ternary complex.

Brg1 occupies the P4.2 promoter in erythroid progenitors

To determine whether Brg1 was associated with the P4.2 promoter in living cells, ChIP analysis was carried out. As shown in Figure 3(A), rabbit polyclonal antibody to Brg1 but not total rabbit IgG precipitated P4.2 promoter fragments from undifferentiated MEL and FVA cells. In contrast, chromatin fragments from the gene's 3'-UTR were not selected by any antibody, confirming the specificity of this analysis and demonstrating that Brg1 binding does not extend outside the gene's promoter and/or coding region.

Brg1 occupancy of the P4.2 promoter decreases with erythroid differentiation

Brand et al. [32] recently identified Brg1 in a MafK-associated protein complex in MEL cell nuclei and showed that Brg1 occupancy of the β -globin promoter and HS2 (hypersensitivity site 2) of the β -globin LCR declined in cells incubated with DMSO for 4 days. We confirmed these results (Figure 3B), analysing Brg1 occupancy before and 48 h after DMSO-induced differentiation, when MEL cells begin to transcribe β -globin and P4.2 RNA. Quantitative ChIP analysis showed that Brg1 occupancy of the P4.2 promoter (Figure 3C) likewise declined with differentiation over a period in which histone H3 and H4 acetylation (Figure 3D) and P4.2 transcription [34,38] increase significantly. Finally, Western-blot analysis of Brg1 in DMSO-induced MEL cells revealed that steady-state levels of this protein were maintained until late stages of differentiation (Figure 3E). These results indicate that the decline in Brg1 occupancy of P4.2 with erythroid differentiation is not caused, at least initially, by any change in Brg1 expression.

Enforced expression of Brg1 in MEL cells represses transcription of P4.2 gene expression

Although ChIP analysis showed that Brg1 was associated with the P4.2 promoter *in vivo*, the question of whether it regulates P4.2 gene expression was not resolved. To investigate, FLAG epitope-tagged Brg1 was expressed in MEL cells under the control of the EF1 α (elongation factor 1 α) promoter and polyclonal populations of cells transduced with this plasmid or the parental vector were selected. As shown in Figure 4(A), expression of the

Figure 3 Reduction in Brg1 occupancy at the P4.2 promoter with erythroid differentiation

(A) ChIP analysis of Brg1 occupancy in undifferentiated MEL and FVA cells. Chromatin fragments immunoprecipitated by rabbit polyclonal antibody to Brg1 or normal rabbit IgG were used in PCR with primers for the P4.2 promoter (top) or its 3'-UTR (bottom). Ethidium bromide-stained PCR products are shown. MEL cells induced to differentiate with 1.5% DMSO for 2 days were also subjected to quantitative ChIP analysis. Mean values ($\times 10^2$) $\pm$ S.E.M. for Brg1 occupancy are plotted for HS2 (β -globin hypersensitivity site 2), Ey gene promoter (Ey) and β -globin promoter (β -globin) in (B) and at the indicated positions in the P4.2 gene in (C). Mean occupancy as a percentage of input from three or more independent real-time PCR assays is shown above bars. (D) Quantitative ChIP analysis of acetylated histone H3 and H4 content in the P4.2 promoter in undifferentiated (U) and DMSO-induced (D) MEL cells. Fold increases in means are shown above bars. (E) Western-blot analysis of Brg1 protein (top) and actin (bottom) in whole-cell extracts from MEL cells treated with 1.5% DMSO for the indicated days.

transfected Brg1 protein was detected using an antibody to the FLAG epitope, although total Brg1 protein, representing the combination of endogenous mouse and transfected human Brg1, was increased only slightly. In contrast, Tal1, GATA-1 and Inl1 protein levels were unchanged by Brg1 overexpression. Although use of polyclonal populations may have underestimated the magnitude of the effect, this increase in Brg1 expression significantly reduced steady-state levels of P4.2 mRNA in cells incubated for 3 days without (Figure 4B) or with (Figure 4C) 1.5% DMSO, while Brg1 transduction had no discernible effects on morphological signs of differentiation and cellular proliferation and had little or no effect on *GPA* (glycophorin A) and *EKLF* gene expression (Figure 4C). Thus enforced expression of Brg1 inhibited endogenous P4.2 gene expression in MEL cells relatively selectively without affecting their differentiation programme.

Brg1 represses P4.2 promoter activity in an HDAC-dependent manner

Having established that Brg1 was recruited to the P4.2 promoter by the E box-GATA binding complex and inhibited P4.2 gene expression when overexpressed, we asked whether Brg1 regulated P4.2 transcription. To investigate, a P4.2 promoter-luciferase reporter and plasmid vector expressing wild-type Brg1 were introduced into differentiating MEL cells. As shown in Figure 5(A),

Figure 4 Repression of P4.2 transcription by enforced expression of Brg1 in MEL cells

(A) Western-blot analysis of transfected FLAG-tagged Brg1, total Brg1 (endogenous mouse + transfected human), and endogenous Tal1, GATA-1 and Ini1 proteins in nuclear extracts from MEL cell transductants. (B) Quantitative real-time RT (reverse transcriptase)–PCR analysis of P4.2 and β -globin mRNA levels in undifferentiated Brg1- (B) and vector-transduced (V) MEL cells. (C) Northern-blot analysis of indicated mRNAs in Brg1-transduced MEL cells following incubation with 1.5% DMSO for 3 days. The levels of mRNAs in Brg1-transduced cells relative to vector controls are shown under autoradiographs. Ethidium bromide-stained 18 S and 28 S rRNA served as loading controls (bottom).

enforced expression of Brg1 effected a significant and dose-related inhibition of DMSO-induced P4.2 promoter activity. In contrast, enforced expression of the ATPase-defective K798R mutant increased reporter expression at low plasmid concentrations, while slightly inhibiting DMSO-induced P4.2 promoter activity at higher concentrations. These results make squelching an unlikely explanation for Brg1-directed repression and are compatible with a role for ATPase activity in this action. At least in these transiently transfected cells, the K798R mutant may have acted as a dominant-negative inhibitor of endogenous SWI/SNF proteins.

We then explored the mechanism by which Brg1 repressed P4.2 transcription. First, as shown in Figure 5(B), the potent HDAC inhibitor TSA (trichostatin A) increased by more than 10-fold transcription from the luciferase reporter gene in undifferentiated MEL cells. TSA also attenuated the inhibitory effects of Brg1 overexpression in DMSO-induced cells, with only a slight reduction (~15%) in P4.2 promoter activity observed with 50 nM TSA (Figure 5C) compared with the approx. 80% decrease seen in the compound's absence. These studies suggest that repression of P4.2 transcription in Brg1-transfected cells results from direct inhibition and are consistent with a mechanism involving histone deacetylation.

Brg1 interacts with mSin3A and HDAC2 in erythroid progenitors

Given the possible contribution of HDAC activity to Brg1-mediated repression of the P4.2 promoter, we investigated whether Brg1 was associated with the nuclear co-repressor mSin3A and HDAC2 in erythroid cells, both of which have been reported to contribute to Brg1-directed repression [14,15]. As shown in Figure 6, mSin3A and HDAC2, but not GATA-1, co-immuno-

Figure 5 HDAC-dependent repression of P4.2 promoter activity by Brg1

(A) Dose-dependent repression of P4.2 promoter activity by Brg1. A luciferase reporter construct containing 1.7 kb of the P4.2 promoter was co-transfected into DMSO-treated MEL cells without (unfilled bar) and with expression plasmids encoding Brg1 (filled bar) or Brg1^{K798R} (hatched bar). Cell lysates were prepared 48 h after transfection and assayed for luciferase activity. P4.2 reporter activities in Brg1-transfected cells were related to those in cells not transfected with Brg1. Plotted is mean luciferase activity relative to control $\pm$ S.E.M. for three independent experiments. (B) De-repression of P4.2 promoter activity by TSA. The P4.2 promoter-reporter construct was transfected into MEL cells in the absence (unfilled bar) or presence (filled bar) of TSA at the indicated concentrations. (C) Attenuation of Brg1-directed repression of P4.2 promoter activity by TSA. The P4.2 promoter-reporter construct was co-transfected without (unfilled bar) or with (filled bar) 880 ng of the Brg1 expression plasmid into MEL cells, which were then treated with 1.5% DMSO and the indicated concentrations of TSA.

Figure 6 Association of mSin3A and HDAC2 with Brg1 in MEL cells

Nuclear extracts from undifferentiated MEL cells were incubated with goat polyclonal antibody to Brg1 or normal goat IgG. Immune precipitates were captured with Protein G-agarose beads and assayed by Western-blot analysis for the indicated proteins.

precipitated with Brg1 from erythroid cell nuclear extracts. While these results do not rule out mSin3A and HDAC2 being recruited to the P4.2 promoter by DNA-bound Brg1, they are consistent with Brg1, mSin3A, and HDAC2 contributing to a single preformed complex.

Enforced expression of Brg1 increases loading of Brg1, mSin3A and HDAC2 and decreases histone acetylation at the P4.2 promoter

Finally, quantitative ChIP analysis was used to determine whether the P4.2 promoter was occupied by Brg1, mSin3A, and HDAC2 in intact cells. Significant loading of Brg1, mSin3A, and HDAC2 was detected at the P4.2 promoter in MEL cells (Figure 7A) and,

Figure 7 Increased loading of Brg1, mSin3A and HDAC2 and decreased histone acetylation at the P4.2 promoter in Brg1-transfected MEL cells

Quantitative ChIP analysis was carried out to determine protein occupancy (A), histone acetylation (B) and total histone H3 content (C) at the indicated locations in Brg1- (B) and vector-transduced (V) MEL cells. Mean levels (value $\times 10^2 \pm$ S.E.M. for Brg1, mSin3A and HDAC2 occupancy (A), acetylated histone H3 (AcH3) and acetylated histone H4 (AcH4) (B) and total histone H3 (H3) (C) are plotted for the P4.2 promoter and 3'-UTR. Mean values are shown above the bars. * $P < 0.01$; ** $P < 0.001$.

while the magnitude of the effect could have been underestimated by the use of polyclonal populations, Brg1, mSin3A and HDAC2 protein occupancy was significantly higher in Brg1-transfected cells ('B') than vector controls ('V'). In contrast, negligible association of these proteins was detected with the P4.2 gene's 3'-UTR (Figure 7A), the promoter of the embryonic β -like globin gene *Ey* (results not shown), and the promoter of the ribosomal *S16* gene (results not shown), demonstrating the specificity of these findings. Finally, the levels of acetylated histone H3 and, especially, acetylated histone H4 (Figure 7B) but not total histone H3 (Figure 7C) at the P4.2 promoter were significantly decreased in Brg1-transfected cells relative to controls, consistent with increased recruitment of an HDAC. Thus mSin3A and HDAC2 occupancy of the P4.2 promoter with Brg1 in undifferentiated MEL cells has consequences for the level of histone acetylation.

DISCUSSION

SWI/SNF complexes have been characterized in erythroid cells and have been shown to bind the β -globin proximal promoter *in vitro* and *in vivo* [16,18,28,29]. The mechanism by which Brg1 regulates transcription of this or other erythroid genes, however, is poorly understood. Our studies suggest that this SWI/SNF protein is recruited to the P4.2 promoter by a multiprotein complex that otherwise functions as an enhanceosome. The present study establishes that this ternary complex can recruit Brg1 with a co-repressor and HDAC, with the effect of inhibiting transcription of a gene subsequently activated by this same complex.

Whole-genome expression analysis has shown that SWI/SNF complexes regulate the transcription of fewer than 5% of all genes [9,39,40]. While the factors responsible for this selectivity are not entirely understood, association of these complexes with specific regions of DNA must be a prerequisite. Evidence for site-specific recruitment of SWI/SNF complexes comes from the independence of transcription of SWI/SNF-regulated genes from their neighbours [9] and the interaction of these proteins with individual transcriptional activators and repressors [11,13,41–43]. Snf5/Ini1, Swi1, BAF57, BAF60a and Swi2/Snf2, for example, have been reported to interact with GCN4 (positive general control of transcription-4), GAL4-VP16 or Sp1 [4,10,11,16,31,41,42], and recruitment of SWI/SNF complexes *in vivo* has been shown to require specific DNA-binding proteins or DNA sequence elements [29,44]. Although interaction with a single transcription factor may be sufficient at some promoters, Brg1 recruitment to the P4.2 gene requires a multiprotein complex that binds an extended sequence motif. Thus, while Emerson and co-workers [4,16,31] reported that this SWI/SNF protein would interact with GATA-1, our EMSA and DNA affinity capture assays found that only the ternary complex could recruit Brg1 to the P4.2 promoter, with GATA-1 unable to do so by itself (Figures 1 and 2).

A critical issue in SWI/SNF recruitment has to do with the order of loading, i.e. whether regulatory proteins bind DNA and then direct these complexes to promoters or the SWI/SNF complex interacts with chromatin first to facilitate binding of transcription factors to DNA. Considering that the E box–GATA-binding complex assembles only when all five components are present and the E box- and GATA-binding sites are both intact [33,34], our studies are more compatible with a protein holocomplex recruiting Brg1 and then binding DNA. The requirement for recruiting Brg1 to the β -globin promoter for the –90 CACCC site, which binds the erythroid transcription factor EKLF, and the –32 TATA box suggests that such a mechanism is not restricted to the P4.2 gene [29]. Except in special cases, therefore, a single DNA-binding protein cannot be the only factor conferring SWI/SNF dependence [9,45]. Otherwise, the number of genes that utilize SWI/SNF complexes in transcription would be far greater than experimentally determined [9,39,40]. Recruitment by multiprotein complexes is likely to be an important mechanism in ensuring specificity in SWI/SNF modulation of transcription.

The inverse relationship between P4.2 promoter occupancy and gene expression is compatible with Brg1 negatively regulating P4.2 transcription in erythroid progenitors, and overexpression of Brg1 in MEL cells, in fact, significantly inhibited DMSO-induced P4.2 promoter activity and endogenous gene expression. Recent studies have established that a SWI/SNF complex inhibits cyclin D1 expression via an HDAC-dependent mechanism and that Brg1 represses the *cad* promoter through the mediation of mSin3A and HDAC2 [14,46]. It is significant, therefore, that Brg1 recruited these same co-repressors to the P4.2 promoter in erythroid progenitors and that inhibition of DMSO-induced P4.2 promoter activity was relieved by an HDAC inhibitor. Moreover, enforced expression of Brg1 effected a 2-fold increase in mSin3A and HDAC2 loading at the P4.2 promoter in association with a 5-fold decrease in P4.2 mRNA levels. These results suggest that Brg1 repression of P4.2 transcription in erythroid progenitors involves recruitment of specific co-repressors and active inhibition of the transactivating activity of the E box–GATA complex. They do not exclude a contribution by this SWI/SNF protein's ATP-dependent nucleosomal remodelling function, however, and an ATPase-defective Brg1 mutant actually stimulated P4.2 promoter activity in transient transfection assays (Figure 5A). Unfortunately, our inability to stably express this mutant in cells (results not shown), similar to others' experience [5], prevented a more definitive

determination of the importance of nucleosomal remodelling to Brg1-directed repression of erythroid-expressed genes. Further, these studies do not prove that the decline in Brg1 occupancy is solely responsible for induction of P4.2 transcription, and it is very likely that the increased expression of this gene with erythroid differentiation reflects transcriptional activation by E box–GATA and other DNA-binding complexes in addition to relief from repression. That such a mechanism is active on genes besides P4.2 is suggested by the 2.5- to 6-fold reduction observed in Brg1-transduced MEL cells for mRNAs from additional genes with E box–GATA elements in their promoters and/or intronic regulatory regions (J. Xie, Z. Xu and S. J. Brandt, unpublished work).

ChIP analysis revealed that Brg1 also occupied the *GPA* and *EKLF* promoters in erythroleukaemia cells (results not shown). The fact that Brg1 overexpression had little to no effect on their transcription (Figure 4), however, suggests a level of regulation beyond Brg1 recruitment, which may relate to the existence of different Brg1-containing SWI/SNF complexes [47–50]. The PBAF (polybromo, Brg1-associated factors) complex, for example, activates nuclear hormone receptor-mediated transcription, while the related BAF complex does not [47]. A candidate for a mediator of such a selective repression in erythroid progenitors is the OSA/BAF250 protein, which is associated with the PBAF, but not BAF, complex [17,47,48], has been reported to inhibit the function of a *Drosophila* proneural E box–GATA-binding complex [37] and is present in the P4.2 promoter-binding complex in erythroid cells (Z. Xu and S. J. Brandt, unpublished work). Irrespective of the mechanism, Brg1 occupancy must not by itself dictate the outcome of transcription.

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