Roles of Brahma and Brahma/SWI2-Related Gene 1 in Hypoxic Induction of the Erythropoietin Gene

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RUNNING TITLE

SWI/SNF Is Required for Hypoxic Induction of Erythropoeitin
SUMMARY

Upon hypoxia the human erythropoietin (EPO) gene is transactivated by the heterodimeric hypoxia-inducible factor 1 (HIF-1). Mammalian SWI/SNF is a chromatin-remodeling complex involved in modulation of gene expression. We demonstrate that Brahma (Brm) and Brahma/SWI2-Related Gene 1 (Brg-1), alternative ATPase subunits of SWI/SNF, potentiate reporter gene activation mediated by HIF-1, in an ATPase-dependent manner. Brm was more potent than Brg-1 in the reporter gene assays. Simultaneous depletion of both Brm and Brg-1 by siRNAs significantly compromised the transcription of the endogenous EPO gene triggered by hypoxia. While knocking down Brm alone resulted in a moderate reduction in transcription of the Erythropoietin (EPO) gene, depletion of Brg-1 resulted in an augmentation of transcription of both the EPO gene and the Brm gene, indicating Brm can compensate for loss of Brg-1. Chromatin Immunoprecipitation (ChIP) and sequential ChIP (re-ChIP) analysis showed that both Brm and Brg-1 associate with the enhancer region of the EPO gene in vivo in a hypoxia-dependent fashion and that each is present in a complex with HIF-1. Brm and Brg-1 were also recruited to the promoter of the vascular endothelial growth factor (VEGF) gene in a hypoxia-dependent fashion, although hypoxic induction of VEGF transcription was not affected by depletion of either or both of Brm and Brg-1. Together these studies reveal a novel role for SWI/SNF in activation of transcription of the EPO gene; indicate an important communication and compensation between Brm and Brg-1, and indicate that the requirement for SWI/SNF during hypoxic induction is gene-specific.
INTRODUCTION

Tissue oxygen concentration is an important regulatory stimulus for many physiological and pathological processes (1). Adaptation to hypoxia depends in part on appropriate alterations in the expression of a number of physiologically relevant genes. Induction of the erythropoietin (EPO) gene by hypoxia is central to the regulation of the oxygen-carrying capacity of the blood (2). Hypoxic induction of genes encoding angiogenic growth factors such as vascular endothelial growth factor (VEGF) leads to new blood vessel formation during development, wound repair, and tumor growth (3-5). Most if not all mammalian cell types share a common mechanism of oxygen sensing and signal transduction (6), enabling hypoxia-induced activation of the transcription factor hypoxia-inducible factor 1 (HIF-1) composed of HIF-1α and Arnt (7). The HIF-1α subunit is regulated by hypoxia both at the level of transactivation and protein stability (8-11).

In general, gene expression is not determined by the simple additive influences of individual transcription factor binding sites. Adjacent sites interact with each other to produce effects which range from repressive to highly synergistic. Multiprotein complexes involving transcription factors, coactivator proteins, and other proteins are thought to integrate signals and create the specificity and control required for precise regulation of gene transcription.

Despite similarities among hypoxia-responsive genes, there are important differences. While EPO expression can be induced as much as 100-fold, other responses to hypoxia are less robust. Hypoxic inducibility can be modulated by other environmental stimuli as well as tissue-specific cues (12). Transcriptional regulation of the EPO gene is achieved by the concerted action of
several transacting factors interacting with the proximal promoter region and with the 3’ untranslated region of the gene (13-17). In the 3’ untranslated region there is a hypoxia-responsive-enhancer element located approximately 120 bp 3’ from the polyadenylation site. This enhancer element is functionally tripartite. One site binds HIF-1 (17). The second site is necessary for transactivation of the EPO gene mediated by HIF-1, but factors interacting with this site have not yet been described. The third is a binding site for the orphan receptor, hepatocyte nuclear factor 4 (HNF4) (18). In addition, a binding site for Smad4 was recently identified that partially overlaps the HNF4 binding site (19). The coactivator protein p300 has been shown to interact with HIF-1 and to be required for enhanced transcription of the EPO and VEGF genes in response to hypoxia (20). Both the HIF-1 site and the adjacent, functionally critical nuclear HNF4 site, are required for high-affinity binding of p300 and formation of a hypoxically inducible multiprotein complex (21).

The regulation of transcription initiation requires that transcription factors function in the context of eukaryotic chromatin. Several classes of chromatin remodeling enzymes have been identified that facilitate transcription from chromatin template in vivo, including histone acetyltransferases (HATs) and ATP-dependent remodeling enzymes. One important ATP-dependent chromatin remodeling factor is the SWI/SNF multisubunit complex, that was first identified in yeast and is highly conserved among eukaryotes (22). The human SWI/SNF complexes contain either Brg-1 or Brm as an essential ATPase subunit, together with a variable number of Brg-1-associated factors (BAFs). Interestingly, Brg-1 and Brm are highly homologous, yet they appear to direct very different cellular pathways. They associate with different promoters during cellular proliferation and differentiation and in response to specific signaling pathways, via their preferential interaction with different classes of transcription factors (23). Gene knockout studies
in mice have demonstrated that mice homozygous for a null mutation in the Brg-1 gene are embryonic lethal, whereas Brm homozygous null mice are viable, but have increased body weight. This suggests that the Brg-1 protein may functionally replace Brm in SWI/SNF complexes (24-26). It was also shown that Brm could compensate for Brg-1 functionally in the regulation of retinoblastoma tumor suppressor activity (27), indicating that Brm and Brg-1 activities are partially redundant.

We provide evidence here that both Brm and Brg-1 specifically potentiate the transcription of the EPO gene mediated by HIF-1, by associating with its 3’ enhancer region. Furthermore, we demonstrate that Brm and Brg-1 can compensate for each other functionally to potentiate hypoxia-induced transcription of the EPO gene. To our knowledge, this is the first reported investigation on the roles of ATPase-containing chromatin-remodeling factors in transcription activation by HIF-1, and it reveals a novel mechanism through which Brm and its homologue Brg-1 functionally compensate each other to facilitate chromatin remodeling.
EXPERIMENTAL PROCEDURES

Plasmid Constructs and Vectors-- pBJ5-BRG-1 and pBJ5-BRG-1 (K785R) were kind gifts of Dr. Myles Brown. pCG-Brm and pCG-Brm (K749R) were described previously (28). Reporters 6 X HRE-SV40-luc and EPO enhancer-SV40-luc were kind gifts from Dr. Greg Semenza and Dr. Franklin Bunn respectively. The pBos-hHIF-1α plasmid was described previously (29).

Cell Culture and antibodies-- The SW-13 adrenal carcinoma and Hep3B cell lines were grown in Dulbecco's modified Eagle medium (Invitrogen) with 10% fetal calf serum (Omega), L-glutamine (Invitrogen), fungizone, and penicillin-streptomycin (Invitrogen) at 37 °C and 5% CO₂. The Brm (N-19), Brg-1 (H-88) and HIF-1α (H−206) antibodies used for the ChIP assays were purchased from Santa Cruz Biotechnology. The Brm antibody used for Western blotting was purchased from BD Transduction Laboratories. Antibody to Ac-K5, K8-H4 was purchased from Upstate Biotechnology. The affinity-purified rabbit polyclonal antibody to ARNT was described previously (30).

Transient Transfections and Reporter Gene Assay- Expression plasmids were transfected into SW-13 cells cultured in 12 well-plates by using the GenePorter 2 (Gene Therapy System) transfection reagent. After 24 h, some of the plates were exposed to hypoxia (1% O₂, 94% N₂ and 5% CO₂) for 24 h. Cells were then harvested and lysed in Passive lysis buffer (Promega). Luciferase activities were measured using the Dual-luciferase system (Promega) with the protocol recommended by the manufacturer. All transfection experiments were performed in triplicate.
RNA interference in Hep3B cells— SiRNA for Brm (siBrm) was prepared using the Dicer siRNA Generation Kit (Gene Therapy System) according to the protocol recommended by the manufacturer. Briefly, Brm cDNA between +141 and +733 relative to the translation start site was amplified by PCR using a forward primer (5’-CCTGGACCTCCAAGTGTCTC-3’) and a reverse primer (5’-GGCCAGATGGTCTGTTGTAG-3’). The amplicon was used as the template for in vitro transcription by T7 RNA polymerase. The double-strand RNA so produced was cleaved into 22 bp fragments by the Dicer enzyme. The siRNA for Brg-1 (siBrg-1), the siRNA for both Brm and Brg-1 (siB/B) and the scrambled RNA oligo (SCX) and their respective complementary oligos were synthesized by Qiagen and annealed according to the manufacturer’s protocol. The sense sequences of siBrg-1, siB/B and SCX are

r(GCCCAUGGAGUCCAU)d(TT), r(GCUGGAGACAGCAGAAG)d(TT) and r(UUCUCGAACGUGCUCACGU)d(TT). Hep3B cells cultured in 6-well plates were transfected at 30% cell confluency using the Oligofectamine transfection reagent (Invitrogen).

Reverse Transcription and Real-time PCR— Cells transfected with siRNAs were harvested twenty-four hours or forty-eight hours after transfection. For measuring mRNA of EPO and VEGF, some of the plates were exposed to hypoxia 72 h after transfection and cultured for further 24h. Total RNA was prepared from transfected cells using the RNeasy Micro kit (Qiagen). 1.5 µg of total RNA was reverse-transcribed using a Super script III Reverse Transcriptase kit (Invitrogen). Real-time PCR was performed using the SYBR Green Master Mix (Applied Bio-Science). The primer sets for RT-PCR are as follows: 5’-TGGAAATTCTGCAAGAGCGG -3’ and 5’-CCACGGTTGCTTTGGTCTTAA -3’ for Brm; 5’-AAGAAGACTGAGCCCGACATTC -3’, 5’-CCGTTACTGCTAAGGCCCTATGC -3’ for Brg-1; 5’-CCACGGTGCTGAACATGCT -3’, 5’-TCGAACACCTGCTGGATGAC -3’ for the ribosomal
36B4 gene; 5’-ATGTGGATAAAGCCGTCAGTGG-3’, 5’-TGATTGTTCGGAGTGGAGCAG-3’ for EPO; and 5’-TACCTCCACCATGCCAAGTG-3’, 5’-ATGATTCTGCCCTCCTCCTTC-3’ for VEGF.

**Chromatin Immunoprecipitation Assay**—The procedure was performed using a kit purchased from Upstate according to the protocol recommended by the manufacturer. Briefly, Hep3B cells were treated with hypoxia or 100 µM CoCl_2_ for the indicated times. Cross-linking was achieved by adding formaldehyde to a final concentration of 1% at 37°C for 10 min. Cells were washed twice with ice-cold phosphate-buffered saline and collected in 1 ml of ice-cold phosphate-buffered saline. Cells were pelleted at 700 × g at 4 °C and resuspended in 0.3 ml of cell lysis buffer (50 mM Tris-HCl (pH 8.1), 10 mM EDTA, 1% SDS, Roche Complete protease inhibitor mixtures), and incubated on ice for 10 min. Cell lysates were sonicated to give a DNA size range from 200 to 900 bp. Samples were centrifuged for 10 min at 4 °C. Supernatants were diluted 10-fold with dilution buffer (16.7 mM Tris-HCl, pH 8.1, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, 0.01% SDS, and Complete protease inhibitor mixture). The solutions were precleared with 80 µl of salmon sperm DNA/protein A agarose slurry for 30 min at 4 °C, and then treated with antibodies overnight at 4 °C. Immune complexes were collected using 60 µl of a salmon sperm DNA/protein A agarose slurry. The beads were pelleted and washed sequentially in the following buffers: low salt wash buffer (20 mM Tris-HCl (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100); high salt wash buffer (20 mM Tris-HCl (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), LiCl wash buffer (10 mM Tris-HCl (pH 8.1), 0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA), and TE buffer (twice). Immuno-complexes were extracted from the beads with 1% SDS-0.1 M NaHCO₃. Cross-linking was reversed by heating the eluates at 65 °C overnight. The eluates were then digested with
proteinase K at 45 °C for 1 h. The solutions were extracted with phenol-chloroform-isoamyl alcohol. DNA was purified by ethanol precipitation. The 3’ enhancer regions (+39 to +295 from the 3’ end of the exon 5), 5’ promoter region (-368 to +18 from the transcription start site) of the EPO gene and 5’ promoter region (-1136 to –889 from the transcription start site) of the VEGF gene were amplified by PCR. The primer sequences for the enhancer, promoter of EPO gene and promoter of VEGF gene were 5’-AATCCCCTGGCTCTGTCCC -3’ and 5’-CCTTGATGACAATCTCAGCGC -3’, 5’-GACCCAGCTACTTTGCGGAACTCAGC-3’ and 5’-TGGCCCAGGGACTCTGCGGCTCTGG-3’, and 5’-GTAGGTTTGAATCATACGCAGG-3’ and 5’-GCACCAAGTTTGTGGAGCTGA-3’, respectively. Re-ChIP assays utilized a similar protocol, except that the primary immunocomplex obtained with the HIF-1α antibody was eluted by 10 mM dithiothreitol with agitation at 37 °C for 30 min. The eluate was diluted 50 times with buffer (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) and immunoprecipitated with the second antibodies.
RESULTS

Transcriptional induction of the EPO gene is associated with modifications of histones at the EPO promoter- Histone-modifying complexes are thought to cooperate with histone-remodeling complexes to reconfigure chromatin, thereby establishing a local chromatin structure that is permissive for the subsequent assembly of an active preinitiation complex (PIC) at the promoter (31, 32). To examine if hypoxia-induced expression of the EPO gene is associated with modifications of histones at its promoter, we performed ChIP assays using an antibody directed to acetylated lysines at positions 5 and 8 of histone H4 at different time points after induction with 100 \( \mu \)M CoCl\(_2\), a hypoxia-mimicking reagent. As shown in Fig. 1B, histone H4 was acetylated at lysine 5 and/or 8 as early as 35 min after induction by CoCl\(_2\). The acetylation reached a peak at 50 min and then decreased. Taken together, these results suggest that EPO promoter may have a nucleosomal structure that undergoes changes upon being stimulated by hypoxia, and imply that histone-remodeling complexes, such as SWI/SNF, may be involved in hypoxic induction of the EPO gene.

Brm and Brg-1 Potentiate Reporter Gene Transcription Mediated by HIF-1- To test the potential involvement of ATP-dependent mammalian SWI/SNF chromatin-remodeling complexes in the transcriptional activation of the EPO gene, transient transfections were carried out in the Brg-1- and Brm-deficient human adrenal carcinoma cell line SW-13, with EPO enhancer-SV40-luc (Fig. 2A), a reporter in which the luciferase gene is driven by an EPO 3’ enhancer fragment containing all the cis-DNA elements described above and a minimal SV40 promoter. We found that both Brm and Brg-1, alternative ATPase subunits of SWI/SNF, enhanced the reporter luciferase activity in a hypoxia-dependent manner (Fig. 2B). Since the activity of this reporter is
relatively weak and the expression of HIF-1α appeared to be relatively low in SW-13 cells, we also co-transfected a HIF-1α expressing plasmid together with Brg-1 and/or Brm along with the reporter in most transfections in order to obtain maximal expression of the reporter gene. Whereas transfection of HIF-1α alone only marginally increased the reporter activity, this activity was further increased markedly by cotransfection with either Brg-1 or Brm. The expression of the reporter was in some instances also enhanced to some extent by Brm and Brg-1 even under normoxic conditions, most probably due to the over expression of HIF-1α leading to its activity even under these conditions. Interestingly, when Brm and Brg-1 were co-transfected, we did not observe a further enhancement of the reporter activity, indicating that the two factors do not enhance EPO transcription in an additive or synergistic manner. We found that Brm was more potent than Brg-1 with regard to enhancing reporter gene transcription (Fig. 2C). However, the transcription of the reporter reached a saturated state with lower amounts of transfected Brg-1 than of Brm, suggesting either that the affinity of the Brg-1-containing SWI/SNF complex to the HIF-1 transcriptional complex is greater than that of the Brm-containing SWI/SNF complex, or that more of the Brm-containing SWI/SNF complex than the Brg-1-containing SWI/SNF complex associate with the HIF-1 transcription complex. The capabilities of Brm and Brg-1 to enhance the transcription of the reporter are dependent on their ATPase activities, since the mutated derivatives deficient for ATPase activities (Brm-M and Brg-1-M in Fig. 2C) exhibited much reduced efficiencies at enhancing the reporter gene expression.

Brm and Brg-1 Act Specifically through the HIF-1 Binding Site- To confirm that SWI/SNF acts specifically through the HRE, we performed transient transfection assays using 6X HRE-SV40-Luc, a reporter in which a luciferase gene is driven by 6 randomly concatenated HREs and the minimal SV40 promoter (Fig. 3A). We obtained very similar results with this reporter to those
using the EPO enhancer-SV40-Luc reporter. As shown in Fig. 3B, both Brm and Brg-1 substantially enhanced the reporter activity in a hypoxia-dependent manner and the mutated versions lacking ATPase activities lost most of their capability to augment reporter gene transcription.

*Over-Expression of Coactivators Possessing HAT activity Cannot Compensate for Lack of Brm and Brg-1 with Regard to HIF-1-dependent Transcription*- Members of the p160 coactivator family and CBP/p300 have been shown to be essential for expression of hypoxia-responsive genes mediated by HIF-1 (20, 33). In yeast, Swi/Snf and Gcn5, a histone acetyltransferase, have partially redundant roles in the control of SUC2 transcription, suggesting a functional overlap between the two different processes of nucleosome remodeling, and histone acetylation (34). To investigate if the above coactivators can compensate for SWI/SNF in regulating of transcription of the EPO gene, we transfected plasmids expressing those coactivitors together with 6X HRE-SV40-Luc. As shown in Fig. 3C, those coactivators failed to potentiate the reporter activity in the absence of Brm or Brg-1, indicating that they are incapable of substituting for SWI/SNF in regulating of transcription of the EPO gene and that their functions might be SWI/SNF-dependent. However, when SRC-1 was co-transfected with Brm or Brg-1, the reporter activities were further enhanced, indicating the coactivators cooperate in an additive way.

*Brm or Brg-1 Is Required for Hypoxia-Induced Transcription of the Endogenous EPO Gene*- In order to further examine the roles played by Brm and Brg-1 in hypoxia-induced transcription of the EPO gene, we used RNA interference to deplete endogenous Brm or Brg-1 individually or together. First, we studied the role of Brm using a pool of small interference RNA oligos (siBrm). Endogenous Brm was knocked down at both the mRNA and protein level in the human hepatoma Hep3B cell line transfected with siBrm, while the level of Brg-1 mRNA was not
affected (Fig. 4A). Depletion of Brm resulted in a moderate reduction in transcription of the endogenous EPO gene triggered by hypoxia (Fig. 4B). The expression of endogenous Brg-1 was knocked down by a single small interfering RNA oligo nucleotide (siBrg-1) at both the mRNA and protein level in Hep3B cells. Interestingly, the level of Brm mRNA was increased by this treatment (Fig. 4C). Also surprisingly, the level of EPO mRNA in the presence of hypoxia was enhanced by depletion of endogenous Brg-1 (Fig. 4D). Since Brm is more effective than Brg-1 in enhancing the HIF-1-mediated transcription of the reporter genes, the enhancement might result from the increased amount of Brm compensating for the lack of Brg-1. Brm may take the place of Brg-1 in SWI/SNF complexes, and such complexes may potentiate transcription of the EPO gene more efficiently than SWI/SNF containing Brg-1. Finally, we depleted Brm and Brg-1 simultaneously using a small interference RNA oligo nucleotide which recognizes both Brm and Brg-1 mRNAs (siB/B). As shown in Fig. 4E, both Brm and Brg-1 were knocked down at the mRNA and protein level, and this resulted in a significant reduction in transcription of the EPO gene induced by hypoxia (Fig. 4F). To investigate whether SWI/SNF potentiates transcription of HIF-1-regulated genes in general, we examined the transcription of VEGF. Interestingly, though the transcription of VEGF is induced by hypoxia via HIF-1, none of the three siRNAs affected the levels of its mRNA induced by hypoxia (Fig. 4B, D and F, the right panels), indicating that SWI/SNF does not function during transcriptional activation of this gene. Taken together, these results suggest that EPO is a target gene of SWI/SNF and the requirement for SWI/SNF is dependent on the nature of the gene promoter and/or enhancer. Brm and Brg-1 appeared to functionally compensate for each other with regard to potentiating transcription of the EPO gene, and their functions are ATPase-dependent.
Brm and Brg-1 Are Recruited to the 3’ Enhancer Region of the EPO gene in Response to Hypoxic Stimulation- Studies in yeast demonstrate that SWI/SNF complexes can localize to specific gene promoters, disrupting nucleosomal DNA to modulate transcription (35-37). The precise mechanism through which SWI/SNF regulates gene promoters in mammals is unresolved, however it was reported recently that in mammalian cells, SWI/SNF complexes can be recruited by transcription factors to specific gene promoters in vivo (38, 39). The observed requirements of Brm and Brg-1 for transcription of the EPO gene coupled to the known role of SWI/SNF in chromatin remodeling suggested that they might be associated with HIF-1 in vivo and be recruited in a hypoxia-dependent manner to the 3’ enhancer region of the EPO gene. The complex containing HIF-1, HNF-4, and SWI/SNF might then interact with the transcriptional initiation complex at the 5’ promoter through a DNA-looping mechanism. To test this hypothesis, we performed ChIP and Re-ChIP assays. A pair of primers spanning 250 bp DNA of the 3’ region of the EPO gene encompassing the functional HRE were used for PCR (Fig. 5A). In these studies, Hep3B cells were treated or untreated with hypoxia for 3h, followed by formaldehyde cross-linking. The cells were lysed, sonicated and then subjected to immunoprecipitation by HIF-1α, Arnt, Brm and Brg-1 antibodies. Antibodies against HIF-1α, Arnt, Brm and Brg-1 efficiently precipitated the 3’ enhancer region of the EPO gene in a hypoxia-dependent fashion (Fig. 5B), indicating that each associates with this region after hypoxic induction. We obtained the same results using Hep3B cells treated with CoCl2, a hypoxia-mimicking reagent (data not shown). To investigate whether Brm or Brg-1 associate with HIF-1 containing templates in vivo, we performed Re-ChIP analysis. Briefly, cross-linked chromatin from CoCl2-treated Hep3B cells was first treated with the HIF-1α antibody. The resulting immunocomplex was then eluted and subjected to immunoprecipitation with Arnt, Brg-
and Brm antibodies. We found that the 3’ enhancer region of the EPO gene present in the first immunocomplex was pulled down again by the last three antibodies, indicating that Brg-1 and Brm (and Arnt) are each associated with HIF-1α at the gene enhancer (Fig. 5C). Interestingly, though depletion of Brm or/and Brg-1 did not affect the levels of VEGF mRNA induced by hypoxia, ChIP analysis showed that both Brm and Brg-1 were recruited to the promoter of VEGF gene in a hypoxia-dependent fashion (Fig. 5D).
DISCUSSION

Erythropoietin (EPO) plays a central role in regulating the oxygen-carrying capacity of the blood. In mammalian systems, the EPO gene is the most hypoxia-responsive gene found so far, and can be induced by hypoxia up to 100-fold. Transcriptional regulation is achieved by the concerted action of several transacting factors interacting with the 5’ proximal promoter region and with the 3’ untranslated region of the EPO gene, which are widely separated from each other.

Coactivators harboring HAT activity such as the p160 proteins and p300, have been shown to be involved in transcription of the EPO gene. This gene therefore provides an ideal system to study the roles played by coactivators and chromatin-remodeling complexes, and to study communication between promoter and enhancer.

In this study, we found that lysine 5 and/or lysine 8 of H4, which are targets of p300 and Src-1 in vitro, were acetylated after hypoxic induction in the promoter region of the EPO gene. This prompted us to investigate the role of SWI/SNF in the transcription of the gene, since the histone acetyltransferases and ATP-dependent remodeling enzymes are usually coupled, and cooperate with each other in regulation of gene transcription. For example, the bromodomains of Brm and Brg-1 are known to bind acetylated lysine in histones with high affinity (40), indicating that HAT coactivators may create a preferred target for the SWI/SNF complex. As we hypothesized, we found that both Brm and Brg-1 could substantially enhance expression of an EPO enhancer-driven reporter gene in a hypoxia-dependent manner in transient transfection experiments. We obtained similar results using 6xHRE-SV40-Luc, a reporter in which luciferase is driven by 6 randomly concatenated HREs, indicating that SWI/SNF complexes act specifically through the HRE. Though much less efficiently, some enhancement of the reporters’ transcription was
observed with the ATPase-abolished mutants of Brm and Brg-1, which are unable to carry out chromatin remodeling. It is possible that Brm and Brg-1, besides participating in nucleosomal remodeling, can facilitate the formation and stabilization of transcription initiation complex independently of their ATPase activities. In fact, we previously obtained similar results when we carried out transient transfection assays using a luciferase reporter driven by a cluster of xenobiotic response elements, in that we observed that ATPase-defective mutants of Brm and Brg-1 could enhance 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin induced reporter gene transcription to some extent (39). Although some studies in yeast suggest that activations of certain genes can occur via either nucleosome remodeling or histone acetylation, recent studies in mammalian systems indicate they may fulfill different roles in alleviating nucleosomal repression. Coincident with this notion, we found that none of the three members of the p160 family of coactivators, nor CBP could substitute for Brm and Brg-1 with regard to HIF-1 mediated transcription.

Importantly, we observed that Brm and Brg-1 are required for hypoxia-induced EPO transcription in vivo and they appeared to compensate for each other functionally. Depletion of both Brm and Brg-1 by siRNA treatment significantly diminished the transcription of the EPO gene. Knocking down Brm on its own resulted in a moderate reduction of gene transcription, while knocking down Brg-1 on its own resulted in an augmentation of gene transcription. This last finding may be attributed to our interesting observations that Brm appears to be more potent than Brg-1 with regard to HIF-1 mediated transcription, and that the expression of Brm mRNA is increased when Brg-1 is depleted. It is of interest that the Brg-1 protein was known to be increased in Brm-null mice, indicating that compensatory up-regulation of the ATPase subunits also appear to occur in the whole animal.
Though the transcription of VEGF is also mediated by HIF-1, our data indicate that Brm and Brg-1 are not essential for its HIF-1-dependent transcription. Nevertheless, our ChIP analysis show that both Brm and Brg-1 are recruited to the promoter of VEGF gene in a hypoxia-dependent fashion. It is of interest that although Brm and Brg-1 are not required for hypoxic induction of the VEGF gene, they are nevertheless recruited to the VEGF promoter upon hypoxia treatment. It is possible that these coactivators are routinely recruited by HIF-1 irrespective of whether they are required or not. It is possible that the VEGF gene maintains an open chromatin structure that is accessible to transcription factors even under normoxic conditions. The modest hypoxic induction of the VEGF gene may be due to the relatively high level of transcription occurrence under these conditions.

Our ChIP assays demonstrate recruitment of Brm and Brg-1 to the enhancer region of the EPO gene, indicating that Brm and Brg-1 may directly regulate the transcription of the EPO gene. Using Re-ChIP analysis, we found that Brm and Brg-1 are each present in a complex that also contains HIF-1, suggesting that Brm and Brg-1 may be recruited to the enhancer by an “enhancersome” consisting of HIF-1, HNF-4, p300 and other coactivators. It remains to be elucidated if HIF-1 can interact with Brm and Brg-1 directly.

In conclusion, our observations indicate that the EPO gene is a novel target gene of the SWI/SNF chromatin-remodeling complex, but that the requirement for this complex is gene specific. To our knowledge, this is the first reported investigation on the roles of ATPase chromatin-remodeling factors in transcriptional activation by HIF-1, and it reveals a novel mechanism through which Brm and its homologue Brg-1 might compensate for each other functionally to fulfill chromatin remodeling.
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(1999) Nature 399, 491-496

FOOTNOTES

5 The abbreviations used are: Brm, Brahma; Brg-1, Brahma/SWI2-Related Gene 1; EPO, erythropoietin; HIF-1, hypoxia-inducible factor 1; HIF-1α, hypoxia-inducible factor 1α; Arnt, aryl hydrocarbon receptor nuclear translocator; CBP, CREB binding protein; VEGF, vascular endothelial growth factor; BAFs, Brg-1-associated factors; HNF4, hepatocyte nuclear factor 4; PIC, preinitiation complex; HRE, hypoxic response element; HAT, histone acetyltransferase; ChIP, chromatin immunoprecipitation.

ACKNOWLEDGMENTS

This work was supported by grant CA28868 from the NCI, and by a fellowship from the University of California Toxic Substances Research and Teaching Program to Feng Wang.
Figure Legends

Fig. 1. **Histones at the EPO promoter are modified upon hypoxic induction.** A, schematic representation of the genomic structure of the EPO gene. Rectangles represent exons, (numbered I to V) with empty rectangles representing untranslated regions. The arrows indicate the primers used in PCR. B, Hep3B cells were treated with 100 μM CoCl2, harvested at the indicated time points, and subjected to ChIP analysis using an antibody directed to acetylated lysines at positions 5 and 8 of histone H4.

Fig. 2. **Brm and Brg-1 enhance transcription of an EPO enhancer-driven reporter.** A, schematic representation of EPO enhancer-SV40-Luc. B, SW-13 cells cultured in 12 well-plates were transfected with 0.25 μg of the reporter together with 25 ng Renilla luciferase reporter driven by the Herpes simplex virus thymidine kinase promoter, 0.25 μg of HIF-1α or the empty pBos vector, and 0.4 μg of Brm and/or Brg-1. C, SW-13 cells were cultured and transfected with HIF-1α and as described in B, except for they were cotransfected with different amount of Brm or Brg-1 or their mutated derivatives deficient for ATPase activity (Brm-M and Brg-1-M). 24 h after transfection, the cells were treated with hypoxia for another 24 h, then subjected to luciferase assay. Firefly luciferase activities were normalized with Renilla luciferase activities. RLU, relative luciferase units.

Fig. 3. **Brm and Brg-1 enhance the transcription of a reporter driven by HREs.** A, schematic representation of 6xHRE-SV40-Luc. B, SW-13 cells cultured in 12 well-plates were transfected with 0.25 μg of the reporter together with 25 ng Renilla luciferase reporter driven by the Herpes simplex virus thymidine kinase promoter, and 1.0 μg of Brm and/or Brg-1 or their mutated derivatives deficient for ATPase activity (Brm-M and Brg-1-M). C, SW-13 cells were
cultured and transfected with the reporters as in B, except that they were cotransfected with 0.25 μg of HIF-1α and 1.0 μg of each coactivators or 0.5 μg of Brm or Brg-1. 0.5 μg of SRC-1 was used when it was cotransfected with Brm or Brg-1. 24 h after transfection, the cells were treated with hypoxia for another 24 h, then subjected to luciferase assay. Firefly luciferase activities were normalized with Renilla luciferase activities. RLU, relative luciferase units. + and * indicate statistically significant differences (p< 0.001) and (p<0.01) respectively.

Fig. 4. **The effects of reduction of Brm and/or Brg-1 by siRNAs on induction of the EPO gene by hypoxia.** Hep3B cells were transfected with siBrm (A and B), siBrg-1 (C and D), siB/B (E and F), and the scrambled RNA duplex as control. To examine the expression of the mRNAs for Brm and Brg-1, the cells were harvested 48h after transfection and the total RNA isolated and used for reverse transcription and real-time PCR. To examine the expression of the Brm and Brg-1 proteins, the cells were harvested and the whole cells extracts were prepared 72 h after transfection. Western blot were done using the whole cells extracts and anti-Brm (A and E) or anti-Brg-1 (C and E) antibodies. To test the expression of EPO and VEGF mRNA, the cells were treated with 1% O₂ for another 24 h 72 h after transfection, and then total RNA were isolated and subjected to reverse transcription and real-time PCR (B, D and F). Each of the real-time PCRs was done three times and one representative result was selected. The result is an average from three real-time PCR reactions with the same template. All these four genes’ mRNA levels were normalized to that of the constitutively expressed 36B4 gene encoding a ribosomal subunit. * indicates statistically significant difference (p<0.01).

Fig. 5. **Brm and Brg-1 are recruited to the enhancer of the EPO gene in a hypoxia-dependent fashion.** A, schematic representation of the human EPO enhancer and the locations of the primers used to amplify the enhancer region. B, Hep3B cells cultured under 21% O₂ or 1%
O$_2$ for 4 h were cross-linked and subjected to sonication and immunoprecipitation with the indicated antibodies. C, ChIP was first carried out using the HIF-1$\alpha$ antibody, and the immunocomplex was eluted by 10 mM dithiothreitol. The aliquots of the diluted elution were immunoprecipitated with Arnt, Brm, or Brg-1 antibodies as indicated. The precipitated chromatin was amplified by PCR using the primers flanking the enhancer of EPO gene. D, ChIP was performed as in B, but the precipitated chromatin was amplified by PCR using the primers for the promoter of the VEGF gene.
Fig. 1

A.

B.

α-Ac-K5/K8(H4)

Input
A. Epo enhancer-SV40-Luc

B. RLU

Fig. 2
Fig. 2
A. 6xHRE-SV40-Luc

B.

Fig. 3
C.

Fig. 3
A.

Fig. 4
Fig. 4
C.

Fig. 4
Fig. 4

D.

Epo/H36B4

VEGF/H36B4

SCX siBrg-1 SCX siBrg-1

SCX siBrg-1 SCX siBrg-1

Normoxia
Hypoxia

*
Fig. 4
Fig. 4
Fig. 5

A. Diagram showing the location of Exon 5, Primer F, HRE, and HNF-4 binding site.

B. Western blot analysis showing the effect of different oxygen levels on HIF-1α and other proteins (Arnt, Brm, Brg1). 

C. ChIP assay showing the interaction of HIF-1α with Arnt, Brm, and Brg1 before and after CoCl$_2$ treatment.
D. O$_2$  21%  1%

HIF-1α
Arnt
Brm
Brg1
Input

Fig. 5
Roles of brahma and brahma/SWI2-related gene 1 in hypoxic induction of the erythropoietin gene
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*J. Biol. Chem.* published online September 3, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M409002200

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