TITLE: Differential activities of murine SIM1 and SIM2 on a hypoxic response element: Crosstalk between bHLH/PAS transcription factors

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RUNNING TITLE: Activities of murine SIM1&2 on a hypoxic response element.
ABSTRACT

The basic-helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) protein family comprise a group of transcriptional regulators that often respond to a variety of developmental and environmental stimuli. Two murine members of this family, Single Minded 1 (SIM1) and Single Minded 2 (SIM2), are essential for post-natal survival but differ from other prototypical family members such as the dioxin receptor (DR) and hypoxia inducible factors, in that they behave as transcriptional repressors in mammalian one hybrid experiments and have yet to be ascribed a regulating signal. In cell lines engineered to stably express SIM1 and SIM2, we show that both are nuclear proteins that constitutively complex with the general bHLH/PAS partner factor, ARNT. We report that the murine SIM factors, in combination with ARNT, attenuate transcription from the hypoxia inducible Erythropoietin (EPO) enhancer during hypoxia. Such cross talk between coexpressed bHLH/PAS factors can occur through competition for ARNT, which we find evident in SIM repression of DR induced transcription from a xenobiotic response element reporter gene. However, SIM1/ARNT, but not SIM2/ARNT, can activate transcription from the EPO enhancer at normoxia, implying the SIM proteins have the ability to bind hypoxia response elements and affect either activation or repression of transcription. This notion is supported by co-immunoprecipitation of EPO enhancer sequences with the SIM2 protein. SIM protein levels decrease with hypoxia treatment in our stable cell lines, although levels of the transcripts encoding SIM1 and SIM2 and the approximate 2h half lives of each protein are unchanged during hypoxia. Inhibition of protein synthesis, known to occur in cells during hypoxic stress in order to decrease ATP utilisation, appears to account for the fall in SIM levels. Our data suggests the existence of a hypoxic switch mechanism in cells which coexpress HIF and SIM proteins, where upregulation and activation of HIF-1α is concomitant with attenuation of SIM activities.
INTRODUCTION

The bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim homology) protein family is an expanding group of transcription factors with diverse roles in early development and adaption to environmental stress. For example, two hypoxia inducible factors, HIF-1α and HIF-2α, are essential for murine embryonic angiogenesis and catecholamine production, as well as adaption to hypoxic stress in adult animals (1-4). The related dioxin (aryl hydrocarbon) receptor (DR) has a poorly understood role in embryonic liver and kidney vascularisation (5), but a well defined function of inducing drug metabolising enzymes when animals are challenged by environmental pollutants such as dioxins and polycyclic aromatic hydrocarbons. (For a recent review, see (6)). More recently, bHLH/PAS proteins such as CLOCK and BMAL/CYCLE/MOP3 have been found to be critical for the central pacemaker function of the suprachiasmatic nucleus that generates circadian rhythms (7,8). Other bHLH/PAS proteins have been demonstrated to be essential for embryonic development, but in contrast to the above factors, are not known to be regulated by environmental signals. Such transcription factors include Drosophila Single Minded (dSIM) and Tracheal (TRH), which function in specifying central nervous system midline cells or tubular structures such as airway passages and salivary glands, respectively (9-11). Two mammalian SIM proteins have been described thus far, SIM1 and SIM2, which are amongst the rare members of this family reported to be transcriptional repressors (for recent review, see (12)).

Murine SIM1 and SIM2 show high amino acid identity in their N-termini (approximately 90% identity in the bHLH and PAS regions), but are completely divergent in their C-termini (13,14). They demonstrate similar expression patterns at the tissue level, being found in the brain, kidney, lung and skeletal muscle (14-19). On closer examination these expression patterns can be overlapping yet distinct at the cellular level. For example, Sim1 is expressed in cells immediately adjacent to the ventral midline cells of the diencephalon expressing Sim2 (14). Targeted gene deletions have shown the separate genes expressing these factors to be essential for survival. Both Sim1−/− and Sim2−/− mice die shortly after birth: Sim1−/− pups lack critical neuroendocrine secreting cells of the hypothalamus (20) while Sim2−/− pups suffer from a breathing defect for which the details are yet to be published (C.Fan, pers.comm., 21). Disruption of one allele of human Sim1 resulted in severe early onset obesity in one female patient (22), and haploinsufficiency causes hyperphagia and obesity in the mouse model (23). While these proteins evidently perform critical biological functions, little is
known of their mechanisms of action. Despite considerable effort, direct target genes for mammalian SIMs have yet to be clearly elucidated (20,24), while in contrast, a number of target genes of the dSIM protein have been characterised, including *Toll, Slit, breathless* and *dSim* itself (25-28).

The promoters of dSIM target genes contain dSIM recognition motifs termed the Central Midline Enhancer (CME) element, the core of which is 5′-ACGTG-3′. Heterodimerisation with a general bHLH/PAS partner protein, termed ARNT, is obligatory for DNA binding by the SIM proteins, the hypoxia inducible factors and the DR (13,15,24,29-33). Consistent with the ACGTG motif being at the heart of dSIM response elements, *in vitro* PCR site selection using dSIM/ARNT heterodimers isolated a core sequence of 5′-GT(C/A)CGTG-3′ (34). Somewhat paradoxically, the CME sequence is also bound by the *Trachealess* (*Trh*) gene product and functions as an enhancer in TRH target genes (27,35,36). The close relationship between the DNA binding basic regions from the bHLH domains of dSIM (KEKSNAARRR) and TRH (KEKSRADAARSRR) explain this overlap of activity. By using PAS domain "swap mutants", it has recently been shown that *Drosophila* target gene specificities for dSIM and TRH are conferred by their PAS domains, presumably by interacting with discerning cofactors or collaborating transcription factors to activate distinct promoters (35). The murine SIMs, which harbour identical (SIM1) or almost identical (one conservative amino acid change, SIM2) basic regions to dSIM (see Figure 1), have been shown to bind the CME and control the expression of synthetic reporter genes containing repeats of the CME taken from the *Drosophila Toll* gene (37).

Intriguingly, the basic region of murine HIF-1α is identical to that of TRH, while the HIF-2α basic region differs by a single amino acid (Figure 1). Not surprisingly then, the core sequence of Hypoxia Response Elements (HREs), 5′-ACGTG-3′, is identical to that of the CME.

The high similarity between basic regions and consequently DNA recognition sites for a number of bHLH/PAS proteins presents the possibility of cross coupling and/or interference of gene regulation between these factors. In particular, the two hypoxia inducible factors and the two SIM proteins, all of which are essential for survival in mammals and have overlapping expression patterns, seemingly have a high potential for binding the same DNA sequences *in vivo*. In order to investigate the interplay of these factors on known mammalian enhancer sequences, we have analysed the influence of SIM1 and SIM2 on reporter genes driven by the hypoxia inducible enhancer of the *erythropoietin* (*EPO*) gene (38). We have created stable cell lines that
express Myc-epitope tagged forms of either mouse SIM1 or SIM2 and show these proteins to be exclusively nuclear. The SIM1/ARNT heterodimer is able to activate the reporter gene controlled by the EPO enhancer whereas the SIM2/ARNT heterodimer represses the hypoxic induction of the reporter gene by competing with the HIF-1α/ARNT heterodimer for the HRE sequence. To investigate the possibility of a hypoxic switch, whereby the hypoxic stabilisation of HIF-1α might be coupled to a hypoxic destabilisation of the SIM proteins, we examined the levels of SIM transcript and protein during hypoxia and conducted [35S]-methionine pulse chase labelling experiments to measure the half lives of SIM1 and SIM2. The levels of both SIM proteins were decreased with hypoxic treatment, but as the transcript and protein half lives were unaltered by oxygen deprivation, this decrease most probably reflects the broad spectrum inhibition of protein synthesis that occurs during hypoxia.

**EXPERIMENTAL PROCEDURES**

*Construction of expression vectors.* A mammalian expression vector for murine SIM2 was generated by inserting the KpnI/NotI fragment from SIM2pBSK (16) into similarly digested pEF-bos-cs (39). Murine SIM1 and SIM2 expression vectors for production of stably transfected cell lines were generated by PCR incorporation of two consecutive epitope tags from c-myc with a three alanine spacer (EQKLISEEDLAAAEQKLISEEDL) at the carboxy-terminus of the SIM1 and SIM2 cDNA’s and subsequent insertion of each cDNA into pEF/IRESpuro (40), creating pEF/SIM1(Myc)2/IRESpuro and pEF/SIM2(Myc)2/IRESpuro. Human Arnt containing a PCR introduced carboxy-terminal dual haemagglutinin (Ha) epitope tag was subcloned into pEF-bos-cs (39) to generate pARNT(Ha)2/EF-bos. The KpnI/SpeI(blunt) fragment from SIM2pBSK and the EagI(blunt)/XbaI fragment from pDR-NLS/CIN4 (41) were inserted into KpnI/XbaI digested pEF/IRESpuro (40) to generate an expression plasmid for the chimeric protein SIM2/AD, for production of a stably transfected 293T cell line. The SIM2/AD protein contains the first 408 residues of SIM2 fused to amino acids 493-834 from the murine DR, followed by two nuclear localisation sequences, two Ha and a six Histidine tag at the C-terminus. A constitutively active deletion mutant of the murine DR that lacks sequence from the ligand binding domain (DRΔLBD) (42) was expressed from the pEF-bos-cs vector.
ARNT603 lacks the C-terminal transactivation domain (43) and was expressed from the mammalian pBNSEN vector (44).

Cell culture and production of stable cell lines expressing Myc-epitope tagged SIM1 and SIM2. Human embryonic transformed kidney cell line 293T was routinely maintained at 37°C, 5%CO2 in Dulbecco’s modified Eagle’s medium (DMEM, Gibco/BRL) supplemented with 10% fetal bovine serum (complete medium) (JRH Biosciences). To produce stably transfected cell lines, 293T cells in 10cm dishes were transfected with 5µg of blank pEF/IRESpuro, pEF/SIM1(Myc)2/IRESpuro, pEF/SIM2(Myc)2/IRESpuro or pEF/mSIM2/AD/IRESpuro using the liposomal transfection reagent DOTAP (Boehringer Mannheim) according to manufacturer’s instructions. Initial selection with 1µg/ml puromycin (Sigma) began 24h after transfection and continued for two weeks. Following this, several single antibiotic resistant colonies were expanded and the rest of the cells were pooled and similarly expanded in media containing 20µg/ml puromycin. Where specified, cells were subjected to a hypoxic microenvironment using anaerobic sachets (Oxide, <1%O2), for a duration of 17 hours.

Immunofluorescence. Stably transfected monoclonal 293T cell lines were seeded onto coverslips and grown for 48h before formaldehyde fixation as previously described (45). Cells were washed once in phosphate-buffered saline (PBS), before being blocked in PBS with 5% skim milk powder for 30 mins, then washed once in PBS and incubated for 2h with 9E10 monoclonal antibody to detect Myc-tagged proteins. After two washes with PBS, cells were incubated for 45 min at room temperature with fluorescein isothiocyanate-conjugated sheep anti-mouse secondary antibody (Silensus) diluted 1:30 in PBS. Following two PBS washes, cells were incubated with bisbenzimide stain (Hoechst 33258; 1µg/ml;Sigma) for 2 min and washed once in PBS before being dried and coverslips mounted onto slides as previously described (45). Cells were examined using a Ziess microscope.

Transient Transfections. The luciferase reporter plasmids pHRE-LUC (29) and pXRE-LUC (46) have been described previously and were used in conjunction with the internal control plasmids pRL-SV40 and pRL-TK (Promega), respectively and the control pGL3-Promoter-LUC vector (Promega). Stably transfected polyclonal cells were plated onto 24-well plates at 3x10^4 cells/well and after 12-24h transfections were performed using DOTAP (Boehringer Mannheim) as per the manufacturer’s instructions. Each transfection contained either 20ng/well pHRE-LUC or pGL3-Promoter-LUC with 50ng/well internal control pRL-SV40, or 200ng/well pXRE-LUC and 100ng/well internal control pRL-TK, together with 200ng/well of each expression vector.
plasmid or blank expression plasmid if necessary to normalise the amount of DNA transfected. Where specified, cells were subjected to a hypoxic environment (<1% O₂) using anaerobic sachets (Oxide), beginning 12 hours after transfection. Cells were washed with PBS 24-48 h post-transfection, lysed with Passive Lysis Buffer (Promega) and extracts analysed for luciferase activity using the Dual Luciferase Reporter assay (Promega) according to the manufacturer’s instructions. Transient transfections performed to produce whole cell extracts for subsequent immunoblotting involved 48h transfection with 2µg of expression plasmid into 293T cells in 6cm diameter dishes using DOTAP as per the manufacturer’s instructions, whilst those for chromatin extract preparation involved 48h transfection of polyclonal 293TSIM2 cells in 175cm² flasks with 20µg of reporter plasmid using FuGENE 6 (Boehringer Mannheim) as per the manufacturer’s instructions.

**Metabolic labelling.** Stably transfected polyclonal pools of 293T cells expressing either SIM1 or SIM2, or containing the blank expression vector in the control line, were seeded into 6cm diameter dishes prior to being washed and incubated for 20min at 37°C with methionine/cysteine-free DMEM (Gibco/BRL) to deplete intracellular pools of methionine and cysteine. The cells were pulse labelled for 40min in the methionine/cysteine-free medium supplemented with 250µCi/ml [35S]methionine/cysteine (Geneworks) and then washed twice and incubated in complete medium for 2h, 4h and 8h chases. Hypoxic treatments began with the chase. Cells were washed twice with cold PBS, lysates prepared and immunoprecipitations performed as described below. Following sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, 7.5% gel) of the immunoprecipitates, gels were dried and analysed using a PhosphorImager (Molecular Dynamics). Labelled bands were quantitated using Image Quant (Molecular Dynamics).

**Immunoprecipitation.** Preparation of whole cell extracts (WCE) (47) and nuclear extracts using Nonidet P-40-Ficoll lysis buffer (41) were performed as previously described. For experiments involving hypoxic treatment, cells were removed from incubation at 37°C in normoxia or hypoxia, media aspirated and cells lysed immediately in cell lysis buffer containing 25µM MG132 (BioMol) to prevent protein degradation via the ubiquitin-proteasome system and 100µM 2,2’-dipyridyl (Aldrich) for hypoxic samples only. Protein concentrations were determined by Bradford assay. Immunoprecipitations with 150-160µg lysate using an anti-ARNT polyclonal rabbit serum raised against residues 1-140 of human ARNT (ARNT51) and an anti-Myc 9E10 monoclonal antibody were performed essentially as previously described (48) with minor variation. The binding buffer A contained 150mM KCl and lysates were incubated with antibody for 2h at 4°C.
Immunoprecipitates were eluted in 0.5% SDS, 0.05M β-Mercaptoethanol for 5 minutes at 50°C, boiled in SDS buffer, separated by SDS-PAGE on a 7.5% gel, and then transferred to nitrocellulose.

**Immunoblotting.** WCE were prepared as described above, with 5-20µg of WCE being subjected to SDS-PAGE (7.5% gel) and then transferred to nitrocellulose using a semi-dry blotter (Hoefer). Proteins were detected with the anti-Myc 9E10 monoclonal antibody, the anti-Ha 12CA5 monoclonal antibody, anti-HIF-1α polyclonal serum raised against residues 786-826 of human HIF-1α (HIF55) or anti-ARNT polyclonal serum (ARNT51).

**Chromatin Immunoprecipitation Assay.** Chromatin extracts were prepared from polyclonal 293TSIM2 stable cells (approximately 2.5x10^7 cells per immunoprecipitation) essentially as previously described with a few alterations (49). To solubulise chromatin and shear DNA, six 30 second sonication pulses interspersed with 1 minute rest periods were performed on ice using a Sonifier Cell Disruptor (Branson Sonic Power B30). An aliquot of chromatin extract was collected for the input sample and the remainder precleared by incubation with Protein A Sepharose (Amersham Pharmacia). Immunoprecipitation overnight at 4°C followed, with 5µg of 12CA5 or 9E10 antibodies. Each sample was incubated with 250µl of a 50% Protein A Sepharose slurry for 1 hour at 4°C and the immunocomplexes subsequently washed six times with 0.1% SDS, 1% Triton X-100, 2mM EDTA, 0.14M NaCl, 1mM PMSF, 0.1% Sodium deoxycholate, 0.02M Tris-HCl, pH 8, three times with .5M Lithium Chloride, 1% NP40, 1% Sodium deoxycholate, 1mM EDTA, 0.1M Tris-HCl, pH 8, twice with 1mM EDTA, 0.01M Tris, pH 8 and eluted twice with 1% SDS, 0.05M Sodium bicarbonate. One tenth of the eluate was subjected to SDS-PAGE and western analysis using the 9E10 antibody and the DNA was purified from the remainder using a previously described protocol [http://mcardle.oncology.wisc.edu/farnham/protocols/](http://mcardle.oncology.wisc.edu/farnham/protocols/). Eluted DNA was resuspended in 30µl of TE and 2µl was added to each PCR using primers designed to amplify the HRE sequences in the reporter plasmid.

**Northern Analysis.** Poly A+ RNA was isolated from 293T control, 293TSIM1, 293TSIM2 stable cell lines essentially as previously described (50), except that cells were removed from normoxic or hypoxic incubation, the media aspirated and cells immediately lysed in cell resuspension buffer with 1% SDS and 200µg/ml Proteinase K (Roche) to minimise reoxygenation. 5µg of each poly A+ RNA was analysed by Northern blot with probes specific to SIM1, SIM2 and β-actin, using Rapid-hyb (Amersham Pharmacia) and PhosphoImager visualisation.
RESULTS

SIM1 and SIM2 are nuclear proteins.

Several mammalian bHLH/PAS proteins are signal regulated and exhibit cytoplasmic to nuclear translocation upon activation. This has been best demonstrated in the case of the DR, which in its latent form is found in the cytoplasm bound with hsp90 and the co-chaperones p23 and XAP2 (for a review, see (6)). Upon ligand binding, a nuclear localisation signal (NLS) within the DR is unmasked and the complex translocates into the nucleus (41,51). In the case of HIF-1α, a C-terminal NLS is hypoxically regulated to enhance nuclear uptake of the protein under low oxygen conditions (52). To assess the activities of SIM1 and SIM2 proteins and to characterise their cellular location, we created stable monoclonal cell lines, derived from human embryonic kidney (HEK) 293T cells, which express Myc-epitope tagged SIM1 and SIM2. Western blotting of cell extracts from these cell lines with an anti-Myc antibody showed expression of Myc tagged SIM1 and SIM2, while extract from the control cell line stably transfected with blank expression vector revealed a complete lack of background staining (Figure 2A). SIM1 and SIM2 belong to class I bHLH/PAS proteins, which characteristically bind the molecular chaperone hsp90 and undergo cytoplasmic/nuclear shuttling before forming active heterodimers with ARNT. While SIM1 and SIM2 are known to dimerise with ARNT (13,16,33) and in vitro translated SIM1 has been shown to bind hsp90 (15), their cellular location has not previously been analysed. Immunohistochemical analysis of these cells using the anti-Myc antibody revealed that both SIM1 and SIM2 are exclusively nuclear (Figure 2B, panels iv and vi). Consistent with the Western blotting results, the control cell line stably transfected with blank expression vector showed no background staining (Figure 2B, panel ii). This nuclear localisation is unchanged during hypoxic treatment of the cells (data not shown). These results reveal the SIM proteins are unusual mammalian class I bHLH/PAS proteins as they are constitutively nuclear, which is consistent with the observation that dSIM is a nuclear protein unless ectopically expressed in the absence of the Drosophila ortholog of ARNT, a protein termed TANGO (53,54).

SIM1/ARNT but not SIM2/ARNT heterodimers can activate the EPO enhancer.
The DNA binding basic regions of dSIM, SIM1 and SIM2 are highly similar to those of the hypoxia inducible factors HIF-1α and HIF-2α (see Figure1), and the minimal core nucleotides of the CME enhancer recognised by dSIM are identical to the minimal core nucleotides present in the HRE of the EPO gene (i.e. 5'-ACGTG-3') (26,55).

To investigate whether the SIM proteins might invoke a response on the HRE sequence in normoxia, we transfected our SIM1 and SIM2 expressing cell lines with an expression vector for ARNT together with a luciferase reporter gene carrying 4 tandem copies of the 18 nucleotide HRE sequence from the EPO gene (29,55). In the SIM1 cell line, activity of the reporter gene was increased approximately 5 fold over that seen for the equivalently transfected control cell line in an HRE-dependent manner (Figure 3A). In stark contrast, no significant increase in reporter gene activity was observed in the SIM2 cell line. These results suggest that either SIM1 harbours a transactivation domain, in analogy to dSIM, or SIM1 recruits ARNT to the response element where ARNT provides a transactivation function. A similar trend was found on transfection of ARNT2, instead of ARNT, with SIM1 (data not shown). As SIM1 has previously been reported to contain a transcription repression domain in the C-terminus (13) we wished to assess the importance of ARNT for the activation seen in SIM1 cells. We therefore repeated the experiment with a C-terminally truncated ARNT603 protein, which lacks a transactivation domain (43), coexpressed with SIM1. While the SIM1/ARNT heterodimer was able to activate the HRE reporter gene, the SIM1/ARNT603 heterodimer was devoid of activity, exhibiting only the background reporter activity observed in control or SIM2 expressing cells (Figure 3A). Figure 3B illustrates successful expression of ARNT and ARNT603 proteins in these transient transfection experiments. This data demonstrates that in the SIM1/ARNT heterodimer, the ARNT transactivation domain, rather than SIM1, is functioning to induce transcription. In contrast, the SIM2/ARNT heterodimer was unable to activate transcription from the HRE reporter gene.

SIM2 can repress hypoxic activation of the EPO enhancer.

SIM2 has been reported to harbour an active transcription repression domain in its C-terminus (33) and unlike the SIM1/ARNT heterodimer, the SIM2/ARNT heterodimer is silent on a reporter gene carrying HRE sequences from the EPO enhancer in normoxia. Previously, expression of SIM2 in COS-7 cells has been shown to inhibit hypoxic activation of a reporter gene in a one-hybrid experiment using ARNT anchored to DNA via a GAL4 DNA binding domain (13,33). In order to test whether SIM/ARNT heterodimers could block hypoxia
induced transcription from the EPO enhancer we used the 293TSIM1 and 293TSIM2 stable cell lines to measure the influence of the SIMs on activity of the hypoxically induced HRE reporter gene. As previously established, the HRE sequences mediate strong hypoxia induced activity, which is absent for the control reporter gene lacking HRE sequences (29) (Figure 4A). This effect is predominantly due to formation of an active HIF-1α/ARNT complex rather than a HIF-2α/ARNT complex, as western blotting shows a large increase in HIF1α protein, but no detectable expression of HIF-2α, when 293T cells are subjected to hypoxia (data not shown). In the presence of SIM2, and to a lesser extent SIM1, the hypoxic induction of the HRE reporter gene is attenuated but not completely ablated, suggesting that SIM/ARNT complexes are competing with HIF-1α/ARNT complexes for control of the reporter gene. This competition could be via two possible mechanisms; either by SIM sequestering ARNT from HIF-1α to merely decrease the concentration of HIF-1α/ARNT complexes in the cell, or by SIM/ARNT complexes forming and then binding the HRE sequence to additionally block access of HIF-1α/ARNT to the reporter gene. To distinguish between these possibilities for SIM2, we created a chimera termed SIM2/AD, where the C-terminal repression domain of SIM2 was replaced by the constitutively active C-terminal activation domain of the DR. Western analysis using an anti-Ha antibody shows clear expression of the Ha epitope tagged SIM2/AD chimera in cell extracts from the 293TSIM2/AD stable cell line compared to control cells (Figure 4B). In combination with ARNT, the SIM2/AD chimera was able to potently activate the HRE reporter gene at normoxia (Figure 4A), thus establishing that the bHLH/PAS region of SIM2 can also recognise and bind the HRE sequence when dimerised with ARNT. To further support this observation, experiments employing chromatin immunoprecipitation protocols were performed using DNA extracted from the SIM2 stable cell line transiently transfected with the HRE reporter. The Myc-tagged SIM2 protein is specifically immunoprecipitated from the DNA extracts using an anti-Myc but not an anti-Ha antibody (Figure 4C). Using primers designed to PCR amplify the HRE sequences in the reporter plasmid, HRE sequences were found to be enriched in the anti-Myc immunoprecipitated pool compared to that from the control anti-Ha immunoprecipitated pool, establishing that SIM2 is bound to HRE sequences within the cell (Figure 4D).

**SIM proteins compete with HIF-1α for binding to ARNT.**
To examine the interplay between HIF-1α and the SIM proteins for binding to their common partner factor, ARNT, we performed a series of co-immunoprecipitation experiments. The lysates from control, SIM1 and SIM2 cell lines that had been cultured in normoxia or hypoxia for 17 hours were immunoprecipitated with an anti-ARNT antibody. The antibodies specifically immunoprecipitated ARNT (Figure 5A) and each immunoprecipitate contains equivalent amounts of ARNT, as seen by Western Blot (Figure 5B). Interestingly, less HIF-1α appears to be associated with ARNT in the SIM stable cell lines compared to the control line as visualised by Western blot of the immunoprecipitate using an anti-HIF-1α antibody (Figure 5B). Similarly, use of an anti-Myc antibody in Western analysis suggests less SIM/ARNT complexes are present in hypoxia when compared to the normoxic extracts (Fig 5B), showing that HIF-1α and SIM proteins can compete to bind limiting amounts of the ARNT partner protein.

**SIM1 and SIM2 can block function of the DR by sequestering ARNT.**

As SIM2 can repress the function of hypoxically activated HIF-1α by competitively dimerising with ARNT and binding the HRE sequence, we sought to determine the ability of the SIM proteins to repress function of the bHLH/PAS DR. For these studies we have used a constitutively active form of the DR which contains a deletion of the ligand binding domain, termed DRΔLBD, which thus dimerises with ARNT to bind the xenobiotic response element (XRE) in the absence of activating ligands (42). The XRE contains a core of 5'-TNGCGTG and the basic region of the DR differs markedly from those of the SIMs and hypoxia inducible factors (see Figure 1), thus the SIM/ARNT or HIF/ARNT heterodimers do not interact with the XRE sequence (15,34). Consistent with this notion, expression of the SIM2/AD chimera which harbours the DR C-terminus did not increase the activity of an XRE reporter gene (Figure 6). Expression of DRΔLBD was able to potently activate the reporter gene by partnering endogenous ARNT, while coexpression of either SIM1 or SIM2 with DRΔLBD silenced this activity (Figure 6). We interpret these results as indicating that the SIM proteins were able to competitively dimerise with endogenous ARNT, rendering DRΔLBD to exist as a monomer and therefore unable to bind the XRE enhancer. Consistent with this model, cotransfection of an ARNT expression plasmid together with DRΔLBD and either SIM1 or SIM2 was able to overcome the silencing of the reporter gene. Importantly, overexpression of ARNT alone was unable to activate the reporter gene (Figure 6). These
results indicate that in situations where ARNT is limiting, the SIM proteins are capable of repressing transcription mediated by other bHLH/PAS proteins by sequestering ARNT.

**SIM1 and SIM2 protein levels decrease after prolonged hypoxic treatment.**

The ability of the SIM1 and SIM2 proteins to dimerise with ARNT and bind the HRE sequence suggests that these proteins may cross couple or interfere with gene regulation by the hypoxia inducible factors HIF-1α and HIF-2α. *In situ* hybridisation studies and Northern analysis show that while expression of the *Sim* genes is tissue restricted (13-16), *HIF-1α* expression is ubiquitous and *HIF-2α* is expressed broadly with substantially high levels in endothelial cells, the carotid body and lung tissue (29,31,56). Some tissues, such as kidney, skeletal muscle and lung, are reported to express at least three of these factors. Given that all four proteins dimerise with ARNT and recognise the same core DNA sequence, this creates a problem of correctly placing each transcription factor complex at its cognate target gene. The HIF-1α protein is known to be present in very low levels at normoxia due to an extremely short half life of <5 minutes (57). Treatment with hypoxia dramatically increases protein levels by stabilising the protein half life to approximately 30 minutes (57). Against this background, we sought to analyse the levels of SIM1 and SIM2 protein with the idea of investigating a potential hypoxic switch mechanism, which might see target gene control affected by hypoxic stabilisation of HIF-1α concomitant with hypoxic destabilisation of the SIM proteins. We examined the levels of all three proteins in lysates from our stable cell lines after a 17 hour hypoxic treatment. Western analysis confirms that stabilisation of HIF-1α in low oxygen conditions is comparable in the control and SIM stable cell lines, whilst the level of Myc-tagged forms of both SIM1 and SIM2 decreased in hypoxia and ARNT levels remained unchanged (Figure 7).

**SIM transcript levels and protein half life are unchanged in hypoxia.**

In order to determine the mechanism by which hypoxic conditions result in a decreased amount of SIM proteins, we first examined the transcripts encoding both SIM1 and SIM2. Poly A+ RNA was isolated from control, SIM1 and SIM2 stable cell lines that had been incubated in either normoxia or hypoxia. Northern
analysis of the poly A+ RNA with either a SIM1 or SIM2 specific probe shows that the levels of these two transcripts do not change with hypoxic treatment of the cells, nor do the levels of the loading control transcript encoding β-actin (Figure 8A). As the levels of HIF-1α protein are controlled by rapid protein turnover in normoxia through the ubiquitin proteasome system (57-59), we investigated the turnover of the SIM proteins in normoxia and hypoxia. Polyclonal pools of SIM1, SIM2 or control stable cell lines were pulse labelled with [35S]-methionine and whole cell extracts taken at increasing time points during the chase period for immunoprecipitation and analysis of the SIM proteins. As expected, immunoprecipitations from pulse labelled control cells showed the absence of any background radiolabelled bands (Figure 8B). In contrast, immunoprecipitations with the anti-Myc antibody produced sharp radiolabelled bands for SIM1 and SIM2 at several time points. Quantitation by PhosphorImaging analysis found that both SIM1 and SIM2 have half lives of approximately 2 hours (Figure 8B). Repeating the experiment during hypoxic treatment of cells revealed that these half lives were not altered by the low oxygen levels. The finding that neither RNA or protein stabilities of SIM1 and SIM2 are affected during hypoxia suggests that the point of control of SIM protein levels may be the translation process. This is consistent with one of the general cellular responses to hypoxia being a broad scale decrease in protein synthesis. For example, a decrease in the rate of total protein synthesis in NHIK 3025 cells of 30-40% has been reported to occur within one hour of hypoxic treatment (60). Similarly, incubation of isolated rat hepatocytes in 5% oxygen results in a dramatic inhibition of protein synthesis (61). Given the relatively short half lives of SIM1 and SIM2, the overall level of both proteins will be susceptible to any changes in translation efficiency during hypoxic stress.

**DISCUSSION**

Gene targeting experiments have established that murine SIM1 and SIM2 are both essential for mice to survive immediately following birth. As with dSIM, the murine SIMs appear to have key neurological functions. Both are expressed in brain regions, with SIM1 critical for terminal differentiation of neuroendocrine secreting neurons in distinct hypothalamic nuclei. SIM2 has an undefined role in breathing reflexes, while its location in the Down’s Syndrome critical region of the human genome, coupled with learning defects found in mice overexpressing SIM2 (62,63), suggest it may play some role in the complex etiology of Down’s
Syndrome. Despite the fundamental biological roles of the SIM proteins, extremely little is known of their mechanisms of action. To begin to address questions relating to SIM1 and SIM2 activities, we have created stable cell lines expressing these proteins and begun to analyse their biochemistry and transcription controlling abilities.

SIM1 and SIM2 are constitutively nuclear proteins and form heterodimers with the general bHLH/PAS partner factor ARNT, which is also constitutively nuclear in most mammalian cells. In contrast to other bHLH/PAS proteins, such as the DR and the hypoxia inducible factors, we find no evidence that SIM1 or SIM2 are activated by environmental or physiological signals. It therefore seems that, unlike the ubiquitously expressed DR and HIF-1α, the activities of SIM1 and SIM2 are controlled by their temporal and spatial expression patterns and that of potential cofactors. This notion is in agreement with a model previously proposed by Ward et al. (54) for function of Drosophila SIM.

Analyses of the transcription controlling activities of SIM1 and SIM2 in our cell lines has shown that these proteins, in conjunction with ARNT, can bind to a prototypical hypoxia responsive enhancer and affect reporter gene activity. In a mechanism similar to that proposed for the aryl hydrocarbon receptor repressor and the DR (64), SIM proteins compete with the hypoxia inducible factors for partnership with ARNT and DNA binding sites. The SIM1/ARNT heterodimer induces transcription via the ARNT C-terminal transactivation domain, a result initially unexpected as SIM1 has previously been reported as repressing transcription from a transactivating GAL4/ARNT chimera in a mammalian cell one hybrid assay (13). Our results are, however, consistent with a recent report where the SIM1/ARNT heterodimer was found to activate a reporter gene containing CME elements from the Drosophila Toll gene (37). The difference in activities for SIM1 in these two situations may be due to alterations occurring in SIM1 structure once it is directly bound to DNA, with the findings of this study involving a more native context than the GAL4/ARNT one hybrid. In support of this idea, it has recently been proposed that a number of transcription factors, such as nuclear hormone receptors and the PIT1 POU-domain protein, undergo differing allosteric modifications in structure according to subtle variations in DNA sequences to which they bind, thus resulting in switches from activation to repression of transcription (65,66). In contrast to SIM1, SIM2 represses the transactivation function of the ARNT C-terminus, a result consistent with that of Moffett and Pelletier (37) as well as the repressive function found for SIM2 on the
GAL4/ARNT chimera in the one hybrid assay (13,33). It will now be important to analyse the activities of SIM1 and SIM2 in other promoter contexts to explore the idea that they may behave as either transcriptional activators or repressors, dependent on promoter context.

We have observed that both SIM1 and SIM2 can successfully negate the strong activity of a constitutively active mutant of the DR by sequestering ARNT, which is an obligate partner for DR function. SIM1 has previously been shown to block function of the wild type DR in a similar manner (15). Yeast two hybrid assays indicate that SIM1/ARNT heterodimers are stronger than liganded DR/Arnt heterodimers, whilst SIM2 has at least the same, or greater affinity for ARNT as the liganded DR (13,15,32). This provides an explanation for complete ablation of XRE driven reporter activity when the SIM proteins were coexpressed with the DR. In contrast, the dimerisation of HIF-1α with ARNT is of similar strength to that of the SIM proteins and ARNT (13), and as such it might be predicted that the SIMs would have a lesser ability to oppose HIF-1α function by sequestering ARNT. This seems to be the case in Figure 4A, where SIM1 and SIM2 only attenuate hypoxic induction of the HRE reporter gene. Indeed, this is explained by the presence of HIF-1α/ARNT heterodimers in hypoxia in the SIM stable cell lines (Figure 5), indicating that not all of the available pool of ARNT is sequestered by the SIM proteins. The competition between HIF, SIM1 and SIM2 for binding to ARNT, and resultant interference with HIF-1α dependent transcription (seen in Figure 4A), is apparent in the decrease in the levels of HIF-1α protein co-immunoprecipitated with ARNT in the SIM stable cell lines (Figure 5). Further complexity occurs with the presence of ARNT2, a protein closely related to ARNT but expressed primarily in brain regions where ARNT is low or lacking (32,56). ARNT2 is very likely the physiological partner for SIM1 in the hypothalamus (24), although it has not been established whether or not this is the case in other tissues. We have performed our reporter gene assays with ARNT2 as the partner for the SIM proteins, but found no differences in activities compared with those when ARNT is the expressed partner (data not shown).

A complex interplay of gene regulation may occur between the two SIM proteins and the two hypoxia inducible factors, HIF-1α and HIF-2α, due to the fact that all four dimerise with ARNT and can bind the same DNA sequence. During hypoxia, it is well established that protein synthesis decreases rapidly in an effort to reduce the ATP demand of the cell in unfavourable conditions of compromised oxidative phosphorylation (67).
Specific stress response proteins escape translational arrest through the employment of alternative translation strategies. For example, VEGF is efficiently translated in hypoxia through a cap-independent internal ribosome entry site (68). HIF-1α protein levels are also markedly increased in hypoxia through rapid protein stabilisation and continued association of the HIF-1α transcript with polysomes in low oxygen, resulting in efficient translation of the transcript (69). The decrease in the level of both SIM1 and SIM2 proteins observed in hypoxia appears to be at the translational level, as our results show the level of transcripts encoding both proteins and the half life of each protein is unchanged in low oxygen conditions. A hypoxic switch can therefore be understood to operate in cells where SIM1/2 are coexpressed with HIF-1α, in that HIF-1α is specifically stabilised and activated in hypoxia, concomitant with a decrease in the levels of SIM1 and SIM2 proteins, leading to the activation of hypoxic target genes.

In summary, we have shown that the murine SIM factors are nuclear proteins that are capable of binding mammalian HRE sequences in combination with ARNT, to result in attenuation of hypoxic reporter gene transcription in hypoxia. This repression occurs through competition of the HIF-1α and the SIM proteins for binding to ARNT but also for DNA binding sites. In contrast to HIF-1α which rapidly accumulates in low oxygen conditions, SIM protein levels decrease in hypoxia, most probably as a result of general translational inhibition in hypoxia stressed cells. Such complex interplay between the bHLH/PAS proteins in cells where the factors are coexpressed may enable the cell to adapt its response to multiple environmental and developmental signals.

REFERENCES


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FIGURE LEGENDS

FIG. 1. Alignment of basic regions from the bHLH of selected bHLH/PAS proteins. Abbreviations are; Drosophila Single Minded (dSIM), Murine Single Minded 1 (SIM1), Murine Single Minded 2 (SIM2), Drosophila Trachealess (TRH), Murine Hypoxia Inducible Factor-1α (HIF-1α), Murine Hypoxia Inducible Factor-2α (HIF-2α), Murine Dioxin Receptor (DR). The residue number of the first amino acid of each basic region in the context of its whole protein is indicated in brackets and residues differing from the dSIM basic region sequence are underlined.

FIG. 2. SIM1 and SIM2 are nuclear proteins in human embryonic kidney cells. A, Western blot (WB) analysis of Myc-epitope tagged forms of SIM1 and SIM2 in whole cell extracts. Protein extracts (20µg) from the 293T control, 293TSIM1 and 293TSIM2 stable cell lines were separated by SDS-PAGE (7.5%) gel, transferred to nitrocellulose membrane and immunoblotted with 9E10 (anti-Myc) monoclonal antibody. B, immunofluorescence of 293T control (i,ii), 293TSIM1 (iii,iv), 293TSIM2 (v,vi) stable cell lines. Cells were seeded onto coverslips and fixed with 1% formaldehyde. Nuclei were visualised using bisbenzimide stain (i,iii,v). Myc-tagged proteins were detected with anti-Myc antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse secondary antibody (ii,iv,vi).

FIG. 3. Transcription of a hypoxia inducible EPO enhancer containing reporter gene is activated by the SIM1/ARNT but not the SIM2/ARNT heterodimer. A, SIM1 and SIM2 expressing or control 293T cell lines
were transiently cotransfected with a luciferase reporter gene controlled by 4 consecutive HRE sequences from the EPO enhancer and expression plasmids for either full length or C-terminally truncated (ARNT603) forms of human ARNT, together with Renilla internal control luciferase. Luciferase activity was normalized against the internal control and results are depicted as fold induction over reporter gene activity in the presence of ARNT alone in the control cell line. Results shown are the average of three experiments performed in triplicate ± standard deviation. B, Western blots (WB) of whole cell extracts (20µg) prepared from 293T cells transfected with either blank vector or expression plasmids for ARNT and ARNT603. Proteins were detected with polyclonal antisera raised against an ARNT amino-terminal epitope.

FIG. 4. The SIM2/ARNT heterodimer binds and represses hypoxic induction of transcription from EPO enhancer sequences. A, subconfluent 293T stable cell lines expressing either SIM1, SIM2 or SIM2/AD or control (Ctrl) were cotransfected with luciferase reporter plasmids as indicated and an expression vector for full length ARNT. Hypoxic treatment began 12h after transfection and continued for 17 hours. Luciferase activity was normalized against the internal Renilla luciferase control and results are depicted as fold induction over activity of pHRE-LUC at normoxia in the presence of ARNT. Results shown are representative and depict one experiment performed in triplicate ± standard deviation. B, schematic and expression of the SIM2/AD chimera.

The location of the SIM2 bHLH motif, SIM2 PAS and the DR C-terminal activation domains are specified. NLS, 2x nuclear localisation signal, Ha, 2x haemagglutinin epitope tag, HIS, 6x Histidine tag. For western blot(WB) analysis, whole cell extracts (15µg) from 293T control and 293TSIM2/AD stable cell lines were separated by SDS-PAGE (7.5%) gel, transferred to nitrocellulose membrane and immunoblotted with 12CA5 (anti Ha) monoclonal antibody. C, Chromatin extracts from 293TSIM2 cells transiently transfected with pHRE-LUC were immunoprecipitated with 12CA5 (anti-Ha) or 9E10 (anti-Myc) antibodies and the eluates were analysed by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-Myc antibody. D, DNA purified from ChIP assay in C was examined by PCR amplification using primers designed to amplify the HRE sequences in pHRE-LUC, where –ve contains no DNA and input contains DNA isolated from the chromatin extract. Results shown are representative of independent experiments performed in triplicate.
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FIG. 5. HIF-1α and SIM proteins compete for binding to ARNT. A, whole cell extract (150µg) from 293T control cells was immunoprecipitated with pre-immune serum (PI) or anti-ARNT serum. The immunoprecipitates were separated by SDS-PAGE (7.5% gel), transferred to nitrocellulose membrane and western blotted (WB) with anti-ARNT serum. B, coimmunoprecipitation of SIM and HIF proteins with ARNT. Lysates (150µg) from normoxic (N) or hypoxic (H, <1% O₂, 17h) 293T control, 293TSIM1 and 293TSIM2 stable cell lines, were immunoprecipitated with anti-ARNT serum and immunoprecipitates were equally divided, separated by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-HIF-1α serum, anti-Myc antibody or anti-ARNT serum as indicated. Results shown are representative of three independent experiments.

FIG. 6. SIM1 and SIM2 mediated repression of transcription induced by the DR is relieved by the addition of ARNT. 293T cells were cotransfected for 48h with the xenobiotic response element (XRE) containing reporter plasmid and expression vectors as indicated and then assayed for luciferase activity. Luciferase activity was normalized against a Renilla luciferase internal control and results are depicted as fold induction over reporter gene activity in the absence of expression vectors. Results shown are the average of three experiments performed in triplicate ± standard deviation.

FIG. 7. SIM protein levels decrease with hypoxic treatment. Whole cell extracts (10µg) from normoxic (N) or hypoxic (H, <1% O₂, 17h) 293T control, 293TSIM1 and 293TSIM2 stable cell lines were analysed by SDS-PAGE (7.5%), transferred to nitrocellulose and immunoblotted (WB) with anti-HIF-1α serum, anti-Myc antibody or anti-ARNT serum. Results shown are representative of three independent experiments.

FIG. 8. SIM transcripts and protein half lives are unaltered by hypoxic treatment. A, Northern analysis of poly A⁺ RNA (5µg) isolated from normoxic (N) or hypoxic (H, <1% O₂, 17h) 293T control, 293TSIM1 or 293TSIM2 stable cell lines using probes specific for SIM1 (S1), SIM2 (S2) or β-actin. B, stable 293T control, 293TSIM1 and 293T SIM2 cell lines were labelled with [35S]methionine/cysteine for 40mins and then either harvested (0h) or chased with complete media for 2, 4 or 8h. Hypoxic treatments began at the 0h timepoint and continued throughout the chase. Whole cell lysates (160µg) were immunoprecipitated with the anti-Myc
antibody, immunoprecipitates were separated using SDS-PAGE (7.5% gel) and dried gels were subjected to PhosphorImager analysis for quantification of labelled bands. Graphed results depict the average cpm at each time point from two separate experiments, whilst below are two representative experiments.

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Figure 1

Basic Region

dSIM (1) M K E K S K N A A R T R R
SIM1 (1) M K E K S K N A A R T R R
SIM2 (1) M K E K S K N A K R T R R
TRH (52) R K E K S R D A A R S R R
HIF-1α (18) R K E K S R D A A R S R R
HIF-2α (15) R K E K S R D A A R C R R

DR (28) E G I K S N P S K R H R D
Figure 2

A

WB  
α-Myc  
83kDa

Control  SIM1  SIM2

B

i) ii)  
iii) iv)  
v) vi)
Figure 3

A

B
Figure 4

A

pGL3-LUC  ▪ SV40 Luciferase

pHRE-LUC  ▪ 4xHRE ▪ SV40 ▪ Luciferase

Fold Induction

35
30
25
20
15
10
5
0

Cell Line:

Ctrl  Ctrl  SIM1  SIM2  SIM2/AD

pGL3-LUC — pHRE-LUC

B

SIM2  DR  AD

bHLH  PAS  NLS/Ha  HIS

SIM2/AD

WB

α-Ha  83kDa

Control  SIM2/AD

C

IP:  α-Ha  α-Myc

WB

α-Myc  83kDa

D

α-Ha IP  α-Myc IP  +ve  Input

500bp
**Figure 5**

### A

![Western Blot Image](image)

**IP:**
- PI
- ARNT

**WB**
- α-ARNT

- 83kDa

### B

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**WB**
- α-HIF
- 175kDa

**WB**
- α-Myc
- 63kDa

**WB**
- α-ARNT
- 83kDa
Figure 6

Fold Induction

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Legend:
- pXRE-LUC
- 2xXRE
- TK
- Luciferase
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