Differential Activities of Murine Single Minded 1 (SIM1) and SIM2 on a Hypoxic Response Element

CROSS-TALK BETWEEN BASIC HELIX-LOOP-HELIX/Per-Arnt-Sim HOMOLOGY TRANSCRIPTION FACTORS*

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The basic helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) protein family comprises 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 postnatal 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 that 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 approximately 2-h 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 utilization, appears to account for the fall in SIM levels. Our data suggest the existence of a hypoxic switch mechanism in cells that coexpress hypoxia-inducible factor and SIM proteins, where up-regulation and activation of hypoxia-inducible factor-1α is concomitant with attenuation of SIM activities.

The basic helix-loop-helix/Per-Arnt-Sim homology (bHLH/PAS) 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 vascularization (5) but a well-defined function of inducing drug-metabolizing enzymes when animals are challenged by environmental pollutants such as dioxins and polycyclic aromatic hydrocarbons. (For a recent review, see Ref. 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 Tracheless (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 among the rare members of this family reported to be transcriptional repressors (for a recent review, see Ref. 12).

Murine SIM1 and SIM2 show high amino acid identity in their N termini (~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), whereas Sim2−/− pups suffer from a breathing defect for which the details are yet to be published (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 characterized, 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′-ACGGT-3′. Heterodimerization 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 Tracheless (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 (KEKSKNAARTTR) and TRH (KEKRSDAARSRR) explains 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 harbor identical (SIM1) or almost identical (one conservative amino acid change, SIM2) basic regions to dSIM (see Fig. 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 differs by a single amino acid (Fig. 1). Not surprisingly then, the core sequence of hypoxia response elements (HREs), 5′-ACGGT-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 discerning cofactors or collaborating transcription factors to activate distinct promoters (35). The murine SIMs, which harbor identical (SIM1) or almost identical (one conservative amino acid change, SIM2) basic regions to dSIM (see Fig. 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 differs by a single amino acid (Fig. 1).

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HIF-1α might be coupled to a hypoxic destabilization of the SIM proteins, we examined the levels of SIM transcript and protein during hypoxia and conducted [35]S]methionine pulse-chase labeling experiments to measure the half-lives of SIM1 and SIM2. The levels of both SIM proteins were decreased with hypoxic treatment, but since 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/NcoI 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 epo taggs from c-myc with a three-amino-acid spacer (EQKLISEEDL) at the carboxyl terminus of the SIM1 and SIM2 cDNAs and subsequent insertion of each cDNA into pEF-IRESpuro (40), creating pEF/SIM1(Myc)/IRESpuro and pEF/SIM2(Myc)/IRESpuro. Human ARNT containing a PCR-introduced carboxyl-terminal dual hemagglutinin (HA) epitope tag was subcloned into pEF-bos-cs (39) to generate a preNT/H1/EF/bos. The KpnI/SphI/Blnl/fragment from SIM2pBSK and the EagI/Blnl/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 493–805 from the murine DR, followed by two nuclear localization sequences, two nuclear export sequences, a six-histidine tag, and the C terminus. A constitutively active deletion mutant of the murine DR that lacks sequence from the ligand binding domain (DR/ARNT) (42) was generated by PCR and cloned into the pEF-bos-c vector. ARNT is identical to that of TRH, which is present in the murine DR, followed by two nuclear localization sequences, two nuclear export sequences, a six-histidine tag, and the C terminus. A constitutively active deletion mutant of the murine DR that lacks sequence from the ligand binding domain (DR/ARNT) (42) was generated by PCR and cloned into the pEF-bos-c vector. ARNT is identical to that of TRH, which is present in the murine DR, followed by two nuclear localization sequences, two nuclear export sequences, a six-histidine tag, and the C terminus. A constitutively active deletion mutant of the murine DR that lacks sequence from the ligand binding domain (DR/ARNT) (42) was generated by PCR and cloned into the pEF-IRESpuro vector (44).
hypoxic environment (<1% O₂) using anaerobic sachets (OXOID), beginning 12 h after transfection. Cells were washed with PBS 24–48 h post-transfection and lysed with Passive Lysis Buffer (Promega), and extracts were analyzed for luciferase activity using the Dual Luciferase Reporter assay (Promega) according to the manufacturer’s instructions. Transfections performed to produce whole cell extracts for subsequent immunoblotting involved 48 h transfection with 2 μg of expression plasmid into 293T cells in 6-cm diameter dishes using DOTAP as per the manufacturer’s instructions, whereas those for chromatin extract preparation involved 48-h transfection of polygonal 293TSIM2 cells in 175-cm² flasks with 20 μg of reporter plasmid using FuGene 6 (Roche Molecular Biochemicals) as per the manufacturer’s instructions.

Metabolic Labeling—Stably transfected polygonal pools of 293T cells expressing either SIM1 or SIM2 or containing the blank expression vector in the control line were seeded into 6-cm diameter dishes prior to expressing either SIM1 or SIM2 or containing the blank expression vector. The cells were pulse-labeled for 40 min in the methionine/cysteine-free medium supplemented with 250 μCi/ml [35S]methionine/cysteine (Geneworks) and then washed twice and incubated in complete medium for 24-48, and 8-h chases. Hypoxic treatments began with the chase. Cells were washed twice with cold PBS, lysates were prepared, and immunoprecipitations were performed as described below. Following SDS-PAGE (7.5%) gel of the immunoprecipitates, gels were dried and analyzed using a PhosphoImager (Molecular Dynamics, Inc., Sunnyvale, CA). Labeled bands were quantitated using ImageQuant (Molecular Dynamics).

Immunoprecipitation—Preparation of whole cell extracts (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, medium was aspirated, and cells were lysed immediately in cell lysis buffer containing 25 mM MgCl₂ (BioMoL) to prevent protein degradation via the ubiquitin-proteasome system and 100 μM 2,2'-dipyridyl (Aldrich) for hypoxic samples only. Protein concentrations were determined by Bradford assay. Immunoprecipitations with 150–160 μg of lysate using an anti-ARNT polyclonal rabbit serum raised against residues 1–140 of human ARNT (ARNT91) and an anti-Myc 9E10 monoclonal antibody were performed essentially as previously described (48) with minor variation. The binding buffer A contained 150 mM KCl, and lysates were incubated with antibody for 2 h at 4 °C. Immunoprecipitates were eluted in 0.5% SDS, 0.05 M β-mercaptoethanol for 5 min at 50 °C, boiled in SDS buffer, separated by SDS-PAGE on a 7.5% gel, and then transferred to nitrocellulose.

Immunoblotting—Whole cell extracts were prepared as described above, with 5–20 μg of whole cell extracts being subjected to SDS-PAGE (7.5% gel) and then transferred to nitrocellulose using a semidyry blotter (Hoefer). Proteins were detected with the anti-Myc antibody and fluorescein isothiocyanate-conjugated sheep anti-mouse secondary antibody (ii, iv, and v).

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 Ref. 6). Upon ligand binding, a nuclear localization 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 characterize 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 (Fig. 2A). SIM1 and SIM2 belong to class I bHLH/PAS proteins, which characteristic bind the molecular chaperone hsp90 and undergo cytoplasmic/nuclear shuttling before forming active heterodimers with ARNT. While SIM1 and SIM2 are known to dimerize with ARNT, while SIM1 and SIM2 are known to dimerize with ARNT, they can also dimerize with each other, as shown by co-immunoprecipitation experiments (not shown). This observation is consistent with previous studies showing that SIM1 and SIM2 can form heterodimers in vitro [see Ref. 4].

Northern Analysis—Poly(A)⁺ RNA was isolated from 293T control, 293TSIM1, and 293TSIM2 stable cell lines essentially as previously described (50), except that cells were removed from normoxic or hypoxic incubation, the medium was aspirated, and cells were immediately frozen in cell resuspension buffer with 1% SDS and 200 μg/ml Proteinase K (Roche Molecular Biochemicals) to minimize reoxygenation. 5 μg of each poly(A)⁺ RNA was analyzed by Northern blot with probes specific to SIM1, SIM2, and β-actin, using Rapid-hyb (Amersham Biosciences) and PhosphorImager visualization.
be 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. Whereas 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 (Fig. 3A). Fig. 3B illustrates successful expression of ARNT and ARNT603 proteins in these transient transfection experiments. These data demonstrate 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 harbor 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 the 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) (Fig. 4A). This effect is predominantly due to formation of an active HIF-1α/ARNT complex rather than a HIF-2α/ARNT complex, since Western blotting shows a large increase in HIF-1α 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 DB. 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 with control cells (Fig. 4B). In combination with ARNT, the SIM2/AD chimera was able to potently activate the HRE reporter gene at normoxia (Fig. 4A), thus establishing that the bHLH/PAS region of SIM2 can also recognize and bind the HRE sequence when dimerized 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 (Fig. 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 com-

ARNT (13, 16, 33) and in vitro translated SIM1 has been shown to bind hsp90 (15), their cellular location has not previously been analyzed. Immunohistochemical analysis of these cells using the anti-Myc antibody revealed that both SIM1 and SIM2 are exclusively nuclear (Fig. 2B, panels iv and vi). Consistent with the Western blotting results, the control cell line stably transfected with blank vector showed no background staining (Fig. 2B, panel ii). This nuclear localization 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, since 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 Fig. 1), and the minimal core nucleotides of the CME enhancer recognized 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 four 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 ~5-fold over that seen for the equivalently transfected control cell line in an HRE-dependent manner (Fig. 3A). In stark contrast, no significant increase in reporter gene activity was observed in the SIM2 cell line. These results suggest that either SIM1 harbors 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). Since SIM1 has previously...
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 12 h after transfection and continued for 17 h. 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 of independent experiments. B, coimmunoprecipitation of SIM and HIF proteins with ARNT. Lysates (150 μg) from normoxic (N) or hypoxic (H, <1% O2, 17 h) 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. 5. HIF-1α and SIM proteins compete for binding to ARNT. A, whole cell extract (150 μg) from 293T control cells was immunoprecipitated with preimmune serum (P1) 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 (150 μg) from normoxic (N) or hypoxic (H, <1% O2, 17 h) 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.
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 dimerize 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 hybridization 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 dimerize with ARNT and recognize 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 min (57). Treatment with hypoxia dramatically increases protein levels by stabilizing the protein half-life to ~30 min (57). Against this background, we sought to analyze 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 stabilization of HIF-1α concomitant with hypoxic destabilization of the SIM proteins. We examined the levels of all three proteins in lysates from our stable cell lines after a 17-h hypoxic treatment. Western analysis confirms that stabilization of HIF-1α in low oxygen conditions is comparable in the control and SIM stable cell lines, whereas the level of Myc-tagged forms of both SIM1 and SIM2 decreased in hypoxia, and ARNT levels remained unchanged (Fig. 7).

**SIM Transcript Levels and Protein Half-life Are Unchanged in Hypoxia**—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 (Fig. 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-labeled with [35S]methionine, and whole cell extracts were taken at increasing time points during the chase period for immunoprecipitation and analysis of the SIM proteins. As expected, immunoprecipitations from pulse-labeled control cells showed the absence of any background radiolabeled bands (Fig. 8B). In contrast, immunoprecipitations with the anti-Myc antibody produced sharp radiolabeled bands for SIM1 and SIM2 at several time points. Quantitation by PhosphorImager analysis found that both SIM1 and SIM2 have half-lives of ~2 h (Fig. 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 nor 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 NIH 3T3 cells of 30–40% has been reported to occur within 1 h 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, whereas 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 that 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 analyze 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 have 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, since 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 system. 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 analyze 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, while SIM2 has the same or greater affinity for ARNT than 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 dimerization 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 Fig. 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 (Fig. 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 the resultant interference with HIF-1α-dependent transcription (seen in Fig. 4A), is apparent in the decrease in the levels of HIF-1α protein co-immunoprecipitated with ARNT in the SIM stable cell lines (Fig. 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 dimerize 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 unfavorable conditions of compromised oxidative phosphorylation (67). Specific stress response proteins escape translational arrest through the employment of alternative translation strategies. For example, vascular endothelial growth factor 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 stabilization 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, since 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 stabilized 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.

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