Functions of the Per/ARNT/Sim Domains of the Hypoxia-inducible Factor*

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The heterodimeric transcription factor hypoxia-inducible factor (HIF) plays an important role in the progression of a number of processes in which O2 availability is compromised and, as such, has become an increasingly attractive therapeutic target. Although tremendous progress has been made in recent years in unraveling the mechanisms underlying O2-dependent regulation of HIF through its O2-dependent degradation domain and C-terminal transactivation domain, our understanding of the contributions of other structural elements, particularly the Per/ARNT/Sim (PAS)-A and PAS-B domains, to the activity of HIF is incomplete. Using insights derived from the recently determined solution structures of the HIF PAS-B domains as a starting point, we have explored the function(s) of the HIF-2α PAS domains via mutational analysis. In contrast to recent models, our data reveal that both PAS domains of the HIF-α subunit are necessary for heterodimer formation but are not required to mediate other HIF functions in which PAS domains have been implicated. Because disruption of individual PAS domains compromises HIF function independent of the mechanism of HIF induction, these data demonstrate the potential utility of targeting these domains for therapeutic applications.

A pathway to sense and respond to changes in O2 availability is expressed in virtually every mammalian cell, contributing to a host of developmental, physiological, and pathophysiological processes (reviewed in Ref. 1). This ubiquitous hypoxic response pathway involves changes in gene expression mediated through the induction of hypoxia-inducible transcription factors (HIFs). HIFs are obligate heterodimers composed of single copies of α- and β-subunits, the latter of which is also called the aryl hydrocarbon receptor nuclear translocator (ARNT) (2). The mammalian genome contains three HIF-α genes, HIF-1α, HIF-2α (also called endothelial Per/ARNT/Sim (PAS) domain protein 1 or HIF-like factor), and HIF-3α (3–5), that share similar domain structures but likely serve nonoverlapping physiological roles (6, 7).

Although the HIF-β subunit is essentially insensitive to O2 availability, both the accumulation and activity of the HIF-α subunit are acutely induced in response to low O2 levels (reviewed in Ref. 8). Briefly, multiple proline residues within the oxygen-dependent degradation domain of the α-subunit are selectively hydroxylated under normoxic conditions (10, 11) and subsequently recognized by the product of the von Hippel-Lindau tumor suppressor gene (pVHL). pVHL is a component of a ubiquitin-protein ligase complex that tags the α-subunit for rapid proteasomal degradation (12–16). A second independent O2-dependent hydroxylation activity directed toward an asparagine residue within the C-terminal transactivation domain of the α-subunit blocks coactivator recruitment under normoxic conditions (17). By virtue of their utilization of O2 as a substrate, the prolyl (18–20) and asparaginyl hydroxylases (21, 22) that regulate HIF are potential “oxygen sensors” in the pathway.

In addition to the protein domains that mediate O2-dependent regulation, both HIF subunits are members of the basic helix-loop-helix (bHLH)-containing PAS domain family of transcription factors. Each HIF subunit contains two PAS domains designated PAS-A and PAS-B. PAS domains frequently mediate protein-protein interactions and are often regulated as a consequence of ligands or cofactors that bind within their hydrophobic cores (reviewed in Ref. 23), although no such function has yet been reported for the HIF PAS domains. One or both of the HIF-α PAS domains have been functionally implicated in heterodimer formation (24, 25), nuclear localization (26), HIF stabilization via heat shock protein 90 (hsp90) association (27–30), and possibly target gene specificity via interactions with currently unidentified factors (31). Past difficulties in using only sequence data to define the boundaries of low homology PAS domains, as well as reliance on uncharacterized recombinant protein and nonphysiological overexpression of HIF constructs, has complicated the interpretation of some of the data underlying these models.

PAS domains are usually about 120 amino acids in size and fold into a five-stranded antiparallel β-sheet flanked by several α-helices. Recently, we employed NMR spectroscopy to determine the solution structure of the HIF-2α PAS-B domain, showing that it adopts this characteristic mixed α/β fold (32). In addition, NMR spectroscopy revealed a direct in vitro association between the PAS-B domains from the two HIF subunits mediated through interactions between their solvent exposed central β-sheets (32, 33). In this study we present evidence supporting the physiological relevance of the observed interaction between the PAS-B domains of the two HIF subunits and demonstrate that both PAS domains of HIF-2α are significantly involved in heterodimer formation in vivo. Although the HIF-2α PAS domains may also contribute subtly to nuclear localization, the presence of the PAS domains had little effect on the sensitivity of HIF-2α to the hsp90 antagonist geldanamycin (GA). In addition to challenging previously held models of PAS domain contributions to HIF biology, our results provide...
an experimental framework to investigate the feasibility of exploiting PAS domains as targets to disrupt HIF function.

**EXPERIMENTAL PROCEDURES**

Generation of Stable Cell Lines—Mutations and deletions to the HIF-2α cDNA were generated by PCR and confirmed by DNA sequencing. Each construct was cloned into the pcDNA3.1 vector (Invitrogen) in which the cytomegalovirus promoter was replaced by the human β-actin promoter and 5′-untranslated region (34) to better approximate O2-sensitive responses in HIF-α transcription/translation (35, 36). The resulting constructs were cotransfected with the pPUR vector (BD Biosciences) conferring puromycin resistance. Stably transfected cells were selected in the presence of 10 μg/ml puromycin. A second round of clonal selection was performed to ensure that each line contained a homogeneous population of cells.

Cell Culture—Cell lines were maintained in HyQ Dulbecco’s modified Eagle’s medium/Ham’s F-12 1:1 medium (HyClone) supplemented with 5% fetal bovine serum (Gemini Bio-Products, Inc.) and penicillin/streptomycin (Cellgro; 100 U/ml and 100 μg/ml, respectively) in the presence of 5.0% CO2 at 37 °C. The cells were exposed to hypoxia within a humidified hypoxic chamber (Coy Laboratory Products, Inc.) filled with 1% O2, 5.0% CO2 (balance N2) at 37 °C. Geldanamycin (Alexis Biochemicals) was prepared as 150X stocks in Me2SO.

For luciferase reporter assays, the cells were plated onto 24-well plates (7.5 × 104 cells/well) 24 h prior to transfection. The cells were transfected with 50 ng of the 3×HRE-tk-luc luciferase reporter construct (3) using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s protocol. After 3 h the medium was changed, and after an additional 2 h, the cells were incubated for 12 h under normoxic or hypoxic (1.0% O2) conditions. The luciferase assays were performed according to Ref. 37.

Western Blot Analysis—The samples were resuspended in SDS sample buffer, and the proteins were resolved by SDS-PAGE prior to Western blot analysis. Mouse monoclonal antibodies to HIF-2α (EP190b) and ARNT (Hbeta234) were obtained from Novus Biologicals, Inc., and mouse monoclonal antibodies to phospho-ATF-2 and Annexin I were obtained from Santa Cruz Biotechnology, Inc. Immune complexes were detected by enhanced chemiluminescence using peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.).

**RESULTS**

**Generation of Stable Cell Lines Expressing HIF-2α Constructs**—Improvements in secondary structure prediction methods (39) and an increase in the number of known PAS domain structures have greatly improved our ability to accurately predict the boundaries for each HIF PAS domain. Such information is critical when assessing domain function because inappropriate truncations can potentially lead to unintended disruption of neighboring structural elements or the introduction of large unfolded regions within a protein upon partial domain removal. Recently, we determined the solution structure of the HIF-2α PAS-B domain by NMR spectroscopy and demonstrated an interaction with the ARNT PAS-B domain mediated by the surface of its central β-sheet (32). We surmised that this in vitro interaction reflected a physiologically relevant interaction likely contributing to heterodimerization of the HIF subunits. However, when recombinant fragments encompassing the bHLH-PAS domains of the HIF subunits were employed in in vitro gel shift assays, deletion of the HIF-2α PAS-B domain had virtually no effect on HIF assembly/DNA binding (data not shown and Ref. 25). Careful examination of numerous HIF-1α and -2α protein fragments expressed from heterologous systems under a variety of conditions revealed that, although soluble, the recombinant HIF-α PAS-A domains were always unfolded as indicated by NMR methods.

**Electrophoretic Mobility Shift Assay**—Annealed double-stranded oligonucleotides derived from the vascular endothelial growth factor promoter sequence: TACACAGTGCATACGTGGGTTTCCACAGGT-CGTCT (4) were 32P-labeled by T4 DNA polynucleotide kinase. Nuclear extracts (0.5 μg/μl) were incubated on ice for 5 min in buffer containing 10 mM Tris/Cl (pH 7.5), 1 mM MgCl2, 1 mM EDTA, 2 mM dithiothreitol, 0.05 μg/μl herring sperm DNA (Invitrogen), and protease inhibitor mixture (Sigma) followed by the addition of the radiolabeled probe and incubation at room temperature for an additional 20 min. Protein-bound DNA complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel.

**Coimmunoprecipitation Assays**—100 μl of 1 μg/μl nuclear lysate was added to 650 μl of immunoprecipitation buffer (10 mM Tris/Cl, pH 7.5, 2 mM EDTA, 0.1% Triton X-100, 1 mM phenylmethylenolsulfon fluoride, and 1% skim milk) containing 15 μl of protein G-agarose beads (Roche Applied Science) and 5 μl of anti-ARNT antiserum elicited in rabbits against the first 140 residues of the human ARNT protein. Following incubation for 2 h at 4 °C, the beads were washed twice with immunoprecipitation buffer and resuspended in SDS sample buffer, and the immunoprecipitated proteins were resolved by SDS-PAGE prior to Western blot analysis.

**Quantitative Real Time Reverse Transcription-PCR Analysis**—Total RNA samples were independently prepared three times using RNA STAT-60 (Tel-Test, Inc.) for each cell line incubated under normoxic or hypoxic (1% O2; 12 h) conditions. First strand cDNA was prepared from 2 μg each of total RNA sample using SuperScript reverse transcriptase (Invitrogen). Quantitative real time reverse transcription-PCR was performed in triplicate for each sample with the 7900HT detection system and software Prism (Applied Biosystems, Inc.) using primers designed against the mouse GLUT1 (CGTCTGGCGATCCTTATTG; GAGGCCCAACAGTCTGCAATG) or β-actin (CATCGTGCCGCCTCTA; CACCCCATAGGAGTCTTCTG) genes. The data were deemed acceptable if the standard deviation was less than 0.4 for each triplicate set. We calculated relative expression (to β-actin) for GLUT1 using the comparative threshold cycle method. The relative induction by hypoxia was calculated by dividing the hypoxia level of expression by the normoxia level of expression within each set. The data for each gene is the mean of three values determined from the three independently harvested sets.

**REFERENCES**

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Contribution of PAS Domains to HIF Function

To better assess the function of the HIF-2α PAS domains under more physiological conditions, we generated a series of HIF-2α constructs (Fig. 1A) containing specific deletions of the PAS domains. Because such large deletions could have unforeseen effects on other HIF domains, we also made a HIF-2α construct containing three point mutations (Q320E/V336E/Y340T) designed to abrogate the observed in vitro interaction between the HIF-2α and ARNT PAS-B domains without causing disruption of the PAS fold itself (PAS-B mut) (32). The wild type and mutant HIF-2α constructs were introduced into the Ka13 CHO cell line. The Ka13 cell line contains an unidentified genetic lesion that eliminates expression of endogenous HIF-α, although the regulatory pathways that govern hypoxic induction of the α-subunit remain intact (40). Therefore, the Ka13 cell line is well suited for examining HIF activity in a background absent of endogenous HIF expression, allowing one to monitor HIF-α variants expressed at physiologically relevant levels rather than relying on gross overexpression of HIF-α at levels that may overwhelm endogenous regulatory factors.

Each HIF-2α construct was cotransfected with a plasmid conferring puromycin resistance to allow for the selection of individual clones in which the HIF-2α expression vector was stably integrated into the genome. For each construct, multiple puromycin-resistant cell lines were selected, and HIF-2α expression levels were assessed by Western blot analysis. To best mimic physiologically meaningful levels of HIF-α expression, we compared the O2 responsiveness of a luciferase reporter gene (3×HRE-tk-luc) governed by multiple HIF response elements (HREs) in clones expressing different levels of wild type HIF-2α. We chose two independently selected, stable wild type HIF-2α-expressing cell lines for additional study that faithfully recapitulated the magnitude of reporter induction under hypoxia observed in a CHO cell line (C4.5) (40) expressing endogenous HIF-1α (Fig. 2). Similarly, two cell lines for each mutant HIF-2α construct were subsequently chosen for further study on the basis of comparable levels of HIF-2α accumulation under hypoxia (Fig. 1B).

Both HIF-2α PAS Domains Are Required for HIF Activity—Having generated pairs of stably transfected cell lines for each construct with similar HIF-2α protein accumulation levels (Fig. 1B), we examined the effects of PAS domain mutations and deletions on HIF activity. Each cell line was transfected with the HIF-responsive 3×HRE-tk-luc reporter and incubated under normoxic or hypoxic conditions (Fig. 2). As expected, no induction of the HIF reporter was observed in the parental Ka13 cell line lacking HIF-α expression, whereas a >10-fold increase in reporter gene induction was observed for the C4.5 cell line, reflecting the hypoxic induction of endogenous HIF activity. A similar induction of reporter gene expression was observed in each of the two cell lines expressing the wild type HIF-2α subunit, confirming that the expression levels of HIF-2α are appropriate to recapitulate physiologically relevant HIF responses. In contrast, deletion of either or both HIF-2α PAS domains

(data not shown) that may complicate interpretation of both PAS-A and PAS-B function in a variety of in vitro assays.

![FIGURE 1. Generation of stable cell lines expressing HIF-2α constructs. A, schematic representation of human HIF-2α constructs. The mutations introduced to the surface of the central β-sheet surface in the PAS-B mut construct (Q320E/V336E/Y340T) are indicated by solid circles, whereas deleted regions are denoted by inverted triangles. B, HIF-2α protein accumulation in HIF-2α-expressing stable cell lines. Following incubation for 12 h under hypoxia (1% O2), total HIF-2α protein levels were assessed by Western blot analysis for the parental Ka13 cell line and two independently selected clones of Ka13 stably transfected with each HIF-2α construct.](http://www.jbc.org/Downloaded from)

![FIGURE 2. HIF-2α PAS domains are required for HIF activity. Cell lines were transiently transfected with the 3×HRE-tk-luc HIF reporter gene and incubated under normoxic or hypoxic (1% O2) conditions for 12 h. The values represent the average luciferase activity of three replicates with the bars indicating standard error.](http://www.jbc.org/Downloaded from)
Contribution of PAS Domains to HIF Function

complete blocked HIF activity (Fig. 2) despite equivalent induction of total protein levels (Fig. 1B).

our prior NMR data indicated that the PAS-B domains from the two HIF subunits interact with one another via the exposed face of the β-sheets on the α-subunit (32, 33). We tested this hypothesis by mutating PAS-B residues whose side chains extend out from the β-sheets toward the protein-protein interface. To minimize changes to the β-sheet backbone, these residues were replaced with amino acids that changed the chemical composition of the side chain without altering the α- and β-carbon positions. One such variant PAS-B domain containing three altered residues (Q322E/M338E/Y342T) maintained the PAS domain fold but severely weakened association with the ARNT PAS-B domain in vitro (32). In agreement with the spectroscopic data, when placed in the context of the full-length HIF-2α (PAS-B mut), even these subtle mutations have a substantial effect on HIF activity, reducing expression of the HIF reporter gene by >50% (Fig. 2). The relative induction of the HIF reporter in the stably transfected cell lines mirrors previous results obtained upon transient transfection of HIF constructs (32). A similar pattern of HIF-2α function was observed for hypoxic induction of the endogenous gene, GLUT1 (TABLE ONE). Together these data indicate that both PAS domains are required for HIF activity and that disruption of a PAS domain interaction observed by NMR has functional consequences in this model system.

The HIF-2α PAS Domains Do Not Mediate the Sensitivity of HIF to a hsp90 Antagonist—Although both PAS domains are required for HIF function in the cell culture models, the underlying mechanistic role(s) played by these domains remain speculative. For example, although we have suggested that an in vitro interaction between the HIF PAS-B domains reflects a physiologically relevant contribution to subunit dimerization in vivo (32, 33), previous studies have concluded that the HIF-α PAS-B domain does not substantially contribute to heterodimerization or DNA binding (24, 25). Furthermore, alternative functions for the HIF PAS domains have been proposed, including contributions to nuclear localization (26) and HIF-α stabilization mediated by interaction with hsp90 (27–30).

To further investigate the underlying defect in HIF activity resulting from PAS domain alterations, we examined the various stable cell lines in greater detail. hsp90 has been reported to associate with HIF-α, protecting the subunit from pVHL-independent degradation (28, 41). Antagonists such as GA that block hsp90 function destabilize HIF-α and are currently being investigated as anticancer agents due in part to their effects on HIF (42). Recently, several groups have reported that hsp90 associates with the HIF PAS domains using in vitro translation systems or overexpression of HIF fragments in cell culture (29, 30).

Because HIF-2α protein accumulated to similar levels in all of our cell lines, we suspected that the effects observed upon PAS mutation/deletion did not stem from alterations in hsp90 association. Consistent with previous reports, treatment of the cell lines expressing wild type HIF-2α with GA resulted in a decrease in HIF-2α protein accumulation (Fig. 3A), although we did observe some variability even between clones expressing the same construct. The reduction in HIF-2α accumulation was accompanied by a decrease in HIF activity as measured by 3×HRE-tk-luc reporter expression (Fig. 3B). Likewise, both accumulation and the residual activity of the PAS-B mut construct remained sensitive to GA (Fig. 3), although again we did observe some variability between the two clones. In fact, despite deletion of either or both PAS domains, HIF-2α stability remains sensitive to GA (Fig. 3A). These results were surprising in light of the reported requirement of the PAS domains for mediating hsp90 association and instead suggest that other elements of the HIF transcription factor may substantially contribute to hsp90-dependent HIF responses.

Both HIF-2α PAS Domains Contribute to HIF Heterodimerization—Because protein accumulation appears to be unaffected by alterations to the PAS domains, we next investigated the ability of each of the HIF-2α

| Cell line | Fold hypoxic induction (mean ± S.D.) | p value |
|-----------|-------------------------------------|---------|
| Ka13 (No HIF) | 0.91 ± 0.14 | 0.34 |
| Wild type 1 | 3.17 ± 1.20 | 0.04 |
| Wild type 2 | 2.39 ± 0.99 | 0.07 |
| PAS-B mut 1 | 1.17 ± 0.36 | 0.45 |
| PAS-B mut 2 | 1.50 ± 0.42 | 0.11 |
| PAS-A del 1 | 1.50 ± 1.35 | 0.56 |
| PAS-A del 2 | 1.67 ± 0.91 | 0.27 |
| PAS-B del 1 | 0.64 ± 0.07 | 0.001 |
| PAS-B del 2 | 0.72 ± 0.19 | 0.07 |
| PAS-AB del 1 | 0.72 ± 0.34 | 0.22 |
| PAS-AB del 2 | 0.84 ± 0.28 | 0.39 |

### Table One

Hypoxic induction of GLUT1 mRNA

- **Wild type 1**: 2.39 ± 0.99 (p = 0.07)
- **Wild type 2**: 1.17 ± 0.36 (p = 0.45)
- **PAS-B mut 1**: 1.50 ± 0.42 (p = 0.11)
- **PAS-B mut 2**: 1.50 ± 1.35 (p = 0.56)
- **PAS-A del 1**: 1.67 ± 0.91 (p = 0.27)
- **PAS-A del 2**: 0.64 ± 0.07 (p = 0.001)
- **PAS-B del 1**: 0.72 ± 0.19 (p = 0.07)
- **PAS-B del 2**: 0.72 ± 0.34 (p = 0.22)
- **PAS-AB del 1**: 0.84 ± 0.28 (p = 0.39)
- **PAS-AB del 2**:

Because HIF-2α protein accumulated to similar levels in all of our cell lines, we suspected that the effects observed upon PAS mutation/deletion did not stem from alterations in hsp90 association. Consistent with previous reports, treatment of the cell lines expressing wild type HIF-2α with GA resulted in a decrease in HIF-2α protein accumulation (Fig. 3A), although we did observe some variability even between clones expressing the same construct. The reduction in HIF-2α accumulation was accompanied by a decrease in HIF activity as measured by 3×HRE-tk-luc reporter expression (Fig. 3B). Likewise, both accumulation and the residual activity of the PAS-B mut construct remained sensitive to GA (Fig. 3), although again we did observe some variability between the two clones. In fact, despite deletion of either or both PAS domains, HIF-2α stability remains sensitive to GA (Fig. 3A). These results were surprising in light of the reported requirement of the PAS domains for mediating hsp90 association and instead suggest that other elements of the HIF transcription factor may substantially contribute to hsp90-dependent HIF responses.

**Both HIF-2α PAS Domains Contribute to HIF Heterodimerization**—Because protein accumulation appears to be unaffected by alterations to the PAS domains, we next investigated the ability of each of the HIF-2α...
subunits to localize to the nucleus. Following incubation under hypoxic conditions, the cells were lysed, and soluble cytosolic proteins were separated from salt-extracted nuclear proteins. As shown in Fig. 4A, wild type HIF-2α resides almost exclusively in the nuclear fraction upon hypoxic treatment. Deletion of one or both of the PAS domains did result in modest retention of HIF-2α in the cytosolic fraction (Fig. 4A). Despite an intact oxygen-dependent degradation domain, much of the HIF-2α protein escapes O2-dependent degradation under normoxia. As has been observed previously (43), wild type HIF-2α resides primarily in the nucleus even under normoxic condition, whereas the defect in localization for the HIF-2α variants is amplified under normoxia (Fig. 4B). Although this subtle defect in HIF-2α nuclear localization is unlikely to account for the total absence of hypoxic HIF activity upon PAS domain deletion, these data suggest that the PAS domains may influence the distribution of HIF within the cell. However, unlike previous studies suggesting that PAS domains suppress nuclear localization by masking a cryptic nuclear localization sequence within the bHLH (26), our results are instead consistent with the PAS domains promoting HIF localization to the nucleus.

Because the loss of hypoxic HIF activity upon PAS domain deletion/mutation does not appear to primarily stem from a defect in nuclear localization, we next examined the contributions of the HIF-α PAS domains to heterodimer formation through the use of a coimmunoprecipitation assay. Nuclear lysates from the hypoxia-treated cell lines were incubated in the presence of anti-ARNT antiserum. Antibody-bound ARNT was precipitated from the lysates, and the amount of associated HIF-2α that coimmunoprecipitated was measured by Western blot analysis. As shown in Fig. 5A, ARNT was able to efficiently coimmunoprecipitate wild type HIF-2α, indicating a strong association between these two HIF subunits. Deletion of either PAS domain was sufficient to block heterodimer formation in this assay (Fig. 5A) in accordance with a complete lack of HIF activity (Fig. 2). The lack of heterodimerization for the PAS deletion constructs was not due simply to a partial reduction in HIF-2α protein levels in the input lysates because we have still observed coimmunoprecipitation of wild type HIF-2α by ARNT when HIF-2α input levels were reduced more than 10-fold (data not shown). Mutations to the HIF-2α PAS-B domain that attenuate the in vitro interaction with the ARNT PAS-B domain also significantly compromise heterodimerization as measured by coimmunoprecipitation (Fig. 5A), consistent with a partial attenuation in HIF function (Fig. 2). The defect in heterodimerization of the PAS variants was likewise reflected in a corresponding loss of HIF-2 DNA binding activity (Fig. 5B).

**FIGURE 4.** Deletion of the PAS domains influences the nucleocytoplasmic distribution of HIF-2α. The cell lines were incubated under hypoxic (1% O2) (A) or normoxic conditions (B) for 12 h. Soluble cytoplasmic (C) and salt-extracted nuclear (N) proteins were separated and analyzed by Western blot analysis with antibodies raised against HIF-2α or ARNT. Antibodies that recognize the transcription factors p-ATF-2 and annexin I were used to assess the integrity of the nuclear and cytoplasmic samples, respectively.

**FIGURE 5.** Both HIF-2α PAS domains are required for heterodimerization with ARNT. A, heterodimerization of HIF-2α and ARNT requires both HIF-2α PAS domains. ARNT was immunoprecipitated (IP) from nuclear extracts prepared from each stable cell line (top left panel). The levels of HIF-2α protein that coimmunoprecipitated with ARNT were assessed by Western blot analysis (bottom left panel). Input levels are indicated in the panels on the right. B, the DNA binding activity of HIF-2 from nuclear lysates is dependent upon both HIF-2α PAS domains. The ability of HIF-2 to bind a DNA fragment derived from the HIF-responsive vascular endothelial growth factor promoter was assessed by an electrophoretic mobility shift assay. A HIF-2-containing complex previously verified by supershift experiments using an antibody to HIF-2α is indicated.
Although these data strongly support the predictions stemming from the NMR studies, it remains formally possible that the β-sheet surface altered in the PAS-B mutant construct mediates a protein-protein interaction with a factor other than the ARNT PAS-B domain. Our model predicts that alterations to individual PAS domains compromise some of the interactions between the HIF subunits. We suspected that overexpression of one of the subunits might still drive the equilibrium toward HIF heterodimer formation, and consequently enhance HIF activity, through the remaining elements that contribute to subunit association (i.e. bHLH). To test this hypothesis, we overexpressed ARNT by transfecting each cell line with a full-length wild type ARNT expression construct. In the absence of HIF-2α, overexpression of ARNT had no effect on 3×HRE-tk-luc reporter gene induction (Ka13; Fig. 6A). However, overexpression of ARNT was able to fully restore the defect in HIF-2 activity stemming from the three point mutations in the HIF-2α PAS-B domain (Fig. 6A). This increase in HIF activity was not due to an ARNT-mediated increase in HIF-α accumulation (Fig. 6B). Even the deletion of individual HIF-2α PAS domains could be partially compensated by ARNT overexpression, although the activity of the HIF-2α PAS-AB deletion construct was not rescued (Fig. 6A). These results emphasize another key point; recovery of HIF activity upon ARNT overexpression indicates that mutation or deletion of individual PAS domains does not lead to unintended global defects of HIF structure that preclude HIF function altogether. These constructs are otherwise capable of interacting with other factors (i.e. coactivators) necessary to induce transcription of target genes.

These results fully support the physiological relevance of the interaction between the HIF PAS-B domains observed by NMR and suggest that the primary contribution of both HIF-α PAS domains to HIF function resides in their ability to mediate heterodimerization. This experimental system, reliant upon proper definitions of domain boundaries as well as physiologically meaningful protein expression levels, provides a valuable resource for addressing the function(s) of PAS domains and demonstrates that disruption of PAS domain function can significantly suppress HIF activity, highlighting the potential utility of targeting these domains for future therapeutic applications.

**DISCUSSION**

In this study we examined the contributions of both α-subunit PAS domains to the function of HIF-2 through the use of a cell-based model system reliant on physiologically meaningful levels of protein expression and activity. Our data demonstrate that both PAS domains are required for HIF activity and that disruption of a putative protein-protein interface employed by these domains can appreciably impair the hypoxic response in cells. In contrast to previous reports, these data provide the first evidence that the HIF PAS-B domains do in fact contribute significantly to the formation of the HIF heterodimer and demonstrate that the NMR models of HIF PAS domain interactions have predictive value in vivo. However, the PAS domains were neither necessary nor sufficient to confer HIF sensitivity to hsp90. Interestingly, we did observe a change in the nucleocytoplasmic distribution of HIF-2α upon PAS deletion/mutation, although the underlying cause of this defect will require additional study. Although these partial (~50%) reductions in nuclear HIF-α levels may contribute to some of the loss of HIF activity, the defects observed in heterodimer formation are substantially greater and therefore likely contribute as much if not more to the observed phenotype. Although we have not repeated these experiments for HIF-1, early indications are that the HIF-1α PAS domains will perform in a similar fashion (32).

In light of recent findings emphasizing the O2-dependent regulation of HIF-α through the oxygen-dependent degradation domain and C-terminal transactivation domain, why is it important to continue to study the function(s) of the HIF PAS domains? PAS domains have been identified in over 3000 proteins throughout all kingdoms of life. Because these domains often serve as components of signal transduction pathways or medically relevant transcription factors (23), it is important to understand the mechanisms by which PAS domains associate with other factors and the contribution of these interactions to protein func-
tion and regulation. Recent structural studies of several PAS domains, including those of HIF, have provided new insights with which to re-examine models of PAS domain structure and function. A common theme emerging from these PAS domain studies is that the solvent-exposed surface of the β-sheet mediates important protein–protein interactions, and mutations to these surfaces often impair PAS function as we observed for HIF (32). Given the absolute dependence on these PAS domain interactions for HIF function, it is appropriate to consider strategies that would target these domains for therapeutic purposes.

Within a physiological context, HIF has been recognized as an important contributor to the progression of a number of disease states. For example, increased levels of HIF have been associated with tumor aggressiveness, resistance to conventional therapies, and mortality for a variety of human cancers (reviewed in Ref. 44). Initially purified using the promoter of the erythropoietin gene (45), HIF has since been found to mediate the hypoxic induction of many factors that promote many aspects of tumorigenesis including metabolic adaptation, angiogenesis, changes in cell proliferation/survival, and cellular invasion and metastasis (44). HIF levels can be induced in cancers in response to intratumoral hypoxia resulting from rapid and uncontrolled cell growth as well as insufficient oxygen delivery to tortuous vasculature frequently associated with tumors (46). Alternatively, HIF can be induced independent of oxygen availability as a consequence of genetic alterations that alter signaling pathways or regulatory factors and result in constitutive accumulation and activation of the HIF-α subunit (44). As a result, small molecules that interfere with HIF function irrespective of the mechanism of HIF induction might have the broadest utility. In addition to small molecule inhibitors of the relevant signaling pathways, a number of small molecules have been shown to effect HIF levels, albeit indirectly, including inhibitors of topoisomerase I, hsp90, microtubule polymerization, and chromatin remodeling factors (reviewed in Refs. 44 and 47). Unfortunately these compounds impinge upon many cellular pathways, making it difficult to assign the relative contribution of HIF inhibition to observed phenotypes. The absence of any compound that directly and specifically targets HIF itself has made it difficult to unambiguously assess the benefits of a HIF-based therapeutic approach in relevant model systems.

Although critical for regulating HIF function, regions such as the oxygen-dependent degradation domain and C-terminal transactivation domain do not adopt a well-defined structure, particularly in the absence of their binding partners, likely restricting the ability to identify useful small molecules that directly recognize these elements (48–50). However, PAS domains can assume a well-folded tertiary structure to mediate protein–protein interactions. Although protein–protein interactions themselves are notoriously difficult to directly disrupt by small molecules, PAS domains, even those not yet shown to bind endogenous ligands, are often predisposed to bind small molecules within their core leading to conformational changes that propagate to the domain surface and disrupt association with other factors (51). We have reasoned that the HIF PAS domains may provide attractive targets for mediating HIF activity regardless of its mode of induction. This study demonstrates the predictive utility of information divined from the HIF PAS domains structures and provides an experimental framework that defines the functional roles of these domains. As such, these findings provide the necessary foundation to investigate the ability to exploit these domains in therapeutic strategies.

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