Aryl Hydrocarbon Nuclear Translocator (ARNT) Promotes Oxygen-independent Stabilization of Hypoxia-inducible Factor-1α by Modulating an Hsp90-dependent Regulatory Pathway*

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Hypoxia-inducible factor-1 (HIF-1) is a potent cellular survival factor contributing to tumorigenesis in a broad range of cancers. The functional transcription factor exists as a heterodimeric complex consisting of HIF-1α and the aryl hydrocarbon receptor nuclear translocator (ARNT). Association of HIF-1 with ARNT is required for its activity; however, no other role has been ascribed to this interaction. We demonstrated previously that pharmacologic inhibition of Hsp90 by geldanamycin (GA) impairs HIF transcription and promotes VHL (von Hippel-Lindau)-independent degradation of the protein, thus implicating Hsp90 as an essential interacting partner for HIF. In this study, we further explore the physiological role for Hsp90 in HIF function. We establish that the PAS (Per-ARNT-Sim) domain of HIF is required both to promote association with Hsp90 and confer sensitivity to GA. Coincidentally, this domain also associates with ARNT. Overexpression of ARNT in a VHL-deficient background resulted in substantially increased HIF-1 protein concomitant with increased protein stability. Conversely, down-regulation of endogenous ARNT protein by RNA interference decreased the steady-state HIF protein. ARNT-mediated stabilization of HIF is specific for the Hsp90-dependent pathway, as ARNT was unable to protect HIF from VHL-mediated degradation. We establish that the ability of ARNT to up-regulate HIF and diminish HIF sensitivity to GA is due to its ability to compete for the Hsp90 binding site on HIF. These data elucidate novel functions for ARNT and Hsp90 in regulating HIF function and further illustrate that cofactor association may significantly impact upon the sensitivity of Hsp90 clients to chaperone inhibitors.

Hypoxia-inducible factor-1α (HIF-1α)3 is a component of a transcriptional complex activated under hypoxic conditions, resulting in the induction of a plethora of target genes that collectively confer cellular adaptation to hypoxia. HIF-1 is comprised of a labile α subunit that is targeted for normoxia-dependent degradation by the ubiquitin ligase VHL (1, 2), whereas its β subunit, HIF-1β, or ARNT, is constitutively expressed. Therefore, the activity of this complex is exquisitely dependent upon the limiting expression of the α subunit. Under hypoxic conditions, prolyl hydroxylation of HIF is inhibited (3–5), thereby preventing VHL from targeting HIF for degradation. This leads to the significant accumulation of HIF protein and a concomitant increase in HIF transcriptional activity due to the productive formation of HIF-1α/ARNT heterodimers.

HIF plays a crucial role in tumor progression, and the protein is overexpressed in a broad range of primary tumors and metastases (6). In the majority of these instances, overexpression is due to constitutive stabilization of the protein by hypoxia and/or aberrant signaling pathway-induced mechanisms. HIF-1α levels may also accumulate as a result of genetic loss of VHL, as observed in hereditary VHL syndrome (7) in which afflicted patients are predisposed to the formation of angio- genic tumors and are especially prone to developing renal cell carcinoma (RCC) (8). Therefore, both hereditary and sporadic RCC represent one of the best characterized models for genetic inactivation of VHL and subsequent HIF dysregulation. The poor prognosis of metastatic RCC illustrates the importance of developing pharmacologic means to interrupt the HIF signaling pathway.

HIF-1α interacts with the molecular chaperone Hsp90 (9). Hsp90 plays a pivotal role in mediating the proper folding and subsequent activation of its numerous “client” proteins (for reviews see Refs. 10 and 11). Hsp90 also cooperates with the proteasomal pathway to eliminate misfolded cellular proteins (12). The antibiotic geldanamycin (GA) associates with Hsp90 and modulates its chaperone function (13, 14), thereby accelerating the degradative activity associated with Hsp90 (15). The ability of GA to promote the proteasome-dependent degradation of numerous oncogenic client proteins (13, 16) is thought to contribute to its potent antitumor properties (17, 18). Although utilization of Hsp90 antagonists has implicated the chaperone in HIF regulation (19), the specific physiological role for Hsp90 in this process still remains largely unknown.

HIF is a member of a superfamily of proteins containing Per-ARNT-Sim (PAS) homology domains. PAS domains confer sensor qualities to their respective proteins, allowing them to rapidly respond to diverse environmental signals. Stimulation of the PAS domain alters its conformation and, in doing,

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¶ The abbreviations used are: HIF, hypoxia inducible factor; Ahr, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CHX, cycloheximide; GA, geldanamycin; HA, hemagglutin A; HLH, helix-loop-helix; Hsp90, heat shock protein 90; PAS, Per-ARNT-Sim homology domain; RCC, renal cell carcinoma; RNAi, RNA interference; RRL, rabbit reticulocyte lysate; sRNA, small interfering RNA; VHL, von Hippel Lindau.

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16128
ARNT Modulates Hsp90-dependent HIF Regulation

16129

ALTERS COFACTOR ASSOCIATION, thus allowing transmittance of the signal. Hsp90 has been identified as a PAS-interacting partner of a number of proteins from this family, such as Drosophila Sim (20) and the aryl hydrocarbon receptor (Ahr) (21, 22). In the case of Ahr, Hsp90 association maintains the receptor in a latent ligand-competent state. Upon presentation of the ligand dioxin, Ahr is released from Hsp90, whereupon it forms a complex with ARNT and translocates to the nucleus. The activity of Ahr is regulated by its interaction with either Hsp90 or ARNT. HIF also interacts with ARNT and Hsp90. We demonstrate herein that the PAS domain of HIF is required for Hsp90 association and GA-mediated destabilization. Furthermore, we show that ARNT competes with Hsp90 for HIF association. Therefore, fundamental properties of HIF are, in large part, influenced by its choice of PAS-associated binding partner. These data may have clinical implications in that cofactors may impact upon client sensitivity to Hsp90 antagonists.

MATERIALS AND METHODS

Plasmids and DNA Manipulation—Transfections with full-length HIF in VHL-deficient cells utilized a cytomegalovirus-driven HA-tagged HIF plasmid provided by Dr. D. Livingston (Dana-Farber Cancer Institute, Boston, MA). This construct served as a template for PCR amplification and subcloning of HIF into the pcDNA3.1 vector (Invitrogen) and mutagenesis of HIF-sensitive prolines, as described (19). This proline-mutated HIF plasmid served as the template for all subsequent PCR reactions to create HIF deletion constructs. All PCR-amplified HIF constructs were constructed with primers containing BamHI (5') and XbaI (3') restriction sites. PCR amplification was performed with Easy-A enzyme (Invitrogen), the PCR products were gel-purified and cloned into the TA cloning vector (Invitrogen), and BamHI/XbaI digested inserts were subcloned into pcDNAHA. The vector pcDNAHA is a modified form of pcDNA3 containing a BamHI sequence immediately downstream from the HA tag (22), kindly provided by Dr. G. Clark (NCI, National Institutes of Health, Rockville, MD). ARNT plasmid was generously supplied by Dr. G. Perdew (Pennsylvania State University, State College, PA). ARNT deletion constructs were similarly created and subcloned into pcDNAFLAG, a derivative of pcDNA3 containing a BamHI site immediately downstream from a FLAG epitope. All constructs were confirmed by DNA sequence analysis. FLAG-tagged VHL was constructed as previously described (19). Murine ARNT 2 and ARNT 3 constructs were generously provided by Dr. Y. Fuji-Kuriyama (Tohoku University, Sendai, Japan).

Cell Culture and Treatments—COS cells were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM glutamine, 5 mM HEPES, and penicillin/streptomycin. 786-O cells were obtained from Sigma, and PS-341 was obtained from Millennium Pharmaceuticals. Transfections were performed using FuGENE (Roche Applied Science) according to the manufacturer's specifications, and cells were harvested 16–20 h following transfection.

Extract Preparation and Immunoblot Analysis—For nuclear extract preparation, cells were washed with phosphate-buffered saline and overlaid with low salt lysis buffer, followed by incubation with Nonidet P40, as described (19). For preparation of total protein extracts, cells were lysed with TNE buffer (50 mM Tris, 1% Nonidet P40, 1 mM EDTA, 100 mM NaCl) and clarified by centrifugation. Equal amounts of protein were subjected to SDS-PAGE, transferred to nitrocellulose, blocked in 5% milk, and incubated with the indicated primary antibodies, followed by detection with horseradish peroxidase-linked secondary antibodies and enhanced chemiluminescence substrates (Pierce). The antibodies used were HIF-1α (Transduction Labs), HA (Cell Signaling Technology), topoisomerase II (Sigma), Hep90 (StressGen), and ARNT (Santa Cruz Biotechnology).

In Vitro Translation and Immunoprecipitation—HIF and ARNT constructs were expressed by coupled in vitro transcription and translation in rabbit reticulocyte lysate (RRL), utilizing conditions recommended by the manufacturer (Promega). For in vitro HIF and ARNT co-immunoprecipitations, HIF constructs were translated in RRL in the presence of [35S]methionine (ICN), FLAG-tagged ARNT constructs were expressed separately in the absence of label, and aliquots (15 μl) of these reactions weremixed for 2 h at 4 °C in TNES buffer, whereupon HIF was immunoprecipitated with FLAG M2 antibody. For Hep90 immunoprecipitations, labeled translated HIF proteins were incubated with Hep90 antibody in buffer (100 mM NaCl, 0.2% Tween, and 20 mM Tris), followed by the addition of 5% BSA-blocked protein G beads (Invitrogen).

Half-life Determination—VHL-deficient 786-O cells were transfected with a 1:1 ratio of HA-tagged HIF and either empty vector or ARNT plasmid. Following transfection, cells were either left untreated or treated for 30 min with 2 μM GA and then incubated with 40 μg/ml CHX for the indicated times. HIF-1 protein was immunodetected from nuclear extracts, the films were quantitated by densitometric analysis, and the values were graphed semi-logarithmically.

siRNA Treatment of Cells—Exponentially growing 786-O cells were plated onto 6-cm dishes and grown to 60% confluence the day before transfection. ARNT siRNA was synthesized (Qiagen), and the duplexes were resuspended to 20 μM. For transfections, siRNAs were diluted with Opti-Mem (Invitrogen), incubated with LipofectAMINE 2000 (Invitrogen) according to the manufacturer, and diluted to a final concentration of 100 nM. The cells were incubated with siRNA complexes for 4 h, whereupon the media was replaced. The nonspecific control siRNA was a fluorescein-labeled, nonsilencing duplex (Qiagen). Transfection of cells with HIF was performed after replacement of the siRNA-containing media and allowed to proceed for 18 h.

RESULTS

The PAS Domain of HIF Associates with Hsp90 and Confers GA Sensitivity to the Protein—Previous reports document an interaction between HIF and Hsp90 (9, 24), but the significance of this interaction is unclear. Based upon numerous examples of the essential functions performed by Hsp90 with other clients, there is a strong likelihood that several functions of HIF are similarly dependent upon Hsp90. In support of this notion, we previously demonstrated that Hsp90 antagonists such as GA reduce the transcriptional activity of HIF and promote its degradation (19). To explore the physiological role(s) of Hsp90 in HIF function, a series of HIF constructs was created to identify the domains of HIF required for conferring sensitivity to GA. A schematic representation of these expression constructs is shown in Fig. 1A. All constructs containing residues subject to modification by prolyl hydroxylases were mutated to render HIF resistant to VHL (3, 4). After confirming the expression of these constructs in COS cells (Fig. 1B), the sensitivity of these proteins to GA or proteasome inhibition was assessed. As shown in Fig. 1C, expression levels of all HIF proteins were increased following treatment with the proteasome inhibitor PS-341, indicating that they are regulated by the proteasome. Although most of the proteins were sensitive to GA, two were notably resistant. The first lacks both the helix-loop-helix (HLH) and PAS domains (residues 1–350), whereas the second consists of the HLH domain (residues 1–100). These data suggest that the PAS domain (residues 101–350) is minimally required to confer GA sensitivity to HIF. Interestingly, the PAS domain alone was inherently unstable in vivo, and the addition of the HLH domain increased its expression level (Fig. 1B; compare PAS with HLH/PAS). This suggests that the HLH domain may impart stability to the protein, possibly by affecting protein conformation. In support of this notion, the HLH/PAS protein appeared more sensitive to GA treatment compared with the PAS domain alone (Fig. 1C).

Proteins sensitive to GA-mediated destabilization are also Hsp90-interacting. It was therefore of interest to correlate the GA sensitivity of HIF proteins with their ability to associate with Hsp90. These experiments were performed in RRL for several reasons. First, our experience has suggested that the HIF-Hsp90 association in cells is very weak and/or transient. Second, because HIF is a nuclear protein, it is unknown whether Hsp90 remains associated with HIF in the nucleus, and, therefore, the majority of the nuclear protein may not be complexed with the chaperone. Third, the in vivo expression levels of the HIF constructs varied, and in vitro translation allows normalization, thereby eliminating several of the vari-
ables that might occur within a cellular context. Finally, RRL has abundant chaperone proteins and is therefore ideally suited to chaperone-client studies. HIF proteins were translated in vitro in the presence of 35S, and HIF association with Hsp90 was determined as described. As shown in Fig. 1D, all of the HIF proteins were capable of associating with Hsp90 except for the expression construct lacking both the HLH and PAS domains. This result is consistent with a previous report demonstrating the interaction of Hsp90 with the N-terminal half of HIF comprising the HLH/PAS domain (24).

ARNT Up-regulates HIF, Stabilizes HIF Protein, and Attenuates Its Sensitivity to GA—During the course of these experiments, it was observed that cotransfection of ARNT and HIF in the HIF-1- and VHL-deficient RCC line 786-O resulted in a marked increase in nuclear HIF expression. No HIF was detected in cytoplasmic extracts (data not shown), suggesting that this expression represents total cellular HIF levels. To determine the potency of the ARNT-mediated HIF up-regulation, 786-O cells were transfected with a constant amount of HIF and increasing amounts of ARNT plasmid. HIF levels were elevated with as little as 0.2 μg of ARNT, or one-fifteenth the amount of HIF plasmid used (Fig. 2A).

Because HIF and ARNT heterodimerize via their HLH/PAS domains (9, 18), and because we now show that the PAS domain of HIF also mediates association with Hsp90, we postulated that ARNT was modulating an Hsp90-dependent pathway for HIF degradation. To test this hypothesis, 786-O cells were transfected with HIF in the presence or absence of ARNT, and the levels of HIF were monitored in vivo in the presence or absence of GA. As shown in Fig. 2B (upper panel), GA treatment promotes almost complete elimination of HIF protein from nuclear extracts. However, co-expression of ARNT not only up-regulates the basal level of HIF but also modestly protects the protein from the destabilizing effects of GA. To determine whether this effect was specific to an Hsp90-dependent pathway, we next examined whether ARNT could similarly protect HIF from the destabilizing effects of VHL. VHL recognizes hydroxylated proline residues on HIF C-terminal to the PAS domain (5), and, because we demonstrate the inability of this region to associate with Hsp90 (Fig. 1D), ARNT overexpression was predicted to have no effect upon this pathway. As shown in Fig. 2B (lower panel), VHL expression resulted in a marked decrease in HIF levels, regardless of whether ARNT was co-expressed. Therefore, although ARNT appears to protect HIF from the effects of GA, it affords no protection from the destabilizing effects of VHL.
The ARNT protein is one member of a family of related ARNT proteins. ARNT 2 is closely related to ARNT and, although it is primarily expressed in neural tissues (25), is capable of heterodimerizing with HIF and inducing transcription (26, 27). ARNT 3 has less homology to ARNT and ARNT2 (28) and is reported to stimulate significantly less HIF-dependent transcription than ARNT (28, 29), while another report demonstrates a complete inability of ARNT3 to associate with HIF (27). To test whether other ARNT-related family members could similarly up-regulate HIF protein, 786-O cells were transfected with HIF and either ARNT, ARNT 2, or ARNT 3. As shown in Fig. 2C, both ARNT and ARNT 2 were equally capable of up-regulating HIF, whereas ARNT 3 had no discernable effect upon HIF levels. These data suggest that a productive association between HIF and ARNT is required for HIF up-regulation.

Because ARNT was able to partially protect HIF from the effects of GA, we presumed that ARNT was regulating HIF at the posttranslational level. To confirm this supposition, we determined whether the ARNT-mediated up-regulation of HIF correlated with an increase in HIF stability. 786-O cells were transfected with HIF with or without ARNT in the presence or absence of GA, and HIF stability was assessed by the addition of CHX. As shown in Fig. 2D (left panel), in the absence of GA, ARNT increased the stability of HIF protein and extended its half-life at least 3-fold. Although treatment with GA (right panel) decreased the half-life of HIF 2-fold in the absence of ARNT, it was unable to significantly destabilize HIF in the presence of ARNT.

To further understand the nature of the ARNT-mediated stabilization of HIF, we examined whether ARNT interfered with GA-mediated HIF ubiquitination. We previously demonstrated that ubiquitinated forms of HIF could be detected following treatment with GA and a proteasome inhibitor (19). Therefore, 786-O cells were cotransfected with HIF and either empty vector or ARNT, and cells were treated simultaneously with GA and PS-341 for 30 min with 0.5 μm PS-341 and 2 μm GA. Total lysates were prepared, HIF was immunoprecipitated with anti-HA antibody, and blots were probed with anti-HIF antibody. HIF-Ub, ubiquinated HIF. F, left panel, 786-O cells were transfected with either a nonspecific siRNA (NS) or with ARNT siRNA (S), and ARNT levels were assessed in nuclear lysates with anti-ARNT antibody. Right panel, cells were transfected first with either the nonspecific or the specific siRNA, followed by HIF transfection, and nuclear HIF levels were monitored. topo, topoisomerase.

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If HIF expression is exquisitely dependent upon ARNT levels in 786-O cells, then the down-regulation of endogenous ARNT should reduce HIF protein. RNA interference was employed to assess the effects of ARNT down-regulation upon HIF expression. We first verified that the introduction of ARNT RNAi into 786-O cells down-regulated endogenous ARNT protein (Fig. 2F, left panel). HIF expression was then assessed in 786-O cells that had been transfected with either a nonspecific RNAi or with an RNAi specific for ARNT. HIF expression from ARNT RNAi transfected cells was severalfold less compared with expression in cells transfected with a nonspecific RNAi (Fig. 2F, right panel).
The HLH/PAS Domain of ARNT Is Minimally Required for Up-regulation of HIF and Attenuation of GA Sensitivity—Collectively, the data suggest that ARNT modulates an Hsp90-dependent pathway for HIF degradation. To further explore this issue, we examined which ARNT domains are required for HIF up-regulation. A series of FLAG-tagged ARNT deletion constructs was created (Fig. 3A) to assess the requirement of specific domains of ARNT for HIF up-regulation. The relative expression of these constructs was verified in COS cells, as shown in Fig. 3B. 786-O cells were transfected with HIF, and the indicated ARNT constructs and HIF expression was determined from nuclear extracts. As shown in Fig. 3C, both full-length ARNT and ARNT HLH/PAS (residues 1–450) up-regulated HIF protein, demonstrating that the ARNT-mediated effects upon HIF are specific and require both the HLH and PAS domains of ARNT. We next sought to identify the minimal HIF domains involved in this up-regulation. 786-O cells were transfected with the indicated HIF proteins in combination with ARNT or ARNT HLH/PAS, and HIF protein levels were assessed (Fig. 3D). Although neither the HLH nor the ΔHLH/PAS HIF proteins were up-regulated by ARNT, the HLH/PAS HIF protein was up-regulated equally well by either full-length ARNT or HLH/PAS ARNT. These data establish that the HLH/PAS domain of HIF is up-regulated by the HLH/PAS domain of ARNT. To confirm whether ARNT HLH/PAS also modulates an Hsp90-dependent pathway, we tested the ability of this protein to attenuate the GA sensitivity of HIF. As shown in Fig. 3E, the ARNT HLH/PAS protein protected HIF from the destabilizing effects of GA, demonstrating that the HLH/PAS domain of ARNT is required for its Hsp90-dependent effects upon HIF stability.

The HLH/PAS Domain of ARNT Associates with the PAS Domain of HIF and Competes for Its Hsp90 Binding Site—Our data strongly suggested that ARNT was modulating an Hsp90-dependent pathway for HIF regulation, and we next considered whether ARNT affected the HIF-Hsp90 interaction. One likely scenario would involve competition between ARNT and Hsp90 for an overlapping binding site on HIF, which is a model that has previously been suggested to account for the ARNT-dependent release of Hsp90 from the PAS proteins Ahr (21, 30) and Sim (20). A similar model for HIF seemed likely because of the fact that the PAS domain of HIF associated with Hsp90, the HLH/PAS domain of HIF was up-regulated by ARNT or HLH/PAS ARNT, and the HLH/PAS domain of HIF has previously been shown to interact with ARNT (31). We first tested the ability of the ARNT expression constructs to associate with HIF. Consistent with previous reports, we demonstrate that HLH/PAS ARNT is capable of associating with full-length HIF in vitro, and we detected a previously unreported weaker association with PAS ARNT (Fig. 4A). The HLH/PAS domain of ARNT is required for association with HIF, as the C-terminal portion of ARNT lacking these domains is unable to interact with HIF. We next determined which of the HIF deletion constructs interact with full-length ARNT. We demonstrate that the PAS domain of HIF is the minimal region required for interaction...
with ARNT (Fig. 4B). To further delineate the domains involved in HIF-ARNT association, HIF deletion constructs were incubated with either full-length ARNT or HLH/PAS ARNT, and association between these proteins was assessed (Fig. 4C). We demonstrate that the PAS domain of HIF associates with either full-length ARNT or HLH/PAS ARNT. Collectively, these results demonstrate that the PAS domain of HIF is primarily responsible for mediating the interaction with ARNT (although the basic HLH domain imparts stability in vivo), while the HLH/PAS domain of ARNT most strongly associates with HIF.

Having established the importance of the PAS domain in ARNT association, we next explored whether ARNT could directly mediate the association of HIF with Hsp90. To answer this question, full-length HIF was translated in the presence of label, incubated with an unlabeled excess of the indicated ARNT proteins, and HIF was detected from Hsp90 immunoprecipitates. A similar in vitro approach was previously utilized to document the ability of ARNT to displace Hsp90 from Sim (20). The data illustrate that both full-length ARNT and the HLH/PAS domain are equally able to displace HIF from Hsp90 (Fig. 4D). Even though the PAS domain of ARNT is capable of associating with HIF (Fig. 4A), this association appeared weaker than those interactions involving the HLH/PAS domain of ARNT, thus offering an explanation as to why this protein does not displace HIF from Hsp90. Therefore, these data establish that the HLH/PAS domain of ARNT, in associating with the PAS domain of HIF, effectively competes with Hsp90 for its binding site on HIF. These data can be summarized in the schematic model, shown in Fig. 5, in which Hsp90 association with HIF results in a latent, potentially unstable complex, whereas ARNT-mediated displacement of the chaperone facilitates the development of a transcriptionally active, stable HIF complex.

**DISCUSSION**

The favorable completion of Phase I 17-allylamino-17-desmethylgeldanamycin (17-AAG) clinical trials signals that therapeutic use of Hsp90 inhibitors is closer to becoming a reality. However, the physiological roles played by Hsp90 in client regulation are often not clearly defined. This lack of information may compromise optimal targeting of respective clients. We and another group previously identified HIF-1 as a GA-sensitive target destabilized by a pathway independent of VHL or oxygen tension (19, 32). Ironically, although Hsp90 was inferred to play an important role for HIF, no clear function
ARNT Modulates Hsp90-dependent HIF Regulation

The PAS domain of proteins typically functions as a sensor of environmental stimuli. It is therefore not surprising that this domain interacts with multiple cofactors that serve to regulate protein activity in response to cellular signals. Hsp90 has been identified as a PAS-associated binding partner for both Sim and Ahr (20–22), and the association of Hsp90 with either protein correlates with maintenance of the client in a latent state. In the case of Ahr, ligand-dependent heterodimerization of Ahr with ARNT prevents the formation of Hsp90-Ahr complexes due to competition for overlapping binding sites within Ahr (21). Because HIF is not a ligand-binding protein, its activation more closely mirrors that of Sim, a non-ligated HLH/PAS transcription factor found in Drosophila. Hsp90 releases its hold upon Sim following heterodimerization with either ARNT or Per. Analogous to this model, we find that ARNT competitively displaces Hsp90 from HIF in vitro. In agreement with a previous report that HIF associates with the HLH/PAS domain of ARNT (9), we now demonstrate that the PAS domain of HIF is critical for its interaction with ARNT. This agrees with a previous report documenting the necessity of the HLH/PAS domain of HIF for ARNT interaction (31). Importantly, we also establish that the PAS domain of HIF associates with Hsp90 and that HIF forms mutually exclusive complexes with either ARNT or Hsp90.

ARNT is not associated with Hsp90 (33), but it has been proposed to promote the release of Hsp90 from ligand-activated Ahr (21) and Sim (20). The ability of ARNT to displace HIF from Hsp90 has not been previously documented, nor have the in vitro effects of ARNT-mediated Hsp90 dissociation of PAS-interacting proteins been explored. We now report that ARNT profoundly affects the expression of HIF in a VHL-deficient RCC model. ARNT overexpression leads to a significant increase in HIF expression, concomitant with an increase in protein stability. Collectively, our data suggest that ARNT modulates an Hsp90-dependent pathway for HIF, with consequent effects upon protein stability. This assertion is corroborated by the following data: 1) ARNT overexpression attenuates the destabilizing effects of GA upon HIF and correlates with decreased GA-mediated HIF ubiquitination; 2) ARNT does not affect VHL-dependent HIF degradation; 3) the ability of ARNT to up-regulate HIF is dependent upon the HLH/PAS domain of ARNT, which is the minimal region required for productive HIF association; competitive Hsp90 displacement, and attenuation of GA sensitivity; 4) a strong association between HIF and ARNT is required for HIF stabilization, a notion validated by the identical up-regulation of HIF by the highly homologous ARNT 2 protein that was shown to productively associate with HIF (26, 27), whereas the less homologous ARNT 3 protein, incapable of inducing a HIF-dependent hypoxic response (27), failed to elevate HIF; 5) the weaker complex between HIF and the PAS domain of ARNT was unable to displace HIF from Hsp90; and 6) the HLH and ∆HLH/PAS HIF proteins were insensitive to GA and were correspondingly not up-regulated by ARNT in vivo.

Interestingly, ARNT stabilized HIF in the absence of GA, which suggests that, in 786-O cells, the Hsp90-dependent pathway for HIF degradation is constitutively active. In support of this premise, although 786-O cells lack VHL (2), we demonstrate that transfected HIF protein is quite unstable. This suggests that alternate, possibly Hsp90-dependent degradation pathways play a previously unappreciated role in regulating HIF turnover. We found that ARNT does not dramatically increase HIF levels in all cell lines tested (data not shown), suggesting that a complex set of factors regulate the basal activity of the Hsp90-dependent degradative pathway and/or the affinity of HIF for cofactors such as ARNT. In 786-O, HIF-2 is abundantly expressed (2), and the ability of this protein to heterodimerize with ARNT likely reduces the amount of ARNT available to HIF, which may, in part, explain why the introduction of exogenous ARNT has such a pronounced effect upon HIF levels.

The in vitro and cellular studies presented here collectively provide a framework for understanding the physiological function of Hsp90 in HIF regulation. We now demonstrate that Hsp90 maintains HIF in a latent, ARNT-competent state. We also describe a novel role for ARNT in mediating HIF function. In addition to its role as a required cofactor for HIF-1α activation, we now highlight its ability to actively displace Hsp90 from HIF, which must logistically precede or occur concomitantly with HIF activation. Our data highlighting the previously unappreciated role for Hsp90 in the regulation of HIF stability adds yet another layer of complexity to HIF function and suggests that Hsp90-dependent pathways may play prominent roles in regulating HIF turnover in instances of VHL inactivation. Our findings may also have broad clinical ramifications in that specific cofactor association may influence the accessibility of Hsp90 clients such as HIF to the chaperone and, thus, modulate their sensitivity to pharmacologic intervention by Hsp90 antagonists.
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