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Review

The hypoxia-inducible factors: key transcriptional regulators of hypoxic responses

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Abstract. Oxygen depravation in mammals leads to the transcriptional induction of a host of target genes to metabolically adapt to this deficiency, including erythropoietin and vascular endothelial growth factor. This response is primarily mediated by the hypoxia-inducible factors (HIFs) which are members of the basic-helix-loop-helix/Per-ARNT-Sim (bHLH/PAS) transcription factor family. The HIFs are primarily regulated via a two-step mechanism of HIF post-translational modification, increasing both protein stability and transactivation capacity. This review aims to summarise our current understanding of these processes, and discuss the important role of the HIFs in the pathophysiology of many human diseases.

Key words. Oxygen; hypoxia; hypoxia-inducible factor; bHLH/PAS; transcription; hydroxylation.

Introduction

Oxygen homeostasis in mammals is tightly regulated, necessitated by the need to maintain sufficient levels for critical oxygen-dependent processes, whilst minimising the production of reactive oxygen species (ROS) that are capable of causing oxidative damage to DNA, lipids and protein. In a state of hypoxia, where oxygen demand exceeds supply, a physiological response is mounted which increases the capacity of blood to carry oxygen to tissues, and alters cellular metabolism, for example facilitating ATP production by anaerobic glycolysis. The hypoxia-inducible factors (HIFs) are key transcriptional regulators of this hypoxic response in both adult and embryonic organisms. In addition, these factors have been implicated in the pathophysiology of many major human diseases, including cancer, myocardial infarction, ischaemia and preeclampsia.

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HIF discovery and classification

The discovery of HIF was enabled by the identification of a minimal hypoxically responsive element (HRE) in the 3′ enhancer of the erythropoietin gene [1]. Subsequent analysis identified HIF as a phosphorylation-dependent protein which binds the major groove of DNA under hypoxic conditions [2]. Purification of this DNA-binding factor revealed HIF was a heterodimeric complex consisting of a novel protein, HIF-1α, and the aryl hydrocarbon nuclear translocator (ARNT, also termed HIF-1β), previously identified as a binding partner of the dioxin/aryl hydrocarbon receptor (DR/AhR) [3–5]. Subsequently, HIF-1α has been independently cloned as a binding partner of both ARNT and p300/CBP [6, 7].
HIF-1α and ARNT belong to a class of transcription factors termed basic helix-loop-helix (bHLH)/PAS proteins, grouped by two conserved domains (fig. 1). The basic region consists of approximately 15 predominantly basic amino acids responsible for direct DNA binding. This region is adjacent to two amphipathic α helices, separated

by a loop of variable length, which forms the primary dimerisation interface between family members [8]. The PAS domain, named after the first three proteins in which it was identified (Per, ARNT and Sim), encompasses 200–300 amino acids containing two loosely conserved, largely hydrophobic regions of approximately 50 amino acids, designated PAS A and PAS B [9]. This domain forms a secondary dimerisation interface between family members in addition to other roles, for example ligand and chaperone binding in the dioxin receptor (DR) [10]. Despite not directly binding DNA, the PAS domain has also been reported to confer target gene specificity to the *Drosophila* proteins Trachealess (Trh) and Single minded (Sim) [11]. The mechanism by which this occurs remains unknown, as does the full extent of functions played by the PAS domain in the HIFs. All known members of the bHLH/PAS family function as dimers, with ARNT and its paralogs the ubiquitous partners. HIF-1α homologs are highly conserved and function in similar roles in organisms other than mammals, including *Drosophila* [12, 13] and fish [14, 15]. HIF regulation mechanisms between organisms are also conserved, which, as will be discussed, predominantly involves a two-step mechanism of posttranslational regulation involving both protein stability and transactivation (fig. 2).

ARNT is an obligate heterodimeric partner for HIF-1α, as well as additional bHLH/PAS proteins such as the DR. The requirement of ARNT in multiple signalling pathways has therefore prompted investigation of competition for ARNT binding. Although several studies have demonstrated the capacity for functional interference between the dioxin and hypoxic signalling pathways [16, 17], at least one study indicates that any cross-talk between these pathways does not occur through competition for ARNT [18]. Hence, the role of competition for ARNT by other bHLH/PAS proteins in vivo remains unclear.

The chaperone Hsp90 binds the PAS domain of the DR and maintains it in a ligand-responsive cytoplasmic state [19, 20]. Similarly, Hsp90 coimmunoprecipitates with the bHLH/PAS domain of HIF-1α but is not detectable translocating into the nucleus [21]. This chaperoning role may explain the requirement for Hsp90 in both heat and hypoxia-induced HIF-1α accumulation, as well as a recent report implicating Hsp90 in a novel HIF-1α degradation pathway [21–23].

Additional HIFs and expression patterns

A closely related protein, HIF-2α [also termed endothelial PAS (EPAS), HIF-like factor (HLF), HIF-related factor (HRF) and member of PAS superfamily 2 (MOP2)] [24–27], was identified shortly after HIF-1α was cloned. HIF-2α shares 48% amino acid sequence identity with HIF-1α and accordingly was found to heterodimerise with ARNT and bind HREs [24, 25]. Deletion analysis has demonstrated both HIF-α proteins share a common functional domain architecture (fig. 1). In addition to the amino-terminal bHLH and PAS domains, the HIF-αs possess two transactivation domains (TADs), separated by a region termed the inhibitory domain (ID), which is responsible for normoxic repression of TAD activity. Overlapping the amino-terminal TAD (N-TAD) is an oxygen-dependent degradation domain (ODDD), which confers normoxic instability to the HIFα- proteins (fig. 2) [28–31].

RNA expression patterns have indicated that both HIF-1α and HIF-2α are largely ubiquitously expressed in human and mouse tissues in an oxygen-independent manner [24–26, 32, 33]. Analysis of cell-type-specific expression patterns, however, indicate that in contrast to ubiquitous HIF-1α, HIF-2α messenger RNA (mRNA) is pre-

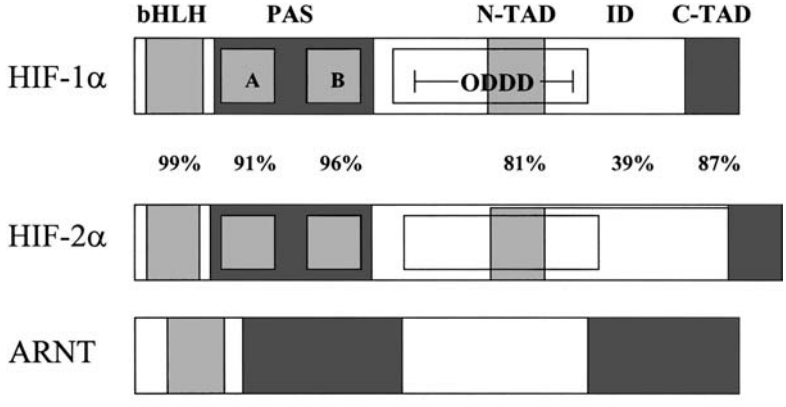

Figure 1. HIF-1α, HIF-2α and ARNT domain structure. HIF-1α, HIF-2α and ARNT are basic helix-loop-helix/Per-ARNT-Sim homology (bHLH/PAS) transcription factors, grouped by conserved amino-terminal bHLH and PAS domains. In addition to the carboxy-terminal transactivation domain (C-TAD), similar to ARNT, HIF-1α and HIF-2α also possess an additional amino-terminal transactivation domain (N-TAD), an inhibitory region (ID) that negatively regulates TAD activity and an oxygen-dependent degradation domain (ODDD) that mediates oxygen-regulated stability. Amino acid similarity between domains of HIF-1α and HIF-2α are given.

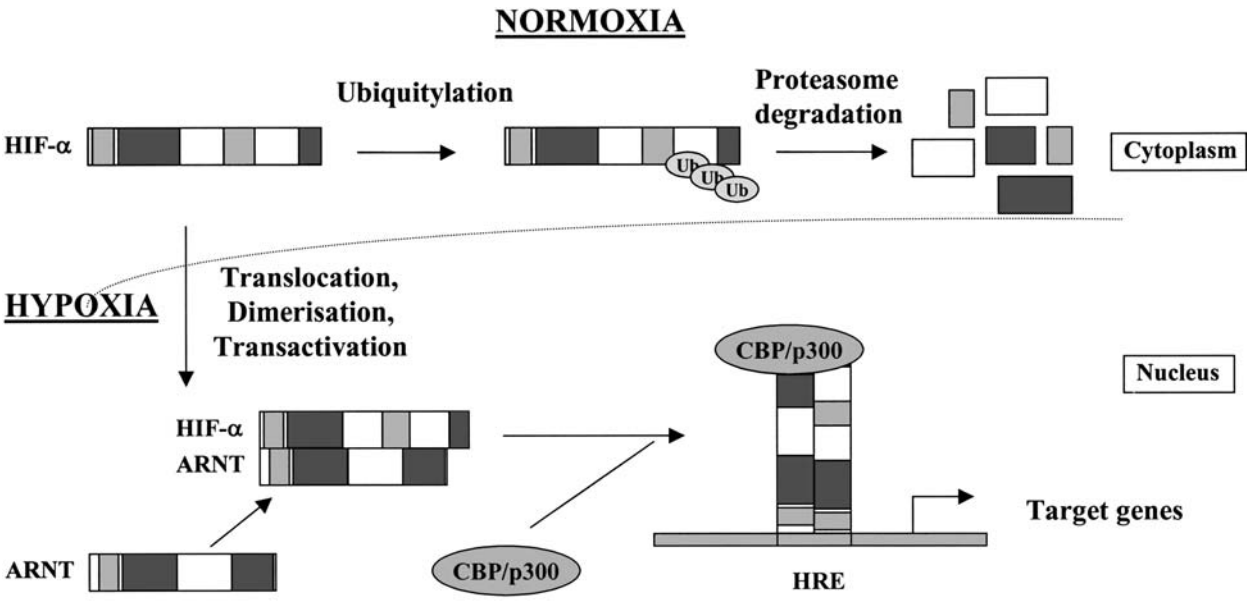

Figure 2. Overview of hypoxically regulated gene expression by HIF-α. In normoxia, HIF-α protein is transcriptionally inactive and rapidly degraded by the ubiquitin/proteasome pathway. Under hypoxia, however, HIF-α becomes stabilised, translocates into the nucleus and heterodimerises with ARNT. This transcriptionally active complex then associates with hypoxia response elements (HREs) in the regulatory regions of target genes, binds transcriptional coactivators (p300/CBP) and induces target gene expression.

dominantly expressed in specific cell types such as endothelial, epithelial, neuronal, fibroblast and macrophage cells [24, 26, 33, 34].

A third HIFα gene has also been discovered, designated *HIF-3*α. Like the better-characterised HIF-1α and HIF-2α, it is expressed in a variety of tissues, dimerises with ARNT, binds to HRE DNA sequences and upregulates reporter expression in a hypoxia-inducible and ARNT-dependent manner [35]. A splice variant of HIF-3α, termed inhibitory PAS (IPAS), has recently been identified [36]. IPAS possesses no endogenous transactivation capacity, but appears to act as a dominant-negative regulator of HIF, interacting with the amino-terminal region of HIF-1α and preventing DNA binding. IPAS is predominantly expressed in the Purkinje cells of the cerebellum and corneal epithelium, and antagonises HIF-dependent angiogenesis despite tissue hypoxia [36]. This alternately spliced HIF-3α transcript is also hypoxically induced in the heart and lung and may contribute to a negative feedback loop for HIF activity in these tissues [37].

HIF splice variants

In mice, two HIF-1α mRNA transcripts (I.1 and I.2) are produced from different promoters (as opposed to alternate splicing) [38]. These transcripts are both efficiently translated independently of oxygen, but differ in that whereas I.1 encodes a protein lacking the first 12 amino-terminal amino acids and is expressed in a tissue-restricted manner, I.2 is ubiquitously expressed and encodes a full-length protein. Despite these differences, no specificity in DNA binding or transactivation capacity has been observed [39, 40]. Interestingly, the I.1 transcript is specifically upregulated in the elongated spermatids of the testes, and after T cell antigen receptor (TCR)-triggered activation of T lymphocytes, although the reason for this remains unclear [41, 42]. Several splice variants have also been identified in humans. One such example is a HIF-1α splice variant, present in skin and several cell lines, which lacks exon 14 [43]. This leads to a frame shift and encodes a shorter protein (736 amino acids) which, although still hypoxically inducible, lacks a carboxy-terminal TAD (C-TAD) and hence is less active than wild-type HIF-1α. [43]. A dominant-negative isoform lacking exons 11 and 12 has also been reported [44]. The resultant protein is 516 amino acids long, stable in normoxia and displays no transactivation or hypoxia-induced nuclear translocation [44]. Similarly, a zinc-induced splice variant lacking exon 12 also acts as a dominant negative, inhibiting HIF activity by binding to ARNT and preventing its nuclear accumulation, possibly accounting for the inhibitory effect of zinc [45]. A naturally occurring antisense transcript complementary to the 3′ untranslated region of HIF-1α has also been reported [46]. This transcript is overexpressed in nonpapillary kidney tumour cells at normoxia, and is hypoxically inducible in lymphocytes where there is a concomitant decrease in HIF-1α mRNA [46].

HIF degradation

The normoxic turnover of HIF-α is very rapid, resulting in essentially no detectable HIF-α protein under normoxic conditions [4, 47, 48]. This normoxic instability is controlled by the central 200-amino acid ODDD that overlaps the N-TAD [48]. The rapid accumulation of HIF-1α and HIF-2α that occurs in hypoxia is mediated by increased protein stability. In contrast, oxygen tension does not have a major affect on HIF-α transcription or translation [32, 48–51]. Similarly, oxygen does not significantly affect ARNT mRNA or protein levels, which are constitutively expressed [48, 49, 51].

The normoxic instability of HIF-α is mediated by polyubiquitylation and subsequent degradation by the proteasome (fig. 2). This has been demonstrated by the use of proteasomal inhibitors or mutation of the E1 ubiquitin activating enzyme [48, 52]. Thus, HIF-α is polyubiquitylated under normoxia with the level of ubiquitylation decreasing in hypoxia [48, 52, 53]. In further support of this, HIF-1α physically interacts with the 20S proteasomal subunit PSMA7 [54].

The von-Hippel-Lindau (VHL) tumour suppressor protein is a component of an E3 ubiquitin-protein ligase complex containing elongins B and C, Cul2 and Rbx1, and it is this capacity by which VHL mediates the proteasomal degradation of HIF-1α and HIF-2α [55]. VHL's role in the normoxic degradation of HIF-α was initially implied by the upregulation of hypoxically responsive mRNAs in VHL-deficient cell lines [56, 57]. The VHL/HIF link was confirmed by the presence of normoxically stable HIF-1α in VHL-deficient cells, and subsequently restored normoxic protein instability upon VHL transfection [58, 59]. VHL is able to exert this effect by binding to amino acids 557–571 or 380–417 of HIF-1α in normoxia (amino acids 517–534 and 383–418 in HIF-2α) via its β domain, while the α domain binds elongins. Ubiquitin is then transferred to unspecified HIF residues, marking the protein for proteasomal destruction [59–63]. In addition, VHL is required for the correct assembly of an extracellular fibronectin matrix [64].

It has emerged that the binding of VHL to HIF in normoxia, and hence the major mechanism by which HIF protein instability is conferred, is mediated by the irreversible hydroxylation of two proline residues (P402 and P564 in HIF-1α, P405 and P530 in HIF-2α) [65–68]. These residues are hydroxylated only in normoxia, enabling the high-affinity binding of VHL to HIF [69] (fig. 3). The identification of egl9, a HIF prolyl-hydroxylase in *Chaenorhabditis elegans*, enabled the cloning of three mammalian homologs designated prolyl hydroxylase domain containing (PHDs) 1, 2 and 3, or HIF prolyl-hydroxylases (HPHs 3, 2 and 1, respectively) [70–74]. A widely expressed fourth PHD/HPH has recently been identified [75].

The reason there are at least four PHD/HPHs remains unclear; however, differences in activity, expression patterns and subcellular localisation may enable a graded or tissue-specific response to hypoxia [70, 71]. At least one PHD/HPH is also present in *Drosophila* that mediates the normoxic instability of the HIF-1α homolog Similar (Sim a) [70]. Despite the similarity to previously characterised prolyl hydroxylation of collagen, HIF-1α and HIF-2α do not possess the hydroxylation consensus sequences identified in collagen, and collagen prolyl hydroxylases are unable to hydroxylate HIF-1α peptides [65, 66, 76]. Thus, the HIF PHD/HPHs represent a novel family of hydroxylases related to, but not functionally redundant with, collagen hydroxylases.

The PHD/HPHs are 2-oxoglutarate-dependent enzymes that require oxygen (O_2) for hydroxylation. They contain iron bound to two histidine and one aspartic acid residue which, when maintained in its ferrous state by ascorbate, binds dioxygen. One oxygen is transferred to the target proline residue of HIF; the second reacts with 2-oxoglutarate to produce succinate and carbon dioxide. Hence, the absence of oxygen leads to no enzyme activity, non-modification of HIF proline residues and no VHL/HIF binding, resulting in stabilised HIFα- protein. Therefore, it is likely the PHD/HPHs function as a direct oxygen sensor in cells that directly modulate HIF in response to physiological oxygen concentration.

The fact that HIF-1α degradation is suppressed by inhibiting either cellular transcription or HIF-1α activity implies that HIF-1α may upregulate a target which degrades it [77]. This may at least partially explain the observed reduction of HIF-1α protein during an extended period of hypoxia [77]. Given that some of the PHD/HPHs are reported HIF-1α target genes, the PHD/HPHs may represent a way by which HIF-1α self-regulates its expression [70, 71].

The regulation of VHL binding by proline hydroxylation represents the major mechanism by which HIF protein levels are controlled. The fact that HIF-α is still somewhat labile in hypoxia, however, where VHL cannot bind, implies the presence of additional mechanisms that influence degradation. One such mechanism involves p53. The *p53* tumour suppressor gene encodes a multifunctional transcription factor that regulates cellular responses to diverse stimuli, including hypoxia. p53 is susceptible to proteasomal degradation and is dependent upon its physical interaction with HIF for hypoxic stabilisation [78]. This interaction has been localised to two HIF-1α motifs adjacent to the proline hydroxylation sites and occurs primarily with a dephosphorylated form of HIF-1α induced in hypoxia [79, 80]. Mdm2 is an E3 ubiquitin-ligase associated with p53 degradation. In contrast to the action of HIF stabilising p53 in hypoxia, p53 conversely targets HIF-1α for Mdm2-mediated ubiquitylation and degradation, possibly through HIF-1α repre-

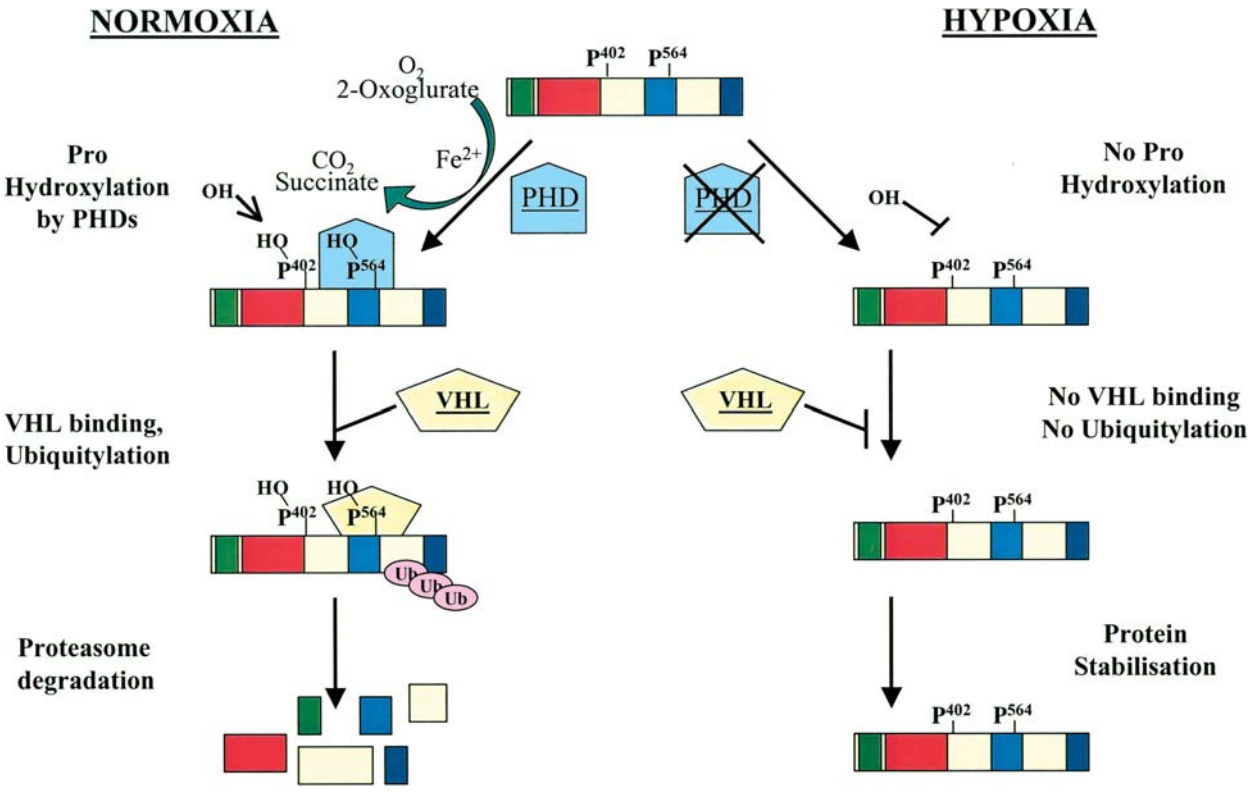

Figure 3. The oxygen-dependent HIF-1α/HIF-2α degradation pathway. In normoxia, HIF-prolyl-4-hydroxylases (PHD) hydroxylate specific proline residues of HIF-1α (P402 and P564) and HIF-2α (P405 and P530) in an oxygen, 2-oxoglutarate and iron-dependent manner. Hydroxylated HIF-α proteins then bind to the von-Hippel-Lindau (VHL) protein, the HIF-α recognition component of an E3 ubiquitin ligase complex. HIF-α is subsequently ubiquitylated, and degraded by the proteasome. In hypoxia, PHD/HPH activity is blocked due to oxygen deficiency, preventing HIF-α proline hydroxylation and VHL binding, and resulting in stabilised HIF-α protein.

senting a better Mdm2 target than p53 [81]. This is demonstrated by p53-deficient cell lines having increased HIF protein and decreased HIF ubiquitylation in hypoxia, and expression of the E6 oncoprotein, which promotes p53 degradation, increasing HIF-1α stability with the hypoxia mimetic cobalt chloride [81]. Recently, the Jun activation domain-binding protein (Jab1) was shown to bind HIF-1α, increasing protein stability and hypoxic reporter activity via competition with p53 for HIF-1α binding [82]. It is tempting to speculate that p53 primarily mediates slow hypoxic degradation of HIF-1α, whilst VHL mediates rapid normoxic degradation.

Transcriptional activation of HIF

Modulation of transactivation domain function is a second major mechanism by which HIF activity is controlled, whereby transactivation domains are repressed at normoxia but active under hypoxia. As discussed previously, HIF-1α and HIF-2α possess two transactivation domains, the N-TAD and C-TAD [29, 30, 83]. The TADs function through recruitment of the general coactivators CBP/p300, SRC-1 and TIF2 [7, 83–87]. The physical interaction of ARNT with CBP/p300 has also been reported [88]. Overexpression of the nuclear redox regulator Ref1 potentiates the hypoxic induction of a reporter gene driven by an N-TAD or C-TAD containing HIF-1α protein, probably by providing an appropriate reductive environment that enhances the ability of HIF to recruit coactivators [83, 86, 89]. These coactivators physically link HIF to the transcriptosome and function as histone acetyltransferases to perform the chromatin remodelling required for transcription. The structure of the cysteine/histidine-rich 1 (CH1) domain of p300 or CBP bound to the C-TAD of HIF-1α has recently been solved [90, 91]. Alanine-scanning mutagenesis of the HIF-1α C-TAD has also revealed key amino acids required for transactivation and p300/CBP binding [92].

The ability of CBP/p300 to bind HIF-1α is inhibited by p35srj (also called cited2). This factor competes with HIF-1α, and other transcription factors, for binding to the CH1 domain of CBP/p300 and hence blocks coactivator recruitment [93]. Interestingly, p35srj is itself activated by HIF-1α under hypoxia and hence may represent a negative feedback mechanism [93]. Unexpectedly, however,

the p35srj mouse knockout displays similarities to VEGF and HIF-1α knockouts that would be consistent with an activating role, with decreased levels of hypoxically responsive mRNAs and an embryonic lethal phenotype displaying cardiac malformation and neural tube defects [94]. Thus, whilst p35srj may have an important role modulating HIF-1α activity, the exact nature of this role remains unclear.

Via a mechanism analogous to proline hydroxylation, Lando and co-workers demonstrated that the C-TADs of both HIF-1α and HIF-2α are hydroxylated in an oxygen-dependent manner (fig. 4) [95]. Similar to proline hydroxylation, modification of the C-TAD occurs at normoxia and involves an O_2, iron and 2-oxoglutarate dependent hydroxylase. In contrast to the control of protein stability, however, this hydroxylation modifies an asparagine residue (N803 in HIF-1α and N851 in HIF-2α) and functions to inhibit the association of HIF-1α and HIF-2α with CBP/p300 at normoxia [95, 96]. Alanine mutation of the asparagine therefore permits coactivator binding at normoxia and full transactivation capacity. In the context of full-length protein however, mutation of both hydroxylated proline and asparagine residues is required for the generation of a protein with full constitutive activity [95]. Thus, hypoxic induction of both HIF-1α and HIF-2α involves a two-step mechanism of increased protein stability and transcriptional activity, both mediated by O_2-dependent hydroxylation.

A yeast two-hybrid screen of the ID and C-TAD of HIF-1α identified FIH-1 (factor inhibiting HIF) as a HIF (and VHL) binding protein that negatively regulates HIF-1α activity [97]. It was subsequently discovered that FIH-1 was in fact a novel O_2, iron and 2-oxoglutarate dependent asparaginyl hydroxylase responsible for regulating HIF-α C-TAD activity [98, 99]. Other asparaginyl hydroxylases with specificity for epidermal growth factor-like domains have previously been characterised, but do not appear to hydroxylate HIF-1α [100, 101]. Despite the identification of at least four PHD/HPHs that regulate HIF-α protein stability via proline hydroxylation, at present there is only one demonstrated asparaginyl hydroxylase. Homology searches, however, have identified other related hu-

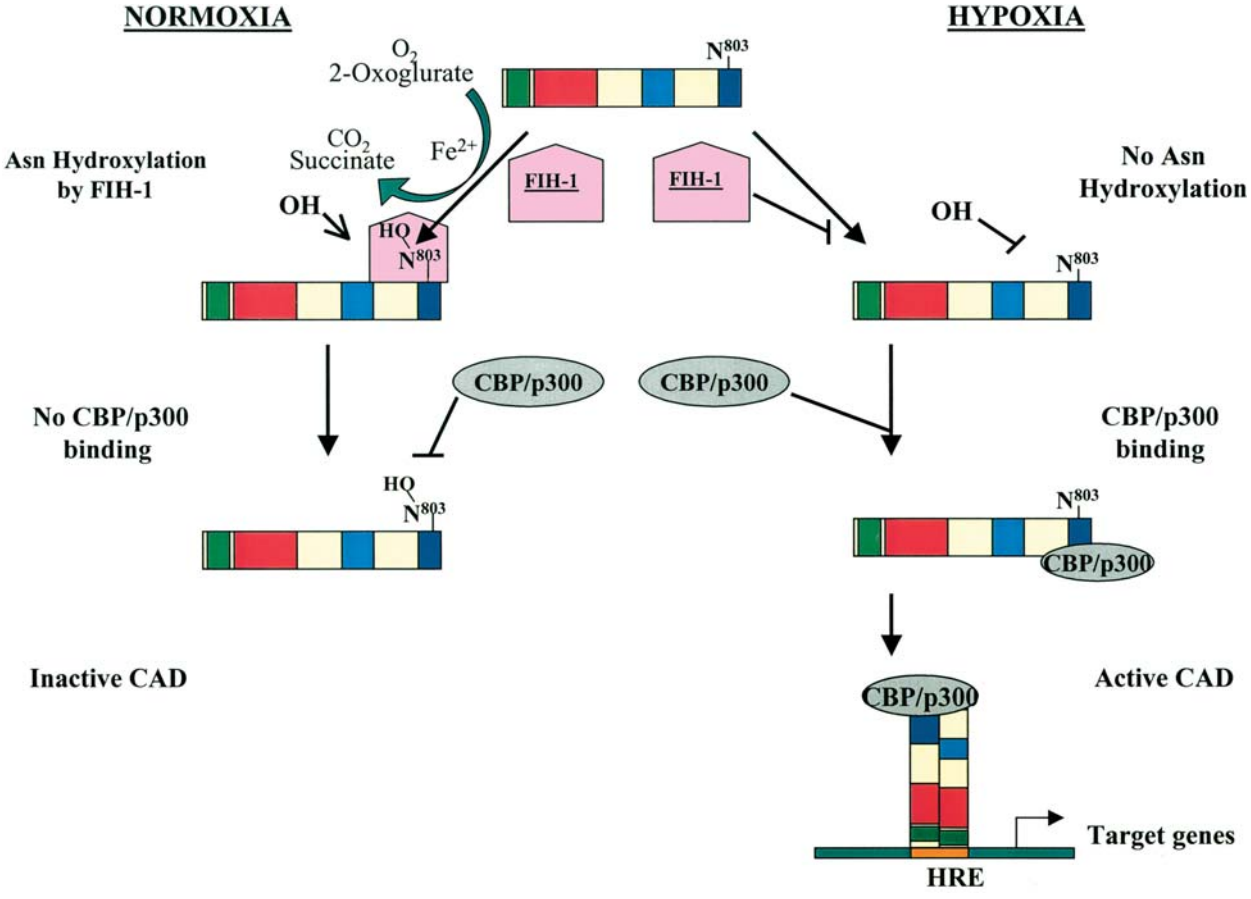

Figure 4. Oxygen-regulated transcriptional activation of HIF-1α and HIF-2α. In normoxia, an oxygen, 2-oxoglutarate and iron-dependent HIF-α asparaginyl hydroxylase (FIH-1) binds and hydroxylates specific asparagine residues of HIF-1α (N803) and HIF-2α (N851). This blocks the recruitment of transcriptional coactivators (p300/CBP) by the carboxy-terminal transactivation domain (C-TAD), resulting in transcriptionally inactive HIF-α. In hypoxia, FIH-1 activity is blocked due to oxygen deficiency, resulting in no asparagine hydroxylation, and consequently enhanced coactivator recruitment and target gene induction.

man expressed sequence tags (ESTs) in addition to homologs conserved in different species throughout evolution [97, 98]. In contrast to other asparaginyl hydroxylases, which produce an erythro-isomer, FIH-1 hydroxylates the asparagine β carbon to produce a threo-isomer [102].

Although proline hydroxylation and VHL association play a critical role in HIF regulation, and complete stabilisation of HIF-α protein results in full activity, the regulation of transcriptional activity by FIH-1 is likely to be crucial under most physiological conditions. For example, in VHL-deficient cells, or when HIF-α is grossly overexpressed, the high levels of stable HIF-α protein appear to saturate the FIH-1 enzyme, resulting in the majority of HIF-α being nonhydroxylated and transcriptionally active [103–105]. Under more physiological conditions, where HIF-α is only partially stabilised, such as mild hypoxia or growth factor induction, FIH-1 is not saturated and exerts an important role in regulating the transcriptional activity of the stabilised protein [95].

Alternate mechanisms of HIF regulation

Western and immunohistochemical analysis demonstrates the marked upregulation of HIF-1α and HIF-2α protein levels under hypoxia in both cell lines and mammalian tissue. Interestingly, however, several reports of HIF protein expression at normoxia, in accordance with various nonhypoxic stimuli reported to increase HIF activity, imply more diverse roles for HIF than solely regulating a hypoxic response. For example, immunohistochemistry has shown normoxic HIF-1α expression in distinct cell types within diverse tissues [106]. Normoxic expression of HIF-1α has also been reported in pulmonary arterial smooth muscle cells and in the midpiece of spermatozoal tails [41, 47]. Furthermore, high levels of HIF-2α have been reported in nonhypoxic bone-marrow macrophages, fibroblasts, endothelial cells and epithelial cells [33, 34].

Despite the central importance of hydroxylases in sensing oxygen tension and regulating HIF activity, an array of cytokines and growth factors have also been implicated in HIF control. These include insulin, insulin-like growth factors 1 and 2, fibroblast growth factor 2, epidermal growth factor, platelet-derived growth factor, transforming growth factor-β1, thrombin, angiotensin 2, hepatocyte growth factor, tumour necrosis factor-α and interleukin 1-β [107–117]. Despite this diversity, many of these factors act upon HIF via common kinase pathways, increasing HIF-1α stability and/or translation.

Nitric oxide (NO) and carbon monoxide (CO) are also implicated in modulating HIF activity. The effects reported for NO and CO, however, vary, most likely due to cell-specific differences and the fact that a transient increase in HIF-1α activity is often observed prior to a prolonged decrease in activity. Thus, NO and CO are reported as both activators of HIF-1α via increased protein accumulation [118–123] or inhibitors [124–127]. A role for ROS has also been suggested in HIF control, although again, there is conjecture regarding whether these ROS have an activating [115, 117, 128, 129] or inhibitory [130] effect.

The use of kinase and phosphatase inhibitors has demonstrated the importance of phosphorylation in HIF regulation [131]. To date, however, only the oxygen-independent phosphorylation of HIF-2α Thr844 has been identified [132]. Members of the mitogen-activated protein kinase (MAPK) pathway have been implicated in increasing the activity of HIF in response to various stimuli. Examples of MAPK, and ultimately HIF, stimuli include Kaposi's sarcoma-associated herpes virus G-protein-coupled receptor [133], the organomercurial compound mersalyl [134], and various cytokines and growth factors [108, 111]. In addition, HIF 1α and HIF-2α transactivation during hypoxia requires p42/p44 MAPKs [135–137] and extracellular regulated kinases (ERKs) [138, 139]. Furthermore, p42/p44 MAPK and ERK1 mediate the in vitro phosphorylation of HIF-1α [138, 140].

Likewise, the phosphatidylinositol 3 kinase (PI3K/AKT) pathway is involved in HIF activation in response to growth factors [108, 109, 111, 113, 141–143], NO [120], vanadate [129] and mechanical stress [144]. The restoration of the Akt phosphatase PTEN (phosphatase and tensin homolog deleted on chromosome 10) into PTEN-deficient cells ablates hypoxic and insulin-like growth factor (IGF)-induced HIF activity, and blocks the increased stability of HIF-α caused by Akt activation [145]. Despite this, the hypoxic stabilisation and activation of HIF-1α has been reported to occur independently of PI3K [146]. In addition, the GTPase Rac1 and diacylglycerol kinase (DGK) have been implicated in HIF-1α hypoxic activation [147, 148], whilst HIF translation is increased by the membrane-linked nonreceptor tyrosine kinase Src1 [149] and the receptor tyrosine kinase Her2 signalling through a PI3K-dependent pathway [141].

HIF translocation

HIF-1α undergoes nuclear accumulation during hypoxia, or normoxia with overexpression or proteasome inhibition, in an ARNT-independent manner [52, 137, 150]. Conversely, HIF-1α is shuttled back into the cytoplasm during reoxygenation [84, 151]. Despite being required for activity, however, nuclear translocation per se is not sufficient to upregulate reporter gene expression, nor protect HIF-1α from degradation [84, 152]. As with the DR and ARNT, a constitutively active nuclear localisa-

tion sequence (NLS) is situated in the bHLH domain and able to mediate the nuclear translocation of chimeric proteins [84]. Addition of the PAS domain, however, abrogates this effect and indicates that it is a second hypoxically regulated carboxy-terminal NLS which mediates the translocation of full length HIF-1α, demonstrated by mutation of Lys719 [84]. Interaction with the p14ARF tumour suppressor protein induces nucleolar relocalisation of HIF-1α, thereby inhibiting transactivation [153]. As is becoming apparent with many aspects of HIF-α regulation, the control of nuclear localization is regulated at multiple levels that may provide a mechanism to activate target genes in a tissue-specific manner.

HIF target genes

HIF-1, 2α/ARNT heterodimers bind to HREs with the core consensus (A/G)CGTG in the regulatory regions of target genes (listed in table 1) to upregulate expression [1, 202–204]. Many of these genes can be grouped by function. For example, the capacity of red blood cells to transport oxygen is increased through genes involved in erythropoiesis. These genes includes the erythrocyte growth and survival factor erythropoietin, and various iron-metabolising genes that control the major erythropoietic rate-limiting step of haem production. Many pro-angiogenic genes, such as vascular endothelial growth factor (VEGF), are also direct HIF targets, as are genes associated with glucose uptake and glycolysis. Thus, HIF regulates both short-term responses to hypoxia, such as erythropoiesis and glycolysis, and longer-term responses such as angiogenesis. In addition to these classes of genes, however, other targets identified do not appear to fall into the above categories. Thus, it appears that HIF may regulate a more diverse range of processes than originally believed, including adipogenesis [205], apoptosis [193], B lymphocyte development [206] and carotid body formation [207]. Despite apparently similar modes of regulation and DNA binding specificity, no bone fide target genes have as yet been identified for HIF-2α or HIF-3α, though several studies implicate HIF-2α in VEGF induction [208–210].

Despite the central importance of HREs to the hypoxic upregulation of target genes, it is apparent that in many cases, HREs alone are not sufficient for hypoxic inducibility [183, 211]. Synergistic cooperation between HIF-1α and a number of other transcription factors has been observed, including Smad3 [212], HNF4 [213], ATF1/CREB1 [156, 183, 202, 214] and AP1 [215, 216]. Thus, whilst the HREs confer hypoxic inducibility, additional elements may be required to assemble a fully functional transcription complex in vivo.

Table 1. Characterised HIF-1α target genes.

HIF-1α target genes	References
Erythropoiesis iron metabolism	
Ceruloplasmin	[154]
Erythropoietin	[1, 155, 156]
Transferrin	[157]
Transferrin receptor	[158–160]
Vascularisation	
Vascular endothelial growth factor	[161–163]
Leptin	[164, 165]
Endothelin-1	[166–168]
Flt-1	[169]
Plasminogen activator inhibitor-1	[170, 171]
Inducible nitric oxide synthase-2	[172, 173]
Intestinal trefoil factor	[174]
Heme oxygenase-1	[175]
Adrenomedullin	[176, 177]
α_{1B}-adrenergic receptor	[178]
Glucose uptake/glycolysis	
Glucose transporter-1,3	[179, 180]
Aldolase-A/C	[181, 182]
Enolase-1	[156, 181, 182]
Lactate dehydrogenase-A	[181–183]
Pyruvate kinase M	[181]
Glyceraldehyde phosphate dehydrogenase	[184]
Phosphofructokinase L	[181]
Phosphoglycerate kinase 1	[181]
6-phosphofructo-2-kinase/fructose-2,6-bisphosphate-3	[185]
Hexokinase1,2	[186, 187]
Adenylate kinase-3	[188]
Carbonic anhydrase-9	[189]
Various	
VL30	[190]
Insulin-like growth factor 2	[110]
Insulin-like growth factor binding protein-1,2,3	[110, 191]
P35srj	[93]
P21	[93, 156]
ETS-1	[192]
NIP-3	[193–195]
DEC1/2	[196]
Collagen prolyl hydroxylase	[197]
Tyrosine hydroxylase	[107, 198]
TGF-β3	[199]
Cyclooxygenase-2	[200]
Presenilin-1,2	[200, 201]

Nonredundancy of HIFs

Despite HIF-1α and HIF-2α sharing close similarity in terms of amino acid sequence, domain architecture, DNA-binding capacity and hypoxic activation pathway, HIF-1α and HIF-2α deficient mice manifest distinct phenotypes. Hence, HIF-1α and HIF-2α have nonredundant functions. HIF-1α –/– embryos die by embryonic day 11 (E11) as a result of defective vascularisation, cardiovascular malformation and the failure of neural tube closure due to mesenchymal cell death. HIF-1α –/– ES cells also show reduced proliferation and lower levels of hypoxically induced HIF target genes [217, 218]. Furthermore, HIF-1 +/– mice develop normally, but display impaired

physiological responses to prolonged hypoxia, including reduced polycythemia, right ventricular hypertrophy, aberrant vascular remodelling and pulmonary hypertension [207, 219]. Selective deletion of HIF-1α from the cartilaginous growth plate results in hypoxically induced apoptosis, lack of chondrocyte growth arrest and skeletal deformation [220].

A vascular phenotype has also been reported for HIF-2α deficient mice. In contrast to HIF-1α –/– embryos in which vascularization is impaired, however, embryonic lethality in HIF-2α –/– mice occurs by E12.5 due to inadequate blood vessel fusion and remodelling [221]. In a second HIF-2α knockout, however, embryonic lethality occurred at E12.5-E16.5 due to insufficient catecholamine production by the organ of Zuckerkandl, the embryonic precursor to the carotid body, resulting in deregulated heart beat and death by bradycardia [222]. Embryonic lethality was rescued by addition of the noradrenalin precursor DOPS to the mother's diet; however, mice died within 24 h of birth due to discontinued supply [222]. Lastly, a third HIF-2α knockout phenotype has been described in which mice not dying by E13.5 due to cardiac failure, possibly the same phenotype as noted by Tian and co-workers, die shortly after birth from respiratory distress syndrome (RDS). This lethality is a result of the failure of alveolar type II cells in the lung to produce sufficient levels of surfactant [210]. Whilst the reason for these divergent HIF-2α –/– phenotypes remain unclear, although they are probably related to the use of different genetic strains of mice and targeting strategies, HIF-2α nonetheless appears to play important roles in development that are different from HIF-1α.

In addition to knockout phenotypes, other differences between the function of HIF-1α and HIF-2α have been noted. One such difference is the resistance of HIF-2α –/– ES cells to hypoglycaemic, but not hypoxically induced, apoptosis. HIF-1α –/– ES cells, however, are resistant to apoptosis initiated by both hypoxia and hypoglycaemia [223, 224]. This may indicate a more pronounced role for HIF-2α in response to environmental stresses other than strictly oxygen. PI3K inhibitors have also been reported to inhibit HIF-1α, though not HIF-2α, protein induction in hypoxia [225]. HIF-2α was also reported to activate reporter expression more strongly from a VEGF promoter than HIF-1α [33, 110]. Lastly, the renal carcinoma cell line 786-0, which expresses a nonfunctional truncated form of VHL and detectable levels of HIF-2α, though not HIF-1α, has also been used to demonstrate differences between the HIF proteins, in this case in regard to tumorigenic activity [103, 104]. Specifically, stabilised HIF-1α expression, through P564 mutation, does not increase tumour growth in subcutaneously injected immunocompromised mice, in contrast to constitutive HIF-2α expression, which promotes tumour development [103].

Mechanisms that lead to phenotypic differences in HIF-α activity, however, remain elusive. One mechanism of differential regulation between the HIF-α proteins is the presence of a Ref1-regulated cysteine residue in the basic region of HIF-2α that must be in a reduced state for DNA binding. This cysteine is replaced by serine in HIF-1α, where DNA binding is constitutive [89].

HIF and disease

As previously stated, hypoxia and the HIFs themselves have been implicated in the pathophysiology of many major human diseases and as such, its manipulation may prove crucial in the therapeutic management of these states. In order for solid tumour growth to occur, tumours must increase oxygen delivery to cells via angiogenesis, and increase the rate of glycolysis, known as the Warburg effect [226]. This in turn produces glycolytic end products such as lactate and pyruvate, which have been reported to cause normoxic HIF-α accumulation and hence a potential positive feedback loop [227]. Given the importance of HIF in the activation of genes essential to these processes, it is not surprising that both HIF-1α and HIF-2α have been strongly implicated in tumour progression and grade, conferring a selective advantage to tumour cells.

Hypoxic conditions within tumours may result in increased HIF stability and activity, or, HIF overexpression may result from oncogenic activation by Src or Ras [149, 228, 229]. The overexpression of one or both HIF-α proteins has been found in invasive bladder cancer [230], brain tumours [231, 232], breast cancer [233, 234], cervical cancer [235], non-small-cell lung cancer [236], non-Hodgkin's lymphoma [237], oropharyngeal cancer [238, 239], pancreatic cancer [240] and numerous other tumours including colon, skin, gastric, prostate and renal clear cell carcinomas [34, 241]. In addition, comparison of HIF-1α (or ARNT) positive and deficient cells when subcutaneously injected or xenografted into immunocompromised mice identifies HIF-1α as a positive factor for tumorigenesis [242–244]. Furthermore, a correlation between HIF overexpression and poor prognosis or treatment resistance has been noted in many of these studies, often in concert with additional genetic alterations such as the absence of functional bcl2 [238] or p53 [245].

The VHL protein was previously discussed as a HIF-α binding component of an E3 ubiquitin ligase complex that mediates HIF-α normoxic degradation. VHL disease results from mutation of the VHL tumour suppressor protein and is a hereditary cancer syndrome characterised by the development of tumours in multiple organ systems. These most commonly include the retina, cerebellum, spinal cord, kidney, pancreas, epididymis and adrenal gland [246, 247]. In most cases, VHL disease is mani-

fested as a consequence of deregulated HIF expression [104, 248]. An exception is type 2C VHL mutations, which retain the ability to downregulate HIF but demonstrate an increased risk of pheochromocytoma, possibly due to defective fibronectin matrix assembly or an inability to regulate unidentified factors [249]. One candidate is Jade1, a protein of unknown function expressed highly in the kidney which interacts with, and is stabilised by, VHL [250].

Preeclampsia, a pregnancy disorder in which trophoblasts fail to invade the myometrium and cause vascular remodelling during placentation, may also be associated with HIF overexpression. During the first 10 weeks of development, hypoxic conditions activate HIF, which acts upstream of transforming growth factor (TGF)-β3, preventing trophoblast differentiation. An increase in placental oxygen levels is then believed to decrease HIF-α expression and enable trophoblast invasion. A failure of this normoxic HIF-1α/TGF-β3 downregulation to occur causes the maintenance of trophoblasts in an immature, noninvasive state, resulting in reduced uteroplacental perfusion [251]. Similarly, analysis of ARNT –/– placentas reveals aberrant trophoblast differentiation. [252].

HIF activity has also been demonstrated in the physiological response to ischaemia, with sheep and rat models of myocardial and cerebral ischaemia increasing HIF-1α expression and inducing target genes such as VEGF and glycolytic enzymes [253–255].

Such studies indicate that therapeutic strategies to treat ischaemic diseases such as stroke and heart disease may involve HIF activation. As proof of principle, one potential mechanism to upregulate HIF is the macrophage-derived peptide PR39, which decreases HIF ubiquitin-proteasome dependent degradation, thereby increasing angiogenesis in vivo [256]. A recent study has also demonstrated that transgenic mice overexpressing HIF-1α in skin basal keratinocytes show increased expression of VEGF and vascularization, without the vascular leakage and inflammation noted with the overexpression of VEGF alone [257]. Interestingly, deletion of the HRE within the VEGF promoter reduced VEGF expression in the spinal cord, causing adult onset motor neuron degeneration in a manner similar to the neurodegenerative disease amyotrophic lateral sclerosis [258]. Identification of the importance of hydroxylation to HIF regulation also suggests targeting of proline and asparaginyl hydroxylases as potential strategies for increasing HIF activity.

In contrast to ischaemic disease, where the activation of HIF may be advantageous, therapeutic strategies to treat cancer and preeclampsia would aim to develop agents that inhibit HIF activation. One example is a HIF-1α C-TAD polypeptide that competes for p300 binding and decreases the expression of VEGF and tumour growth in mice [259]. Several small molecule inhibitors of the HIF transcriptional activation pathway have also been identified [260].

Conclusion

Rapid advances in understanding the molecular nature of hypoxic responses have led to the elucidation of oxygen sensors, hydroxylation mechanisms that relay this information to key proteins (HIF-1α an HIF-2α) and the identity of hypoxically regulated target genes that counteract oxygen depravation. Despite this, many questions remain unanswered, including the functions of HIF-3α, the mechanisms of nonredundancy between HIF-1α and HIF-2α, and the identity of additional targets of HIF, the PHD/HPHs, FIH-1 and VHL. These processes have direct relevance to both development and human disease and will, in all probability, aid the formulation of effective therapeutic agents.

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