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review

The hypoxia-inducible-factor hydroxylases bring fresh air into hypoxia signalling

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Metazoans rapidly respond to changes in oxygen availability by regulating gene expression. The transcription factor hypoxia-inducible-factor (HIF), which controls the expression of several genes, 'senses' the oxygen concentration indirectly through the hydroxylation of two proline residues that earmarks the HIF-α subunits for proteasomal degradation. We review the expression, regulation and function of the HIF prolyl hydroxylases or prolyl hydroxylases domain proteins, which are genuine oxygen sensors.

Keywords: cell signalling; hypoxia inducible factor; oxygen sensing; prolyl hydroxylase domain protein

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Introduction

All organisms respond to changes in their environment by activating networks of signal transduction pathways that are regulated by post-translational modifications and that culminate in changes in gene expression. Studies of the adaptation to low O_2 availability have now identified the hypoxia-inducible-factor (HIF) transcription factor, and a further mechanism of signal transduction involving a 'new' post-translational modification that is inherently O_2-dependent: hydroxylation on proline residues. This review summarizes our current knowledge of the prolyl hydroxylase domain-containing proteins (PHDs) that catalyse HIF prolyl hydroxylation.

The hypoxia-inducible-factor transcriptional complex

HIF is central to O_2 homeostasis during embryonic development and postnatal life in both physiological and pathophysiological processes such as tumour growth, ischaemia and tissue repair (Semenza, 1998). Indeed, HIF regulates the transcription of many genes involved in cellular and systemic responses to hypoxia. HIF consists of one of the three O_2-regulated HIF-α subunits (HIF-1α, HIF-2α and HIF-3α) and the constitutively expressed HIF-1β subunit. The most extensively studied isoform is the ubiquitous HIF-1α.

HIF activation is a multistep process involving HIF-α stabilization, nuclear translocation, hetero-dimerization, transcriptional activation and interaction with other proteins. Several steps in this activation process are independently regulated by O_2. However, O_2-dependent regulation of the proteasomal degradation of HIF-α is the most crucial step (Huang *et al*, 1996; Salceda & Caro, 1997).

Looking for the oxygen sensor

In well-oxygenated cells, HIF-α is an exceptionally short-lived protein (half-life less than 5 min at 21% O_2) and steady-state levels are very low. By contrast, reduced O_2 availability induces HIF-α accumulation by relaxing its ubiquitin-proteasome degradation. A central oxygen-dependent degradation domain (ODDD) mediates HIF-α proteolytic degradation. The mechanism by which O_2 deprivation increases HIF-α stability remained obscure until Ratcliffe's group showed that ubiquitylation and proteasomal degradation of HIF-α requires pVHL, the product of the von Hippel–Lindau tumour suppressor gene (Maxwell *et al*, 1999). pVHL is the HIF-α recognition component of a multiprotein complex, which functions as a ubiquitin E3 ligase (Kim & Kaelin, 2003). Subsequent studies have shown that stabilization of HIF-α is due to a disruption of the pVHL/HIF-α interaction under hypoxic conditions. Two laboratories simultaneously reported that O_2-dependent hydroxylation of two conserved proline residues (Pro402 and Pro564 in human HIF-1α) in the ODDD triggers pVHL binding and thus HIF-1α proteasome targeting (Ivan *et al*, 2001; Jaakkola *et al*, 2001). Within months of these initial reports, the PHDs—the enzymes catalysing the hydroxylation reaction—were identified (see Fig 1; Bruick & McKnight, 2001; Epstein *et al*, 2001).

The family of HIF prolyl hydroxylases

Epstein and colleagues identified egg-laying abnormal-9 (EGL-9) as the HIF prolyl hydroxylase in *Caenorhabditis elegans* and they described a family of three human and mouse *phd* genes that are homologous to *egl-9* (Epstein *et al*, 2001). Unfortunately, several acronyms were coined to describe these genes. PHD1, PHD2 and PHD3 (used in the initial paper and in this review) have also been called EGL nine homologue 2 (EGLN2), EGLN1 and EGLN3, or HIF prolyl hydroxylase 3 (HPH3), HPH2 and HPH1, respectively. Each of these homologues has a conserved gene structure, which suggests duplication in the lineage leading to vertebrates (Taylor, 2001).

The PHDs belong to the superfamily of iron- and 2-oxoglutarate-dependent dioxygenases. These enzymes need O_2 as a co-substrate, which provides the molecular basis for their O_2-sensing function. In the hydroxylation reaction, one oxygen atom is added to a peptidyl proline to form hydroxyproline, whereas the other is used in a coupled decarboxylation reaction that converts 2-oxoglutarate to

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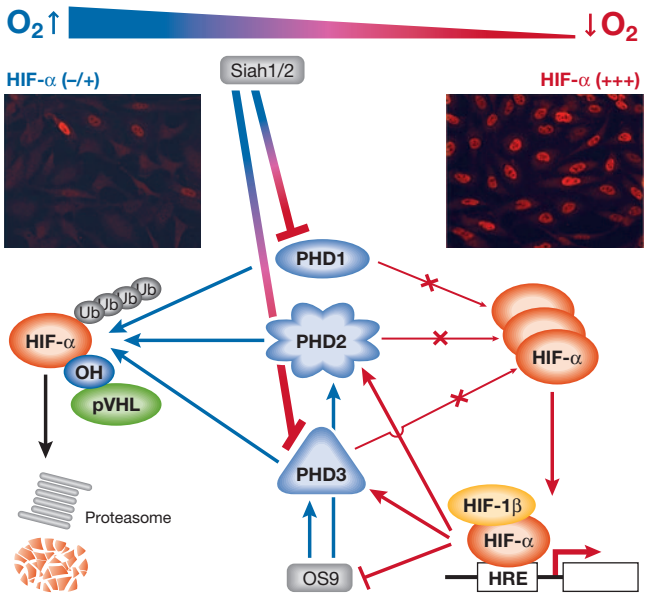

Fig 1 | Prolyl-hydroxylase-domain proteins regulate hypoxia inducible factor-α in response to O_2 availability. Under aerobic conditions (blue arrows), prolyl-hydroxylase- domain proteins (PHDs) hydroxylate hypoxia inducible factor-α (HIF-α), which allows the von Hippel–Lindau protein (pVHL) to bind and thus target HIF-α to the proteasome. Likewise, by binding to PHD2 and PHD3, OS9 promotes HIF-α hydroxylation. A decrease in O_2 availability (red arrows) inhibits the PHDs; HIF-α accumulates and induces the expression of target genes. In addition, Siah 1 and 2 trigger PHD1 and PHD3 degradation under hypoxic conditions. Interestingly, hypoxia controls PHD2, PHD3, OS9 and Siah 1 and 2 expression by a feedback loop mechanism. Immunofluorescence inserts show expression of HIF-1α in HeLa cells at 20% O_2 (left) and 1–2% O_2 (right). HRE, hypoxia response element.

succinate; thus, PHDs also require 2-oxoglutarate as a co-substrate. Likewise, PHDs use Fe^{2+} and ascorbate as co-factors. Fe^{2+} is crucial for activating O_2 and as a template for the orderly binding of reactants. Ascorbate seems to act by reducing Fe^{3+}, which binds to the active site of the enzyme after the decarboxylation reaction, and is therefore necessary for its re-activation (Fig 2).

Substrate specificity. PHDs hydroxylate two proline residues in a conserved LxxLAP sequence motif. *In vitro* studies have assigned their relative activities as PHD2>>PHD3>PHD1 (Huang *et al*, 2002). However, these results are different from those of another report, which showed similar specific activities for PHD2 and PHD3, and a lower activity for PHD1 (Tuckerman *et al*, 2004).

The two HIF-1α prolines are differentially hydroxylated by the PHDs (Epstein *et al*, 2001; Chan *et al*, 2005). All three PHDs can hydroxylate HIF-1α Pro564, but only PHD1 and PHD2 are able to hydroxylate HIF-1α Pro402. Furthermore, the PHD1 and PHD2 K_m values for a Pro402-containing peptide were about 20–50 times higher than those for a Pro564-containing peptide, which suggests important differences between Pro402 and Pro564 (Huang *et al*, 2002).

Despite the *in vitro* analysis, the existence of three highly conserved PHDs in mammalian cells raised important questions as to whether and in what way each PHD contributes to HIF regulation *in vivo*. It is now well established that all three PHD proteins regulate HIF-α (Appelhoff *et al*, 2004). However, the contribution of each PHD is dependent on its relative abundance, which supports the theory that HIF-α hydroxylation is a non-equilibrium reaction. We have shown that PHD2 has a dominant role, as it is the rate-limiting enzyme that sets the low steady-state levels of HIF-1α in normoxia (Berra *et al*, 2003). Carmeliet's group have also shown that the knockdown of *phd2* is lethal in mice, whereas *phd1* or *phd3* knockout mice have no important defects (P. Carmeliet, personal communication). Conversely, we have detected a role for PHD1 and PHD3 in HIF-1α degradation during long-term hypoxic stress (A.G., J.P. and E.B., unpublished data).

Tissue distribution and alternative splicing. PHD1, PHD2 and PHD3 are expressed in all tissues albeit at different levels (Cioffi *et al*, 2003; Lieb *et al*, 2002). PHD2 and PHD3 messenger RNAs are subjected to alternative splicing (Hirsila *et al*, 2003), although no enzyme activity has been detected for any of the alternatively spliced forms. Therefore, changes in the splicing pattern can be expected to influence the production and activity of the enzymes.

Localization. Intracellular localization of the PHDs has been reported by using chimeric proteins fused to the green fluorescent protein (Metzen *et al*, 2003). PHD1 is present exclusively in the nucleus, PHD2 is located mainly in the cytoplasm and PHD3 is distributed homogeneously in the cytoplasm and nucleus. Despite its mostly cytoplasmic localization, PHD2 is able to shuttle between the cytoplasm and the nucleus. Indeed, our data indicate that PHD2 accumulates in the nucleus after 4 h of incubation with leptomycin B, which inhibits nuclear export (A.G., D. Roux, J.P. and E.B., unpublished data). We have previously shown that HIF-1α can be degraded in the nucleus and cytoplasm (Berra *et al*, 2001b); therefore, PHD2 has all the attributes to target HIF-1α degradation in both cellular compartments.

Regulation of PHD expression. PHD expression is regulated in three ways. First, there is hypoxia/HIF-dependent regulation as the expression of PHD2 and PHD3 is transcriptionally induced by hypoxia, which promotes a negative feedback loop (Berra *et al*, 2001a; Berra *et al*, 2003; Epstein *et al*, 2001). Indeed, hypoxic induction of PHDs is HIF-1-dependent (del Peso *et al*, 2003). Silencing of HIF-1α or HIF-2α results in decreased hypoxia-induced PHD3 expression. By contrast, PHD2 is not affected by HIF-2α (Aprelikova *et al*, 2004). Second, there is hypoxia-dependent/HIF-independent regulation. Similar to HIF-α, PHD1 and PHD3 are also degraded by the proteasome (Nakayama *et al*, 2004). Degradation of PHDs by Siah 1 and 2, their specific E3 ligases, is enhanced by hypoxia and Siah 1 and 2 are transcriptionally upregulated during hypoxia in a HIF-1-independent manner. Third, physiological stimuli other than hypoxia can also regulate the expression of PHDs. Indeed, PHD1 mRNA is upregulated by estrogens in the ZR75-1 breast cancer cell line (Seth *et al*, 2002). SM20, the rat orthologue of human PHD3, is induced in response to p53 activation (Madden *et al*, 1996). Nerve growth factor withdrawal also induces SM20 in PC-12 cells, as do platelet-derived growth factor and angiotensin II in smooth muscle cells (Lipscomb *et al*, 2001). Likewise, overexpression of the *Drosophila melanogaster* cyclin-dependent protein kinase complex CycD/Cdk4 increases the

expression of Fatiga (the *Drosophila* homologue of PHD) at the post-transcriptional level (Frei & Edgar, 2004).

Regulation of PHD activity. PHDs are effective O_2 sensors. In keeping with their K_m values for O_2 (230–250 μM), which are slightly above the atmospheric concentration, the activity of PHDs is tightly regulated by the full range of O_2 concentrations from normoxia (21% O_2) to the lowest (<0.1% O_2) hypoxic level (Hirsila *et al*, 2003). Nevertheless, as mentioned above, prolonged hypoxia can unexpectedly enhance PHD activity. Whether PHD accumulation during hypoxia and/or extra mechanisms are responsible for this re-activation is unknown. Whatever the explanation, it is noteworthy that prolonged hypoxia would limit HIF-1 activation, which supports the regulatory feedback loop we proposed previously (Berra *et al*, 2001a).

Although the availability of O_2 serves as a general determinant of PHD activity, several further parameters regulate this activity in a more complex manner to subtly adapt HIF function in a variety of dynamic microenvironments. Depletion of intracellular ascorbate and competitive inhibitors of 2-oxoglutarate lead to PHD inhibition. Similarly, normoxic stabilization of HIF-1α by some oncogenes is mediated by the inhibition of prolyl hydroxylation—such as in Ras^{Val12} and v-Src activation (Chan *et al*, 2002). Mutations in the tumour suppressor succinate dehydrogenase (SDH) have also been linked to PHDs (Selak *et al*, 2005; Lee *et al*, 2005); succinate, which accumulates as a result of SDH mutations, inhibits PHD activity. Thus, succinate transmits an oncogenic signal from mitochondria to the cytosol. Interestingly, an independent study that links mitochondria to PHD activity was reported simultaneously: HIF-1 activity is repressed by mRpL12, the *D. melanogaster* homologue of the mammalian mitochondrial ribosomal protein MRPL12a (Frei *et al*, 2005).

The role of reactive oxygen species (ROS) in the control of HIF-1α stability is also important. This role remains controversial despite the identification of PHDs as the oxygen sensors. This is mainly owing to the fact that hypoxia concomitantly inhibits PHD activity and induces the production of ROS by mitochondria. An interesting explanation for this duality of action arose from the work of Metcha-Gregoriou and colleagues, who showed that the accumulation of ROS in *JunD*$^{-/-}$ cells decreases the availability of Fe^{2+}, which reduces the activity of PHDs (Gerald *et al*, 2004). Therefore, any stress capable of inducing a persistent boost of free radicals should affect the stability of HIF-1α by indirectly inhibiting PHDs. Another source of controversy arose from blocking respiration through the inhibition of mitochondria, which prevents HIF-1α stabilization in moderate hypoxia. One school of thought, which we favour, points to the redistribution of O_2 in the cell, whereas another proposes the abrogation of ROS (for a discussion see Hagen *et al*, 2003; Doege *et al*, 2005; Kaelin, 2005).

In addition, an increasing number of proteins such as OS9 seem to bind to PHDs and to regulate their activity (Baek *et al*, 2005). Several second messengers also modify PHD activity (Appelhoff *et al*, 2004; Hirota *et al*, 2004; Temes *et al*, 2005).

Functions of PHDs. PHDs are pivotal components of the signalling pathways that are elicited to assure O_2 homeostasis. So far, more than 70 HIF target genes have been identified, including genes involved in the development and function of the vascular system, erythropoiesis, energy metabolism, pH regulation, cell proliferation and migration. This represents an elegantly adaptive mechanism for survival, but

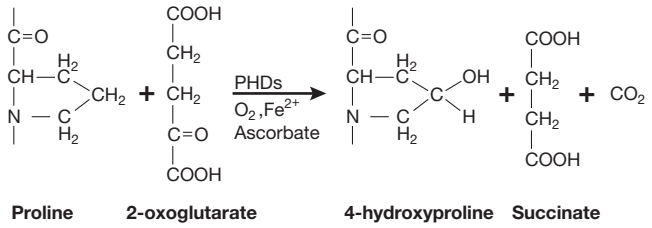

Fig 2 | Catalytic function of the prolyl-hydroxylase-domain proteins. These enzymes need O_2 and 2-oxoglutarate as co-substrates, and Fe^{2+} and ascorbate as co-factors. The hydroxylation reaction forms hydroxyproline and succinate. PHDs, prolyl-hydroxylase-domain proteins.

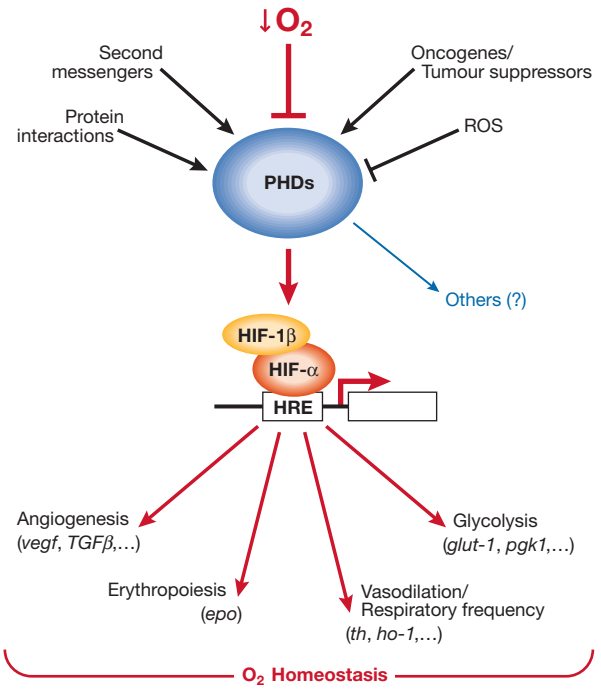

Fig 3 | Schematic model of prolyl-hydroxylase domain regulation and function. Several physiological stimuli, in addition to O_2 availability, regulate prolyl hydroxylase domain (PHD) activity, which promotes fine-tuning adaptation to the microenvironment. PHDs, by modulating hypoxia-inducible factor-α (HIF-α) stability and thus HIF activity, are pivotal in O_2 homeostasis. Nevertheless, PHDs might have a role in other physiological and pathological processes. We still do not know whether PHDs drive these functions through a HIF-dependent mechanism or through hydroxylation of new PHD substrates. HRE, hypoxia response element; ROS, reactive oxygen species.

these target genes are also implicated in the pathogenesis of several serious diseases including myocardial ischaemia, stroke, pulmonary hypertension, pre-eclampsia, and almost every type of cancer. Therefore, PHDs, by modulating HIF-α stability and thus HIF activity, are at the heart of these pathophysiological processes.

Apart from their known function in the cellular response to low O_2 availability, some data suggest that PHDs have a role in several other physiological and pathophysiological processes, such as the control of cell size. Indeed, loss of function in Fatiga suppresses the growth

but not the proliferation function of CycD/Cdk4 in *D. melanogaster* (Frei & Edgar, 2004). Likewise, *C. elegans* lacking *egl-9* show an egg-laying defect and are resistant to an otherwise paralytic *Pseudomona aeruginosa* toxin. Further analysis is necessary to explain definitively whether PHDs drive these functions through a HIF-dependent mechanism or through hydroxylation of new PHD substrates. We have shown the regulatory mechanisms and function of PHDs in Fig 3.

Protein hydroxylation

Besides prolyl hydroxylation, HIF-1α is hydroxylated on an asparagine residue (Asn803)—in its carboxy-terminal transactivation domain—by factor inhibiting HIF (FIH-1; Lando *et al*, 2002). FIH-1, which could be subjected to the same regulatory mechanisms as the PHDs, suppresses HIF transcriptional activity under normoxic conditions by blocking its association with the coactivator p300/CBP.

In addition to HIF-α, prolyl hydroxylation has long been known to be important for collagen biosynthesis through stabilization of the triple-helical conformation of collagen fibres (Uitto & Lichtenstein, 1976). Furthermore, recent reports implicate the LxxLAP motif in the pVHL-dependent ubiquitylation of subunit 1 of RNA polymerase II (Hanson *et al*, 2003), and the hydroxylation of iron regulatory protein 2 (IRP2) in its interaction with the ubiquitin machinery (Kuznetsova *et al*, 2003). With database predictions of new iron- and 2-oxoglutarate-dependent dioxygenases such as AlkB (Welford *et al*, 2003), these data hint that protein hydroxylation extends beyond the HIF system and might be widely involved in cell signalling.

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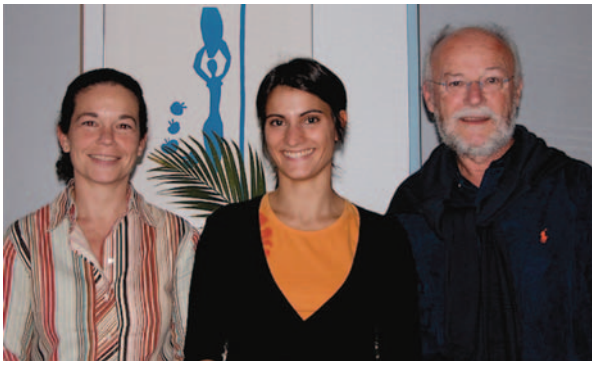

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