HIF prolyl and asparaginyl hydroxylases in the biological response to intracellular O₂ levels

Norma Masson and Peter J. Ratcliffe*

The Henry Wellcome Building of Genomic Medicine, Roosevelt Drive, Oxford OX3 7BN, UK

*Author for correspondence (e-mail: pjr@well.ox.ac.uk)

Summary

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that plays a crucial role in mediating cellular responses to oxygen. Oxygen availability influences multiple steps in HIF activation and recent studies have indicated that at least two steps in this process are governed by a novel mode of signal transduction involving enzymatic hydroxylation of specific amino acid residues in HIF-α subunits by a series of 2-oxoglutarate (2-OG)-dependent oxygenases. These enzymes are non-haem iron enzymes that use dioxygen in the hydroxylation reaction and therefore provide a direct link between the availability of molecular oxygen and regulation of HIF. Prolyl hydroxylation regulates proteolytic destruction of HIF-α by the von Hippel-Lindau ubiquitin ligase complex, whereas HIF-α asparaginyl hydroxylation regulates recruitment of transcriptional coactivators. The involvement of at least two distinct types of 2-OG-dependent oxygenase in oxygen-regulated transcription suggests that these enzymes may be well suited to a role in cellular oxygen sensing.

Key words: Hypoxia inducible factor-α, Prolyl hydroxylation, Asparaginyl hydroxylation, von Hippel-Lindau protein, Ubiquitylation

Introduction

The cellular response to lack of oxygen has attracted particular interest because of its central involvement in medical pathophysiology. Depending on severity, hypoxia alters almost every aspect of cellular physiology, and this complexity presents formidable problems for understanding how the relevant signals are generated and transduced. Nevertheless, studies of one particular response, oxygen-sensitive regulation of the haematopoietic growth factor erythropoietin, have provided considerable insights into these processes through the definition and analysis of a transcription factor termed hypoxia-inducible factor (HIF) (Semenza and Wang, 1992). Unexpectedly, this system proved to be widely operative in mammalian cells (Maxwell et al., 1993) and has many other transcriptional targets. HIF targets include genes involved in angiogenesis, apoptosis, vasomotor control and energy metabolism, as well as erythropoiesis (reviewed by Wenger, 2000). In keeping with this, the system is implicated in the pathophysiology of many human diseases (for reviews, see Brahimi-Horn et al., 2001; Maxwell et al., 2001; Semenza, 2000a).

HIF binds to a core pentanucleotide DNA sequence (RCGTG) in the hypoxia-response elements (HREs) of target genes. The DNA-binding complex is a heterodimer of HIF-α and HIF-β subunits (Wang et al., 1995). HIF-β subunits are constitutive nuclear proteins that have different dimerisation partners in other systems of gene regulation (for a review, see Gu et al., 2000). HIF-α subunits have a specific function in hypoxia-inducible gene regulation and are the targets of the oxygen-sensitive signalling pathway.

As was originally observed in studies of erythropoiesis and erythropoietin, induction of HIF and HIF-target genes by hypoxia is closely mimicked by exposure of cells to cobaltous ions (for a review, see Ebert and Bunn, 1999) and by exposure to specific iron chelators (Wang and Semenza, 1993). Together with the specificity of the response to hypoxia, as opposed to other metabolic stresses, these findings led to the concept of a specific oxygen sensor, and different types of ferroprotein were postulated to perform this function (for reviews, see Bunn and Poyton, 1996; Semenza, 1999). Interestingly, HIF is also activated by growth factors, oncogenes and tumour suppressor mutations that promote cell survival or proliferation, thus effecting a potential link between the growth of metabolizing tissues and the provision of an oxygen supply (reviewed by Maxwell et al., 1999).

In keeping with the complexity of this task, HIF-α exists as multiple isoforms with different biological properties. Three principal isoforms (HIF-1α, HIF-2α and HIF-3α) are encoded by distinct genetic loci, further diversity being generated by alternative promoter usage and splicing patterns (Makino et al., 2002; Wenger, 2002). Assembly of an active HIF complex is a multi-step process involving regulated synthesis, processing and stabilization of HIF-α, nuclear localization, dimerisation and interaction with transcriptional coactivators (for reviews, see Semenza, 2000b; Wenger, 2002). To date, analyses of the regulatory mechanisms underlying HIF activation by hypoxic and non-hypoxic stimuli have emphasized the involvement of different types of pathway and different modes of HIF activation. Proteolysis of HIF-α subunits is strikingly oxygen dependent (Huang et al., 1996; Huang et al., 1998; Pugh et al., 1997; Sutter et al., 2000). In contrast, the rate of HIF-1α translation appears largely independent of oxygen (Gorlach et al., 2000) but is responsive to growth factor and oncogenic stimulation (Karni et al., 2002; Laughner et al., 2001; Treins...
Oxygen-dependent proteolysis of HIF-α

Prolyl hydroxylation and proteolytic capture of HIF-α by the von Hippel-Lindau ubiquitin ligase complex

Under normoxic conditions, HIF-1α subunits have an exceptionally short half-life and steady-state levels are very low (Jewell et al., 2001), whereas increasing severity of hypoxia retards degradation of HIF-1α subunits in a graded manner (Jiang et al., 1996). This proteolytic regulation is mediated by a central oxygen-dependent degradation domain (ODD) that contains N- and C-terminal portions (N-ODD and C-ODD) that can operate independently (Ema et al., 1999; Huang et al., 1998; O’Rourke et al., 1999; Pugh et al., 1997). Proteolytic regulation of HIF-1α and HIF-2α is critically dependent on the von Hippel-Lindau tumour suppressor protein (pVHL), with pVHL functioning as the substrate recognition component of a multi-component ubiquitin E3 ligase (Maxwell et al., 1999; Cockman et al., 2000; Ohh et al., 2000) (Fig. 1).

Recognition that the HIF-α–pVHL protein interaction is suppressed by the classical HIF-activating stimuli of cobaltous ions, iron chelation and hypoxia led to a detailed biochemical analysis of this interaction. These studies demonstrated the existence of two interaction sites for pVHL within the HIF-α ODD, corresponding to the NODD and CODD subdomains, and showed that interaction is regulated by enzymatic hydroxylation at specific prolyl residues (for human HIF-1α, at Pro 402 in the NODD and at Pro 564 in the CODD) (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Since the only previously characterized mammalian prolyl hydroxylases (pro-collagen prolyl hydroxylases) were 2-oxoglutarate-dependent oxygenases (reviewed by Kivirikko and Myllyharju, 1998), it was predicted that the HIF prolyl hydroxylases would also belong to this family of enzymes. Based on conserved structural features (a β-barrel jelly-roll conformation that aligns a 2-histidine-1-carboxylate Fe (II) coordination motif at the catalytic site) (Schofield and Zhang, 1999; Valegard et al., 1998), a candidate approach was used to define the HIF-modifying enzymes. This identified the HIF prolyl hydroxylases as the products of genes related to C.elegans egl-9, a gene that was first described in the context of an egg-laying abnormal (EGL) phenotype (Bruck and McKnight, 2001; Epstein et al., 2001).

In mammalian cells, three isoforms were identified, termed prolyl hydroxylase domain (PHD) enzymes (PHD1-PHD3), and shown to hydroxylate HIF-α in vitro (Bruck and McKnight, 2001; Epstein et al., 2001). These enzymes have an absolute requirement for dioxygen as co-substrate. The overall reaction results in insertion of one oxygen atom into the HIF-α peptide substrate at the prolyl residue, the other generating succinate from 2-OG with the release of CO2. Relatively labile

---

**Fig. 1.** Two independent hydroxylation pathways regulate HIF activity in response to cellular oxygen level. In normoxia, oxygen availability enables PHD-dependent prolyl hydroxylation of the HIF-α ODD. This prolyl hydroxylation allows binding of the VHL E3 ligase leading to ubiquitylation and degradation of HIF-α subunits. Oxygen availability also enables FIH-dependent asparaginyl hydroxylation of the C-TAD, blocking interaction with the p300/CBP co-activator. In hypoxia, the PHD and FIH enzymes are inactive and the lack of hydroxylation results in stable HIF-α able to form a DNA-binding heterodimer with HIF-β and recruit p300/CBP at the C-TAD.
binding of Fe(II) at the 2-his-1-carboxylate centre results in striking sensitivity to inhibition by iron chelators and metals such as Co(II) that can exchange for Fe(II) at this site. As predicted, the activity of the recombinant PHD enzymes is strongly inhibited by cobaltous ions, and iron chelation. Furthermore, reactions conducted in a controlled oxygen environment showed that the activity of the purified enzyme is strikingly sensitive to graded levels of hypoxia in vitro (Epstein et al., 2001). Thus the properties of these non-haem iron enzymes fit those of the postulated ferroprotein sensor underlying the classic characteristics of the erythropoietin/HIF response.

Functional studies of PHD enzymes

In C. elegans the hif-1 gene product is completely stabilized by inactivating mutations in egl-9, providing genetic evidence for the critical function of EGL-9 in the HIF response (Epstein et al., 2001). Similarly, in Drosophila melanogaster, abrogation of a single PHD homologue, termed fatiga, by RNAi or chromosomal deletion leads to striking upregulation of the HIF-1α homologue Similar (Bruck and McKnight, 2001; Lavista-Llanos et al., 2002). Interestingly, among the mammalian enzymes PHD2, but not PHD1 and PHD3, contains an N-terminal zinc finger ‘MYND’ putative protein-interaction domain that is distinct from the catalytic domain and conserved in EGL-9, which suggests that PHD2 is most closely related to the C. elegans gene product (Taylor, 2001). When over-expressed in culture cells each of the mammalian PHD enzymes has the capacity to reduce the HIF transcriptional response in modest hypoxia, presumably by compensating for oxygen-limited hydroxylation (Bruck and McKnight, 2001). However, further studies are required to define the relative importance of the PHD enzymes in the physiological regulation of HIF.

Pharmacological inactivation of the PHDs by 2-OG analogues is efficient by stabilizing HIF-α (Ivan et al., 2002; Jaakkola et al., 2001), but this action is nonspecific with respect to the PHD isoforms. In vitro studies do suggest significant differences in substrate specificity. For instance, PHD3 does not appear to hydroxylate the NODD site in HIF-1α (Epstein et al., 2001), and comparison of enzyme activity in vitro showed that the C0DD sequence is hydroxylated most efficiently by PHD2 (Huang et al., 2002). Interestingly, biochemical purification from rabbit reticulocyte extract identified PHD2 but not the other enzymes, using hydroxylation of the human HIF-1α C0DD as the activity assay (Ivan et al., 2002).

The three enzymes have different tissue distributions (Lieb et al., 2002; Oehme et al., 2002) and, at least under conditions of over-expression, have distinct patterns of sub-cellular localisation (Huang et al., 2002; Metzen et al., 2003). PHD2 mRNA is widely expressed, but is particularly abundant in adipose tissue (Oehme et al., 2002). PHD3 mRNA is also expressed in many tissues, but is most abundant in the heart and placenta (Lieb et al., 2002; Oehme et al., 2002). Likewise, PHD1 mRNA is expressed in many tissues but expression is much increased in the testis (Lieb et al., 2002).

The HIF-α ODD sequences are quite distinct from the typical Pro-Pro-Gly repeats that are targeted by pro-collagen prolyl hydroxylases. Sequence alignment of mammalian HIF-

1α and HIF-2α, at both the NODD and CODD regions, and the Caenorhabditis elegans HIF-1 ODD, reveals a conserved LxxLAP motif (Masson et al., 2001). However, the functional basis for this conservation remains unclear. Analyses of individual mutations at Leu559, Leu562 and Ala563 in the context of the human HIF-1α CODD indicate that all are tolerated at least to some degree in assays of hydroxylation by each PHD enzyme, suggesting that, with the exception of the target prolyl residue, the substrate-recognition determinants are relatively non-stringent (Huang et al., 2002).

Interaction of hydroxylated HIF-α with VHL E3

The mechanism by which insertion of a single oxygen atom into proline governs recognition by pVHL has been analysed in crystallographic studies of the HIF-α-pVHL interaction. The hydroxylated HIF-1α CODD peptide binds the β-domain of pVHL in an extended conformation, making contact at two distinct sites: HIF-1α residues 560-567 containing hydroxyproline (Hyp) residue 564 (site 1), and residues 571-577 (site 2) (Hon et al., 2002; Min et al., 2002). At site 1 the Hyp residue is buried within a pocket in pVHL, the oxygen atom hydrogen bonding with pVHL residues Ser111 and His115 in the floor of the pocket. Although proline would fit into this pocket, it would not permit the hydrogen bonding and would exclude a water molecule that hydrogen bonds to these residues in the unliganded structure. This mechanism is central to the specificity of pVHL for hydroxylated HIF-α. In keeping with this, solution binding assays using peptides of varying length confirm the importance of site 1; site 2 providing only a modest increase in the binding affinity (Hon et al., 2002; Min et al., 2002). Kinetic and competition studies indicate that the ODD binds to the same site with similar affinity (Hon et al., 2002), although more efficient binding of the ODD to the VHL-E3 complex retrieved from cultured cells versus pVHL produced by programmed reticulocyte lysate suggests that there are significant differences between ODD and CODD binding to pVHL that are not yet understood (Masson et al., 2001).

Although the physiological role of pVHL as a critical component of the hypoxia response is clear, the role of HIF dysregulation in the tumour predisposition associated with pVHL inactivation is less clear (Kaelin, 2002). It is therefore of interest that all of the five pVHL residues lining the Hyp-binding pocket are sites for tumour-associated missense mutations (Beroud et al., 1998). This strongly suggests that loss of capture either of HIF-α or of another hydroxylated pVHL substrate directly contributes to the oncogenic process. In keeping with this, overexpression of a peptide containing the hydroxylation site from the HIF-1α CODD at a level sufficient to overwhelm hydroxyproline substrate recognition by pVHL was found to block pVHL tumour suppressor function (Maranchie et al., 2002). In contrast, the same authors found that expression of a transcriptionally active HIF-1α polypeptide, which is stabilized by mutations within the CODD that prevent recognition by pVHL, does not block tumour suppression (Maranchie et al., 2002). This suggests that recognition of a hydroxylated substrate other than HIF-1α is important for pVHL tumour suppressor function. Using a similar strategy, other workers found that stabilized HIF-2α does prevent pVHL tumour suppression (Kondo et al., 2002).
Taken together, these findings suggest that pVHL tumour suppression is dependent on its ability to capture hydroxylated substrates that include, but are not necessarily confined to, HIF-2α polypeptides. In this regard, the recent identification of the RNA polymerase II large subunit as a prolyl-hydroxylated substrate of pVHL is of interest (Kuznetsova et al., 2003).

Function of the intact HIF-α ODD

In addition to the minimal NODD and CODD, other sequences have been implicated in proteolytic regulation either because their inclusion amplifies regulation manifest by the minimal domain or because their deletion or mutation impairs proteolytic regulation of the native HIF-α polypeptide (Huang et al., 1998). Such sequences could affect conformation so as to optimise the presentation of the hydroxylase-recognition or pVHL-binding sites. Alternatively, they may represent points of interaction with other pathways that regulate HIF-α. In this context it is of interest that at least two other post-translation modifications of the HIF-α ODD are known to occur. First, the HIF-1α ODD is heavily phosphorylated, although the functional significance of this remains unclear. For instance, in vitro assays of VHL E3 binding and ubiquitylation at the NODD, phosphorylation-dependent shifts in NODD mobility can be observed but do not appear to affect its properties as a hydroxylase–VHL-E3 substrate (Masson et al., 2001). Nevertheless, mutational studies at other sites within the ODD have implicated certain phosphoacceptor residues as functionally important. One study examined a HIF-1α polypeptide that was disabled at the NODD, and showed that, in this context, phosphoacceptor mutations near the CODD (S551G/T552A) have a stabilizing effect in normoxic cells that is not observed in the case of the phosphomimetic mutations (S551D/T552D) (Sutter et al., 2000). Although this site is outside the minimal functional CODD, it is possible that, in the native molecule, phosphorylation alters the characteristics of hydroxylase–VHL-E3 interaction so as to enhance degradation. Nevertheless, to date, the sites of phosphorylation within the ODD have not been precisely mapped, and it is unclear whether these particular residues are in fact phosphorylated.

Recently, further insights into the function of the ODD have been provided by the recognition of ARD-1 as an ODD-interacting acetyl transferase that acetylates Lys 532 (Jeong et al., 2002). This process apparently promotes interaction of the CODD with VHL, increasing the efficiency of ubiquitylation and degradation, although at present it is not clear whether the effect is to enhance hydroxylation or VHL capture of hydroxylated HIF CODD. ARD-1 mRNA is downregulated by hypoxia, and it has been proposed that resulting downregulation of ODD acetylation in hypoxia inhibits VHL-mediated proteolysis (Jeong et al., 2002). Interestingly, it has also been demonstrated that hypoxia upregulates histone deacetylase mRNAs (Kim et al., 2001). However, it is not yet clear whether oxygen regulation of acetylation pathways represents a distinct interface with oxygen, or whether these pathways connect back in some way to the HIF transcriptional cascade.

Other recent studies have demonstrated the potential for HIF-α carboxylate residues surrounding the CODD to bind metal ions such as Co(II). Although in vitro studies have indicated that binding of cobalt to this site could reduce capture of hydroxylated HIF by pVHL, potentially providing a further explanation for stabilization of HIF-α by cobalt, whether this occurs in vivo, and how it fits with the activity of cobalt on other regulatory domains of HIF-α is unclear (Yuan et al., 2003). Indeed overexpression of PHD3 in vivo appears sufficient to ablate Co(II)-induced stabilization of HIF-1α, suggesting that the dominant mode of action of cobalt in vivo is through inhibition of hydroxylase activity (Cioffi et al., 2003).

Oxygen-dependent transcriptional activation of HIF-α

HIF-1α and HIF-2α contain two transactivation domains (TADs): an N-terminal TAD overlapping the ODD; and a C-terminal TAD that is distinct from the ODD (Jiang et al., 1997; O’Rourke et al., 1999; Pugh et al., 1997) and manifests oxygen-regulated interaction with p300/CBP (Bhattacharya et al., 1999; Ema et al., 1999) (Fig. 1).

Characterization of isolated C-TAD activity showed that, as with the ODD, the action of hypoxia can be mimicked by cobaltalous ions, iron chelators, and the inhibitory 2-OG analogue dimethyl oxalylglycine, indicating the operation of an independent but closely similar oxygen-sensing signal (Pugh et al., 1997; Sang et al., 2002). Mass spectrometric analysis of a HIF-2α C-TAD polypeptide, which was overexpressed and purified from 293T cells, again revealed an oxidative modification, in this case hydroxylation of an asparaginyl residue (Asn851 in human HIF-2α, corresponding to Asn803 in human HIF-1α) (Lando et al., 2002b). The relevant asparaginyl hydroxylase was rapidly demonstrated to be a molecule termed factor inhibiting HIF (FIH) (Hewitson et al., 2002; Lando et al., 2002a) that had first been identified as a protein that can interact with the HIF-α C-TAD and suppress transcription (Mahon et al., 2001). Studies of recombinant protein have established that FIH can hydroxylate the HIF-α C-TAD directly, that the site of hydroxylation is the β-carbon of the asparaginyl residue (McNeill et al., 2002), and that this modification prevents interaction of the HIF-α CAD with the CH-1 domain of the coactivator p300 (Hewitson et al., 2002; Lando et al., 2002a).

It has also been reported that FIH interacts with pVHL and forms a ternary complex with the HIF-α C-TAD (Mahon et al., 2001). Although interaction with pVHL is not an absolute requirement for FIH activity (Hewitson et al., 2002; Sang et al., 2002), these findings fit well with studies of HIF-dependent transcription in pVHL-defective cells. Upregulation of HIF-target gene expression in such cells is essentially complete (Gnarra et al., 1996; Iliopoulos et al., 1996; Maxwell et al., 1999), which implies that all oxygen-dependent controls of HIF, and not just proteolytic regulation, are disabled in the absence of VHL. This suggests that VHL is intimately involved with the hypoxia response, its functions extending beyond those of an E3 ligase.

Structural analyses of FIH and the C-TAD–CH-1 interaction

NMR studies indicate that the non-hydroxylated HIF-1α C-
TAD is disordered in solution but becomes structured on binding to CH-1; HIF-α C-TAD forming α-helices that interact with each side of CH-1 (Dames et al., 2002; Freedman et al., 2002). Asn803 forms part of the helical conformation, and is buried in the interface between the two proteins. Thus it is predicted that β-hydroxylation at this site would destabilise the helix and place the hydroxyl group in an energetically unfavourable hydrophobic environment with no hydrogen-bonding partner, and thus effectively disrupt the CH-1 interaction.

For FIH itself, recently solved crystallographic structures show a dimeric structure that conforms to the predicted β-barrel jelly-roll conformation (Bae et al., 2002; Dann et al., 2002; Hewitson et al., 2002). However, structures of FIH complexed with substrates and inhibitors reveal a number of unusual features (Hewitson et al., 2002). The 2-OG 5-carboxylate binding site differs from the characteristic Arg, Ser/Thr site on the eighth strand of the jelly-roll employed by most previously characterized 2-OG dioxygenases and involves hydrogen bonding to Lys214 (on the fourth strand of the jelly-roll), Thr196, and Tyr145 (Dann et al., 2002; Elkins et al., 2003). Co-crystallisation with HIF-α C-TAD peptides reveals a two-site interaction, changes in the FIH structure upon binding indicating an induced fit (Elkins et al., 2003). The HIF-α C-TAD hydroxylation site itself adopts a largely extended conformation that includes a tight turn, stabilised by hydrogen bonding between the backbone carbonyl of HIF-1α Val802 and NH of Ala804, that projects the side chain of the target Asn 803 towards the active site Fe(II) (Elkins et al., 2003). These unusual structural features of FIH at both the HIF-α C-TAD and 2-OG binding sites may assist design of selective inhibitors. The structure of the FIH–C-TAD complex also suggests a potential interaction with protein phosphorylation. It has been proposed that phosphorylation at a conserved threonine residue (Thr796 in human HIF-1α) promotes transactivation by directly facilitating interaction with p300/CBP (Gradin et al., 2002). The relationship of this residue to the active site of the HIF-α CAD–FIH complex suggests that phosphorylation might also inhibit HIF-α asparaginyl hydroxylation.

Oxygen-dependent subcellular localization of HIF-α
A number of pieces of evidence point to a further mode of HIF regulation involving oxygen-dependent nuclear/cyttoplasmic localization of HIF-α (Kallio et al., 1998). In normoxia, HIF-α levels are low and subcellular localization cannot be directly observed. However, overexpression of HIF-α or HIF-α-GFP fusion proteins that are less rapidly degraded yields detectable protein in normoxic cells and several (Kallio et al., 1998; Kallio et al., 1999), although not all (Hofer et al., 2001), studies have observed that this protein shifts to the nucleus only in hypoxia, implying a distinct oxygen-regulated step. Interestingly, as with the other modes of HIF regulation, the effect of hypoxia is mimicked by iron chelators, which suggests that subcellular localization is also regulated by the same or similar Fe(II)- and 2-OG-dependent oxygenases (Kallio et al., 1998). Indeed, an independently regulated nuclear localization step has also been observed for the Drosophila HIF-α homologue Similar, where the process is governed by the prolyl hydroxylase homologue Fatiga (Lavista-Llanos et al., 2002). In the mammalian system all three HIF-α subunits contain an unusual bipartite nuclear localization signals in the C-terminal portion of the molecule (Luo and Shibuya, 2001). However, in isolation this signal mediates constitutive nuclear import irrespective of oxygen, and current evidence suggests that oxygen-regulated localization of HIF-α involves active nuclear export or cytoplasmic retention in oxygenated cells that is dependent on other sequences within the HIF-α polypeptide that remain to be defined (Groulx and Lee, 2002; Kallio et al., 1998; Lavista-Llanos et al., 2002; Luo and Shibuya, 2001).

Oxygen-dependent splicing of HIF-α
Alternative splicing at the HIF-3α locus generates the inhibitory protein IPAS that inhibits HIF responses by forming non-productive heterodimers with HIF-1α (Makino et al., 2001). It has recently been reported that, in mice exposed to severe hypoxia, activation of this alternative splicing process upregulates IPAS transcripts in certain organs, particularly heart and lung, providing evidence for another step in the HIF pathway that may be controlled by protein hydroxylation (Makino et al., 2001; Makino et al., 2002).

Perspectives
The common involvement of prolyl and asparaginyl hydroxylation by distinct Fe(II)- and 2-OG-dependent oxygenases in different modes of HIF regulation (Fig. 1) suggests that such enzymes may be well suited to a role in cellular oxygen sensing. Clearly the direct incorporation of molecular oxygen at the site of hydroxylation allows a direct interface with the intracellular availability of dioxygen. Furthermore, the requirement for multiple co-factors and co-substrates may contribute to the regulatory potential of these molecules (Fig. 2). Kinetic studies of 2-OG-dependent oxygenases have indicated that binding occurs in the order Fe (II), 2-OG, prime substrate, and then dioxygen (Zhang et al., 2000; Zhou et al., 1998) (Fig. 3). Oxidation of the prime substrate is believed to occur through the generation of a highly reactive ferryl species, and an important feature of the process is that binding of prime substrate precedes that of dioxygen, thus limiting the risk of non-productive and potentially damaging radical formation (reviewed by Prescott and Lloyd, 2000).

Some enzymes of this class also require the presence of ascorbate for full catalytic activity, and the PHD enzymes may have a similar requirement. Although the mechanism is incompletely understood, one action of ascorbate on procollagen prolyl hydroxylase is to reconstitute active enzyme following oxidation of the iron in uncoupled cycles in which 2-OG is decarboxylated without subsequent hydroxylation of the prime substrate; ascorbate may also act to augment an intracellular Fe(II) pool that can replenish the active site Fe(II) (for reviews, see Kivirikko and Myllyharju, 1998; Prescott and Lloyd, 2000). Whether limiting availability of iron or ascorbate contributes to the physiological regulation of HIF is unclear. However, using antisera that recognise the hydroxylated HIF-1α CODD specifically, it has been shown that under conditions of oncogenic stimulation of HIF, hydroxylation at this site is often not complete even in fully oxygenated cell cultures (Chan
the other oxidises 2-oxoglutarate forming succinate and CO₂.

Following binding of dioxygen, one oxygen atom is incorporated into the hydroxylated HIF residue and the other oxidises 2-oxoglutarate forming succinate and CO₂. As HIF hydroxylation is not an equilibrium reaction, the extent of modification at a given oxygen concentration will also be affected by the quantity of available enzyme. Since the PHD enzymes are the products of genes that are strongly inducible and expressed in a tissue-specific manner, this is likely to have an important effect in shaping cellular responses to hypoxia. For instance, both PHD2 and PHD3 mRNAs are strongly induced by hypoxia itself (Epstein et al., 2001). In keeping with this, prior exposure of cells to hypoxia enhances the HIF prolyl hydroxylase activity found in cell extracts, and the rate of HIF-α degradation following a return to normoxia (Berra et al., 2001). Interestingly, PHD1 mRNA has also been reported to be an oestrogen-inducible transcript (Seth et al., 2002), whereas PHD3 has previously been identified in different cell environments and HIF hydroxylase pathways that have the potential to provide precisely tuned physiological responses to hypoxia.

Fig. 2. HIF-dependent responses to O₂ may be modulated by the cellular environment. Multiple points of interaction exist between the cellular environment and HIF hydroxylase pathways that have the potential to provide precisely tuned physiological responses to hypoxia.

Fig. 3. Proposed order of binding of co-substrates and prime substrate by the HIF hydroxylases. Following binding of dioxygen, one oxygen atom is incorporated into the hydroxylated HIF residue and the other oxidises 2-oxoglutarate forming succinate and CO₂.
types as a gene that is induced by p53 (Madden et al., 1996), by stimuli inducing smooth muscle differentiation (Wax et al., 1994) and by nerve growth factor withdrawal (Lipscomb et al., 2001). In future work it will be important to determine whether and in what way these effects on hydroxylase expression patterns affect cellular responses to hypoxia.

Delineation of these HIF-hydroxylation pathways provides new targets for therapeutic intervention. There is increasing evidence that activation of HIF is protective in ischemic/hypoxic disease, and can generate a productive angiogenic response (Elson et al., 2001; Vincent et al., 2000). Direct induction of HIF-1 in vivo has been achieved both by use of NODD and CODD polypeptides that block VHL-mediated degradation (Marancie et al., 2002; Willam et al., 2002) and by 2-OG analogues that inhibit the HIF hydroxylases (Ivan et al., 2002; Jaakkola et al., 2001). Given the extent of the 2-OG superfamily, it is likely that particular attention to the design of specific inhibitors will be required. Nevertheless, emerging structural information defining unusual and specific features of particular hydroxylases suggests that this should be possible.

Finally, it will be of particular interest to determine whether protein hydroxylation has a broad role in signalling responses to hypoxia. Recent evidence suggests that both HIF-3α and the large subunit (Rpb1) of RNA polymerase II are also targeted for VHL-dependent ubiquitylation via prolyl hydroxylation (Kuznetsova et al., 2003; Maynard et al., 2003). Previous studies of PHD1 and PHD3 have implicated these gene products in the regulation of a variety of cellular growth, differentiation and apoptotic pathways, and it is possible that prolyl hydroxylation of non-HIF-α substrates is involved in such responses (Érez et al., 2002; Lipscomb et al., 2001; Madden et al., 1996; Seth et al., 2002; Wax et al., 1994). In addition, more effective database searching enabled by new structural insights suggests the existence of a much larger 2-OG oxygenase family than has been previously recognised (Elkins et al., 2003), raising the possibility that other members of this family function in oxygen-regulated pathways that involve protein hydroxylation.

References

Bae, M.-Y., Ahn, M.-Y., Jeong, J.-W., Bae, M.-H., Lee, Y. M., Bae, S.-Y., Park, J.-W., Kim, K.-R. and Kim, K.-W. (2002). Jabi interacts directly with HIF-1α and regulates its stability. J. Biol. Chem., 277, 9-12.

Beroud, C., Joly, D., Gallou, C., Staroz, F., Orfanelli, M. T. and Junien, C. (1998). Software and database for the analysis of mutations in the VHL gene. Nucleic Acids Res. 26, 256-258.

Berra, E., Richard, D. E., Gothe, E. and Pouyssegur, J. (2001). HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1α degradation. FEBS Lett. 491, 85-90.

Bhattacharya, S., Michels, C. L., Leung, M-K., Arany, Z. P., Kung, A. L. and Livingston, D. M. (1999). Functional role of p35srj, a novel p300/CBP transactivational function of the HLF and HIF1alp in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. EMBO J. 18, 1905-1914.

Epstein, A. C. R., Gleadle, J. M., McNeill, L. A., Hewison, K. S., O’Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. L., Dhandha, A. et al. (2001). C. elegans EGL-9 and mammalian homologues define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell 107, 43-54.

Érez, N., Miliavsky, M., Goldfinger, N., Peles, E., Gudkov, A. V. and Rotter, V. (2002). Falkor, a novel cell growth regulator isolated by a functional genetic screen. Oncogene 21, 6713-6721.

Freedman, S. J., Sun, Z.-Y., Poy, E., Kung, A. L., Livingston, D. M., Wagner, G. and Eck, M. J. (2002). Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1α. Proc. Natl. Acad. Sci. USA 99, 5367-5372.

Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J. R., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D. and Linehan, W. M. (1996). Post-transcriptional regulation of vascular endothelial growth factor mRNA by the product of the VHL tumor suppressor gene. Proc. Natl. Acad. Sci. USA 93, 10589-10594.

Gorlach, A., Camenisch, G., Kvetikova, I., Vogt, L., Wenger, R. H. and Gassmann, M. (2000). Efficient translation of mouse hypoxia-inducible factor-1α under normoxic and hypoxic conditions. Biochim. Biophys. Acta 1493, 125-134.

Gradin, K., Takasaki, C., Fujii-Kuriyama, Y. and Sogawa, K. (2002). The transcriptional activation function of the HIF-like factor requires phosphorylation at a conserved threonine. J. Biol. Chem. 277, 23508-23514.

Groulx, I. and Lee, S. (2002). Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor requires nuclear-cytoplasmic trafficking of the von Hippel-Lindau tumor suppressor protein. Mol. Cell. Biol. 22, 5319-5336.

Gu, Y.-Z., Hogenesch, J. B. and Bradfield, C. A. (2000). The PAS superfamily: sensors of environmental and developmental signals. Annu. Rev. Pharmacol. Toxicol. 40, 519-561.

Hewison, K. S., McNeill, L. A., Riordan, M. V., Tian, Y.-M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M. et al. (2002). Hypoxia inducible factor (HIF) asparagine hydroxylase is identical to Factor Inhibiting HIF (FIH) and is related to the cupin structural family. J. Biol. Chem. 277, 26351-26355.

Huot, T., Desbulllets, I., Hopfi, G., Gassmann, M. and Wenger, R. H. (2001). Dissociating hypoxia-dependent and hypoxia-independent steps in the HIF-1α activation cascade: implications for HIF-1α gene therapy. FASEB J. 15, 2715-2717.

Hon, W. C., Wilson, M. L., Harlos, K., Claridge, T. D., Schofield, C. J., Pugh, C. W., Maxwell, P. H., Ratcliffe, P. J., Stuart, D. I. and Jones, E.
Jiang, B.-H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996). Activation of hypoxia-inducible transcription factor depends primarily on reox-sensitive stabilization of its alpha subunit. J. Biol. Chem. 271, 32253-32259.

Huang, J. E., Gu, J., Schau, M. and Bunn, H. F. (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an oxygen-dependent domain via the ubiquitin-proteasome pathway. Proc. Natl. Acad. Sci. USA 95, 7987-7992.

Huang, J., Zhao, Q., Mooney, S. M. and Lee, F. S. (2002). Sequence determinants in hypoxia inducible factor-1alpha for hydroxylation by the prolyl hydroxylases PHD1, PHD2 and PHD3. J. Biol. Chem. 277, 39792-39800.

Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (2002). Molecular Basis of the VHL Hereditary Cancer Syndrome. Nature Rev. Cancer 2, 673-682.

Kallio, P. J., Okamoto, K., O’Brien, S., Carrero, P., Makino, Y., Tanaka, H., Min, J.-H., Yang, H., Ivan, M., Gertler, F., Kaelin, W. G. J. and Pavletich, N. P. (2002). Inhibition of HIF-1alpha in response to hypoxia is instantaneous. FASEB J. 15, 1312-1314.

Jiang, B.-H., Semenza, G. L., Bauer, C. and Marti, H. H. (1996). Hypoxia-inducible factor 1 alpha levels vary exponentially over a physiologically relevant instantaneous.

Ivan, M., Kondo, K., Yang, H., Kim, W., Vailando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G. J. (2001). HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for oxygen sensing. Science 292, 464-469.

Ivan, M., Haberberger, T., Gervasi, D. C., Michelson, K. S., Gunzler, V., Kondo, K., Yang, H., Sorokin, I., Conaway, R. C., Conaway, J. W. et al. (2002). Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor. Proc. Natl. Acad. Sci. USA 99, 13459-13464.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jeon, J.-G., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.

Kallio, P. J., Wilson, W. J., Bae, M.-K., Ahn, M.-Y., Kim, S.-H., Sohn, T.-K., Bae, M.-H., Yoo, M.-A., Song, E. J., Lee, K.-J. and Kim, K.-W. (2002). Regulation and destabilization of HIF-1alpha by ARD1-mediated acetylation. Cell 111, 709-720.

Jewell, U. R., Kvitikova, I., Scheid, A., Bauer, C., Wenger, R. H. and Kuznetsova, A. V. (2001). HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression. Mol. Cell. Biol. 21, 3995-4004.
Oxygen-regulated HIF hydroxylase pathways

Valegard, K., van Scheltinga, A. C. T., Lloyd, M. D., Hara, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H. J., Baldwin, J. E., Schofield, C. J. et al. (1998). Structure of a cephalosporin synthase. Nature 394, 805-809.

Vincent, K. A., Shyu, K. G., Luo, Y., Magner, M., Tio, R. A., Jiang, C., Goldberg, M. A., Akita, G. Y., Gregory, R. J. and Isner, J. M. (2000). Angiogenesis is induced in a rabbit model of hindlimb ischemia by naked DNA encoding an HIF-1 alpha/VP16 hybrid transcription factor. Circulation 102, 2255-2261.

Wang, G. L. and Semenza, G. L. (1993). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. Blood 82, 3610-3615.

Wang, G. L., Jiang, B.-H., Rue, E. A. and Semenza, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc. Natl. Acad. Sci. USA 92, 5510-5514.

Wax, S. D., Rosenfield, C. L. and Taubman, M. B. (1994). Identification of a novel growth factor-responsive gene in vascular smooth muscle cells. J. Biol. Chem. 269, 13041-13047.

Wenger, R. H. (2000). Mammalian oxygen sensing, signalling and gene regulation. J. Exp. Biol. 20, 1253-1263.

Wenger, R. H. (2002). Cellular adaptation to hypoxia: O2-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O2-regulated gene expression. FASEB J. 16, 1151-1162.

Willam, C., Masson, N., Tian, Y. M., Mahmood, S. A., Wilson, M. I., Bicknell, R., Eckardt, K. U., Maxwell, P. H., Ratcliffe, P. J. and Pugh, C. W. (2002). Peptide blockade of HIFalpha degradation modulates cellular metabolism and angiogenesis. Proc. Natl. Acad. Sci. USA 99, 10423-10428.

Yu, F., White, S. B., Zhao, Q. and Lee, F. S. (2001). HIF-1 alpha binding to VHL is regulated by stimulus-sensitive proline hydroxylation. Proc. Natl. Acad. Sci. USA 98, 9630-9635.

Yuan, Y., Hilliard, G., Ferguson, T. and Millhorn, D. E. (2003). Cobalt inhibits the interaction between hypoxia inducible factor-alpha and von Hippel-Lindau protein by direct binding to hypoxia inducible factor-alpha. J. Biol. Chem. 278, 15911-15916.

Zhang, Z. H., Ren, J. S., Stammers, D. K., Baldwin, J. E., Harlos, K. and Schofield, C. J. (2000). Structural origins of the selectivity of the trifunctional oxygenase clavaminic acid synthase. Nat. Struct. Biol. 7, 127-133.

Zhou, J., Gunisor, M., Bachmann, B. O., Townsend, C. A. and Solomon, E. L. (1998). Substrate binding to the alpha-ketoglutarate-dependent non-heme iron enzyme clavaminic synthase 2: coupling mechanism of oxidative decarboxylation and hydroxylation. J. Am. Chem. Soc. 120, 13539-13540.

Zundel, W., Schindler, C., Haas-Kogan, D., Koong, A., Kaper, F., Chen, E., Gottschalk, A. R., Ryan, H. E., Johnson, R. S., Jefferson, A. B. et al. (2000). Loss of PTEN facilitates HIF-1-mediated gene expression. Genes Dev. 14, 391-396.