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From Erythropoietin to Oxygen: Hypoxia-Inducible Factor Hydroxylases and the Hypoxia Signal Pathway

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Abstract
The regulation of blood red cell production by the hormone erythropoietin (Epo) provides a paradigm for control of gene expression by oxygen. Analysis of this pathway has revealed a widespread system of gene regulation based on a transcriptional complex termed hypoxia-inducible factor (HIF). Hydroxylation of specific prolyl and asparinyl residues in the α subunit of HIF by a series of non-haem iron-dependent dioxygenases has been defined as a novel mechanism of protein modification that transduces the oxygen-sensitive signal.

Blood red cell production is accurately controlled so as to maintain haematocrit within tightly defined limits. Classical experiments performed around 50 years ago established the function of erythropoietin as a blood stream hormone that was produced by the kidneys and liver in response to reduced blood oxygen availability, and which operated on the bone marrow to regulate feedback changes in red cell production [1–3]. This work laid the foundation for purification of erythropoietin [4], cloning of the encoding cDNA [5, 6], and therapeutic application of recombinant erythropoietin in the treatment of renal anaemia [7, 8]. Nevertheless the mechanism(s) underlying the tight control of erythropoietin gene expression by oxygen remained enigmatic.

However, the availability of the cloned erythropoietin gene, together with the demonstration that particular hepatoma-derived cell lines produced erythropoietin in an oxygen-regulated manner in tissue culture [9], set the stage for a molecular approach to dissection of the oxygen responsive signal pathway starting with the gene itself. This presentation will outline progress along this route over the last 10 years, which has recently culminated in the definition of a set of non-haem iron-dependent dioxygenases that link the erythropoietin transcriptional pathway to the availability of molecular oxygen. These findings provide a biochemical framework on which to base a better understanding of physiological responses to hypoxia, as well as a potential route to therapeutic manipulation of these processes.

Studies of Erythropoietin Regulation Define a Widespread Transcriptional Response to Hypoxia Based on Hypoxia-Inducible Factor

A first step in moving along the pathway regulating erythropoietin expression was the definition of an oxygen-regulated DNA control element (the erythropoietin 3′ enhancer) by assay of transfected erythropoietin DNA

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sequences for oxygen-regulated activity in hepatoma cells [10–12]. These studies defined a specific point of interaction between the oxygen-sensitive signalling system (at least as manifest in the erythropoietin-producing hepatoma cells) and the erythropoietin gene locus. Unexpectedly, however, transfection studies of the erythropoietin 3′ enhancer also demonstrated oxygen-regulated activity after introduction into a wide variety of cells, regardless of whether the cells produced erythropoietin, or were derived from an erythropoietin-producing organ. Thus it became clear that the highly specific and sensitive response to hypoxia that was manifest in the erythropoietin-producing tissues was, in fact, a general property of mammalian cells [13].

The next major step along the pathway was provided by the analysis of proteins binding the erythropoietin 3′ enhancer. Work by Semenza and Wang [14] demonstrated that the response was critically dependent on sequence-specific DNA binding of a transcription factor that was termed hypoxia-inducible factor (HIF). Following affinity purification of the HIF DNA-binding complex Wang et al. [15] cloned the encoding cDNAs, and showed that HIF consisted of a heterodimer formed between two basic-helix-loop-helix PAS proteins, HIF-α and HIF-β.

It is now clear that the HIF transcriptional cascade mediates a broad set of systemic and local responses to hypoxia in addition to erythropoietin regulation, and that the system is tightly conserved between mammalian cells and invertebrate model organisms such as *Drosophila melanogaster* [16] and *Caenorhabditis elegans* [17, 18]. HIF target genes encode regulators of a number of medically important processes such as angiogenesis, vasomotor control and cellular proliferation/survival decisions, as well as red cell production [for review see 19]. All these responses, and those of HIF itself, manifest characteristics similar to those originally defined in studies of erythropoietin. Notably, activation by hypoxia could be mimicked by certain transition metals (cobalt II, nickel II, and manganese II) and iron chelators, classical properties of the erythropoietin response that had first led to the proposal that the upstream signal pathway involved the operation of a ferroprotein sensor [20, 21].

Further analysis of the oxygen-sensitive pathway focused on HIF and established a regulatory function for the α-subunit. HIF-α subunits specifically mediate responses to hypoxia, whereas HIF-β subunits are constitutive nuclear proteins that function in a variety of transcriptional systems with alternative dimerization partners. HIF-α is subject to multiple modes of regulation, but the most clearly defined oxygen-regulated controls are post-translational. Analysis of HIF-α polypeptides as fusion proteins has demonstrated several domains of HIF-α that could function independently to convey regulation by hypoxia when linked to the yeast transcription factor Gal4 [22–24]. Two isoforms of HIF-α (HIF-1α and HIF-2α) demonstrate closely similar domain architecture and modes of regulation by oxygen [25–27]. Both possess a central domain that mediates oxygen-dependent proteolytic degradation. This domain can be divided into two subdomains, each of which can independently convey oxygen-dependent proteolysis [24]. Each HIF-α subunit also possesses two transactivation domains: an internal activation domain that overlaps the proteolytic degradation domain, and a C-terminal activation domain that operates in an oxygen-regulated manner that is independent of proteolysis [27, 28]. In addition, at least under conditions of overexpression, HIF-1α demonstrates oxygen-dependent control of nuclear localisation [29]. Thus these studies have demonstrated that HIF activation is a multi-step process that involves three distinct types of post-translational control of HIF-α.

The existence of distinct domains that can mediate either proteolytic or non-proteolytic regulation independent of the remaining HIF sequences defined specific polypeptide sequences that must interact independently with the upstream oxygen-sensitive signalling system. However, though these sequences were studied in detail, the nature of the signal-transducing interaction was not immediately apparent. Since HIF-α subunits are heavily phosphorylated [30], and perturbation of protein phosphatase/kinase pathways can modulate HIF activity [31], it was generally expected that the oxygen-sensitive pathway would involve oxygen-regulated phosphorylation of specific HIF-α residues. Unexpectedly, however, no sites of oxygen-regulated phosphorylation were defined, suggesting the operation of a different mode of signal transduction.

Insights into HIF Regulation from the von Hippel-Lindau Tumour Suppressor

A further step in understanding the hypoxia pathway was provided by the definition of the von Hippel-Lindau tumour suppressor (pVHL) as a critical component of a ubiquitin E3 ligase complex that physically interacts with and targets HIF-α subunits for proteolytic degradation [32, 33]. von Hippel-Lindau disease is a hereditary cancer syndrome in which affected individuals bearing a germline

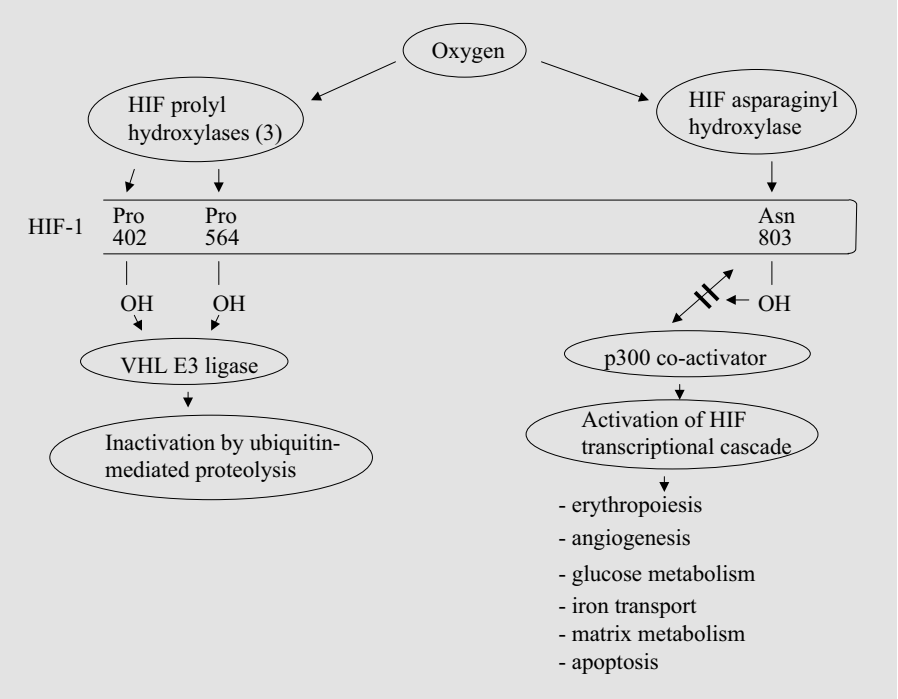

Fig. 1. Regulation of HIF by prolyl and asparaginyl hydroxylation. The residue positions indicated are those in human HIF-1α. In the presence of oxygen, hydroxylation at Pro402 and Pro564 targets HIF-1α for proteolytic destruction via the VHL E3 ubiquitin ligase complex, whereas hydroxylation at Asn803 prevents interaction with the p300 co-activator, thereby blocking transcriptional activity of any HIF-1α that remains intact. In hypoxia both these processes are suppressed allowing assembly of a transcriptionally active HIF complex.

mutation in the *VHL* gene are predisposed to highly angiogenic tumours (haemangioblastomas) of the retina and CNS, phaeochromocytoma, and renal cell carcinoma, following somatic inactivation of the second *VHL* allele. In *VHL* defective cells HIF-α subunits are constitutively stabilised regardless of oxygen levels, resulting in upregulation of HIF target genes, and most probably accounting for the angiogenic phenotype of *VHL*-associated tumours [32].

These findings provided a new focus for analysis of the oxygen-sensitive pathway through studies of the interaction between HIF-α polypeptides and pVHL. Further studies defined two short subsequences that interact with pVHL, corresponding to the independently active proteolytic degradation domains in HIF-α [34–36]. These sequences interact directly with the β-domain of pVHL [33], which provides a link to other components of the multi-component ubiquitin ligase (elongins B and C, Cul2, and Rbx-1), by means of a further interaction between its α-domain and elongin C [37].

Immunoprecipitation experiments using whole cell extracts revealed that capture of HIF-α by pVHL is suppressed by treatment of cells with iron chelators and cobaltous ions, and (when oxygen is excluded from the cell extraction buffers) by hypoxia, indicating that regulation of this interaction accurately reflects the properties of the oxygen-sensitive pathway [32, 38–40].

Further studies showed that the HIF-α/pVHL interaction could be reproduced in vitro using recombinant HIF-α and pVHL, but that the interaction required that the HIF-α polypeptide was pre-incubated with a cell extract in the presence of iron and oxygen. Temperature sensitivity and heat inactivation suggested that this process involved an enzymatic modification, and mutational analysis together with mass spectrometry of modified HIF-α polypeptides showed that the critical modification was hydroxylation of a specific prolyl residue (P564 in human HIF-1α) [39, 40]. Subsequent studies showed that each of the two in HIF-α degradation domains contains a site of prolyl hydroxylation and defined a common LxxLxP motif at the hydroxylation site [36].

The mechanism by which addition of a single oxygen atom to a prolyl residue within a HIF-α degradation motif governs recognition by pVHL has recently been studied by X-ray crystallography of a hydroxylated HIF-α peptide bound to the VCB (pVHL, elongins B and C). These studies reveal a single well-defined hydroxyproline-binding pocket on the surface of the pVHL β-domain [41, 42]. Highly specific discrimination between hydroxylated and non-hydroxylated HIF is achieved by an optimised hydrogen-bonding network between VHL residues in the floor of the binding pocket and the HIF-α hydroxyproline residue, that would be denied to proline.

Oxygen-Dependent Hydroxylation of HIF-α by a Series of 2-Oxoglutarate-Dependent Dioxygenases

Suppression of the HIF-α modifying activity in cell extracts by hypoxia, iron chelators, and certain 2-oxoglutarate analogues that inhibit the pro-collagen prolyl hydroxylases suggested that, like the pro-collagen enzymes, the HIF prolyl hydroxylases would belong to the iron and 2-oxoglutarate-dependent dioxygenase superfamily [39].

Importantly, since these enzymes have an absolute requirement for dioxygen as co-substrate, the findings indicated a direct link to the availability of molecular oxygen. Moreover labile binding of non-haem iron (II) at the catalytic site is associated with inhibition by iron chelators and cobalt (II). Crystallographically characterised members of this family possess a common β-barrel jelly-roll conformation that aligns a 2-histidine-1-carboxylate (HXD...H) iron co-ordination motif at the catalytic site [43]. Based on this model a combined structural prediction/genetics approach was used to identify candidate HIF prolyl hydroxylases as a highly conserved family of proteins related to the *C. elegans Egl-9* (Egl, egg-laying defect) gene product [18, 44]. The identification of a functional HIF system in *C. elegans* together with the existence of viable worms bearing inactivating mutations of the *Egl-9* gene enabled genetic testing of the role of *Egl-9* in HIF regulation. *Egl-9* mutant worms showed constitutive stabilisation of the HIF-α homologue clearly demonstrating a critical function for *Egl-9* in the oxygen-regulated response [18]. *Egl-9* is represented in mammalian cells by three isoforms termed PHD (Prolyl Hydroxylase Domain) 1, 2, and 3. Testing of recombinant forms of these proteins confirmed that each possesses HIF prolyl hydroxylase activity [18, 44].

Characterisation of the recombinant PHD enzyme activity confirmed direct inhibition by iron chelators, and cobaltous ions, and demonstrated striking sensitivity to reduced oxygen availability. Such properties clearly reflect those of erythropoietin and HIF regulation, strongly suggesting that the physiological characteristics of erythropoietin regulation by oxygen is, at least in part, based on the biochemical properties of these enzymes [18]. Interestingly, in addition to the co-substrate requirement for dioxygen, the co-factor requirements for iron and 2-oxoglutarate have the potential to contribute other regulatory signals from cellular iron availability and (since 2-oxoglutarate is a Krebs cycle intermediate) from energy metabolism.

Though these findings likely explain the processes underlying the proteolytic regulation of HIF-α, earlier studies outlined above have shown other modes of regulation of HIF-α with similar characteristics. For instance, non-proteolytic regulation of HIF-α C-terminal transactivation demonstrates similar responses to hypoxia, iron chelators and cobaltous ions [22, 23, 45]. Though these findings suggested the operation of a similar process, the LxxLxP sequence that is targeted for prolyl hydroxylation is not present in this domain. Recently, however, mass spectrometry of this domain has demonstrated hydroxylation of an asparaginyl residue as the regulatory modification that governs interaction with the p300 co-activator and thus the activity of this domain [46]. Interestingly, a protein termed FIH (Factor Inhibiting HIF) that was first identified as a HIF-interacting protein that depressed HIF transcription by unknown mechanisms [47], also conforms to the structural model for 2-oxoglutarate dioxygenases. Recent studies have demonstrated that FIH is, in fact, a HIF asparaginyl hydroxylase that modifies the C-terminal asparaginyl (N803 in human HIF-1α) in an iron- and oxygen-dependent manner [48, 49].

Thus, to date, a total of four dioxygenases mediating two different types of regulatory modification of HIF-α have been defined, and potentially explain many of the characteristics of physiological regulation by hypoxia (figure 1). The findings have pharmacological as well as physiological implications since it may now be possible to activate the HIF response by enzyme inhibition as a therapy against ischaemic/hypoxic disease or even erythropoietin deficiency. Equally, they raise the important questions as to whether these enzymes are all private to the HIF pathway or have other substrates that are important cellular responses to oxygen, and whether any of the regulatory hydroxylations are subject to reversal by other control mechanisms in a manner analogous to phosphatase/kinase signal pathways.

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