COMMENTARY

Enzyme substrate recognition in oxygen sensing: how the HIF trap snaps

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The transcriptional activator HIF (hypoxia-inducible factor) is a focal point of biomedical research because many situations in physiology and in pathology coincide with hypoxia. The effects of HIF activation may be a facet of normal growth, as in embryonic development, they may counterbalance a disease, as seen in the stimulation of erythropoiesis in anaemia, and they may be part of the pathological processes, as exemplified by tumour angiogenesis. The oxygen-sensitive α-subunits of HIF are primarily regulated by the enzymatic hydroxylation that induces rapid proteasomal degradation. The HIFα hydroxylases belong to a superfamily of dioxygenases that require the co-substrates oxygen and 2-oxoglutarate as well as the cofactors Fe^{2+} and ascorbate. The regulation of enzyme turnover by the concentration of the co-substrate oxygen constitutes the interface between tissue oxygen level and the activity of HIF. The HIFα prolyl hydroxylases, termed PHDs/EGLNs (prolyl hydroxylase domain proteins/EGL nine homologues), bind to a conserved Leu-Xaa-Xaa-Leu-Ala-Pro motif present in all substrates identified so far. This recognition motif is present twice in HIF1α, which gives rise to a NODD [N-terminal ODD (oxygen-dependent degradation domain)] containing Pro^{402} of HIF1α and a CODD (C-terminal ODD) where Pro^{564} is hydroxylated. PHD1/EGLN2 and PHD2/EGLN1 hydroxylate both ODDs with higher activity towards CODD, whereas PHD3/EGLN3 is specific for CODD. The reason for this behaviour has been unclear. In this issue of the *Biochemical Journal*, Villar and colleagues demonstrate that distinct PHD/EGLN domains, that are remote from the catalytic site, function in substrate discrimination. This elegant study improves our understanding of the interaction of the oxygen-sensing PHDs/EGLNs with their substrates, which include, but are not limited to, the HIFα proteins.

Key words: EGL nine homologue (EGLN), hypoxia, hypoxia-inducible factor (HIF), oxygen-dependent degradation domain (ODD), prolyl hydroylase domain (PHD), von Hippel–Lindau protein (VHL protein).

Insufficient oxygen availability to cells or tissues, i.e. hypoxia, leads to a number of responses that involve a significant change in the cellular gene expression pattern. The transcriptional activator HIF (hypoxia-inducible factor) is the central component of these responses [1,2]. In hypoxia, HIF is expressed in all nucleated cells. HIF homologues and other proteins involved in this pathway have been identified in all the multicellular species investigated so far, including insects, fish, and mammals. Genetic inactivation of *hif1α* in mice is lethal, as a consequence of impaired cardiac and vascular development. HIF controls more than 100 target genes. The most prominent protein products of these genes are erythropoietin and vascular endothelial growth factor, which is a key component of angiogenesis. Other target genes adapt cellular metabolism to hypoxia. Whereas HIF decreases the activity of the mitochondrial electron-transport chain, it simultaneously stimulates glucose uptake and glycolysis via increased production of glycolytic enzymes and plasma-membrane-bound glucose transporters.

In general, hypoxia occurs when oxygen demand exceeds delivery. This situation may be due to cell proliferation as, for example, in tumour growth. In this case, a vicious circle can develop, because the ensuing activation of HIF leads to angiogenesis, which in turn favours tumour growth. Tissue hypoxia can also result from reduced transport of oxygen by the blood, as in anaemia or diseases associated with ischaemia. The central position of HIF in oxygen homoeostasis makes it an attractive target for therapeutic manipulation. Indeed, HIF inhibitors that are expected to be beneficial in tumour treatment are under development [3]. On the other hand, HIF activators that have the potential to induce endogenous erythropoietin production are considered as a treatment option for anaemia resulting from, for example, chronic kidney disease. However, the death of one patient has halted clinical trials of FG-2216, which is the first substance that has been used in clinical trials as an HIF-activating anti-anaemia drug [4].

The active HIF transcription-factor complex is composed of a β-subunit, which is constitutively present in the cell nucleus, and an oxygen-sensitive α-subunit. Three HIFα isoforms have been described. HIF1α has been analysed in most of the studies and shares many characteristics with HIF2α. The functions of HIF3α are less well established. Three splice forms can be generated from *hif3a*. One splice form, termed IPAS [inhibitory PAS (Per–Arnt–Sim) protein], has been reported to function as an endogenous HIF-inhibitor [5]. While HIFα isoforms are relatively stable in hypoxia, they have a half-life of approx. 5 min in the presence of oxygen. Following oxygen-dependent enzymatic hydroxylation of two distinct proline residues, HIF1α binds pVHL (von-Hippel–Lindau tumour suppressor protein), which leads to ubiquitination and degradation by the 26S proteasome. The HIFα prolyl hydroxylases recognize the conserved amino acid sequence Leu-Xaa-Xaa-Leu-Ala-Pro, which is present twice in the HIF1α molecule so that Pro^{402} and Pro^{564} can be hydroxylated. The domains conferring oxygen sensitivity are, therefore, termed NODD and CODD [N-terminal and C-terminal ODD (oxygen-dependent degradation domains) respectively]. In addition to prolyl hydroxylation, the C-terminal transactivation domain can undergo asparaginyl hydroxylation, which blocks the recruitment of transcriptional co-activators. Thus two separate mechanisms regulate the activity of HIF1: prolyl hydroxylation leads to protein instability, whereas asparaginyl hydroxylation impairs transactivating function [6].

The HIFα hydroxylases belong to a family of 2-oxoglutarate-dependent dioxygenases which are a subgroup of iron-dependent non-haem oxygenases. Three enzymes have been identified

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which hydroxylate HIF1α on distinct proline residues. The enzymes were designated PHD (prolyl hydroxylase domain)-containing enzymes PHD1, PHD2 and PHD3 [7] or EGLN2 (EGL nine homologue enzyme 2), EGLN1 and EGLN3 respectively. The EGLN terminology refers to the relationship of these proteins to the protein egg-laying-deficient 9 of the nematode worm *Caenorhabditis elegans*. The PHDs/EGLNs co-ordinate iron in a His-His-carboxylate motif and are dependent on the presence of oxygen, 2-oxoglutarate and ascorbate. HIF α-subunits were the first substrates of the HIF-α hydroxylases to be identified. The conserved amino acid recognition sequence Leu-Xaa-Xaa-Leu-Ala-Pro is specific for HIFα prolyl hydroxylases, although it has been reported that the enzymes tolerate some degree of variation. The recognition motif has been localized in additional proteins and, indeed, it was shown that PHD1 also hydroxylates Pro^{191} of IκB (inhibitory κB) kinase β, thus interfering with activation of the transcription factor NF-κB (nuclear factor κB) [8]. In addition, Pro^{1465} of the RBP1 (RNA-binding protein 1) subunit of RNA polymerase II undergoes hydroxylation, which induces the binding of pVHL [9]. However, in that report, evidence that the HIFα hydroxylases are responsible was not presented. Furthermore, the PHDs/EGLNs display differential activity towards NODD and CODD of HIF1α. Of note, PHD3/EGLN3 is specific for CODD. Given the close structural relationship of the three PHDs/EGLNs, the molecular basis of substrate discrimination is not obvious.

In this issue of the *Biochemical Journal*, Villar and colleagues [10] identify domains of the PHDs/EGLNs that are responsible for differential binding of the ODDs of HIF-1α. It should be noted that the catalytic domain of these enzymes resides in the C-terminus and shows a very high degree of conservation. Comparison between the crystal structure of the catalytic centre of PHD2/EGLN1 and the other HIFα prolyl hydroxylases does not indicate obvious differences which would predict significant specificity in substrate recognition [11]. Remarkably, however, the N-terminal extension of the enzymes is either not conserved, as between PHD1/EGLN2 and PHD2/EGLN1, or significantly shortened, as in PHD3/EGLN3. Thus Villar and colleagues adopted the idea that the domains that are specific for each of the PHDs/EGLNs should be responsible for substrate discrimination. To this end, these workers deleted N-terminal domains or constructed expression vectors that induce the expression of chimaeric prolyl hydroxylases within transfected cells. Subsequently, it was tested whether the resulting chimaeric proteins interacted with either the NODD or the CODD. Full-length HIF1α molecules, which contained only one ODD, were generated by mutation of Pro^{402}, resulting in a molecule that only comprised the CODD, or vice versa, by mutation of Pro^{564}, leaving only the NODD intact. The results of these experiments indicate that a distinct domain that is remote from the catalytic centre of the PHD/EGLN molecule is responsible for substrate discrimination. With respect to PHD2/EGLN1, amino acids 236–251 are required for binding to the NODD. Because this substrate-binding domain is dissimilar in PHD3/EGLN3, its activity is restricted to the CODD. The evidence provided indicates that this effect is neither due to modification of enzymatic activity nor due to mediation by another component of the enzyme–substrate complex. Therefore, Villar and colleagues have resolved another important piece of the complex HIF puzzle.

It remains to be tested whether the interaction domains identified in their work are also sufficient to induce interaction of the PHDs/EGLNs with HIF2α and HIF3α. With respect to the regulation of these HIFα isoforms, several questions are still open. Because Villar and colleagues used full-length HIF1α in their study, they have not defined which region of HIF1α the PHD/EGLN interaction domain binds. This is interesting with respect to the question as to whether the isolated domains used in *in vitro* studies truly recapitulate the physiological interaction between PHDs/EGLNs and HIFs. This could, for example, impinge on the K_m of the PHDs/EGLNs for oxygen, an issue that has been controversial in the literature. Consequently, it is becoming increasingly clear that the identification of alternative PHD/EGLN substrates will require more than just the identification of a Leu-Xaa-Xaa-Leu-Ala-Pro motif.

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