Cell, Vol. 107, 1–3, October 5, 2001, Copyright ©2001 by Cell Press

Minireview

HIF-1, O_2, and the 3 PHDs: How Animal Cells Signal Hypoxia to the Nucleus

Gregg L. Semenza[1]
McKusick-Nathans Institute of Genetic Medicine
The Johns Hopkins University School of Medicine
Baltimore, Maryland 21287

Hypoxia-inducible factor 1 (HIF-1) is a global regulator of cellular and systemic O_2 homeostasis in animals. A molecular basis for O_2-regulated expression of the HIF-1α subunit has now been determined, providing a mechanism for changes in gene expression in response to changes in cellular oxygenation.

O_2

For all organisms on earth, changes in O_2 concentration represent a fundamental physiologic stimulus. In animals, this stimulus elicits both acute (rapid-onset and short-term) and chronic (delayed-onset and long-term) responses. Intracellular O_2 concentrations are maintained within a narrow range due to the risk of oxidative damage from excess O_2 (hyperoxia), and of metabolic demise from insufficient O_2 (hypoxia). Whereas acute responses often entail changes in the activity of preexisting proteins, chronic responses invariably involve changes in gene expression. A remarkable variety of molecular mechanisms that control the activity of prokaryotic and yeast transcription factors in response to changes in the environmental O_2 concentration have been delineated (for review, see Gilles-Gonzalez, 2001; Poyton, 1999; Taylor and Zhulin, 1999).

HIF-1

In the animal kingdom, the transcriptional activator hypoxia-inducible factor 1 (HIF-1) functions as a global regulator of O_2 homeostasis that facilitates both O_2 delivery and adaptation to O_2 deprivation (reviewed in Semenza, 2000). O_2 delivery can be accomplished by diffusion in a roundworm (which consists of $\sim 10^3$ cells) and HIF-1 may have originally evolved in simple multicellular animals to regulate cellular energy metabolism (glycolysis versus oxidative phosphorylation) according to O_2 availability. In contrast, elaborate erythroid, cardiac, vascular, and respiratory systems are required to adequately supply O_2 to adult mammals (many of which consist of $>10^{13}$ cells) and HIF-1 is required for the establishment and utilization of each of these systems. The number of known HIF-1 target genes continues to increase rapidly and the established roles of the encoded proteins (Figure 1) provides a molecular basis for understanding how HIF-1 controls the various developmental and physiological processes described above. Because of the critical involvement of HIF-1 in development and postnatal physiology, as well as disease pathophysiology (as discussed below), a major goal for investigators in the field has been to understand the molecular mechanisms by which the physiologic signal (reduced O_2 availability) is transduced to the nucleus in the form of increased HIF-1 transcriptional activity.

How Does O_2 Regulate HIF-1 Expression?

HIF-1 is a heterodimer composed of a HIF-1β subunit, which is constitutively expressed, and a HIF-1α subunit, the expression and transcriptional activity of which are precisely regulated by the cellular O_2 concentration (Wang et al., 1995). Both subunits contain basic helix-loop-helix domains that mediate dimerization and DNA binding, as well as a second dimerization motif referred to as the PAS domain based upon its original identification in the PER, ARNT, and SIM proteins. A superfamily of PAS domain proteins has been identified, the majority of which are prokaryotic signal transduction molecules involved in responding to environmental stimuli such as light, O_2 concentration, and redox state (Taylor and Zhulin, 1999). This finding suggested that HIF-1 might be directly regulated by O_2 because the PAS domains of several prokaryotic proteins bind prosthetic groups such as heme. However, a bewildering collection of models has been proposed over time involving signal tranduction via upstream O_2 binding hemoproteins or via the generation of superoxide or other reactive oxygen species (ROS), either by an NAD(P)H oxidoreductase or by the mitochondrial electron transport chain (ETC) (reviewed by Wenger, 2000). Involvement of phosphoinositide-3-kinase, MAP kinase, and receptor tyrosine kinase pathways has also been implicated in the induction of HIF-1 activity by hypoxia. In contrast, recent data indicating that the induction of HIF-1α in response to hypoxia is virtually instantaneous (Jewell et al., 2001) argued against a complex signal-transduction pathway. The lack of consensus that has prevailed is best illustrated by noting that the NADPH oxidoreductase and mitochondrial ETC models were diametrically opposed, as they proposed decreased and increased ROS generation, respectively, under hypoxic conditions. These models were supported by experimental data derived primarily from the use of redox-sensitive fluorescent compounds to measure ROS and from the effects of pharmacologic agents (inhibitors of NADPH oxidase or ETC activity) on the expression of HIF-1 and downstream genes in tissue culture cells. The considerable limitations of these experimental approaches underscored the need for a genetic approach to the problem of hypoxia signal transduction.

VHL

Direct analysis of HIF-1α revealed that the protein is subject to ubiquitination and proteasomal degradation under nonhypoxic conditions (Huang et al., 1998; Kallio et al., 1999; Salceda and Caro, 1997), a process that is inhibited under hypoxic conditions (Sutter et al., 2000). O_2-regulated expression of HIF-1α is mediated by a functional domain of approximately 200 amino acids (around residues 400–600) located C-terminal to the PAS domain. Somatic cell genetics was utilized to demonstrate that renal carcinoma cells derived from patients with von Hippel-Lindau syndrome, which lack expression of the VHL tumor-suppressor protein, constitutively express HIF-1 target genes as a result of constitutive

[1] Correspondence: gsemenza@jhmi.edu

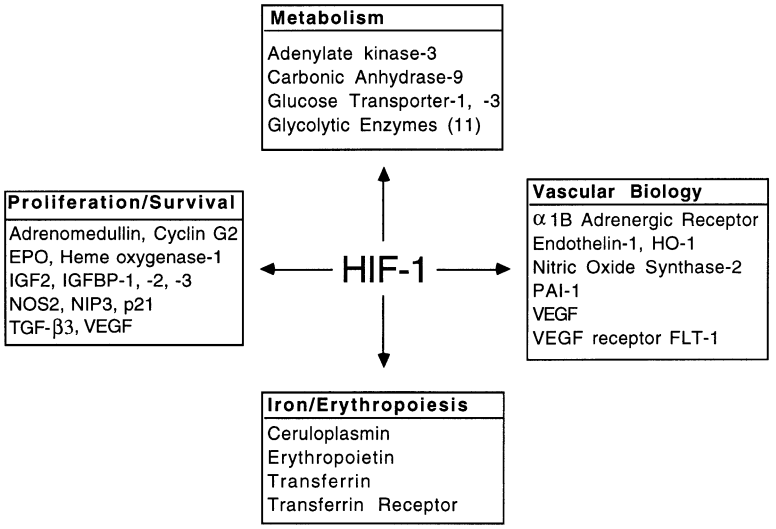

Figure 1. Representative HIF-1 Target Genes and Their Roles in Oxygen Homeostasis

HIF-1-regulated genes include the 11 glycolytic enzymes aldolase A, aldolase C, enolase 1, glyceraldehyde-3-phosphate dehydrogenase, hexokinase 1, hexokinase 2, lactate dehydrogenase A, phosphofructokinase L, phosphoglycerate kinase 1, pyruvate kinase M, and triosephosphate isomerase. Abbreviations: EPO, erythropoietin; HO, heme oxygenase; IGF, insulin-like growth factor; IGFBP, IGF binding protein; NOS, nitric oxide synthase; PAI, plasminogen activator inhibitor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

expression of HIF-1α (Maxwell et al., 1999). Downregulation of HIF-1 activity and target gene expression under nonhypoxic conditions can be restored by stable transfection of a VHL expression vector. Biochemical studies demonstrated, first, that VHL is the recognition component of an E3 ubiquitin-protein ligase that targets HIF-1α for degradation and, second, that interaction with VHL requires the O_2- and iron-dependent hydroxylation of proline residue 564 in HIF-1α by an enzymatic activity distinct from the known procollagen prolyl hydroxylases (Ivan et al., 2001; Jaakkola et al., 2001) (Figure 2). Thus, although these studies represented a major advance in the field, the excitement was tempered by the lack of a molecular characterization of the putative enzyme.

PHDs

In this issue of *Cell*, an elegant series of experiments is reported which utilize a genetic approach in the roundworm *C. elegans* to delineate the molecular basis for regulation of HIF-1α expression by the cellular O_2 concentration (Epstein et al., 2001). In this study, the investigators identified VHL and HIF-1α homologs, and demonstrated that *vhl-1* mutant worms (which lack VHL function) constitutively express HIF-1α, similar to the renal carcinoma cells derived from patients with von Hippel-Lindau syndrome. In vitro-translated worm HIF-1α did not bind to VHL unless it was incubated with worm extract, suggesting a requirement for prolyl hydroxylase (PH) activity, as previously demonstrated in mammalian cells. Mutation of a proline residue (Pro-621) in worm HIF-1α eliminated interaction with VHL, similar to the effect of mutating Pro-564 in human HIF-1α, thus providing further evidence for an evolutionarily conserved mechanism of proline hydroxylation.

To identify a putative PH, the investigators searched the *C. elegans* genome database for sequences that might encode a member of the 2-oxoglutarate-dependent oxygenase superfamily based on the presence of a β-barrel jelly roll motif which contains the catalytic site of these enzymes. This search criterion led to the *egl*-9 gene, which encoded a protein product of previously unknown function. Like *vhl-1* mutants, *egl*-9 mutant worms constitutively expressed HIF-1α. In vitro-

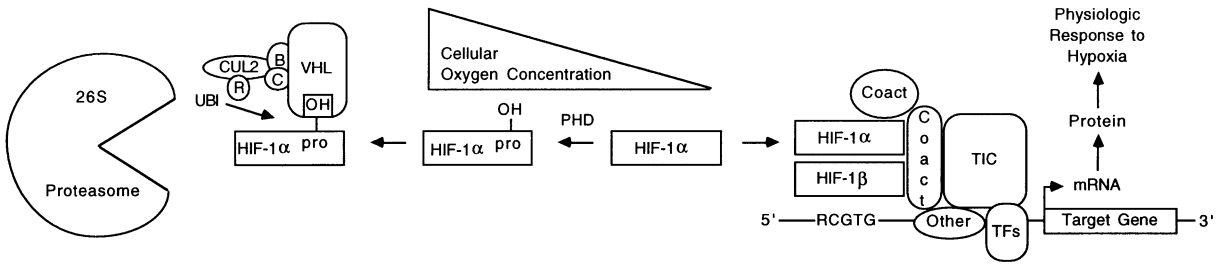

Figure 2. Regulation of HIF-1α Expression by Cellular O_2 Concentration

O_2 availability determines the rate at which HIF-1α is subject to prolyl hydroxylation by PHDs 1-3. Prolyl hydroxylation is required for the interaction of HIF-1α with VHL which recruits elongins B and C, Cullin 2 (CUL2), and RBX1 (R) to constitute a functional E3 ubiquitin-protein ligase complex. Ubiquitination of HIF-1α targets the protein for degradation by the 26S proteasome. Under hypoxic conditions, HIF-1β dimerizes with HIF-1α, which escapes prolyl hydroxylation, ubiquitination and degradation. The HIF-1 heterodimer binds to hypoxia response elements containing the core recognition sequence 5′-RCGTG-3′ and recruits coactivator (Coact) molecules resulting in increased transcription initiation complex (TIC) formation and mRNA synthesis that ultimately results in the production of proteins that mediate physiologic responses to hypoxia. The battery of HIF-1 target genes that are expressed in response to hypoxia is cell-type-specific and is determined by the binding of other transcription factors (TFs) which establish basal rates of transcription.

translated EGL-9 could replace worm extract in stimulating the interaction of VHL with wild-type HIF-1α, but not when Pro-621 was mutated. The ability of EGL-9 to generate HIF-1α containing 4-hydroxyproline was demonstrated directly by HPLC to establish that EGL-9 was indeed a HIF-1α PH.

Using the EGL-9 sequence, the investigators next identified three ubiquitously expressed EGL-9 homologs in mammals, designated PHD (PH domain-containing protein) 1, 2, and 3, each of which hydroxylates human HIF-1α at Pro-564. In contrast, only PHD1 and PHD2 hydroxylate Pro-402, a second site of prolyl hydroxylation and VHL binding identified by the same group. The proline residues that are subject to hydroxylation in both worm and mammalian HIF-1α proteins are embedded within the amino acid motif LXXLAP. Finally, the investigators demonstrated that the ability of in vitro-translated PHD1 to modify HIF-1α (as measured by the binding of HIF-1α to VHL) was dependent upon the ambient O_2 concentration. Thus, whereas the PHD proteins require iron, oxoglutarate, and O_2 as cofactors, these results suggest that O_2 is rate limiting for PH activity, a finding that provides the long-sought molecular basis for the regulation of HIF-1α expression by the cellular O_2 concentration.

The multiplicity of mammalian PHDs in contrast to the single worm enzyme provides justification for the genetic approach in a model organism, as it is likely that studies in mammalian cells would have been frustrated by redundancy. In striking symmetry, there are two additional HIF-1α orthologs in mammals, designated HIF-2α and HIF-3α. The expression of these latter two proteins is also O_2-regulated but, compared to HIF-1α, they appear to have more specialized and tissue-specific functions. The extent to which the three mammalian PHDs have overlapping and unique activities and functions (e.g., K_m for O_2, target proteins for modification) remains to be determined. As described above, preliminary evidence suggests that the three mammalian PHDs may have differing hydroxylase activities toward Pro-564 and Pro-402 of HIF-1α. The activities of these enzymes toward LXXLAP motifs in HIF-2α and HIF-3α remain to be determined. Although this motif is highly conserved, it is probably worth contemplating the possibility that other residues in HIF-1α may also be subject to O_2-dependent hydroxylation. For example, transactivation domain function is also O_2-regulated by mechanisms that remain incompletely understood (reviewed in Semenza, 2000; Wenger, 2000).

The demonstration of direct regulation of HIF-1 by O_2 is a major finding because Epstein et al. (2001) have demonstrated a mechanism by which changes in cellular O_2 concentration can directly result in changes in gene expression, a fundamental advance in cellular physiology. The previously proposed involvement of a variety of signal transduction pathways in the O_2-dependent regulation of HIF-1 activity will need to be reevaluated in the light of these new findings. Furthermore, since prolyl hydroxylation represents a novel posttranslational modification (compare acetylation, phosphorylation, etc.) for regulating protein-protein (i.e., HIF-1α-VHL) interactions, this mechanism may be utilized for the regulation of other protein interactions and other biochemical processes in addition to protein ubiquitination, with the major distinguishing characteristic being that the modification is O_2 regulated. It should be possible to utilize proteomic techniques to identify proteins other than HIF-1α whose expression is increased in *egl-9* mutant worms.

Clinical Relevance

Hypoxia plays an important role in the pathophysiology of ischemic cardiovascular disease, cancer, stroke, and chronic lung disease, which are the most common causes of mortality in the U.S. population. There is a growing body of data indicating that HIF-1 contributes to the pathogenesis of cancer and hypoxic pulmonary hypertension while protecting against the ischemia and infarction (reviewed in Semenza, 2000). Whether targeting HIF-1 for inhibition (in cancer and lung disease) or induction (in ischemic disorders) will be of therapeutic utility remains to be established. The delineation of the evolutionarily-conserved PHD-VHL-HIF-1 pathway has now provided multiple targets and will hopefully lead to the discovery of pharmacologic agents that can be used for proof-of-principle experiments in animal models and as a lead for the development of agents that can be used in clinical trials. Whereas such important clinical applications remain to be demonstrated in the future, the work of Epstein et al. (2001) has now provided us with a much better appreciation of how each cell in a multicellular animal can sense changes in O_2 concentration and send this signal to the nucleus.

References

Epstein, A.C.R., Gleadle, J.M., McNeil, L.A., Hewitson, K.S., O'Rourke, J., Mole, D.R., Mukherji, M., Metzen, E., Wilson, M.I., Dhanda, A., et al. (2001). Cell *107*, this issue, 43–54.

Gilles-Gonzalez, M.A. (2001). IUBMB Life *51*, 165–173.

Huang, L.E., Gu, J., Schau, M., and Bunn, H.F. (1998). Proc. Natl. Acad. Sci. USA *95*, 7987–7992.

Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J.M., Lane, W.S., and Kaelin, W.G., Jr. (2001). Science *292*, 464–468.

Jaakkola, P., Mole, D.R., Tian, Y.M., Wilson, M.I., Gielbert, J., Gaskell, S.J., Kriegsheim, A., Hebestreit, H.F., Mukherji, M., Schofield, C.J., et al. (2001). Science *292*, 468–472.

Jewell, U.R., Kvietikova, I., Scheid, A., Bauer, C., Wenger, R.H., and Gassmann, M. (2001). FASEB J. *15*, 1312–1314.

Kallio, P.J., Wilson, W.J., O'Brien, S., Makino, Y., and Poellinger, L. (1999). J. Biol. Chem. *274*, 6519–6525.

Maxwell, P.H., Wiesener, M.S., Chang, G.W., Clifford, S.C., Vaux, E.C., Cockman, M.E., Wykoff, C.C., Pugh, C.W., Maher, E.R., and Ratcliffe, P.J. (1999). Nature *399*, 271–275.

Poyton, R.O. (1999). Respir. Physiol. *115*, 119–133.

Salceda, S., and Caro, J. (1997). J. Biol. Chem. *272*, 22642–22647.

Semenza, G.L. (2000). Genes Dev. *14*, 1983–1991.

Sutter, C.H., Laughner, E., and Semenza, G.L. (2000). Proc. Natl. Acad. Sci. USA *97*, 4748–4753.

Taylor, B.L., and Zhulin, I.B. (1999). Microbiol. Mol. Biol. Rev. *63*, 479–506.

Wang, G.L., Jiang, B.-H., Rue, E.A., and Semenza, G.L. (1995). Proc. Natl. Acad. Sci. USA *92*, 5510–5514.

Wenger, R.H. (2000). J. Exp. Biol. *203*, 1253–1263.