Genetic Analysis of Hypoxia Signaling and Response in *C. elegans*

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ABSTRACT: During normal development and homeostasis, animals use cellular and systemic strategies to adapt to changing oxygen levels. In mammals, hypoxic tissues secrete growth factors to induce angiogenesis, and individual cells increase anaerobic metabolism in order to sustain basic cellular functions. Many of these critical responses to decreased oxygen availability are regulated by the hypoxia-inducible factors, dimeric transcriptional complexes consisting of α and β subunits. HIFα proteins are specialized for hypoxia response, and oxygen levels regulate their stability and activity. The *C. elegans hif-1* gene is orthologous to mammalian HIFα genes, and *C. elegans* has proven to be a powerful system for the study of hypoxia-inducible factor regulation and function. Mutants lacking *hif-1* function are viable in normoxic or anoxic conditions, but they cannot adapt to hypoxia. Recent genetic analyses in *C. elegans* led to the identification of the evolutionarily conserved enzyme that hydroxylates HIFα in an oxygen-dependent manner. Once modified, HIFα binds the von Hippel–Lindau tumor suppressor protein and is targeted for proteasomal degradation. Here, we briefly review the characterization of *C. elegans hif-1* and interacting genes, and discuss genetic strategies for studying hypoxia signaling and response.

KEYWORDS: bHLH-PAS proteins; *egl-9*; oxygen; transcription; von Hippel–Lindau tumor suppressor

INTRODUCTION

In a multicellular organism, cells and tissues have varying access to oxygen, and cellular adaptation to hypoxia (low oxygen) plays a central role in many important developmental processes. Large animals require circulatory systems to distribute oxygen and nutrients, and angiogenesis is guided by the vascular endothelial growth factor, which is secreted by hypoxic cells.[1] Coronary occlusions, ischemic heart disease, and other pathologies that disrupt the circulatory system can cause immediate and disastrous effects, including hypoxic cell death and irreversible damage to critical tissues. These pathologies can be partially alleviated by hypoxic preconditioning,[2,3] suggesting that specific changes in gene expression can protect cells from short-term oxygen deprivation. Cellular response to hypoxia also plays a central role in tumor growth.[4–6]

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Ann. N.Y. Acad. Sci. 995: 191–199 (2003).

HYPOXIA-INDUCIBLE FACTORS

Many of the physiological responses to decreased oxygen availability are mediated by the sequence-specific DNA-binding hypoxia-inducible factors (HIF).[1] HIF complexes are dimeric, consisting of alpha and beta subunits. The HIFβ subunit is also termed ARNT (aryl hydrocarbon receptor nuclear translocator).[7,8] While ARNT can dimerize with other transcription factors, HIFα is apparently dedicated to hypoxia response. Researchers have isolated three murine HIFα genes (HIF-1α, HIF-2α, and HIF-3α). The HIFα proteins have striking sequence similarities, and the proteins have distinct, but overlapping, expression patterns.[7,9–11] HIF-1α has an essential role in angiogenesis. Mice lacking a functional HIF-1α gene die early in embryogenesis with severe vascular defects.[12,13] Mice that are deficient for HIF-2α (EPAS-1) suffer from bradycardia, which is associated with decreased catecholamine synthesis.[14]

Regulation of HIFα

The stability of HIFα is regulated by oxygen. When cellular oxygen levels are high, a conserved proline residue in the HIFα subunit is hydroxylated. This increases the affinity of the oxygen-dependent degradation domain (ODD) of HIFα for the von Hippel–Lindau tumor suppressor protein (VHL).[15–17] VHL targets HIFα for polyubiquitination and proteasomal degradation.[18,19] The prolyl hydroxylase that modifies HIFα was first identified in *Caenorhabditis elegans* (see below).[20] In hypoxic conditions, prolyl hydroxylation and subsequent degradation of HIFα are inhibited. HIFα translocates to the nucleus, dimerizes with ARNT, and activates the expression of target genes, which act to increase oxygen delivery or implement metabolic adaptation to hypoxia. While oxygen-dependent degradation is an important aspect of HIFα regulation, several studies suggest that other cell-type-specific factors or signaling pathways modulate HIFα activity. These have been reviewed recently elsewhere.[21]

A genetic model system for the study of hypoxia-inducible factor regulation and function has been developed in *C. elegans*. Genetic studies will complement the wealth of biochemical and pharmacological analyses in mammalian cells and will advance our understanding of hypoxia signaling and response.

HYPOXIA RESPONSE IN *C. ELEGANS*

The nematode *C. elegans* is a powerful animal model system for the dissection of evolutionarily conserved signaling pathways. *C. elegans* are small (~1 mm in length as adults), and they can be cultured on agar plates with a bacterial food source. There are two sexes, self-fertilizing hermaphrodites and males, and the generation time is less than 4 days at 20°C. The wild-type embryonic and postembryonic lineages have been extensively documented and are essentially invariant.[22,23] This allows mutant phenotypes to be diagnosed at the level of individual cells. The simplicity and experimental convenience of *C. elegans* make it an attractive model organism for genetic analyses, and a wealth of recent genome-wide studies has increased the power of this system. The sequence of the ~10^8 bp genome was completed in 1998, revealing approximately 19,000 genes.[24] Large-scale studies are under way to characterize the function of every gene by double-stranded RNA interference, full-genome micro-

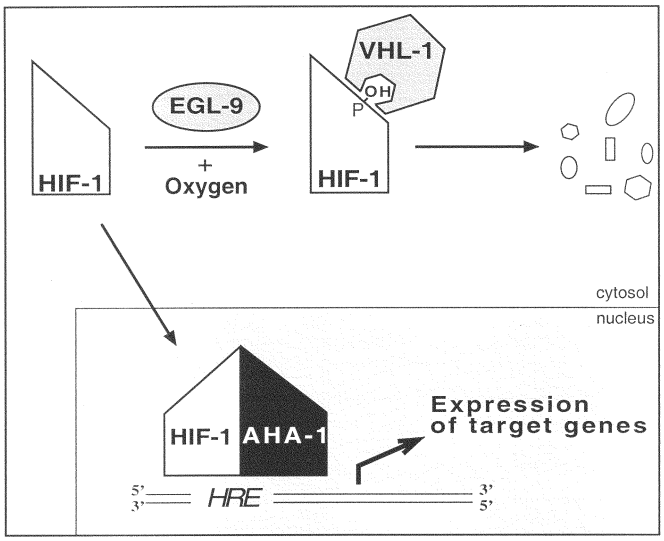

FIGURE 1. Regulation of *C. elegans* HIF-1. HIF-1 protein levels are regulated by oxygen availability. The EGL-9 protein is a member of the 2-oxoglutarate-dependent oxygenase superfamily, and oxygen is a required cofactor for EGL-9 function. EGL-9 acts directly on HIF-1 to hydroxylate the proline residue in the conserved LXXLAP motif. This modification is required for binding of HIF-1 to VHL-1. VHL-1 targets HIF-1 for proteasomal degradation. Under hypoxic conditions, EGL-9 does not efficiently modify HIF-1, and HIF-1 dimerizes with AHA-1 to form a transcriptional complex.[20] The HIF-1 heterodimer binds to the hypoxia response elements (HRE) in target genes to implement changes in gene expression (unpublished data).

arrays, and semiautomated analyses of protein-protein interactions.[25–28] Much of these data are available to the scientific community through dedicated internet sites.[29]

In the wild, *C. elegans* inhabits the soil, where it can encounter hypoxic microenvironments. *C. elegans* is able to maintain a near-normal metabolic rate at environmental oxygen concentrations as low as 2%. In 0.5% or 1% oxygen, the animals must decrease oxygen consumption, but they continue to develop and reproduce.[30] *C. elegans* does not have a complex circulatory system. Any cell in the organism is only a few cell widths from the outer surface of the worm or the intestinal lumen,[22] and oxygen delivery is thought to be accomplished by diffusion. Thus, individual cells must sense and adapt to local environmental oxygen levels. Recent discoveries have revealed that the molecular mechanisms that govern transcriptional responses to hypoxia are, at least in part, conserved between *C. elegans* and humans (see text below and FIG. 1).

C. ELEGANS HIF-1

The *C. elegans hif-1* gene is orthologous to mammalian HIF alpha subunits.[20,31] Like its mammalian cognates, *C. elegans* HIF-1 protein is induced by hypoxia and is rapidly degraded upon reoxygenation. *hif-1* mRNA levels are not dramatically

affected by oxygen concentration.[20,31] Under conditions in which *C. elegans* HIF-1 is stable, it binds AHA-1, the orthologue of mammalian ARNT/HIFβ, to form a complex that can bind DNA sequences containing the hypoxic regulatory element (FIG. 1).[31] Both HIF-1 and AHA-1 are expressed in most, if not all, cells, as assayed by a *hif-1*:GFP reporter gene and AHA-1-specific antibodies. In the intestine, which does not express any bHLH-PAS proteins other than HIF-1 and AHA-1, nuclear localization of AHA-1 is dependent upon HIF-1.[31]

VHL-1 and EGL-9 Mediate the Degradation of HIF-1

Recent studies have identified an evolutionarily conserved enzyme that directly couples cellular oxygen levels to the stability of HIF α subunits. In 2001, two groups demonstrated that a conserved proline in HIF-1α and HIF-2α was hydroxylated in an oxygen-dependent manner and that this modification increased binding of the HIF α subunits to VHL.[15,16] VHL targets the protein for proteasomal degradation.[18,19] In a tour de force published later in the year, Ratcliffe, Maxwell, Pugh, and colleagues identified the enzyme that hydroxylates HIFα. First, they demonstrated that oxygen-dependent hydroxylation of the LXXLAP motif in HIFα proteins and the subsequent interaction with VHL are conserved in nematodes and humans. Modification of *C. elegans* HIF-1 allows direct binding of VHL-1, the orthologue of VHL.[20] The importance of VHL-1 for HIF-1 degradation was further confirmed by isolation and analysis of mutants carrying a deletion in the *vhl-1* gene. These VHL-1-deficient animals express HIF-1 protein at high levels in both normoxic and hypoxic conditions. Second, the authors identified the enzyme that hydroxylates the oxygen-dependent degradation domain of HIF-1 by assaying HIF-1 protein levels in a series of mutant strains, each defective in a different candidate gene. They found that animals carrying loss-of-function mutations in the *egl-9* gene failed to downregulate HIF-1 expression in normoxic conditions. Additional experiments demonstrated that the EGL-9 protein acted directly on HIF-1 to hydroxylate the proline in the LXXLAP motif *in vitro*. Finally, the authors identified mammalian homologues of EGL-9 (termed PHD 1, 2, and 3) and demonstrated that the mammalian PHDs hydroxylate human HIF-1α or HIF-2α *in vitro*.[20] Thus, HIF-1, VHL-1, and EGL-9 appear to be part of an evolutionarily conserved regulatory network that senses hypoxia and implements appropriate transcriptional changes (FIG. 1).

The *egl-9* gene was originally isolated as a gene required for normal egg-laying,[32] and *egl-9* encodes a member of the 2-oxoglutarate-dependent oxygenase superfamily.[33,34] In an independent study, *egl-9* mutants were shown to have decreased sensitivity to cyanide exposure.[35] The cyanide sensitivity of *vhl-1*-deficient animals or *egl-9*;*hif-1* double-mutants has not been reported, but phenotypic and molecular studies indicate that *egl-9* and *vhl-1* are likely to have functions that are independent of their critical role in the oxygen-dependent degradation of HIF-1.

The **hif-1** *Gene Is Required for Adaptation to Hypoxia*

The *hif-1* (*ia04*) mutation is predicted to be a strong loss-of-function allele. It deletes exons 2–4 of *hif-1*, and it introduces an early translational stop codon to the most abundant forms of *hif-1* mRNA.[31] Animals that are homozygous for the *hif-1* mutation exhibit no visible defects under standard laboratory conditions. However,

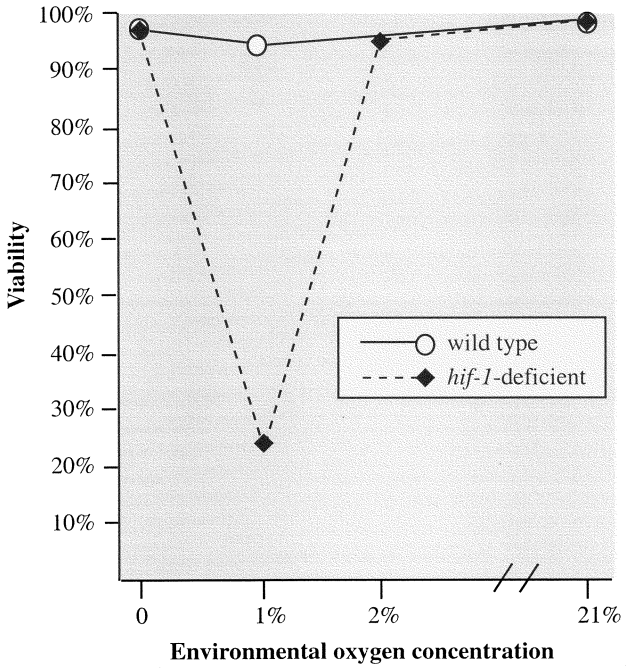

FIGURE 2. Viability of wild-type *C. elegans* and *hif-1* (*ia04*) mutants at varying oxygen levels. This graph summarizes published studies in which the survival of wild-type animals and *hif-1* (*ia04*) mutants was assayed at 21%, 2%, 1%, and 0% oxygen at 20°C.[31,36] The viability of *hif-1*-defective *C. elegans* at oxygen concentrations between 0% and 0.5% has not been reported. The survival curve is unlikely to be linear, and there may be a threshold oxygen concentration for survival. As discussed in the text, wild-type animals do not survive prolonged oxygen deprivation at 28°C.[37]

hif-1-defective animals are unable to adapt to 0.5% or 1% oxygen. While wild-type animals survive and reproduce in 1% oxygen, 66% of *hif-1*-defective animals do not survive embryogenesis in these conditions, and an additional 9% die during larval development (FIG. 2).[31]

Anoxia-Induced Suspended Animation versus Hypoxia

Analyses of *C. elegans hif-1* have revealed a fundamental difference in the molecular mechanisms by which *C. elegans* respond to hypoxia (1% or 0.5% O_2) versus anoxia (0% O_2) (FIG. 2). In anoxic conditions at 20°C, *C. elegans* at all stages arrest development and cell-cycle progression, and they enter a reversible state of "suspended animation". After 24 hours of incubation in anoxia, ~90% of *C. elegans* recover and continue development.[30,36] Padilla, Roth, and colleagues have shown that there is no detectable difference between *hif-1*-defective and wild-type animals in their ability to survive these conditions (FIG. 2).[36] Thus, *hif-1* function is essential in hypoxic conditions in which animals continue to grow and develop, but *hif-1* does

not appear to have an important role in anoxia-induced suspended animation or in subsequent recovery upon reoxygenation.

The ability of *C. elegans* to survive oxygen deprivation decreases at high temperatures.[37] *C. elegans* are commonly cultured at temperatures between 15°C and 25°C. At 27°C, they are much more likely to form dauers, a stress-resistant alternative larval stage.[38] Crowder and colleagues have shown that when adult *C. elegans* are incubated for 20 h at 28°C in an anoxic chamber (5% CO_2, 10% H_2, 85% N_2 gas; oxygen levels measured at below 0.3%), fewer than 5% of adult animals survive.[37] Remarkably, this "hypoxic death" can be suppressed by certain loss-of-function mutations in the *daf-2* insulin-like growth factor receptor gene.[37] These *daf-2* alleles have also been shown to prolong the life of *C. elegans* grown in optimal conditions and to induce dauer formation at 25°C.[39] It will be interesting to learn whether mutation of *hif-1* influences the ability of animals to survive the hypoxic death assay.

Multiple Species of C. elegans hif-1 *mRNAs*

Over 45 *hif-1* cDNAs have been completely or partially sequenced by the *C. elegans* genome project, and 4 forms of *hif-1* mRNAs have been identified.[27] These are diagrammed in FIGURE 3. The three most abundant forms (*a*, *b*, and *c*) contain all of the previously defined domains in HIF-1, including the bHLH, PAS, and oxygen-dependent degradation domains. The *b* form utilizes an alternative splice acceptor site for exon 7, and this adds 6 bases and 2 codons. Both the *c* and *d* forms are rare. The *c* form includes an additional ~600 bp exon (exon 5.5 in FIG. 3). The smaller *d* species of *hif-1* appears to utilize an alternative promoter inside intron 4, and it does

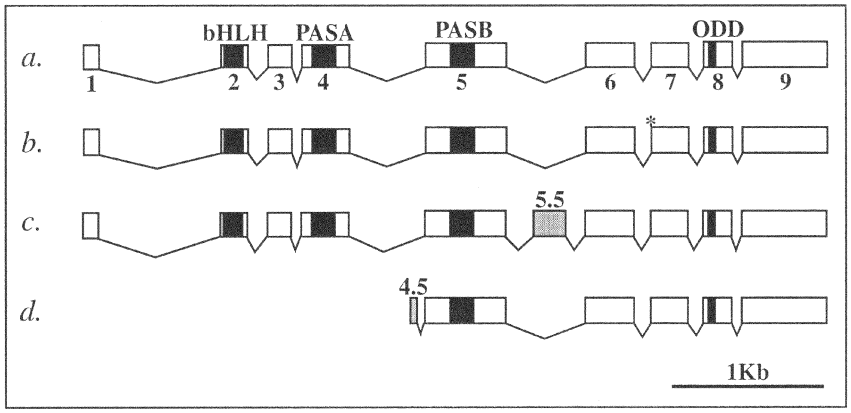

FIGURE 3. Multiple species of *hif-1* mRNAs. Exons are drawn as boxes, and the basic helix-loop-helix (bHLH), PER-ARNT-SIM (PAS), and oxygen-dependent degradation (ODD) domains are indicated in black. The *a* form has been characterized in previous biochemical and molecular studies.[20,31] The *b* form utilizes a different splice acceptor site for exon 7, and it encodes 2 additional amino acids (labeled *). Both the *c* and *d hif-1* mRNAs are rare. The *c* form includes an additional exon (labeled as 5.5). The *d* form appears to utilize an alternative promoter in intron 4, and it includes exon 4.5. Neither the bHLH nor PASA motifs are encoded by the *d* mRNA. This diagram summarizes data from The *C. elegans* Genome Consortium (available at http://www.wormbase.org/).

not encode the bHLH or PASA motifs. The protein encoded by the *a* class of mRNAs is well characterized,[20,31] but the functional and biochemical properties of the alternative gene products have not yet been examined.

Strategies for Genetic Analysis of Hypoxia Signaling and Response in C. elegans

C. elegans lacking *hif-1*, *vhl-1*, or *egl-9* are viable, and this enables full-genome microarrays to characterize hypoxia-induced changes in gene expression. In preliminary studies, we used this approach to clarify the role of HIF-1 in hypoxia response. We identified the following classes: (1) genes that are regulated by hypoxia in wild-type animals, but not in *hif-1* mutants; (2) genes that are regulated by hypoxia, independent of *hif-1* function; and (3) genes that require *hif-1* for basal levels of expression, but are not induced by a 4-h exposure to hypoxia. These data confirm that HIF-1 induces the expression of many genes in response to hypoxia. Further, they demonstrate that HIF-1 does not mediate all transcriptional responses to low oxygen levels. It is not immediately clear which *hif-1*-dependent changes in expression represent direct targets of HIF-1. As a first step towards identifying functionally important HIF-1:AHA-1 binding sites, we are assaying the binding specificity of this complex by gel mobility shift assays. Initial results suggest that the HIF-1:AHA-1 binding site is similar to the mammalian hypoxia response element (unpublished data).

Once direct targets of HIF-1 have been identified, it will be possible to construct GFP reporter genes for use in genetic screens. We have generated a *C. elegans* strain carrying a hypoxia-induced reporter gene that has a restricted expression pattern in wild-type animals in normoxic conditions. In environmental or genetic conditions that increase HIF-1 stability, the expression of the GFP reporter expands to other tissues (unpublished data). This and other reporters will be used to screen for mutations that result in increased or decreased HIF-1 activity. Such genetic analyses may identify additional evolutionarily conserved regulators of the hypoxic response.

ACKNOWLEDGMENTS

C. elegans strains were obtained from the *Caenorhabditis* Genetic Stock Center, which is supported by the National Institutes of Health National Center for Research Resources. The *hif-1* cDNAs were isolated and characterized by the *C. elegans* Genome Consortium. We are thankful to Danielle Thierry-Mieg for drawing our attention to the rare *hif-1* mRNAs. We are grateful to Min Jiang, Stuart Kim, and colleagues at Stanford University, who performed the microarray hybridizations. The hypoxia project in the Powell-Coffman lab is supported by grants from the National Science Foundation (No. 9874456) and from the American Heart Association (Established Investigator Award to J. A. Powell-Coffman).

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