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Structural and functional analysis of hypoxia-inducible factor 1

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Structural and functional analysis of hypoxia-inducible factor 1. Hypoxia-inducible factor 1 (HIF-1) is a basic helix-loop-helix protein that activates transcription of hypoxia-inducible genes, including those encoding: erythropoietin, vascular endothelial growth factor, heme oxygenase-1, inducible nitric oxide synthase, and the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase L, and phosphoglycerate kinase 1. Hypoxia response elements from these genes consist of a HIF-1 binding site (that contains the core sequence 5′-CGTG-3′) as well as additional DNA sequences that are required for function, which in some elements include a second HIF-1 binding site. HIF-1 is a heterodimer. The HIF-1α subunit is unique to HIF-1, whereas HIF-1β (ARNT) can dimerize with other bHLH-PAS proteins. Structural analysis of HIF-1α revealed that dimerization with HIF-1β (ARNT) requires the HLH and PAS domains, DNA binding is mediated by the basic domain, and that HIF-1α contains a carboxyl-terminal transactivation domain. Co-transfection of HIF-1α and HIF-1β (ARNT) expression vectors and a reporter gene containing a wild-type hypoxia response element resulted in increased transcription in non-hypoxic cells and a superinduction of transcription in hypoxic cells, whereas HIF-1 expression vectors had no effect on the transcription of reporter genes containing a mutation in the HIF-1 binding site. HIF-1α and HIF-1β (ARNT) protein levels were induced by hypoxia in all primary and transformed cell lines examined. In HeLa cells, the levels of HIF-1α and HIF-1β protein and HIF-1 DNA-binding activity increased exponentially as cellular oxygen tension decreased, with maximum values at 0.5% oxygen and half-maximal values at 1.5 to 2% oxygen. HIF-1α and HIF-1β (ARNT) mRNAs were detected in all human, mouse, and rat organs assayed and mRNA expression was modestly induced in rodents subjected to hypoxia. HIF-1α protein levels were induced *in vivo* when animals were subjected to anemia or hypoxia. The *HIF1A* gene was mapped to human chromosome 14q21-q24 and mouse chromosome 12.

This paper reviews recent investigations in our laboratory that have attempted to determine the molecular mechanisms by which the transcription factor hypoxia-inducible factor 1 (HIF-1) mediates homeostatic responses to hypoxia in mammals. HIF-1 was first identified [1] as a DNA-binding activity that was induced when cultured cells were exposed to hypoxic conditions (1% O_2) [reviewed in 2–5]. We have continued to utilize cultured cells to study the role of HIF-1, but we have now also begun the more demanding process of analyzing HIF-1 expression *in vivo*.

Identification of target genes for activation by HIF-1 in hypoxic cells

HIF-1 is a basic helix-loop-helix transcription factor that appears to be involved in many important homeostatic responses to hypoxia. Genes whose transcription is activated by HIF-1 include the following: (a) *EPO*, encoding erythropoietin, the primary regulator of erythropoiesis and thus a major determinant of blood O_2-carrying capacity [6]. (b) *VEGF*, encoding vascular endothelial growth factor, the primary regulator of angiogenesis and thus a major determinant of tissue perfusion [7–9]. (c) *ALDA, ENO1, LDHA, PFKL,* and *PGK1*, encoding the glycolytic enzymes aldolase A, enolase 1, lactate dehydrogenase A, phosphofructokinase L, and phosphoglycerate kinase 1, respectively, which provide a metabolic pathway for ATP generation in the absence of O_2 [10–12]. (d) *HO1* and *iNOS*, encoding heme oxygenase 1 and inducible nitric oxide synthase, which are responsible for the synthesis of the vasoactive molecules carbon monoxide and nitric oxide, respectively [13, 14].

Analysis of hypoxia response elements

In the case of the *EPO* and *VEGF* genes, *cis*-acting DNA sequences of 33 base pairs (bp) and 35 bp, respectively, have been identified that are sufficient for hypoxia-induced transcription of reporter genes and thus constitute hypoxia response elements (HREs) [1, 8, 9]. These HREs have in common the presence of a HIF-1 site and flanking sequences that are essential for function. Mutation of the *EPO* sequence 5′-CACAG-3′ located downstream of the HIF-1 site resulted in a complete loss of HRE function. The sequence 5′-(C/A)ACAG-3′ is also present both immediately upstream and downstream of the HIF-1 site in the *VEGF* gene. The factor which recognizes this sequence has not been identified.

For other genes such as *ENO1, HO1, LDHA*, and *PGK1,* the minimal functional HRE sequence has not been precisely determined. However, these HREs are all characterized by the presence of two HIF-1 sites within less than 30 bp. In the case of *ENO1* and *LDHA*, mutation of one site completely eliminated HRE function, whereas mutation of the other site severely diminished, but did not eliminate the transcriptional response to hypoxia, suggesting that the two sites did not function equivalently. In the *ALDA* and *ENO1* genes, sequences outside of the functional HRE were identified that bound HIF-1 *in vitro* but did not contribute to the hypoxic response based upon mutagenesis analysis in transfection assays, indicating that a HIF-1 site is necessary but not sufficient for HRE function. Comparison of the ten HIF-1 binding sites identified within the HREs described above revealed an invariant core sequence 5′-CGTG-3′.

Structural analysis of HIF-1

Protein purification [15] and isolation of cDNA sequences [16] revealed that HIF-1 is composed of two subunits. The HIF-1α

subunit is an 826 amino acid protein whose sequence was not previously reported, whereas the HIF-1β subunit is the protein product of the *ARNT* gene which was previously shown to encode 774 and 789 amino acid isoforms of the aryl hydrocarbon receptor (AHR) nuclear translocator protein [17]. ARNT can therefore dimerize with HIF-1α in cells subjected to hypoxia to form HIF-1 and can also dimerize with AHR in cells exposed to aryl hydrocarbons such as dioxin to form the AHR complex. Additional transcription factors that may utilize ARNT as a dimerization partner include three *Drosophila* bHLH-PAS proteins: single-minded (SIM) [18], similar (SIMA) [19], and trachealess (TRH) [20, 21].

The HIF-1α and HIF-1β (ARNT) proteins share the following structural motifs in common [6, 22]: (a) The bHLH, or basic helix-loop-helix domain, is the hallmark of an extensive superfamily of transcription factors. The HLH domains mediate protein dimerization that is necessary for DNA binding mediated by the basic domains. (b) Whereas the HLH domain is sufficient for dimerization of most bHLH proteins, HIF-1α and HIF-1β (ARNT) contain a second required dimerization domain, PAS, which was originally identified by the presence of related sequences in the *P*ER (which does not contain a bHLH domain), *A*RNT, and *S*IM proteins [17]. All PAS domains contain two internal homology units of approximately 50 amino acids, the A and B repeats, each of which contains an invariant HXXD motif (H, histidine; X, any amino acid; D, aspartate) [16]. For HIF-1α and HIF-1β (ARNT), the HLH and PAS domains together create a functional interface for subunit protein-protein dimerization. (c) The carboxyl half of the HIF-1α and HIF-1β (ARNT) proteins contain one or more potent transactivation domains which are presumed to interact directly or indirectly with components of the transcription initiation complex and thus affect the rate of transcription of genes to which they have bound.

Functional analysis of HIF-1

To demonstrate directly that HIF-1 functions as a transcriptional activator, cultured cells were co-transfected with (a) reporter plasmids containing an HRE from the *ENO1, EPO,* or *VEGF* gene and (b) effector plasmids that allow constitutive expression of HIF-1α and HIF-1β (ARNT) from a cytomegalovirus promoter [6, 9]. Forced expression of HIF-1 activated reporter gene transcription in non-hypoxic cells and caused a superactivation in hypoxic cells. Reporter genes that contained an HRE with a mutation that disrupted HIF-1 binding did not respond to hypoxia or expression of recombinant HIF-1, demonstrating that transcriptional activation required sequence-specific binding of HIF-1. Whereas reporter gene transcription was activated by co-transfected HIF-1α expression vector in a dose-dependent manner, there was no effect of added HIF-1β (ARNT) expression vector, suggesting that HIF-1β (ARNT) is present in excess relative to HIF-1α in both non-hypoxic and hypoxic cells.

To identify HIF-1α sequences required for transactivation of reporter genes in hypoxic cells, we co-transfected a reporter plasmid, an effector plasmid encoding full-length HIF-1β (ARNT) and either full-length or deletion mutants of HIF-1α. Expression of full-length HIF-1α (aa 1-826) resulted in 7- and 29-fold higher levels of reporter gene transcription at 20% and 1% O_2, respectively, than in the absence of expression vectors [6]. Expression of the mutant HIF-1α (aa 1-390) resulted in only 4- and 6-fold increases over control levels at 20% and 1% O_2, respectively. These results indicate that the carboxyl-terminal half of HIF-1α is required for transactivation in hypoxic cells, whereas transactivation in non-hypoxic cells may be partially mediated by other sequences such as the HIF-1β (ARNT) transactivation domain. Analysis of nuclear extracts from transfected cells by gel shift and immunoblot assays revealed that the mutant HIF-1α (aa 1-390) protein was expressed at higher levels than full length HIF-1α, indicating that the deletion specifically affected transactivation [6].

We also constructed an effector plasmid which constitutively expressed a mutant form of HIF-1α that lacked both the basic domain and transactivation domain, such that it could dimerize with HIF-1β (ARNT) but the resulting heterodimer could not bind to DNA or activate transcription. Co-transfection of this dominant-negative mutant form of HIF-1α resulted in a dose-dependent repression of reporter gene transcription in hypoxic cells, thus demonstrating that the endogenous activation of reporter genes in hypoxic cells was also mediated by HIF-1 [6, 9].

HIF-1 expression as a function of cellular oxygen concentration

We performed a series of experiments in collaboration with H. Marti and C. Bauer (Institute of Physiology, Zurich, Switzerland) to determine the relationship between HIF-1 expression and cellular oxygen concentration. HeLa cells were incubated over a range of physiologic and pathophysiologic O_2 concentrations, either in the absence or presence of 1 mM KCN to block oxidative phosphorylation and eliminate any intracellular or extracellular O_2 gradients resulting from O_2 consumption, and nuclear extracts were prepared for gel shift and immunoblot assays. HIF-1α protein, HIF-1β (ARNT) protein, and HIF-1 DNA-binding activity all increased exponentially as cellular O_2 concentrations were decreased from 20% to 0.5% O_2 both in the presence and absence of KCN [23]. For all three parameters, the curves showed a point of inflection at 4 to 5% O_2, the response was half-maximal at 1.5 to 2% O_2, and maximal at 0.5% O_2. The magnitude of the HIF-1α induction was much greater than that of HIF-1β as might be expected given that HIF-1β is present in excess and that HIF-1α is the subunit specific to HIF-1. *In vivo* measurements have revealed that O_2 concentrations in most tissues under normal physiologic conditions are in the range of 2 to 5%, indicating that any decrease in tissue oxygenation would occur along the steep portion of the HIF-1 response curve [24]. If cells *in vivo* respond in a manner similar to HeLa cells in culture, then the induction of HIF-1 expression could provide a means to activate homeostatic transcriptional responses that are proportional to the degree of the inciting hypoxic stimulus.

Expression of HIF-1 in vivo

HIF-1α and HIF-1β mRNAs were detected by blot hybridization in all human tissues assayed, including brain, heart, kidney, lung, liver, pancreas, placenta, and skeletal muscle [24]. In addition, a BLAST search of the expressed sequence tag database (dbEST) identified HIF-1α expression in bone, fetal and adult brain, pancreatic islets, placenta, retina, uterus, and white blood cells. The ubiquitous expression of HIF-1α and HIF-1β mRNA is consistent with the proposed role of HIF-1 in coordinating homeostatic responses to hypoxia throughout the body. To determine whether HIF-1 mRNA expression was induced by hypoxia *in vivo*, rats were exposed to 21% or 7% O_2 for one hour prior to RNA isolation. HIF-1α and HIF-1β mRNAs were detected in

brain, heart, kidney, liver, lung, and spleen [24]. Modest increases in HIF-1 mRNA levels were demonstrated in brain, kidney, and lung tissue from hypoxic rats. In C57BL/6J mice, the basal levels of HIF-1α mRNA were lower than in rats, allowing a clearer demonstration of the induction by hypoxia in brain, kidney, and lung [24].

Genetics of HIF-1

The *ARNT* gene was previously assigned to mouse chromosome 3 and human chromosome 1q21 [25]. *HIF1A* was assigned to human chromosome 14q21-q24 by analysis of somatic cell hybrids and by fluorescence *in situ* hybridization [26]. *Hif1a* was localized by interspecific backcross analysis [26] within a region of mouse chromosome 12 encompassing > 30 cM that demonstrates conservation of synteny with a region of human chromosome 14 extending from *PAX9* at 14q12-q13 to *IGHC* at 14q32.33 [27]. Although the *Ahr* gene also maps to mouse chromosome 12, that locus falls outside the region of conserved synteny and the human *AHR* locus maps to chromosome 2 [reviewed in 27]. Based upon these data, there does not appear to be any clustering of genes encoding bHLH-PAS proteins in the human or mouse genome.

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