Inhibitory PAS Domain Protein (IPAS) Is a Hypoxia-inducible Splicing Variant of the Hypoxia-inducible Factor-3α Locus*

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The inhibitory PAS (Per/Arnt/Sim) domain protein, IPAS, functions as a dominant negative regulator of hypoxia-inducible transcription factors (HIFs) by forming complexes with those proteins that fail to bind to hypoxia response elements of target genes. We have previously observed that IPAS is predominantly expressed in mice in Purkinje cells of the cerebellum and in corneal epithelium of the eye where it appears to play a role in negative regulation of angiogenesis and maintenance of an avascular phenotype. Sequencing of the mouse IPAS genomic structure revealed that IPAS is a splicing variant of the HIF-3α locus. Thus, in addition to three unique exons (1a, 4a, and 16) IPAS shares three exons (2, 4, and 5) with HIF-3α as well as alternatively spliced variants of exons 3 and 6. In experiments using normal mice and mice exposed to hypoxia (6% O2) for 6 h we observed alternative splicing of the HIF-3α transcript in the heart and lung. The alternatively spliced transcript was only observed under hypoxic conditions, thus defining a novel mechanism of hypoxia-dependent regulation of gene expression. Importantly, this mechanism may establish negative feedback loop regulation of adaptive responses to hypoxia/ischemia in these tissues.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF481145, AF481146, and AF481147.

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Mammalian cells adapt to hypoxic conditions through a transcriptional response pathway mediated by the hypoxia-inducible factor-1 (HIF-1) (1). HIF-1 is a heterodimer composed of an α subunit, HIF-1α (2), and the transcription factor Arnt (1). In addition, Arnt dimerizes with the HIF-1α paralogs HIF-2α (3, 4) or HIF-3α (5) in hypoxic cells. Two distinct mechanisms are important for regulation of HIF-1α and HIF-2α activity by oxygen. Under normoxic conditions, HIF-α proteins interact with the von Hippel-Lindau tumor suppressor protein, pVHL (6). pVHL functions as an E3 ubiquitin ligase that targets HIF-α proteins for degradation by the proteasome (7–9). HIF-α-pVHL interaction is dependent upon hydroxylation of critical proline residues within the degradation domain of the HIF-α proteins (10, 11). This posttranslational modification is inhibited under hypoxic conditions, resulting in stabilization of HIF-α protein levels, possibly due to reduced binding of O2 to HIF prolyl hydroxylase enzymes that require Fe(II) and O2 for function (12, 13).

In addition to stabilization of HIF-α protein levels, hypoxia induces the function of the transactivation domains of HIF-α proteins and enhances their ability to interact with transcriptional coactivator proteins (3, 14–16). This interaction has recently been shown to be blocked by hydroxylation under normoxic conditions of a conserved asparagine residue within one of the two transactivation domains of HIF-1α and HIF-2α (17). Asparagine hydroxylation is abrogated under hypoxic conditions (17), and it has been speculated that both the prolyl and asparagine hydroxylases modulating HIF-α function may serve as oxygen sensors in the hypoxia signal transduction pathway. We have previously identified a novel factor, IPAS, that functions as a dominant negative regulator of HIF-α function. IPAS dimerizes with HIF-α proteins and thereby impairs productive interaction between HIF-α and hypoxia response elements of target genes (18). Expression of IPAS in the cornea correlates with low levels of expression of the HIF-1α target gene vascular endothelial growth factor under hypoxic conditions (18). Thus, it is possible that IPAS defines a novel mechanism of negative regulation of angiogenesis and maintenance of an avascular phenotype. Here we demonstrate that IPAS is an alternative splicing product of the HIF-3α locus. Interestingly, accumulation of the IPAS-specific alternative splicing product was hypoxia-inducible in the mouse heart and lung, indicating a previously unknown mode of negative feedback loop regulation of HIF-α-mediated signaling pathways in these tissues.

EXPERIMENTAL PROCEDURES

Data Base Searches—BLAST searches of GenBank™ were performed using the BLAST (19) service at the National Center for Biotechnology Information (NCBI) home page (www.ncbi.nih.gov) using our previously reported mouse IPAS cDNA sequence (18), GenBank™ accession number AF416641.

RNA Preparation and Semiquantitative RT-PCR—Eight-week-old C57Bl/6 mice were exposed to either normoxia or hypoxia (maximally 6% O2) for 6 h, and total RNA samples from various tissues were obtained by the guanidine isothiocyanate method. All animal experiments were approved by the local animal research ethics committee of...
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Fig. 1. Exon organization of the mouse HIF-3α gene. Exons 1a, 4a, and 16 (shaded) are specific for IPAS mRNA, which is also generated by specific portions of exons 3 and 6 (dotted). The basic helix-loop-helix (bHLH), PAS, and transactivation (TAD) domains are indicated.

Fig. 2. Expression of IPAS and HIF-3α mRNAs in mouse heart tissue. IPAS and HIF-3α mRNA expression in mouse heart from control mice (maintained under normoxic conditions (N)) or mice exposed to hypoxia (H) was monitored by RT-PCR using transcript-specific sets of primers (indicated as bars on top of the schematic representations of the transcripts).

Fig. 3. Hypoxia-inducible expression of the IPAS-specific splicing variant of the HIF-3α locus. The use of the IPAS-specific exon 4a was monitored by RT-PCR with various numbers of amplification cycles using exon-specific sets of primers as indicated. RNA was isolated from either heart or lung tissue from control mice (maintained under normoxic conditions (N)) or mice exposed to hypoxia (H). β-Actin mRNA levels were monitored as a reference for semiquantitative analysis.

Fig. 4. Hypoxia-inducible accumulation of the IPAS-specific transcript of the HIF-3α locus. The presence of the IPAS-specific transcript containing a truncated version of exon 6 was monitored. RNA samples from either heart or lung tissue from control mice (maintained under normoxic conditions (N)) and mice exposed to hypoxia (H) were subjected to semiquantitative RT-PCR analysis using exon-specific sets of primers as indicated. β-Actin mRNA levels were monitored as a reference.
Stockholm, Sweden and the Institute of Medical Science, The University of Tokyo, Japan and conducted according to their guidelines. First-strand cDNA was synthesized using an oligo(dT)25 primer and 2 μg of DNase-treated total RNA as a template followed by a semiquantitative PCR analysis for IPAS- and HIF-3α-specific transcript levels relative to β-actin mRNA expression. To ensure that the PCR was in the exponential phase, different PCR cycles (ranging from 27 to 36) were tested, and 33 cycles of amplification were applied in most experiments unless otherwise specified. The identities of the PCR products were confirmed by sequencing. PCR primer pairs and annealing temperatures (ATs) for amplification of the exons were as follows: (i) exons 1a–16 of IPAS: sense (primer1), 5′-AGGCCGACCCCATGGCCTT-3′; antisense (primer2), 5′-TCTTCAGGCTTCCTTGGTC-3′; AT, 58 °C; (ii) exons 1–7 of HIF-3α: sense (primer3), 5′-GGGAGGCTGAGTCTTATG-3′; antisense (primer4), 5′-TCCAAACGCTGATGATTTC-3′; AT, 54 °C; (iii) exon 4a of IPAS: sense (primer5), 5′-GAGGGTTCGTCATGGTACT-3′; antisense (primer6), 5′-TCTTCAGGCTTCCTTGGTC-3′; AT, 49 °C; (iv) exons 6–7 of HIF-3α: sense (primer7), 5′-CAGCTCTAGCAGCATAGAAC-3′; antisense (primer8), 5′-TCCAAACGCTGATGATTTC-3′; AT, 49 °C; and (v) exons 6–16 of IPAS: sense (primer7), 5′-CAGCTCTAGCAGCATAGAAC-3′; antisense (primer9), 5′-AGAGAGGATTCAGTCT-3′; AT, 49 °C.

RESULTS AND DISCUSSION

**IPAS Is an Alternative Splicing Product of the HIF-3α Locus**—Sequencing of mouse genomic DNA revealed that the IPAS mRNA species contains a unique first exon (GenBank™ accession number AF481145) but shares exon 2 with HIF-3α (Fig. 1). We have therefore used the exon numbering of the HIF-3α locus (5) and termed the first exon of IPAS exon 1α of the HIF-3α locus. In addition to exon 1α, IPAS mRNA contains the unique exons 4α (GenBank™ accession number AF481146) and 16 (GenBank™ accession number AF481147). Moreover, a mechanism of IPAS pre-mRNA splice site selection in exon 3 uses an alternative 3′ splice site 14 nucleotides downstream of the HIF-3α 3′ splice site. In a similar fashion in IPAS mRNA exon 6 an alternative 5′ splice site located 87 nucleotides upstream of the HIF-3α 5′ splice site is used (Fig. 1). The inclusion of exon 4α together with the use of the alternative 3′ splice site in exon 3 during IPAS mRNA processing results in a reading frame shift that determines a unique feature of IPAS. In conclusion, the IPAS mRNA is a product of alternative splicing of the HIF-3α locus.

**Accumulation of the IPAS-specific Splicing Product of the HIF-3α Locus Is Hypoxia-inducible**—We next used primers specific for either IPAS or HIF-3α mRNAs (schematically represented in Fig. 2) to monitor by RT-PCR analysis mRNA expression of these two mRNA species in heart tissue from control mice or mice exposed to hypoxia (6% O2) for 6 h. In agreement with earlier observations using RNA blot analysis (18) we observed that hypoxia induces IPAS mRNA expression levels (Fig. 2). Interestingly, this analysis also indicated a corresponding down-regulation of HIF-3α mRNA levels (Fig. 2). RT-PCR analysis of RNA isolated from several control and hypoxic mouse tissues using a number of primers specific for the 5′ and 3′ untranslated regions of IPAS confirmed that we have obtained the full-length IPAS reading frame (18), and, importantly, in similar analyses we have not detected any form of mouse HIF-3α mRNA that is generated by transcription of exon 1α.

We next monitored the use of the IPAS-specific exon 4α in transcripts isolated from control or hypoxic mouse heart and lung tissues. As shown in Fig. 3, a transcript containing the IPAS-specific exon 4α was detected in these tissues only following exposure of the mice to hypoxia. The analysis was performed following different cycles of PCR amplification, demon-
following exposure of mice to rather severe hypoxia, i.e., splicing product of the HIF-3α/H9251 locus in the Mouse Heart—To identify the degree of hypoxia required to induce accumulation of the IPAS-specific splicing product of the HIF-3α gene in mouse heart, mice were exposed for 6 h to increasing degrees of hypoxia ranging from 18 to 6% O2. The IPAS-specific splicing product was first observed following exposure of mice to rather severe hypoxia, i.e. 8–6% O2 (Fig. 5).

In summary, our results demonstrate that the dominant negative regulator of HIF-α function, IPAS, is generated by alternative splicing of the HIF-3α locus. Obviously, our data do not preclude the existence of intermediate splicing variants. In fact, as indicated by a comprehensive search of expressed sequence tag (EST) data bases there are human HIF-3α transcript variants that include the IPAS-specific exon 1a and also contain the IPAS-specific variant of exon 3 but lack the IPAS-specific exon 4a (Fig. 6). It is presently premature to conclude whether these EST sequences reflect splicing intermediates or fully processed mature transcripts. Against this background, it will be important to perform a careful genetic analysis in mice to determine the role of IPAS and possibly HIF-3α in regulation of hypoxia signaling.

Interestingly, accumulation of the IPAS-specific alternative splicing product of the HIF-3α locus is hypoxia-inducible in mouse heart and lung tissues. During maintenance of mice under normoxic conditions, IPAS mRNA is expressed in a very tissue-restricted manner with readily detectable levels only found in the Purkinje neurons of the cerebellum and the cornea epithelium (18). In the case of the latter tissue, the function of IPAS appears to provide a strategy of negative regulation of vascular endothelial growth factor gene expression and angiogenesis. This mode of negative regulation of HIF-1α function is important for the avascular phenotype of the cornea (18). Hypoxia-inducible alternative splicing of the HIF-3α locus resulting in hypoxia-inducible IPAS mRNA expression in the heart and lung suggests that IPAS may modulate hypoxia- or ischemia-dependent adaptive gene regulatory responses in these tissues as well. Thus, in these tissues, hypoxia-inducible accumulation of the IPAS-specific alternative splicing product of the HIF-3α locus not only defines a novel mechanism of hypoxia-dependent gene regulation but also a potential mechanism of negative feedback loop regulation of HIF-α-mediated signaling pathways, which may be of considerable medical interest.

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