Transcriptional Up-regulation of Inhibitory PAS Domain Protein Gene Expression by Hypoxia-inducible Factor 1 (HIF-1)

A NEGATIVE FEEDBACK REGULATORY CIRCUIT IN HIF-1-MEDIATED SIGNALING IN HYPOXIC CELLS*

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The inhibitory PAS (Per/Arnt/Sim) domain protein (IPAS), a dominant negative regulator of hypoxia-inducible transcription factors (HIFs), is potentially implicated in negative regulation of angiogenesis in such tissues as the avascular cornea of the eye. We have previously shown IPAS mRNA expression is up-regulated in hypoxic tissues, which at least in part involves hypoxia-dependent alternative splicing of the transcripts from the IPAS/HIF-3α locus. In the present study, we demonstrate that a hypoxia-driven transcriptional mechanism also plays a role in augmentation of IPAS gene expression. Isolation and analyses of the promoter region flanking to the first exon of IPAS gene revealed a functional hypoxia response element at position −834 to −799, whereas the sequence upstream of the HIF-3α first exon scarcely responded to hypoxic stimuli. A transient transfection experiment demonstrated that HIF-1α mediates IPAS promoter activation via the functional hypoxia response element under hypoxic conditions and that a constitutively active form of HIF-1α is sufficient for induction of the promoter in normoxic cells. Moreover, chromatin immunoprecipitation and electrophoretic mobility shift assays showed binding of the HIF-1 complex to the element in a hypoxia-dependent manner. Taken together, HIF-1 directly up-regulates IPAS gene expression through a mechanism distinct from RNA splicing, providing a further level of negative feedback gene regulation in adaptive responses to hypoxic/ischemic conditions.

Cellular adaptation to hypoxic conditions is accompanied by changes in expression of a panel of genes encoding physiologically relevant proteins (1–3). These genes have been shown to contain hypoxia response elements (HREs)2 in their promoter regions. Under hypoxic conditions the response elements are recognized by a hypoxia-inducible transcription factor (HIF)-1, a heterodimeric complex of the basic helix-loop-helix PAS (Per/Arnt/Sim) domain proteins HIF-1α and HIF-1β (Arnt) (4). In addition, the HIF-1α paralogs HIF-2α (5, 6) and HIF-3α (7) dimerize with Arnt in hypoxic cells to form DNA-binding complexes. Two distinct mechanisms are important for regulation of HIF-1α and HIF-2α activity by oxygen. Under normoxic conditions, HIF-α is targeted by the von Hippel-Lindau protein (pVHL) for ubiquitylation and rapid proteasomal degradation (8, 9). pVHL binding is mediated through hydroxylation of specific prolyl residues located in the central region of HIF-α proteins. Hydroxylation at the 4-position of those prolines of HIF-α enables formation of two hydrogen bonds to pVHL and increases the binding of pVHL to HIF-α by several orders of magnitude (10). This post-translational modification of HIF-α is catalyzed by HIF-prolyl hydroxylases, a set of dioxygenases that require O2, Fe(II), and 2-oxoglutarate as cosubstrates. At low oxygen levels, there is a corresponding decrease in prolyl hydroxylation of HIF-α, resulting in release of pVHL and stabilization of HIF-α protein (11, 12). Stabilized HIF-α then translocates to the nucleus where it dimerizes with Arnt to bind to the HREs of target genes (13). Recently, a similar mode of regulation of HIF-3α at the protein expression level has been suggested (14).

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 (5, 15, 16). Under normoxic conditions this interaction is blocked by hydroxylation of a conserved form of HIF-1α that is not recognized by pVHL, which, in turn, is stabilized. Therefore, stabilization of HIF-1α by hypoxia further augments the activity of HIF-1 complex to the element in a hypoxia-dependent manner.

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2 The abbreviations used are: HRE, hypoxia response element; IPAS, inhibitory PAS domain protein; HIF, hypoxia-inducible factor; Arnt, arylhydrocarbon receptor nuclear translocator; VEGF, vascular endothelial growth factor; MBEC, mouse brain endothelial cell(s); EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; HBS, HIF-1-binding site; CMV, cytomegalovirus; RT, reverse transcription; PGC-1, peroxisome proliferator-activated receptor γ coactivator-1; ChIP, chromatin immunoprecipitation; PBS, phosphate-buffered saline; nt, nucleotide(s).
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asparagine residue within C-terminal transactivation domain of HIF-α (17). Asparagine hydroxylation is abrogated under hypoxic conditions (17), allowing HIF-α to recruit coactivators. Therefore, 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 (18).

As yet another determinant of cellular responsiveness to hypoxia, we have identified IPAS, a dominant negative regulator of HIF-α function (19). IPAS targets HIF-α subunits to form a complex lacking binding activity to the HRE and thereby impairs HIF target gene expression under hypoxic conditions. High expression of IPAS was found in the cornea, which correlates with low level of hypoxia-inducible VEGF expression and an avascular phenotype in this tissue. Thus, IPAS defines a novel mechanism of negative regulation of angiogenesis and tissue-specific control of responsiveness to hypoxic conditions (19). Interestingly, sequencing of the mouse IPAS genomic structure revealed that IPAS is a splicing variant of the HIF-3α locus (20).

An important physiological aspect of IPAS is its hypoxia-inducible expression. In tissues including brain, heart, lung, and skeletal muscle of mice, expression of IPAS mRNA is up-regulated following exposure to hypoxia, suggesting the presence of a negative feedback mode of regulation of hypoxia-inducible gene expression (19). Interestingly, such hypoxia-inducible expression of IPAS is supported at least in part by an alternative splicing mechanism that is observed exclusively under hypoxic conditions in these tissues (20).

Here we demonstrate that IPAS promoter activity is also induced by hypoxia. Functional analyses of the promoter region of IPAS/HIF-3α gene revealed an HRE mediating up-regulation of IPAS gene transcription under hypoxic conditions. HIF-1 binds to the cis-element and plays an essential role in transactivation of the IPAS gene. Thus, the IPAS-mediated negative feedback regulatory circuit in HIF-1-dependent gene regulation involves both hypoxia-dependent alterations in the splicing pattern of IPAS/HIF-3α transcripts as well as transcriptional activation of the IPAS-specific promoter.

EXPERIMENTAL PROCEDURES

Plasmids—For construction of a series of IPAS or HIF-3α promoter-driven luciferase reporter plasmids, various lengths of DNA fragments from the 5′-flanking region of IPAS exon 1a or HIF-3α exon 1 were amplified on mouse genomic DNA templates by PCR with flanking 5′-Kpn1 and 3′-Nco1 enzyme restriction site using Platinum pfx DNA polymerase (Invitrogen). The PCR products were enzyme-digested then cloned into the Kpn1-Nco1 site of pGL3-Basic vector (Promega). Oligonucleotides corresponding to the sequence of the hypoxia-responsive 35-bp region of the IPAS promoter were synthesized and two or three copies of them were inserted in front of luciferase gene to generate reporter plasmids pIPAS/HRE 2x and pIPAS/HRE 3x, respectively. A hypoxia-inducible luciferase reporter containing three tandem repeat of HRE from the erythropoietin gene enhancer, pT811uc/HRE, HIF-1α, or Arnt expression plasmid pCMV4-HIF-1α or pCMV4-Arnt, respectively, and the expression plasmid pcDNA3 HIF-1α/1–396/VP16 encoding a constitutively active form of HIF-1α, have been described elsewhere (19, 21). To generate an IPAS/HIF-3α mini-gene plasmid, a genomic region containing exon 3, exon 4a, exon 4, and corresponding introns was cloned by PCR using bacterial artificial chromosome encompassing cognate mouse genomic fragment as a template and then ligated into pcDNA-3 vector (Invitrogen).

Cell Culture—Primary mouse brain endothelial cells (MBEC) were kindly supplied by Dr. Yihai Cao (Karolinska Institutet) and maintained in Ham’s F-12 medium (Invitrogen) containing 10% fetal calf serum and antibiotics. Mouse hepatoma Hepa-1c1c7 cells were obtained from ATCC and cultured in α-minimum essential medium supplemented with 10% fetal calf serum and antibiotics. MBEC stably integrated with IPAS/HIF-3α mini-gene was established by lipocytation transfection and subsequent selection of the cells by the antibiotics G418 (Promega).

Isolation of Tissue Total RNA—Male C57Bl6 mice (8 weeks old) were exposed to either normoxic (21%) or hypoxic (6%) conditions for 6 h and then sacrificed by cervical dislocation, and the organs were removed. Total RNA was isolated from frozen tissues using RNA WIZ reagent (Ambion). All of the animal experiments were approved by the local animal research ethics committee of Institute of Medical Science, The University of Tokyo, Japan, and conducted according to their guidelines.

RNase Protection Assays—To generate the templates for an RNA probe, PCR was performed on mouse genomic DNA template using Platinum pfx DNA polymerase (Invitrogen) with the following primers; forward primer, 5′-TCTAGACCTCTCCTCTTCCTCGGA-3′; reverse primer, 5′-AAGCTTACGGCCTGACCGCCCAA-3′). The PCR product was digested with HindIII and Xba1 and subcloned into the corresponding site of pcDNA3. The RNA probe was transcribed on the Xba1-digested templates by T7 RNA polymerase-based MAXI script in vitro transcription kit (Ambion) incorporating 32P-labeled UTP. The full-length 202-bp riboprobe was separated and isolated on a denaturing polyacrylamide gel. The assay was performed using RPA III assay kit (Ambion). Briefly, the probe was coprecipitated with 50 µg of total RNA from the normoxic/hypoxic tissues, dissolved in hybridization buffer, and incubated at 42 °C for 18 h, followed by the digestion with the mixture of RNase A and T1. The protected RNA fragments were precipitated, denatured, then separated on an acrylamide gel, and visualized by autoradiography.

RT-PCR—For synthesis of first strand cDNA, 2 µg of DNase-treated total RNA from mouse tissues were reversely transcribed by Superscript II reverse transcriptase (Invitrogen) using oligo(dT)18 primers, according to the manufacturer’s protocol. PCR amplification using 1 µl of the first strand cDNA as a template was carried out in a total volume of 30 µl of mixture composed of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 µM dNTPs, 0.25 µM each of the sense and antisense primers, 1 unit of Ex Taq DNA polymerase (TaKaRa, Japan). Amplification by 33 cycles of a step program (94 °C for 30 s, 51 °C for 30 s, and 72 °C for 1 min) was performed after 3 min of denaturing of the samples at 94 °C. The identities of the PCR products were confirmed by sequencing. Oligonucleotide PCR primers were as follows: exons 1a of IPAS, forward
Hypoxia increases IPAS gene transcription. A, accumulation of the transcripts containing IPAS exon 1a in the heart of the mouse under hypoxic condition. The amount of the mRNA carrying IPAS exon 1a and its upstream sequence in the heart either from control mouse (maintained under normoxic conditions, N) or mouse exposed to hypoxia (H) was determined by RNase protection assay. β-Actin mRNA levels were monitored as a reference for semi quantitative analysis. B, RT-PCR analysis of the expression level of IPAS or HIF-3α-specific transcript. The presence of either IPAS-specific transcript containing exon 1a-exon 2 junction or HIF-3α-specific transcript demonstrating exon 1a-exon 2 connection in the same RNA samples as A was monitored by RT-PCR analysis using the exon-specific sets of primers.
primers flanking the putative HRE within the IPAS gene promoter: forward, CAATAAATCCATTTCTGCCGCA, and reverse, GAGAGGGCGTGGACACTAAGGA. Primers flanking the HRE of the VEGF gene promoter were used as a positive control: forward, AACAAGGGCCTCTGTCTGCCCA, and reverse, TTGTGGCACTGAGAACGGGGGT.

RESULTS

Hypoxia-dependent Up-regulation of IPAS Gene Transcription—To examine the involvement of the IPAS promoter in hypoxia-inducible IPAS gene expression, we performed RNase protection assays for determination of the level of mRNA containing exon 1a of the IPAS/HIF-3α gene. Total RNA extracted from the heart of mice either exposed to normoxic or hypoxic conditions was hybridized with an RNA probe encompassing IPAS exon 1a and its 138-base upstream sequence and then subjected to RNase digestion. The protected mRNA fragment corresponding to the transcript spanning from nt 1 to 62 of exon 1a was readily detected in RNA samples from normoxic mice, indicating basal transcriptional levels of the IPAS gene (Fig. 1A). In the heart from mice exposed to hypoxic conditions, the amount of the protected fragment containing IPAS exon 1a was clearly increased, whereas the level of β-actin mRNA was constant, demonstrating that the transcripts initiated upstream of IPAS exon 1a were specifically up-regulated by hypoxia (Fig. 1A). In excellent agreement with these observations, RT-PCR analysis using primers recognizing IPAS exon 1a and 2 showed up-regulation of the content of exon 1a in the transcripts isolated from hypoxic mouse heart tissue. Interestingly, the same RT-PCR analysis employing a primer pair specific for HIF-3α exon 1 showed only a modest increase of the transcript containing HIF-3α exon 1 (Fig. 1B).

Taken together, these data indicate that hypoxia specifically up-regulates transcription initiating in an upstream of IPAS exon 1a of the mouse HIF-3α.

Localization of the Sequence-mediating Hypoxia-dependent Activation of the IPAS Promoter—To further investigate the mechanism of hypoxia-dependent activation of the IPAS promoter, we generated and cloned into a luciferase expression vector a series of truncated fragments of the 5′-flanking region of the IPAS gene (Fig. 2A). MBEC were transiently transfected with these reporter plasmids and cultured either under normoxic (21% O2) or hypoxic (1% O2) conditions prior to monitoring of cellular luciferase activity (Fig. 2B). Cells transfected with pIPAS/-350 or with pIPAS/-678 luciferase reporter carrying 350- or 678-bp-long segments of the IPAS gene upstream
from the ATG in exon 1a, respectively, failed to show an increase of luciferase expression in response to hypoxia. Reporter constructs containing longer fragments of the IPAS promoter such as pIPAS/-1061, pIPAS/-1220, pIPAS/-1855, pIPAS/-2205, pIPAS/-2748, and pIPAS/-3548, produced similarly low levels of cellular luciferase activity under normoxic conditions. In contrast, under hypoxic conditions, these reporter plasmids showed 2–3-fold higher expression of luciferase, as compared with normoxic cells, indicating that a region encompassing a region between positions −678 and −1061 of the IPAS promoter (Fig. 2B). On the other hand, luciferase reporters fused to DNA fragments encompassing −1.5, 2.7, and 3.5 kb upstream of HIF-3α exon 1 showed only basal levels of luciferase expression even following exposure to hypoxic conditions. Thus, in agreement with the lack of hypoxia-dependent generation of transcripts spanning HIF-3α exon 1, these data suggest that the HIF-3α promoter is not inducible by hypoxia (Fig. 2B). In support of this conclusion, experiments similarly conducted in a similar fashion in a different cell line, Hepa1c1c7 cells demonstrated the same location of the hypoxia-responsive region of the IPAS promoter, i.e. a location between nt −1061 and −678. Moreover, no hypoxia-dependent induction of the HIF-3α promoter was observed in these cells (Fig. 2C). Taken together, these data demonstrated that this sequence localized upstream of IPAS exon 1a mediates hypoxia-dependent induction of IPAS gene transcription.

Effect of HIF-1 on Promoter Activity of the IPAS Gene—To determine the involvement of HIF-1 in the hypoxia-dependent increase of IPAS promoter activity, the same set of luciferase reporters containing IPAS promoter sequences was cotransfected with a HIF-1α expression plasmid into MBEC. It has been described that overexpression of wild type HIF-1α by transient transfection induces HRE-regulated transcription to some extent even under normoxic conditions (23, 24), possibly because of saturation of endogenous pVHL levels and ensuing stabilization of the HIF-1α protein (9). Reporter gene activities by pIPAS/-350 and pIPAS/-678 were not influenced by coexpression of HIF-1α under normoxic culture conditions and not increased upon exposure to hypoxia (Fig. 3A). In contrast, activity of reporter constructs such as pIPAS/-1061, pIPAS/-1220, pIPAS/-1855, pIPAS/-2205, pIPAS/-2748, and pIPAS/-3548 were clearly augmented following coexpression of HIF-1α under normoxic conditions and further increased by hypoxia (Fig. 3A). On the other hand, overexpression of Arnt in the presence of the same panel of reporter plasmids (pIPAS/-1061, pIPAS/-1220, pIPAS/-1855, pIPAS/-2205, pIPAS/-2748, and pIPAS/-3548) in MBEC resulted neither in up-regulation of basal luciferase activity under normoxic conditions nor in further hypoxia-dependent enhancement of the activity of the reporter genes (Fig. 3B). Taken together, IPAS promoter activity was increased by overexpression of HIF-1α, and the region mediating the effect of HIF-1α coincided with the locus responsible for hypoxia-inducible induction of the IPAS promoter.

Determination of the Functional Hypoxia Response Element in the IPAS Gene Promoter—To precisely map the cis-acting element mediating hypoxia-dependent IPAS promoter activation, we generated another set of luciferase reporters carrying different lengths of DNA fragments containing the 5′-flanking region of the IPAS gene and performed transient transfection assays normalized to SV40 early enhancer/promoter-generated Renilla-derived luciferase activities. MBEC transfected with pIPAS/-771 and pIPAS/-799 showed similar levels of modest luciferase activity either under normoxic (21% O2) or hypoxic (1% O2) culture was measured. The means ± S.D. of triplicated experiments are shown.

![Figure 3. Augmentation of the promoter activity of IPAS gene by coexpression of HIF-1α](image-url)

**A** Activation of IPAS Gene Transcription by HIF-1α

| Plasmid          | 21% O2 | 1% O2 |
|------------------|--------|-------|
| pIPAS/-350       |        |       |
| pIPAS/-678       |        |       |
| pIPAS/-1061      |        |       |
| pIPAS/-1220      |        |       |
| pIPAS/-1855      |        |       |
| pIPAS/-2205      |        |       |
| pIPAS/-2748      |        |       |
| pIPAS/-3548      |        |       |

**B** Effect of HIF-1 on Promoter Activity of the IPAS Gene

| Plasmid          | 21% O2 | 1% O2 |
|------------------|--------|-------|
| pIPAS/-350       |        |       |
| pIPAS/-678       |        |       |
| pIPAS/-1061      |        |       |
| pIPAS/-1220      |        |       |
| pIPAS/-1855      |        |       |
| pIPAS/-2205      |        |       |
| pIPAS/-2748      |        |       |
| pIPAS/-3548      |        |       |

The means ± S.D. of triplicated experiments are shown.
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this 35-bp region in front of the luciferase gene produced transcriptional response to hypoxia in a copy number-dependent manner (Fig. 4C). Moreover, removal of the sequences carrying the 35-bp region from hypoxia-responsive pIPAS/−1220, generating a reporter construct named pIPAS/−1220(Δ−891/−799), pIPAS/−1220(Δ−845/−799), and pIPAS/−1220(Δ−834/−799), resulted in loss of the hypoxia-dependent induction response (Fig. 4D). These data demonstrate that the 35-bp region of the IPAS promoter is critical for induction under hypoxic conditions. To further characterize the hypoxia-responsive 35-bp element of the IPAS promoter, we tested the effect of HIF-1α on the function of the region. To this end, we employed the expression plasmid for the constitutively active form of HIF-1α in reporter gene assays using IPAS promoter-luciferase constructs. As a constitutively active form of HIF-1α, we used a chimeric protein of truncated HIF-1α (lacking the degradation domain and endogenous transactivation function) fused to the activation domain of the viral transcription factor VP16: HIF-1α/1–396/VP16. This fusion protein has been shown to activate HRE-driven luciferase gene expression even under normoxic conditions, thus allowing us to test the direct effect of HIF-1α without the influence of other signaling pathways in the hypoxic cells (25). The IPAS promoter-luciferase reporter genes pIPAS/−834, pIPAS/−1061, and pIPAS/−1220 were induced by HIF-1α/1–396/VP16 in normoxia to levels comparable with those observed under hypoxic conditions. In contrast, neither pIPAS/−771 nor pIPAS/−799 responded to coexpression of HIF-1α/1–396/VP16, indicating that the 35-bp element between −834 and −799 is targeted for regulation by HIF-1α (Fig. 4E). In an excellent agreement with these data, the reporter construct lacking the 35-bp element, pIPAS/−1220(Δ−834/−799), failed to respond to constitutively active HIF-1α.

**HIF-1 Binds to the Hypoxia-responsive cis-Element of the IPAS Promoter**—To demonstrate binding of HIF-1 to the hypoxia-responsive region of the IPAS promoter, we performed EMSA using a labeled 35-bp oligonucleotide probe together with nuclear extracts of MBEC cultured either under normoxic or hypoxic conditions (Fig. 5). As shown in previous studies, when 32P-labeled oligonucleotide corresponding to the HRE in the 3′enhancer of erythropoietin (Epo) gene was incubated with nuclear extracts of MBEC treated under hypoxic conditions, a more slowly migrating band was observed, indicating protein-HRE complex formation (Fig. 5, lanes 3–7). Competition by unlabeled oligonucleotides showed that formation of the DNA-protein com-
sequence abolished complex formation, demonstrating the sequence specificity of the formed complex (lane 11). Moreover, addition of the antibody against HIF-1α shifted the complex to the more slowly migrating one, and an antibody for Arnt altered the amount of formed complex, indicating that HIF-1α and Arnt in the hypoxic nuclear extract generated the protein complex binding to the hypoxia-responsive element of the IPAS promoter (lanes 14 and 15). On the other hand, a 35-bp region next to the putative HRE of the IPAS promoter, i.e. a region spanning the promoter sequence −870 to −835, failed to generate any specific protein-DNA complexes even with the hypoxic nuclear extracts, indicating the absence of a HIF-1-binding site (HBS) at this region of the IPAS promoter (lanes 17–20). The hypoxia-responsive 35-bp element of the IPAS promoter contains three classical HBS-like motifs: the region from −810 to −805, −813 to −808, and from −821 to −816 (Fig. 6A, indicated by arrows). To determine the HIF-1-binding sequence of the IPAS promoter, we generated a panel of mutant 35-bp oligonucleotides in which adenine triplets substitute for half-sites of the HBS-like motifs (Fig. 6A) and performed EMSA using those mutants as probes. Oligonucleotide probes carrying consensus 18-nt Epo-HRE or wild type 35-bp HRE of the IPAS promoter formed protein-DNA complexes with the nuclear extracts from MBEC exposed to hypoxic conditions. Mutations of the proximal (−810 to −805 indicated by box) HBS-like motif, resulting in M1, M3, and M5 probes, diminished complex formation, whereas mutations of the distal (−821 to −816) HBS-like motif (M2 and M4 probes) did not influence the DNA binding activity in the hypoxic nuclear extract, demonstrating that HIF-1 binds to the proximal HBS-like motif in IPAS promoter located at positions −810 to −805 of the IPAS promoter (Fig. 6B). Finally, to directly test whether endogenous HIF-1α binds to the HREs of the IPAS gene promoter in vivo, we performed a ChIP assay using MBEC cultured under either normoxic or hypoxic conditions. VEGF represents a HIF-1α target gene with a well characterized HRE in its promoter region thus serving as a positive control. Following cross-linking, an anti-HIF-1α antibody was used to precipitate HIF-1α protein, and the amount of coprecipitated HRE-containing genomic DNA fragments of the IPAS and the VEGF promoter was assessed by PCR. Genomic DNA fragments prior to immunoprecipitation generated similar signals from hypoxic and normoxic MBEC with specific primer pairs either for the VEGF or for the IPAS promoter, indicating optimum sonication and equal input of genomic DNA (Fig. 7, lanes 1 and 2). When precipitated with control rabbit polyclonal antibodies, DNA fragments from both nor-

**FIGURE 5.** HIF-1 binds to the hypoxia-responsive cis-element in the promoter region of IPAS gene. Nuclear extracts (NE) from MBEC were prepared after incubation under either normoxic (N) or hypoxic (H) condition for 6 h. 32P-Labeled oligonucleotide probes encompassing HRE in 3′-enhancer of erythropoietin gene (Epo W18), 35 bp between −834 and −799 nt upstream of the IPAS exon 1a (IPAS/−834/−799), and 28 bp between positions −780 and −835 of IPAS gene (IPAS/−870/−835) were mixed with the nuclear extracts, and the formed protein-DNA complexes were separated on 4% polyacrylamide gel. Molar excess of unlabeled Epo W18 or IPAS/−834/−799 oligonucleotide was used as a specific competitor (S), respectively, and unlabeled randomized 20-mer oligonucleotide was used for nonspecific competition (NS). Anti-HIF-1α or -Arnt antibody was employed for supershift experiments. C, DNA-protein complex; F, free probe.

**FIGURE 6.** Determination of HIF-1-binding site in the hypoxia response region of the IPAS promoter. A, sequence of oligonucleotide probes corresponding to the hypoxia response 35-bp region of the IPAS promoter (Wt) and its various substitution mutants (M1–M5). The hypoxia response region of IPAS promoter contains classical HBS-like motifs (indicated by arrows). Substituted nucleotides in mutant probes are indicated by shading. B, proximal HBS-like motif is involved in HIF-1 binding to the IPAS promoter. Nuclear extracts (NE) from hypoxic MBEC was incubated with 32P-labeled wild type (Wt) or mutant probes (M1–M5) as indicated in A, and protein-DNA complex formation was monitored by polyacrylamide gel electrophoresis. Epo W18 probe was used as positive control for HIF-1-DNA complex formation. C, DNA-protein complex; F, free probe.
moxic and hypoxic MBEC failed to demonstrate any signals (Fig. 7, lanes 3 and 6). In contrast, anti-HIF-1α antibody enriched HRE-containing IPAS and VEGF genomic DNA fragments from hypoxia-exposed cells (Fig. 7, lanes 4 and 5), suggesting that these sequence elements were occupied by HIF-1α protein in hypoxic cells.

**FIGURE 7.** HIF-1α binds to the hypoxia response region of the IPAS promoter in vivo. CHIP assay using MBEC cultured under either normoxic (21% O₂, N) or hypoxic (1% O₂, H) conditions for 6 h. The cells were incubated with formaldehyde for cross-linking, and then DNA was extracted and immunoprecipitated (IP) with anti-HIF-1α antibody (α-HIF-1α) or rabbit polyclonal immunoglobulins (lg). After reversal of cross-linking, DNA was recovered and used in PCR for 35 cycles. Primers flanking the hypoxia-responsive region of the IPAS promoter or the HRE of the VEGF gene enhancer were included in PCRs and generated a 103- or 163-bp product, respectively. For input, DNA from the extract prior to immunoprecipitation (−) was used.

**DISCUSSION**

Here we have demonstrated that IPAS, a dominant negative regulator of hypoxia-inducible transcription factors, is transcriptionally up-regulated under hypoxic conditions. HIF-1 binds to the hypoxia response element in the promoter region of the IPAS gene to mediate hypoxia-dependent activation of gene transcription, thus constituting a negative feedback regulatory loop of HIF-1-dependent gene regulation.

We have previously shown that IPAS mRNA is up-regulated in certain tissues including brain, heart, lung, and skeletal muscle of the mice exposed to hypoxic conditions (19). In such tissues of hypoxic animals, expression of IPAS mRNA dominated over the accumulation of its splicing variant HIF-3α, indicating an involvement of an alternative splicing mechanism in hypoxia-dependent augmentation of IPAS mRNA expression (20). In addition to altered mRNA splicing, we have observed by RNase protection and RT-PCR assays in the same tissues (e.g., heart and lung) of hypoxic mice up-regulation of transcripts initiated upstream of IPAS-specific exon 1a, suggesting that hypoxia-dependent increase of IPAS mRNA levels may also depend on IPAS promoter activation. In fact, isolation and analysis of the IPAS gene promoter by means of luciferase reporter gene assays revealed hypoxia-dependent activation of the promoter. These data suggest that two distinct hypoxia-dependent mechanisms contribute to up-regulation of IPAS
expression under hypoxic conditions, providing the cells with a finely tuned mechanism of regulation of hypoxia responsiveness. In contrast, transcription from the 5’-flanking region of the HIF-3α-specific exon 1 was not up-regulated under hypoxic conditions. Moreover, we have previously shown that HIF-3α-type splicing products were not increased in any of the examined tissues of mice exposed to hypoxia (20). These observations indicate that the central mechanism for regulation of HIF-3α expression may not involve the control of mRNA levels and thus is distinct from the mode of regulation of IPAS expression. In support of this notion, HIF-3α has been shown to be regulated at the protein level by oxygen tension via the ubiquitin-proteasomal pathway targeting the HIF degradation domain (14), which is absent in the IPAS protein. Interestingly, a previous analysis of gene expression profiles of pulmonary arterial endothelial cells demonstrated up-regulation of HIF-3α by hypoxia or by a constitutively active form of HIF-1α (28). However, the microarray analysis in the study used a probe set for HIF-3α recognizing three different isoforms of the gene products of the HIF-3α/IPAS locus, and two of those isoforms contain IPAS exon 1a-type first exon. Therefore, it is possible that the results were confounded by hypoxia-dependent up-regulation of IPAS promoter-driven transcripts. Taken together, such a variety of distinct mechanisms for regulation of the products of the HIF-3α/IPAS locus demonstrate the complexity in regulation of cellular hypoxia responsiveness via hypoxia-inducible transcription factors.

In the sequential deletion analysis of the IPAS promoter, we found a colocalization of the functional sequence mediating the hypoxia inducibility of the promoter and two classical HRE-like sequences. These sequences do not show a complete match to the conventional core HRE, A/GCGTG, found in enhancer/promoter regions of HIF-1 target genes such as the Epo or VEGF genes (22, 29, 30). However, it has been shown that some degenerative HRE-like sequence are capable of mediating hypoxia-inducible gene transcription (31, 32). In fact, sequence-specific binding by the HIF-1 complex to this hypoxia-responsive 35-nt sequence in the hypoxic cells was detected both in vivo and in vitro by ChIP assay and by EMSA, respectively. In addition, EMSA employing mutated 35-nt probes demonstrated HIF-1 binding to the proximal HRE-like sequence. Therefore, we conclude that hypoxia-dependent activation of IPAS promoter involves the HRE-like motif and the transcription factor HIF-1. In strong support of this model, expression of a constitutively active form of HIF-1α by-passed the requirement of hypoxic stimuli for activation of the IPAS promoter and resulted in an induction of reporter gene expression under normoxic conditions. Moreover, removal of the HRE-like sequence motif from the reporter construct decreased this responsiveness to constitutively active HIF-1α to activate gene expression, suggesting that the presence of both the transcriptionally active HIF-1α and the HRE-like sequence is essential for IPAS promoter activation. Since an elevation of IPAS levels leads to negative regulation of the HIF-1 function (19), we propose that HIF-1 itself participates in the negative feedback loop for regulation of hypoxia responsiveness via IPAS. In analogy to our results, it has recently been shown that HIF-1-mediated gene transcription is involved in the enhancement of HIF-1α degradation pathways, which may lead to negative regulation of HIF-1-mediated signal transduction (33–35). Interestingly, besides HIF-1α, transcription-mediated negative feedback regulation has been demonstrated for several conditionally regulated transcription; NF-κB directly activates transcription of its inhibitory molecule IκB for termination of NF-κB activity (36–38); the circadian rhythm regulator Clock/brain muscle Arnt-like factor 1 heterodimer induces another set of clock genes, Period and Cryptochrome, to form an inhibitory complex with Clock for resetting the expressed protein profile in the central clock region of the brain (39, 40), and the arylhydrocarbon receptor induces transcription of the arylhydrocarbon receptor repressor gene in certain tissues, which may lead to a modulation of their detoxification capacity in affected tissues (41). Therefore, such a mode of transcriptional feedback regulation might define an important mechanism for regulation of the fundamental physiology of higher organisms.

It has recently been proposed that transcription and RNA splicing are highly coordinated processes both at the functional and structural levels (27, 42). One well characterized example of such a mode of coordination of distinct gene regulatory mechanisms is the transcriptional coactivator PGC-1; while coactivating peroxisome proliferator-activated receptor γ target gene transcription, PGC-1 promotes splicing of the transcribed RNA. Importantly, the ability of PGC-1 to function in RNA processing appears to require its prior interaction with the promoter region of that gene (26). In a similar fashion, because hypoxic conditions recruit HIF-1 to the promoter of IPAS gene and at the same time enhance the alternative splicing of IPAS (20), one may expect a link between occupation of the promoter by HIF-1 under hypoxic condition and hypoxia-dependent augmentation of alternative splicing. In the present study, we have demonstrated in a promoter swap assay assembling the constitutively active CMV promoter in front of an IPAS mini-gene construct that hypoxia-inducible inclusion of the IPAS-specific exon 4α occurs irrespective of the specific promoter context, suggesting that HIF-1 binding to the promoter and mRNA splicing can be functionally uncoupled and may have evolved either independently or in combination with one another to increase the repertoire of a cell to respond to hypoxia. Plausibly, not only the HIF-1α/IPAS-negative feedback mechanism but also complex regulatory interactions between hypoxia-inducible transcription factors and their related molecules including target gene products may coordinate and regulate transcription, RNA processing, and protein stability to finely tune cellular responses to hypoxia. It will be important both from a basic physiological and a medical point of view to elucidate in molecular detail these complex interactions of hypoxic signal transduction pathways and to examine whether certain other hypoxia target genes, in addition to HIF-3α/IPAS, are regulated by altered splicing pattern under hypoxic conditions.

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