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Biochemical and Biophysical Research Communications 337 (2005) 209–215
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Binding and regulation of hypoxia-inducible factor-1 by the inhibitory PAS proteins ☆

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Received 30 August 2005
Available online 15 September 2005

Abstract

Hypoxia-inducible factor-1 (HIF-1), which consists of oxygen-sensitive HIF-1α and constitutively expressed HIF-1β subunits, activates transcription of genes encoding proteins that mediate adaptive responses to reduced oxygen availability. The mouse inhibitory PAS (Per/Arnt/Sim) domain protein (IPAS) functions as a negative regulator in HIF-mediated gene expression. In this report, we cloned the human orthologs of the mouse IPAS gene, IPASH1 and IPASH2, to further study the regulatory mechanism of HIF-1 by the IPAS proteins. The human IPAS proteins inhibited the transactivation function of HIF-1α under hypoxic conditions. In addition, human IPAS proteins blocked the hypoxia-induced VEGF expression and inhibited cell migration and tube formation of human umbilical vein endothelial cells. Interestingly, both HIF-1α and HIF-1β interacted with the IPAS proteins. Collectively, these results suggest that human IPAS proteins inhibit angiogenesis by binding to and inhibiting HIF-1α and HIF-1β.

Keywords: Hypoxia; Hypoxia-inducible factor-1α; Inhibitory PAS domain protein; Angiogenesis

HIF-1 activates the expression of its target genes that promote angiogenesis, vasculogenesis, glucose metabolism, and apoptosis [1–3]. HIF-1 is a heterodimeric transcription factor composed of HIF-1α subunit and HIF-1β subunit also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) [4,5]. Under normoxic condition, the HIF-1α subunit is subject to rapid degradation through HIF prolyl hydroxylase (HPH)-catalyzed hydroxylation and then von Hippel–Lindau tumor suppressor gene product (pVHL)-mediated ubiquitination [6–8], whereas hypoxia blocks the degradation of HIF-1α and, therefore, leads to accumulation of HIF-1α. Oxygen levels thus can affect the protein stability, subcellular localization, and transcriptional activity of the HIF-α subunit. By contrast, the HIF-1β subunit is constitutively expressed in the nucleus and its activity is not affected by hypoxia. The bHLH-PAS (Per/Arnt/Sim) domains are required for heterodimer formation between the HIF-1α and HIF-1β subunits and for DNA binding. Beside HIF-1α, there are two additional members of the bHLH-PAS superfamily: HIF-2α, referred to as endothelial PAS domain protein 1 (EPAS1), and HIF-3α [9,10].

There have been extensive studies to identify cellular regulators of HIF-1α [11]. One of the recently identified regulators is the inhibitory PAS domain protein (IPAS) [12]. The mouse IPAS gene is a splicing variant of HIF-3α locus [13]. The IPAS protein contains no endogenous

☆ *Abbreviations:* HIF-1α, hypoxia-inducible factor-1α; PAS, Per/Arnt/Sim; IPAS, inhibitory PAS domain protein; ARNT, aryl hydrocarbon receptor nuclear translocator; GST, glutathione-*S*-transferase; HA, hemagglutinin; VEGF, vascular endothelial growth factor.

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doi:10.1016/j.bbrc.2005.09.038

transactivation domain. It acts as a dominant negative regulator of HIF-1α by forming a complex with HIF-1α, which results in failure of HIF-1α binding to hypoxia response elements of its target genes. Expression of the mouse IPAS in the cornea correlates with reduced expression of the VEGF gene under hypoxic conditions. In humans, there are at least three HIF-3α splicing variants that have no transactivation domain [14,15].

In the present study, we cloned the human IPAS genes, IPASH1 and IPAHS2, to further study the regulatory mechanism of HIF-1 by the IPAS proteins. The human IPAS proteins inhibited the transactivation activity of HIF-1α and the expression of VEGF under hypoxic conditions. The results from angiogenesis assay indicated that the human IPAS proteins may inhibit tumor growth and angiogenesis by regulating endothelial cell migration and tube formation. We also found that both HIF-1α and ARNT (HIF-1β) interact with the human IPAS proteins, suggesting the complexity of the angiogenesis-inhibitory mechanism.

Materials and methods

Cell culture. Human embryonic kidney (HEK) 293, 293T, and BOSC 23 [16] cells were maintained in Dulbecco' modified Eagle's medium (DMEM) with 0.375% sodium bicarbonate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) in a humidified 5% CO_2 incubator at 37 °C. Human umbilical vein endothelial cells (HUVECs) were grown on a gelatin-coated 10 cm plate in M199 medium containing 3 ng/ml basic fibroblast growth factor (bFGF), 5 U/ml heparin, and 20% FBS. The HUVECs used in this study were from passages 5 to 8.

Cloning of IPASH1 and IPASH2 cDNAs. IPASH1 and IPASH2 cDNAs were cloned from human fetal brain Marathon-Ready cDNA (Clontech) using polymerase chain reaction (PCR) with gene-specific primers.

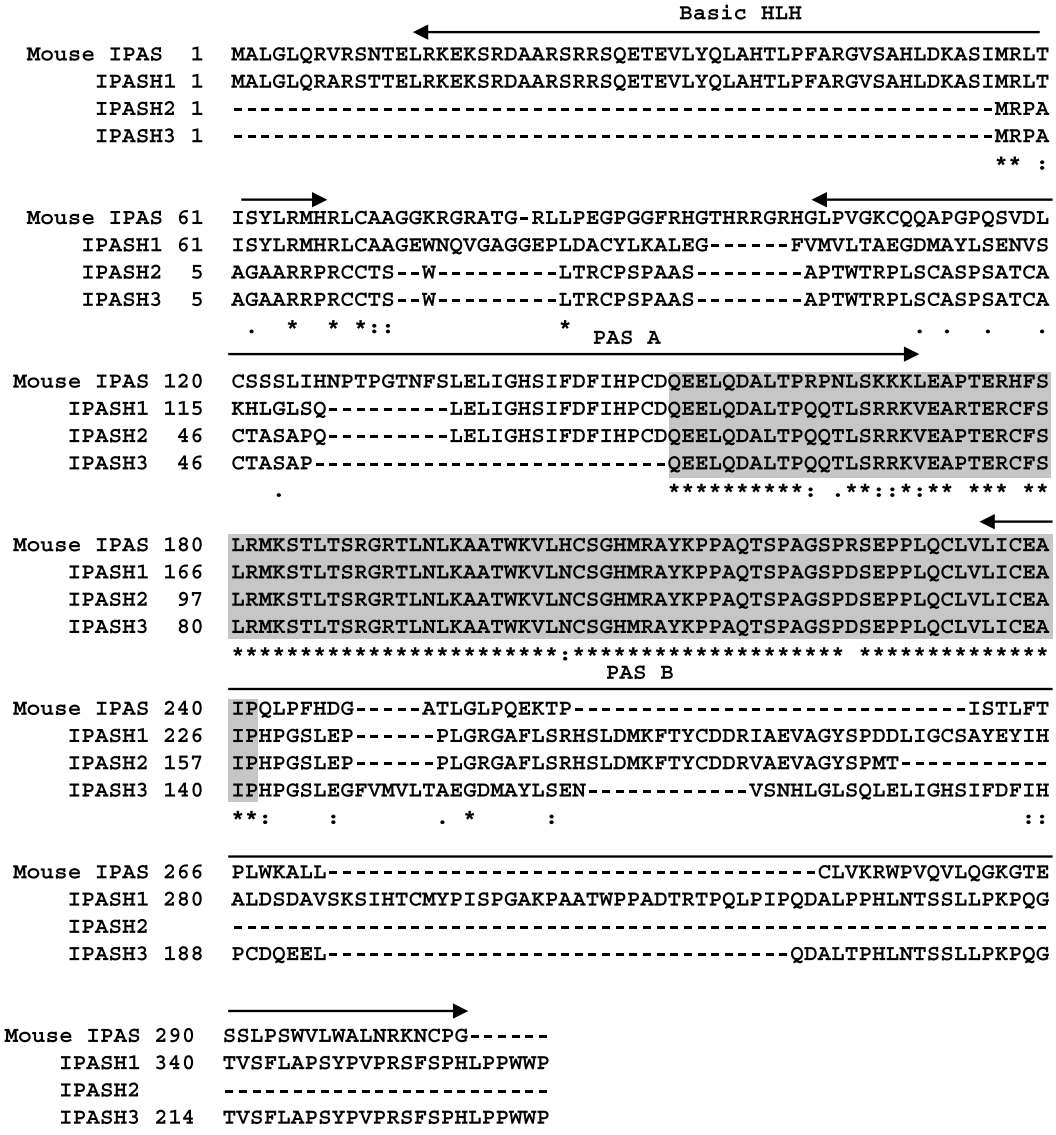

Fig. 1. Sequence similarity between human and mouse IPAS proteins. Identical amino acids (*), conservative substitutions (:), and semi-conserved substitutions (.) are indicated. Dashes indicate deletions to maximize the sequence similarity. Basic HLH and PAS A and B domains are indicated with arrows. Highly conserved PAS domain is indicated with shadows in dark gray.

Plasmid constructions. Glutathione-*S*-transferase (GST) fused-HIF-1α deletion mutant plasmids (HIF-1α 1–340 and 401–826) and GST-ARNT (amino acids, 66–789) plasmid for expression in mammalian cells were constructed by polymerase chain reaction (PCR), followed by cloning into the pEBG plasmid. All mutants were confirmed by DNA sequencing. The N-terminal hemagglutinin (HA)-tagged HIF-1α expression plasmid was constructed by subcloning the full-length HIF-1α cDNA into the pcDNA3-HA plasmid. The full-length HIF-1α cDNA was inserted into *Bam*HI and *Xba*I sites of pFA-CMV containing the GAL 4 DNA binding domain to construct pFA-CMV-HIF-1α. pFLAG/IPASH1 and pFLAG/IPASH2 were constructed in the pFLAG-CMV2 plasmid (Sigma–Aldrich). The luciferase reporter plasmid (pFR-Luc) containing the promoter with five tandem repeats of GAL4 binding sites was purchased from Stratagene. The pHRE-luc reporter plasmid contains four copies of the hypoxia-responsive element of the erythropoietin (EPO) gene, the simian virus 40 promoter, and the luciferase gene in pGL3 promoter plasmid [17].

Transfections. For transient transfection, 1.4×10^6 cells were plated in 60 mm plate, grown overnight, and transfected by either LipofectAMINE (Invitrogen) or calcium phosphate method. Transfection using LipofectAMINE was performed according to the manufacturer's instructions. The calcium phosphate precipitation method was performed as described previously [18].

In vivo binding assays and immunoblotting. Transfected BOSC 23 cells were treated with 125 μM $CoCl_2$ to mimic hypoxic conditions. In each set of transfections, the total DNA concentrations were equalized with appropriate empty plasmids. Unless otherwise specified, all transfected cells were subjected to $CoCl_2$ exposure for 16 h. After 48 h of incubation, preparation of protein extracts, immunoprecipitation, and immunoblot were performed as described previously [19].

Luciferase assay. 293T cells were grown to 50–80% confluence in 60 mm cell culture dish, and were transfected by LipofectAMINE with 1 μg each of reporter plasmid and HIF-1α expression plasmid, and 0.5 μg pCMV/β-gal with or without 1 μg of either pFLAG/IPASH1 or pFLAG/IPASH2. Total amounts of DNA were equalized with empty plasmids. The luciferase activity was determined using an assay system (Promega) with a luminometer as described previously [19].

Determination of vascular endothelial growth factor level in cell supernatant. To determine vascular endothelial growth factor (VEGF) expression levels, we obtained conditioned medium (CM) from control or human IPAS-transfected HEK 293 cells. The amounts of VEGF in the CM were determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Preparation of conditioned medium and cell migration assay. HEK 293 cells were transfected with pcDNA 3.1 (Zeo)-IPASH1, IPASH2, or empty pcDNA 3.1 (Zeo) vector as described above. After transfection, the cells were grown in 10% FBS DMEM under normoxic conditions for 48 h. Transfected cells were then incubated in low serum medium (M199, 1% FBS) for 16 h under hypoxic conditions before the CM was collected. The CM was centrifuged at 500*g* for 10 min and then 800*g* for 15 min to remove cell debris. The resultant CM was directly used for assays.

Endothelial cell migration assays were performed in 48-well microchemotaxis chambers (Neuro Probe, Cabin John, MD) as described [20]. For positive controls, bFGF and VEGF were used with the final concentrations of 5 and 10 ng/ml, respectively. The membrane filers were fixed and stained using Diff-Quick (Baxter Healthcare, McGraw Pa, IL). The number of cells that migrated through the filter was determined by counting two regions of each well under a microscope. Experiments were carried out in triplicate and repeated at least twice.

Tube formation assay. The tube formation assay was performed in 48-well plates. Plates were coated with 150 μl of Matrigel (12 mg/ml; Collaborative Biomedical Products, Bedford, MA) and incubated at 37 °C for 30 min to promote gelling. HUVECs (20,000 cells) in 1% FBS-M199 with CM were added to each well. For positive control, bFGF or VEGF was added to the final concentration of 5 and 10 ng/ml, respectively. All assays were performed in triplicate. After 10 h incubation, chosen fields from each sample were analyzed for imaging.

Results and discussion

Inhibition of the transactivation activity of HIF-1α by the human IPAS proteins

In search of human orthologs of the mouse IPAS gene using NCBI blast homology search program, we identified three EST clones homologous to the mouse IPAS sequence and termed them as IPASH1 (GenBank Accession No. BAD93355), IPASH2 (GenBank Accession No. BAB13819), and IPASH3 (GenBank Accession No. BAB14824), respectively. Sequence comparison revealed that all human IPAS genes are alternatively spliced forms of HIF-3α gene and lacking the open reading frame sequences corresponding to the C-terminal region of HIF-3α. Notably, IPASH1 contains bHLH and PAS domains while both IPASH2 and IPASH3 contain the PAS domain but not the bHLH domain (Fig. 1). We cloned IPASH1 and IPASH2 from human fetal brain cDNA pool to further study the regulatory mechanism of HIF-1α by the human IPAS proteins.

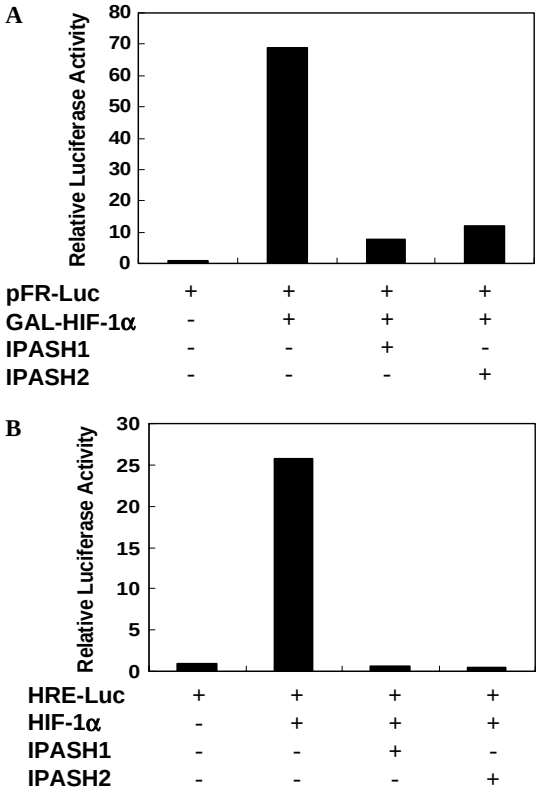

Fig. 2. Both IPASH1 and IPASH2 inhibit HIF-1α transactivation activity. HEK 293T cells were transfected with 1 μg each of the reporter plasmid pFR-Luc (A) or pHRE-Luc (B) together with other plasmids described. pCMV/β-gal was included in all transfections. Cells were treated with 125 μM $CoCl_2$ for 24 h. After 48 h of transfection, equal quantities of cell extracts were used for luciferase assays. The relative fold induction of luciferase activity was determined and normalized to β-galactosidase activity. This is a representative of three independent experiments.

To investigate whether the IPASH proteins are involved in HIF-1α transcriptional regulation, we performed luciferase reporter assays and examined the effects of IPASH1 and IPASH2 proteins on transactivation activity of HIF-1α (Fig. 2). For these assays, the HIF-1α fused with the DNA binding domain of yeast GAL4 and a reporter plasmid (pFR-Luc) carrying 5× GAL4 binding sequences in the promoter region that controls expression of the luciferase gene were used. Together with HIF-1α expression plasmid and the pFR-Luc reporter plasmid, IPASH1 or IPASH2 expression plasmid was co-transfected into 293T cells (Fig. 2A). To mimic hypoxic condition, the transfected cells were then treated with 125 μM $CoCl_2$. While overexpression of HIF-1α was sufficient for stimulation of the reporter gene, co-expression of either IPASH1 or IPASH2 suppressed HIF-1α transactivation activity.

Since IPASH1 contains the bHLH domain while IPASH2 does not, we then used a hypoxia-responsive element (HRE)-driven luciferase reporter system to test if the bHLH domain of IPAS is necessary for inhibition of HIF-1α transactivation activity. As shown in Fig. 2B, transfection of either IPASH1 or IPASH2 expression plasmid blocked HIF-1α-mediated transactivation activity, which indicates that the bHLH domain of IPASH1 is not necessary for inhibition. Taken together, these results suggest that the human IPAS proteins inhibit the HIF-1α transactivation activity through the PAS domain of the IPAS proteins.

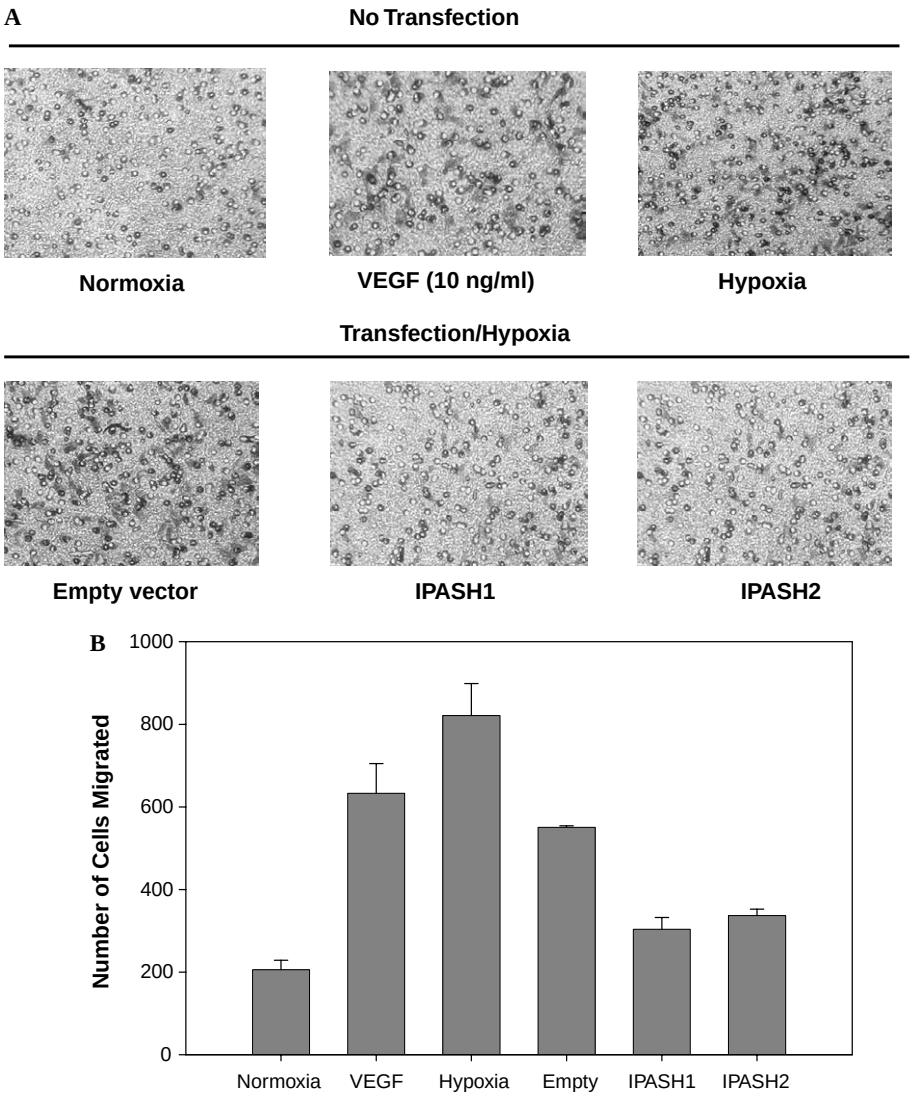

Fig. 3. The human IPAS proteins inhibit endothelial cell migration. Untransfected HEK 293 cells were incubated under normoxic or hypoxic conditions before collecting conditioned medium (CM). The collected CM was used for HUVEC migration assays (A, upper panel). VEGF was used as a positive control with the final concentrations 10 ng/ml, respectively. For assays with transfected HEK 293 cells, cells were transfected with IPASH1, IPASH2, or empty pcDNA 3.1 plasmids (A, lower panel). After transfection, the cells were grown in 10% FBS DMEM under normoxic conditions for 48 h. Transfected cells were then incubated in low serum medium (M199, 1% FBS) for 16 h under hypoxic conditions before the CM was collected. The resulting CM was directly used for assays. Migration assays were performed as described in Materials and methods. The number of migrated cells was determined by counting two regions of each well under a microscope (B).

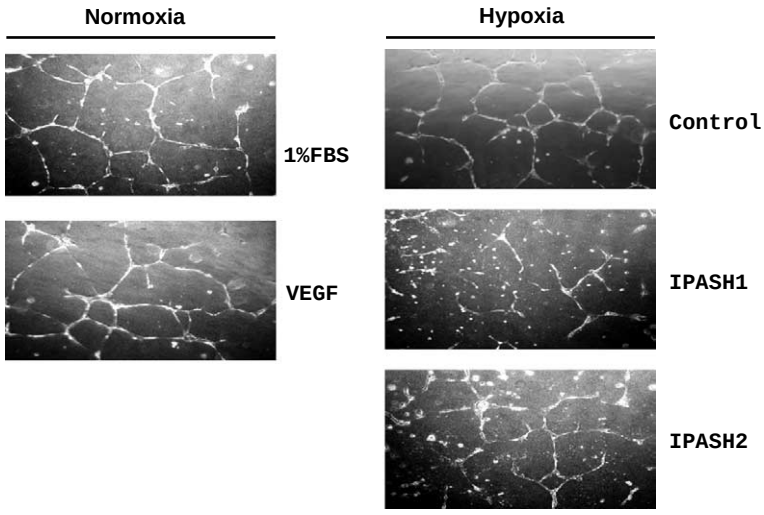

Fig. 4. The human IPASH proteins inhibit HUVEC tube formation. The tube formation assays were performed on 48-well plates as described in Materials and methods. CM was collected as described in Fig. 3. Plates were coated with Matrigel and incubated for 30 min to promote gelling. HUVECs in 1% FBS-M199 with conditioned medium were added to each well and incubated for 12–24 h before collecting data.

The IPASH proteins inhibit HUVEC migration

Lack of oxygen supply, as often observed in cancer tissue, induces angiogenesis which is mediated by HIF-1α. One of the first cellular events occurring during the angiogenesis process is the extravasion and migration of inflammatory cells toward the signal source. The angiogenesis process may result in the vascularization of the tumor, thereby promoting tumor growth. Therefore, it is important to block migration of endothelial cells to inhibit angiogenesis. To investigate whether the human IPAS proteins are related to endothelial migration, we performed in vitro HUVEC migration assays using conditioned medium (CM) obtained from transfected HEK 293 cells (Fig. 3). Transfected cells were incubated in low serum medium (1% FBS) for 16 h before CM was collected. CM was centrifuged to remove insoluble materials and directly used for assays. The migratory effect of CM on HUVECs was tested using Boyden chambers [21]. The effects of normoxia, hypoxia, and VEGF on HUVEC migration were tested with CM from non-transfected HEK 293 cells incubated under appropriate conditions. The level of HUVEC migration upon incubation with hypoxia-treated CM was significantly higher than that with normoxia-treated CM, which was similar to that observed with VEGF (10 ng/ml), suggesting that hypoxia is involved in migration of endothelial cells. CM from human IPAS-transfected cells significantly reduced migration of HUVEC, while the control (CM obtained from empty plasmid-transfected cells) did not show the inhibitory effect. These results indicate that the human IPAS proteins possibly regulate endothelial cell migration.

The IPASH proteins inhibit HUVEC tube formation

Next, to determine whether the human IPAS proteins inhibit the HUVEC tube formation that is a major process of angiogenesis [22], we examined the ability of the human IPAS proteins to regulate the formation of capillary-like structures of HUVECs on Matrigel. CM collected from hypoxia-treated control cells caused an increase in capillary-like structures, when compared to CM from normoxia-treated cells. However, CM from hypoxia-treated IPASH expressing HEK 293 cells, failed to form capillary-like structure (Fig. 4). Therefore, our results clearly indicate that the human IPAS proteins inhibit tube formation of endothelial cells.

Suppression of hypoxic induction of VEGF by the human IPAS proteins

Vascular endothelial growth factor (VEGF) is one of the major target genes of HIF-1α [23]. The protein specifically

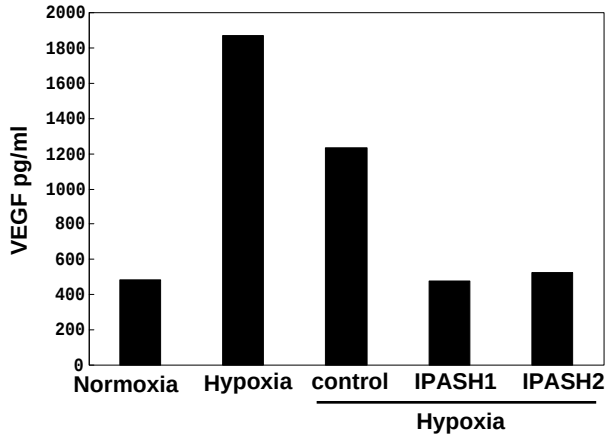

Fig. 5. Effects of the human IPAS proteins on vascular endothelial growth factor (VEGF) expression level from hypoxia-treated HEK 293 cells. Conditioned medium (CM) was obtained after transfection of control or human IPAS expression plasmids into HEK 293 cells. CM from untransfected cells was collected after normoxic or hypoxic treatments. The amount of VEGF in CM was determined using an enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

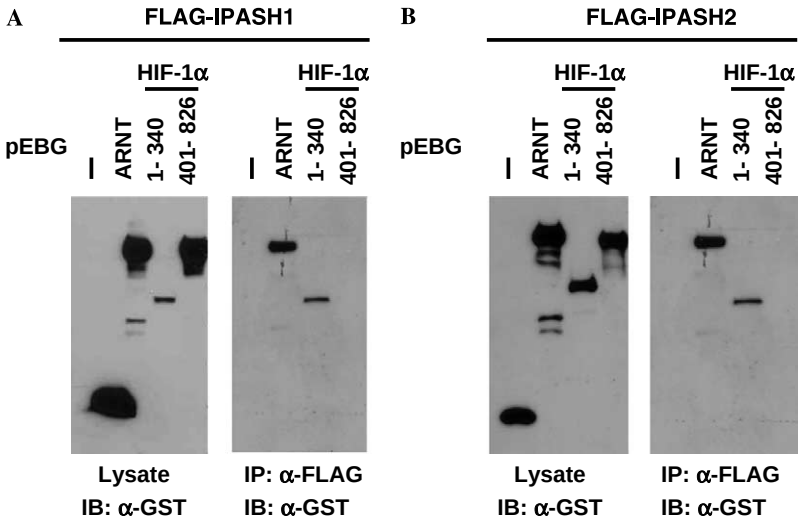

Fig. 6. Both IPASH1 and IPASH2 interact with HIF-1α and ARNT. The in vivo bindings of the human IPASH1 (A) and IPASH2 (B) to HIF-1 subunits were tested with immunoprecipitation assays. Cell lysates were prepared after transfection and immunoprecipitated with anti-FLAG M2 affinity gel. Immunoprecipitates were resolved on SDS–PAGE and immunoblotted with an anti-GST antibody (right panels in A and B). Whole cell extracts were immunoblotted with an anti-GST antibody to show expression levels of proteins (left panels in A and B). IP, immunoprecipitation; IB, immunoblot. Lane 1, pEBG empty plasmid; lane 2, pEBG HIF-1 ARNT; lane 3, pEBG HIF-1α (amino acid residues, 1–340), and lane 4, pEBG HIF-1α (401–826).

recruits endothelial cells into hypoxic and vascular area, and stimulates their proliferation [24]. Since VEGF is a target gene of HIF-1α and a major inducer in endothelia cell migration and tube formation, we suspected that the level of VEGF secreted into CM could be modulated by the human IPAS proteins. To address this question, we measured the VEGF protein levels in CM from HEK 293 cells treated with various conditions using the Quantikine VEGF ELISA kit (R&D System). As expected, CM from the human IPAS-transfected cells incubated at hypoxia contained lower level of VEGF proteins, compared to control CM (Fig. 5), indicating that the human IPAS proteins indeed suppress hypoxia-mediated VEGF induction in HEK 293 cells.

Interaction of the human IPAS proteins with HIF-1α and ARNT

The mouse IPAS protein has been known to interact with HIF-1α but not with ARNT (HIF-1β) [12]. However, the mouse IPAS gene is a spliced variant of HIF-3α that is thought to interact with ARNT [13,25]. To test whether the human IPAS proteins interact with ARNT and HIF-1α, the human IPAS proteins were fused to FLAG tag and used for binding assays (Fig. 6). BOSC23 cells were transiently transfected with FLAG-tagged human IPAS expression plasmids along with GST-HIF-1α (amino acid residues 1–340), HIF-1α (401–826), or ARNT expression plasmids. After 48 h of transfection, the FLAG-fused IPAS proteins were immunoprecipitated with anti-FLAG M2 beads and the co-precipitated HIF proteins were probed by immunoblotting with an anti-GST-antibody. The N-terminal PAS domain (residues 1–340) of HIF-1α was detected in the pulled-down human IPAS protein complexes, while the C-terminal domain (residues 401–826) of HIF-1α was not co-precipitated with the IPAS proteins. In addition, both IPASH1 and IPASH2 were found to interact with ARNT. Taken together, our results indicate that IPASH proteins interact with both HIF-1α and ARNT through the PAS domains.

In summary, we demonstrated that the human IPAS proteins interact with HIF-1α and HIF-1β (ARNT), and inhibit the transactivation activity of HIF-1α under hypoxia-mimicking conditions. Since IPASH1 contains both bHLH and PAS domains while IPASH2 contains only PAS domain, PAS core domain of the IPAS proteins is believed to be crucial for HIF-1α regulation. The results from angiogenesis-related functional assays suggested that the human IPAS proteins might inhibit tumor growth and angiogenesis by regulating endothelial cell migration and tube formation. Our findings that the human IPAS proteins may play roles in negative regulation of angiogenesis by inhibiting HIF-1α could provide an important clue to understand the regulatory functions of the IPAS proteins in hypoxia-induced angiogenesis.

Acknowledgments

We thank Sewook Hwang for technical assistance in tube formation and migration assays. This work was supported by the grant (FG05-23-01) of the 21C Frontier Functional Human Genome Project from The Ministry of Science and Technology (MOST) of Korea.

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