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von Hippel-Lindau protein adjusts oxygen sensing of the FIH asparaginyl hydroxylase

Shan Hua Li[a,d], Yang-Sook Chun[b], Ji-Hong Lim[a], L. Eric Huang[c], Jong-Wan Park[a,b,*]

[a] Department of Pharmacology, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Republic of Korea
[b] Department of Biomedical Sciences, Ischemic/Hypoxic Disease Institute, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Republic of Korea
[c] Department of Neurosurgery, University of Utah School of Medicine, Salt Lake City, UT 84132, USA
[d] Department of Basic Medical Sciences, Medical College, Xiamen University, Daxue Rd 168, Xiamen 361005, Fujian, PR China

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ABSTRACT

Hypoxia inevitably develops in rapidly growing tumors and acts as an important microenvironment that forces changes in tumor behavior. Hypoxia-inducible factor 1α (HIF-1α) is activated during hypoxia and promotes the progression of malignancy by stimulating angiogenesis and by augmenting the ability of tumors to survive. In aerobic conditions, HIF-1α is destabilized by the PHD prolyl-hydroxylases that target HIF-1α for proteolysis via the von Hippel-Lindau protein (pVHL) and further inactivated by the FIH asparaginyl-hydroxylase that precludes the recruitment of transcription coactivators. Although HIF-1α degradation is well understood, little is known about how its transcriptional activity increases gradually in response to decreasing oxygen. In particular, it has been questioned how FIH having a high affinity for oxygen regulates the HIF-1α activity in moderate hypoxia. We here found that the HIF-1α-FIH interaction is disrupted in 1–5% oxygen. Both *in vitro* and *in vivo* binding analyses revealed that pVHL acts as an adaptor for FIH to bind HIF-1α. Furthermore, because the pVHL–FIH interaction depends on oxygen tension, the FIH-mediated inactivation of HIF-1α can be exquisitely regulated according to the severity of hypoxia. Based on these findings, we propose that pVHL fine-tunes the transcriptional activity of HIF-1α in graded oxygen tensions.

1. Introduction

Hypoxia-inducible factor-1 (HIF-1) is composed of HIF-1α and HIF-1β subunits, and is a transcription factor that up-regulates numerous genes essential for adaptation to hypoxia (Wang and Semenza, 1995; Schofield and Ratcliffe, 2004). Of these two subunits, HIF-1α is O_2-dependently regulated, and acts as the primary transactivating factor. HIF-1α possesses an O_2-dependent degradation domain (ODDD) and two transactivation domains (TADs), namely, an N-terminal TAD (NAD) and a C-terminal TAD (CAD) (Pugh et al., 1997). HIF-1α stability and activity are O_2-dependently regulated. When Pro402 and Pro564 residues within its ODDD are hydroxylated by HIF-1-prolyl hydroxylases (PHDs), they serve as recognition motifs for von Hippel-Lindau protein (pVHL) targeting, and HIF-1α is subsequently ubiquitinated and degraded through proteasomes (Ivan et al., 2001; Jaakkola et al., 2001). In addition, the Asn803 residue within CAD can be hydroxylated by factor inhibiting HIF-1 (FIH), which inactivates HIF-1α by blocking the recruitment of p300/CBP co-activator (Lando et al., 2002; Freedman et al., 2002). Furthermore, since PHD and FIH cannot function without O_2, HIF-1α is stabilized and activated under hypoxic conditions.

Both PHDs and FIH belong to the Fe^{2+}/2-oxoglutarate-dependent dioxygenase family. They split O_2 and utilize the oxygen atoms for amino acid hydroxylation and succinate production (Hewitson et al., 2002). Under O_2-deficient conditions, their activities are expected to be limited. Indeed, PHDs are sensitively regulated by O_2 tension, and HIF-1α is stabilized under moderate hypoxia (1–5% O_2) (Hirsilä et al., 2003). In contrast, FIH has a higher affinity for O_2 than PHDs and thus remains active under moderate hypoxia, which suggests that more severe hypoxia is required to deactivate FIH (Koivunen et al., 2004; Stolze et al., 2004).

The above finding poses a question as to how HIF-1α maintains its activity under moderate hypoxia. It is generally believed that NAD contributes to gene expression in moderate hypoxia and that CAD provides further gene expression in severe hypoxia (Pouysségur et al., 2006). This scenario appears reasonable, but it is inconsistent with some aspects. Based on FIH-sensitivity, Dayan et al. (2006) discriminated between CAD- and NAD-dependent

* Corresponding author at: Department of Pharmacology, Seoul National University College of Medicine, 28 Yongon-dong, Chongno-gu, Seoul 110-799, Republic of Korea. Tel.: +82 2 740 8289; fax: +82 2 745 7996.
E-mail address: parkjw@snu.ac.kr (J.-W. Park).

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genes. They demonstrated that FIH-sensitive, CAD-dependent genes are expressed at higher levels in 0.2% O_2 than in 3% O_2. This supported the hypothesis that the FIH repression of CAD can be released only under severe hypoxia. However, it should be noted that almost CAD-dependent genes were significantly induced even under moderate hypoxia in their study. Moreover, several studies analyzed the specific activity of CAD using the Gal4-CAD/Gal4-Luc reporter system, and showed that CAD can be activated in moderate hypoxia (Gu et al., 2001; Yeo et al., 2006; Shin et al., 2008). Given these reports, we hypothesized that there is a hitherto unidentified mechanism responsible for FIH inhibition in moderate hypoxia. We tested the possibility that HIF-1α activity is regulated O_2-dependently through the interaction between FIH and HIF-1α.

2. Materials and methods

2.1. Cell culture

HEK293 embryonic kidney cell-line was obtained from American type culture collection, and VHL(−/−; +/+) RCC4 renal cell carcinoma cell-lines from European collection of cell cultures. All cells were maintained in Dulbecco's modified Eagle's Medium supplemented with 10% FBS. Cells were grown in a humidified atmosphere at 37 °C, and the gas tension was 20% O_2/5% CO_2 for normoxia or 0.3–5% O_2/5% CO_2 for hypoxia.

2.2. Reagents and antibodies

Dimethyloxalylglycine (DMOG) and MG132 were purchased from ALEXIS Biochemicals (San Diego, CA), and [α-^{32}P]CTP (500 Ci/mmol) from GE Healthcare (Piscataway, NJ). Other chemicals were purchased from Sigma–Aldrich (St. Louis, MO). Anti-HIF-1α antisera were raised against aa. 418–698 or 718–826 of human HIF-1α. A mouse monoclonal antibody against hydroxylated Asn803 of HIF-1α was a generous gift from Dr. Myung Kyu Lee (KRIBB, Korea). Anti-pVHL was purchased from BD Biosciences Pharmingen (San Diego, CA), and anti-Flag from Sigma–Aldrich, anti-HA from Roche Ltd. (Basel, Schweiz). Antibodies against FIH, GFP, GST, His-tag, β-tubulin, Gal4(DBD), and HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.3. Expression plasmids, siRNAs and transfection

EPO enhancer-luciferase, Gal4-CAD (aa. 776–826) and VP16-p300/CH1 plasmids were provided by Dr. Eric Huang (University of Utah, USA), and Flag-tagged VHL plasmid by Dr. Hong-Duk Youn (Seoul National University, Korea). The plasmid of stably expressed HIF-1α (sHIF-1α) was made by deleting three degradation motifs (aa. 397–405, 513–553, and 554–595). The plasmids of GFP-tagged CT (aa. 720–826), CT1 (aa.720–781) and CT2 (aa. 782–826) were constructed by inserting corresponding cDNAs into the pcDNA-GFP vector. HA-tagged FIH plasmid was constructed by inserting FIH cDNA into pcDNA-HA. Flag/SBP-tagged VHL1-62, VHL1-155, VHL1-206, and VHL1-213 were constructed by inserting corresponding VHL cDNAs into pcDNA-Flag/SBP. To knock-down FIH, HIF-1α and VHL, synthesized siRNA duplexes were designed and obtained from Invitrogen (Carlsbad, CA). The sequence targeting FIH (NM_017902) corresponds to nucleotides 91–111 of the coding region, HIF-1α (NM_001530) to nucleotides 360–384, and VHL (NM_000551) to nucleotides 581–606. Non-targeting control siRNA was purchased from STpharm Co. (Seoul, Korea) and its sequence is 5′-AUGAACGUGAAUUGCUCAA-3′. To express or knock-down genes, cells at 40% confluence were transfected with plasmid or siRNA using calcium phosphate, Lipofectamine 2000 (Invitrogen) or nucleofection (Amaxa, Walkersville, MD) method. Cells were allowed to stabilize for 48 h before being used in experiments.

2.4. Immunoblotting

Cells were lysed with in a SDS buffer containing 10% β-mercaptoethanol. Proteins were separated on 8–10% SDS/polyacrylamide gel, and then transferred to Immobilon-P membranes (Millipore, Bedford, MA). Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TTBS) for 1 h and then incubated overnight at 4 °C with a primary antibody (1:1000–2000). Membranes were incubated with a HRP-conjugated secondary antibody (1:5000) for 1 h, and HRP was visualized using the ECL+ kit (GE Healthcare).

2.5. Immunoprecipitation

For immunoprecipitation of endogenous proteins, cell lysates (1 mg of protein) were incubated with 5 μl of an antibody or preimmune serum at 4 °C for 4 h. The immune complexes were incubated with protein G-Sepharose beads (GE Healthcare) overnight at 4 °C. For immunoprecipitation of exogenous proteins, HEK293 cells were co-transfected with the indicated plasmids. One day after transfection, cells were maintained under normoxia or exposed to 16 h of hypoxia. Cell lysates were incubated with an antibody or preimmune serum for precipitation at 4 °C for 4 h, and then incubated with protein G beads at 4 °C for 4 h. For two step immunoprecipitation of exogenous proteins, HEK293 cells were co-transfected with Flag-VHL, HA-FIH, and GFP-CAD plasmids. Cell lysates were incubated with Flag-affinity Sepharose beads (Sigma–Aldrich) at 4 °C for 4 h, and after washing out unbound proteins, eluted bound protein from beads with Flag peptide (Sigma–Aldrich) at room temperature for 30 min, and then eluted protein were incubated with HA-affinity sepharose beads (Sigma–Aldrich) at 4 °C for 4 h. After washing, the precipitated proteins were eluted in the denaturing SDS sample buffer, and then subjected to SDS–PAGE and immunoblotting using an antibody for identification.

2.6. Reporter assay

Cells were co-transfected with 1 μg each of reporter plasmid and β-gal plasmid using the calcium phosphate or nucleofection method. pcDNA was added to ensure that the final DNA concentrations in both the control and experimental groups were at similar levels. After stabilized for 24 h, cells were incubated under either normoxic or hypoxic conditions for 16 h, and then lysed to determine luciferase and β-gal activities. Luciferase activity was divided by β-gal activity in each sample to normalize transfection efficiency.

2.7. RNA isolation and semi-quantitative RT-PCR

To quantify mRNA levels, we performed a highly sensitive semi-quantitative RT-PCR. Total RNAs were isolated with TRIZOL regent (Invitrogen). One μg of RNA was reverse-transcribed at 46 °C for 1 h, and the cDNAs were amplified over 15–20 PCR cycles (94 °C for 30 s, 53 °C for 30 s and 70 °C for 1 min) in 20 μL reaction mixture containing 5 μCi [α-^{32}P] dCTP. The PCR products (5 μL) were electrophoresed on a 4% polyacrylamide gel, and dried gels were autoradiographed. The nucleotide sequences of primer pairs were 5′-GGTGAAGTTCATGGATGTCT-3′ and 5′-TCTGCATTCACATTTGTTGT-3′ for VEGF-A; 5′-GTCA TCCTCTTCCATGAGAC-3′ and 5′-AGGTAGATGTGGTGGTCACT-3′ for Aldolase-A; 5′-GACTCTGCACCCAGATTTAG-3′ and 5′-CACAA GGTCTGAGATTCCAT-3′ for LDH-A; 5′-ACACCTTCTACAATGAGCTG-3′ and 5′-CATGATGGAGTTGAAGGTAG-3′ for β-actin.