

Inhibition of the catalytic activity of HIF-1 α -prolyl hydroxylase 2 by a MYND-type zinc finger

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Running title page

- a)** Running title : Regulation of PHD2 activity by MYND-Zn finger
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- c)** The number of text pages (19),
number of tables (0),
number of figures (7),
references (31),
the number of words in the Abstract (191)
the number of words in the Introduction (555)
the number of words in the Discussion (671)
- d)** A list of nonstandard abbreviations used : bHLH, basic helix-loop-helix; HIF-1 α , hypoxia-inducible factor-1 α ; HRE, hypoxia-responsive element; GST, glutathione-S-transferase; MYND, Myeloid translocation protein 8, Nervy, and DEAF1; ODD, Oxygen dependent degradation domain; PAS, Per-Arnt-Sim; PHD, HIF-1 α -specific prolyl hydroxylase; TPEN, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine; VHL, von Hippel Lindau.

Abstract

Hypoxia-induced gene expression is initiated when the hypoxia-inducible factor-1 α (HIF-1 α) subunit is stabilized in response to a lack of oxygen. An HIF-1 α -specific prolyl hydroxylase (PHD) catalyzes hydroxylation of the proline-564 and/or 402 residues of HIF-1 α by an oxygen molecule. The hydroxyproline then interacts with the ubiquitin E3 ligase, von Hippel Lindau (VHL) protein, and is degraded by an ubiquitin-dependent proteasome. PHD2 is the most active of three PHD isoforms in hydroxylating HIF-1 α . Structural analysis showed that the N-terminal region of PHD2 contains a MYND-type zinc finger domain whereas the catalytic domain is located in its C-terminal region. We found that deletion of the MYND domain increased the activity of both recombinant PHD2 protein and in vitro translated PHD2. The zinc chelator, N, N, N', N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) augmented the activity of wild type PHD2-F but not that of PHD2 lacking the MYND domain, confirming that the zinc finger domain is inhibitory. Overexpression of PHD2 lacking the MYND domain caused a greater reduction in the stability and function of HIF-1 α than did overexpression of wild type PHD2, indicating that the MYND domain also inhibits the catalytic activity of PHD2 *in vivo*.

Introduction

Hypoxia is the most common type of cell injury in various human diseases including myocardial infarction, stroke, acute renal failure and solid tumors. However, organisms have evolved mechanisms for adapting to hypoxia. Thus, hypoxia leads to up-regulation of the transcription of genes involved in anaerobic ATP production and oxygen delivery. Hypoxia-inducible factor-1(HIF-1) is a widespread transcription factor that promotes expression of hypoxia-inducible genes such as vascular endothelial growth factor, erythropoietin, glucose transporters and glycolytic enzymes (Masson and Ratcliffe, 2003; Seagroves et al., 2001). It consists of HIF-1 α and HIF-1 β subunits, both of which belong to the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family. Arnt (HIF-1 β) is a partner of the aryl hydrocarbon receptor (AhR), as well as of HIF-1 α and other bHLH-PAS proteins. HIF-1 α is rapidly degraded under normoxic condition by the ubiquitin-proteasome system, whereas the level of Arnt is constant (Huang et al., 1998; Kallio et al., 1999). Hydroxylation of proline-564 and/or 402 residues in the oxygen-dependent degradation domain (ODD) of HIF-1 α initiates its ubiquitination and subsequent proteasomal degradation (Masson and Ratcliffe, 2003). Prolyl hydroxylation of HIF-1 α is catalyzed by a novel HIF-1 α -specific prolyl hydroxylase which requires O₂, 2-oxoglutarate, vitamin C, and Fe²⁺ (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001). The tumor suppressor, von Hippel-Lindau (VHL) protein, which functions as an E3 ubiquitin ligase, interacts with the hydroxylated prolines of HIF-1 α and brings about the assembly of a complex that activates a ubiquitin-dependent proteasome (Maxwell et al., 1999; Min et al., 2002; Ohh et al., 2000). When cells lack oxygen, proline hydroxylation ceases and HIF-1 α protein

accumulates. In mammalian cells, a family of HIF-1 α specific prolyl-4 hydroxylases has been identified and given the acronyms PHD1 (HPH3, EGLN2), PHD2 (HPH2, EGLN1), and PHD3 (HPH1, EGLN3) (Huang et al., 2002; Metzen et al., 2005; Taylor, 2001).

Although all three PHDs hydroxylate prolines of HIF-1 α *in vitro*, there is evidence that PHD2 has the primary role *in vivo* (Appelhoff et al., 2004; Berra et al., 2003; Hirsila et al., 2003; Huang et al., 2002). Thus, experiments employing short interfering RNAs revealed that silencing of PHD2 is enough to stabilize and activate HIF-1 α in normoxic cells (Berra et al., 2003). Moreover PHD2 is the most abundant of the three isoforms in most normoxic cells (Appelhoff et al., 2004). These findings suggest that each PHD has its own specific substrate, and that PHD2 is the major form responsible for hydroxylating HIF-1 α , and therefore the critical oxygen sensor maintaining the low steady state level of HIF-1 α in normoxic conditions (Freeman et al., 2003; Huang et al., 2002; Masson et al., 2004; Masson and Ratcliffe, 2003).

In addition to hypoxia, Co(II) ion and iron chelators, which inhibit the catalytic activity of PHDs, as well as other agents such as growth factors and the oncogenes Ras, active Src and Akt, have been reported to activate HIF-1 α under normoxia (Chan et al., 2002; Karni et al., 2002; Zundel et al., 2000). It is not clear whether these non-hypoxic stimuli repress the catalytic activity of PHD2, to stabilize HIF-1 α , or act in some other way. In this study we investigated whether the activity of PHD2 is regulated. By analyzing the catalytic activity of purified PHD2 and truncated mutants, we found that the N-terminal region of PHD2 contains a MYND-type Zn finger domain which inhibits catalytic activity.

Materials and Methods

Cells, cDNAs and reagents — Human epithelial HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum (Bio Whittaker), gentamicin (5 µg/ml, Life Technologies Gibco BRL) and Fungizone (0.25 µg/ml, Life Technologies Gibco BRL) in humidified air containing 5% CO₂ at 37°C. Cells were made hypoxic by incubation in an anaerobic incubator (Model 1029, Forma Scientific, Inc.) in 5% CO₂, 10% H₂, and 85% N₂ at 37°C or Multi-gas incubator (Model : NU-4950G, NuAire, Inc). We used the following human cDNAs in expression vectors, transfection assays and in vitro transcription and translation experiments; PHD1 (AJ310544), PHD2 (AJ310543), PHD3 (AJ310545), HIF-1α (U22431), VHL (AF010238). The p(HRE)₄-luc reporter plasmid contained 4 copies of the erythropoietin hypoxia-responsive element 5'-GATCGCCCTACGTGCTGTCTCA-3'; nucleotides 3449-3470). Anti-HIF-1α was obtained from Transduction Laboratories. We obtained N, N, N', N'-*tetrakis* (2-pyridylmethyl) ethylenediamine (TPEN) from Calbiochem, and all remaining chemicals from Sigma Chemical Co. Culture media were purchased from Gibco BRL, fetal bovine serum from Bio Whittaker.

Expression of PHDs and HIF-1α — Full-length cDNAs for PHD1, 2, and 3 were cloned from a human lymphocyte cDNA library into pcDNA3.1B(+) (Invitrogen). For *in vitro* transcription and translation, wild type PHD2 (PHD2-F) cDNA and cDNAs for PHD2-182 (amino acids 182-426), PHD2-60 (amino acids 60-426), PHD2-16 (amino acids 16-426) and PHD2-184 (amino acids 184-418) were subcloned into pcDNA3.1B(+) (Invitrogen) or pET21bHis2 (Novagen). For bacterial expression, the

cDNAs for PHD2-F and the catalytic domain, PHD2-184 (amino acids 184-418) were subcloned into pET21bHis2 (+) vector (Novagen) and expressed with C-terminal histidine tags. For transfections, cDNAs for PHD2-F and PHD2-60 were subcloned into pCMV-3xFlag vector (Sigma) and expressed with N-terminal flag tags. We subcloned VHL into pcDNA 3.1/hygro for *in vitro* transcription and translation. A plasmid encoding the HIF- α 401-603aa region (the oxygen-dependent degradation domain (ODD) linked to glutathione-S- transferase (GST)) was kindly provided by Dr. Seong-Eon Ryu (KRIBB, Korea). Peptides (Biotin-DLDLEMLAPYIPMDDDFQLR and Biotin-DLDLEMLA(P-OH)YIPMDDDFQLR) were synthesized by AnyGen Co. Ltd. (Kwangju, Korea). These 20mer peptides contain residues 556 to 575 of HIF-1 α .

Expression and purification of PHD2 protein — The human PHD2 gene (identical to AJ310543) was cloned into the pET21b His2 (+) vector and overexpressed in *E. coli* as histidine-tagged fused proteins and purified by Ni-affinity chromatography. The histidine fusions of full-length PHD2-F (amino acids 1-426) and catalytic domain PHD2-184 (amino acids 184-418) were further purified by gel-filtration chromatography (Hi-Load Superdex200) and concentrated by ultrafiltration. PHD1, 2, 3 or mutants of PHD2 pcDNA3.1B(+) were *in vitro* transcribed and translated from the T7 promoter using a rabbit reticulocyte lysate (Promega).

Measurement of PHD activity by a VHL pull down assay — The *in vitro* VHL pull down assay was performed as described by Jaakkola *et al.* (2001) (Jaakkola et al., 2001). Briefly, [³⁵S]-methionine-labeled VHL protein was synthesized by *in vitro* transcription and translation using the pcDNA3.1/hygro-VHL plasmid, according to the instruction manual (Promega, Cat# L1170). GST-ODD (amino acid 401-603 of human HIF-1 α)

was expressed in *E. coli* and purified with glutathione-uniflow resin according to the instruction manual (BD Biosciences Clontech Cat# 8912-1). Resin-bound GST-ODD (200 µg protein /about 80 µl of resin volume) was incubated in the presence of 2 mM ascorbic acid, 100 µM of FeCl₂ and 5 mM α-ketoglutarate with the indicated amounts of enzyme in 200 µl of NETN buffer (20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF) with mild agitation for 90 minutes at 30 °C. The reaction mixture was centrifuged and washed three times with 10 volumes of NETN buffer. Resin-bound GST-ODD was mixed with 10 µl [³⁵S]-labeled VHL in 500 µl of EBC buffer (120 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.5 % (v/v) Nonidet P-40). After mild agitation at 4°C for 2 h, the resin was washed three times with 1ml of NETN buffer, and proteins were eluted in 3× SDS sample buffer, fractionated by 12 % SDS-PAGE, and detected by autoradiography. The amount of each sample loaded was monitored by staining the GST- ODD with Coomassie blue.

Assay for interaction between VHL and synthetic biotinylated HIF-1α peptides was described in Epstein *et al.*, 2001. Peptide (Biotin-DLDLEMLAPYIPMDDDFQLR) was synthesized by AnyGen Co. Ltd. (Kwangju, Korea). This 20mer peptide contains residues 556 to 575 of HIF-1α. Seven micrograms of biotinylated peptide (Biotin-DLDLEMLAPYIPMDDDFQLR: residues 556 to 575 of human HIF-1α.) was preincubated with PHDs in a final volume of 100µl in NETN buffer containing 2mM ascorbic acid, 100 µM of FeCl₂ and 5mM α-ketoglutarate at 30°C for 90min. ImmunoPure Immobilized Monomeric Avidin (Pierce, Cat# 20227) (30 µl of a 50% slurry) was pretreated with 3mg of bovine serum albumin for 5 minutes at room temperature. The pretreated immobilized monomeric avidin was added to the above hydroxylation reaction mixture, which was incubated with mild agitation for 60 min at

22°C. Avidin-associated peptide washed three times with 1 ml NETN buffer and then mixed with 10 µl of [³⁵S]-labeled VHL in 100µl of EBC buffer with mild agitation at 4°C for 2 hours. The resin was washed four times with 1 ml NETN buffer, and proteins were eluted in BBE buffer (0.1M NaHPO₄, 0.15M NaCl, 2mM D-biotin). Eluted VHL was analyzed by 12% SDS-PAGE, autoradiographed.

Mass spectrophotometric analysis — HIF-1α peptide (Biotin-DLDLEMLAPYIPMDDDFQLR) (400 ng) was incubated with 2 µg of PHD2-184 in a final volume of 10 µl in NETN buffer containing 5 mM ascorbic acid, 100 µM of FeCl₂ and 5 mM α-ketoglutarate at 30 °C for 90 minutes. For MALDI-TOF analyses, α-cyano-4-hydroxycinnamic acid solution was prepared in acetonitrile/water containing 0.1% TFA (50:50, v/v) at a concentration of 10 mg/ml. This matrix solution was used to dilute samples (1:10 ratio) to a final concentration of 1 ng/µl. They were then spotted directly onto the target plate and allowed to air-dry. Mass spectrometric analyses of the samples were performed with a Voyager analyzer (Applied Biosystems).

Results

The catalytic and inhibitory domains of PHD2 — We examined the hydroxylation activity of the PHDs by measuring capture of [³⁵S]-labeled VHL by biotin-labeled HIF-1 α peptide (amino acids 556-575) or GST-ODD (oxygen dependent degradation domain of HIF-1 α , amino acids 401-603) as substrates (Fig. 1A, and B respectively). To compare the HIF-1 α -specific hydroxylation activities of PHD1, 2, and 3, we transcribed and translated each enzyme *in vitro* in a rabbit reticulocyte lysate and confirmed that each of the enzymes was of the expected size. Comparison of the intensity of captured [³⁵S]-labeled VHL with the amount of PHDs synthesized (Fig. 1C) indicated that PHD2 had the highest activity of the three isoforms. The gels were also stained with Coomassie Blue to confirm equal loading of the GST-ODD substrate. In agreement with several other studies, our result confirms that PHD2 is the major HIF-1 α prolyl-4-hydroxylase (Appelhoff et al., 2004; Berra et al., 2003).

Structural analysis using ExPasy programs (<http://us.expasy.org>) predicted that the N-terminal region of PHD2 (amino acids 21 to 58) contained a MYND-type zinc finger domain. The term MYND refers to three proteins: Myeloid translocation protein 8, Nervy, and DEAF1. The C-terminal region of PHD2 contains the conserved catalytic domain (amino acids 294 to 392) of 2-oxoglutarate and Fe (II)-dependent dioxygenases such as collagen prolyl-4 hydroxylase (P4Hc) (Fig. 2A). We constructed a number of deletion mutants of PHD2 and transcribed and translated each of them *in vitro* in the rabbit reticulocyte system. VHL pull down experiments showed that proteins lacking the zinc finger domain, such as PHD-184, PHD2-182, and PHD2-60 had high activity, whereas those that retained the zinc finger domain, such as PHD2-F and PHD2-16 had

less activities (Fig. 2B, and C). These results indicate that the zinc-finger motif is inhibitory.

With the aim of determining the crystal structure of the catalytic domain of PHD2, we cloned a cDNA for amino acids 184 to 418 into a prokaryotic expression vector and expressed it with a histidine tag in *E. coli* (Fig. 2A). Both full-length PHD2-F and PHD2-184 were purified by Ni affinity chromatography and gel filtration chromatography. They had the expected molecular weights and were present in the soluble fraction of *E. coli* (Fig. 3A). Interestingly, when we tested the purified products using the [³⁵S]-labeled VHL pull down assay, the truncated form, PHD2-184, proved to have much higher activity than full-length PHD2-F (Fig. 3B). Our observations confirmed that the catalytic domain occupies the C-terminal half of PHD2 and that the N-terminal half, an inhibitory domain. In order to measure the activity of the recombinant PHD2-184 by detecting hydroxylation of HIF-1 α rather than by visualizing captured VHL, we incubated biotinylated HIF-1 α peptide (amino acids 556 to 575) with PHD2-184, and determined the change in molecular weight of the peptide by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis. Since the peptide contains proline-564, hydroxylation by PHD2-184 increases its molecular weight. HIF-1 α peptide samples treated with PHD2-184 showed a second MALDI-TOF peak that corresponded to an increase in molecular weight of 16 (Fig. 3C). This confirms that the recombinant PHD2-184 hydroxylates HIF-1 α without any other cellular components.

We tested whether the hydroxylation reaction can be reversed. Immobilized HIF-1 α was treated with recombinant PHD2-184 or S-100 fraction of HeLa cells in normoxia for 30 min and then further incubated in anoxic condition for the indicated

periods of time. VHL pull down analysis (Fig. 3D) indicated that lack of oxygen did not reverse the hydroxylation reaction (Masson *et al.*, 2001; Chan *et al.*, 2002). This finding suggests that, to stabilize HIF-1 α , hypoxia can reduce the interaction of VHL with newly synthesized HIF-1 α but not reverse the hydroxylation of preexisting HIF-1 α .

Effects of Zn chelator, TPEN on the activity of PHD2 — To confirm the inhibitory action of the MYND-type zinc finger domain, we treated recombinant PHD2-F and PHD2-184 proteins with the Zn-specific chelator TPEN, and measured their activities. Hydroxylation and VHL pull-down analysis indicated that TPEN increased the activity of recombinant PHD2-F, but not that of PHD2-184 (Fig. 4A and C). It also increased the activity of *in vitro* transcribed and translated PHD2-F but not of PHD2-184 or PHD2-60, which lack the MYND zinc finger domain (Fig. 4B). Although TPEN can also act as an iron-chelating agent, this did not affect the activity of the PHD2 mutants because an excess of iron (100 μ M) was present in the reaction mixtures together with the TPEN (2 or 5 μ M). Moreover the addition of zinc ions reversed the effect of TPEN on PHD2-F (Fig. 4A, B, and C). These observations imply that chelating Zn (II) with TPEN activates the enzyme by incapacitating the MYND zinc finger domain.

Effect of the MYND zinc finger domain on the stability and transactivation of HIF-1 α — The finding that the MYND domain inhibits hydroxylation of HIF-1 α and its interaction with VHL suggested that deletion of the MYND domain would increase VHL-dependent ubiquitination and degradation of HIF-1 α . HeLa cells were transfected with enough HIF-1 α plasmid (1 μ g) to overcome hydroxylation/VHL/ubiquitin-dependent degradation, and Western analysis showed that HIF-1 α protein could be detected even in normoxic condition. Cotransfection with a limited amount (500 ng) of

Flag-tagged PHD2-F plasmid reduced the level of HIF-1 α slightly, whereas cotransfection with the same amount of PHD2-60 had a greater effect in both normoxia (21% O₂) and partial hypoxia (5 % hypoxia), indicating that deletion of the MYND domain increases hydroxylation/VHL dependent degradation of HIF-1 α . Western analyses with Flag antibody showed that the transfected PHD2-F and PHD2-60 were expressed at similar levels. To test whether the transfected PHD2 also affected the level of endogenous HIF-1 α , HeLa cells were transfected with limited amounts (700 ng) of PHD2-F or PHD2-60, and exposed to partial hypoxia (5% O₂). This had a less stabilizing effect on the endogenous HIF-1 α than complete anoxia (0% O₂), indicating that HIF-1 α was still in part being hydroxylated and degraded by VHL. Consistent with the previous results, PHD2-60 was more effective than PHD2-F in destabilizing the endogenous HIF-1 α (Fig. 5B).

To confirm that deletion of the MYND zinc domain results in greater inactivation of HIF-1 α , we transfected HeLa cells with plasmids encoding HIF-1 α and PHD2 together with a hypoxia-inducible luciferase reporter. Transfection with HIF-1 α increases reporter genes even in normoxic condition. PHD2-60 proved to be more effective than PHD2-F in blocking HIF-1 α -dependent induction of the reporter gene (Fig. 6) demonstrating that the presence of the MYND domain limits the hydroxylation/VHL dependent degradation of HIF-1 α *in vivo*.

Discussion

We have shown that purified recombinant PHD2 can hydroxylate HIF-1 α without needing any other polypeptides, unlike collagen proline hydroxylase which consists of two α chains and two β chains. In agreement with several other studies, we confirmed that PHD2 is the major HIF-1 α prolyl-4-hydroxylase. PHD2 shares the conserved catalytic domain of 2-oxoglutarate and Fe (II)-dependent dioxygenases with other prolyl-4 hydroxylases including PHD1, PHD3 and collagen prolyl hydroxylase, but has a unique N-terminal MYND-type zinc finger domain. We have demonstrated that deletion of the MYND-type zinc finger domain increases the activity of both *in vitro* translated PHD2 and recombinant PHD2 protein (Fig. 2 and 3), and that treatment with the Zn chelator, TPEN increases the activity of PHD2-F but not PHD2-mutants which lack the MYND domain (Fig. 4), indicating that the catalytic activity of PHD2 is inhibited by the N-terminal zinc finger domain. Our transfection analyses demonstrated that deletion of the MYND domain destabilized HIF-1 α under both normoxia and hypoxia (5% O₂), and decreased the expression of an HIF-1 α -driven reporter gene. These results suggest that the MYND domain inhibits the hydroxylation activity of PHD2 and the resulting VHL-dependent degradation of HIF-1 α *in vivo* (Fig. 7). It will be of interest to determine whether the mutations affecting the MYND domain found in certain human diseases affect the activity of PHD2 and the stability of HIF-1.

The reduction in the stability of HIF-1 α due to deletion of MYND domain *in vivo* was small compared to the reduction in enzyme activity *in vitro*. This reflects the fact that hydroxylation of HIF-1 α may not be a limiting step for its ubiquitin-dependent degradation *in vivo*. Since the MYND domain inhibits the catalytic activity of pure

recombinant PHD2, this domain may reduce the accessibility of its catalytic domain. Although more work needs to be done, the results of a yeast two hybrid screen suggest that the MYND domain of PHD2 does not interact with the catalytic domain but rather with a component of a specialized cytoplasmic organelle (unpublished data).

MYND is an acronym for the three best-characterized representatives, MYeloid translocation protein 8 (MTG8/ETO) (Wang et al., 1998), Nervy protein, and Deaf-1. The MYND-type zinc finger contains 8 amino acids that can coordinate 2 zinc atoms (Fig. 2A). The common function of this domain is not clear, but many of the proteins, including MTG8/ETO (Lutterbach et al., 1998; Wang et al., 1998), BS69 (Ansieau and Leutz, 2002), m-Bop (Gottlieb et al., 2002), and Mammalian programmed cell death protein 2 (PDCD2/RP8PDCD2) (Scarr and Sharp, 2002), are known to be transcriptional repressors. MTG8 is part of a high molecular weight complex that contains co-repressors and HDACs (Lutterbach et al., 1998; Wang et al., 1998), while BS69 is an adenovirus E1A binding protein that binds to the transactivation domain of the adenovirus type 5 E1A 32 kDa protein (289R) and inhibits its transactivation activity (Ladendorff et al., 2001). The MYND domain of BS69 interacts with the PXLXP motifs of several other cellular and oncoviral-proteins including Epstein-Barr virus, EBNA2, and Myc-related cellular protein, MGA, as well as c-Myb (Ansieau and Leutz, 2002). Its MYND domain also interacts with a co-repressor, N-CoR, and is a component of several transcriptional repressor complexes (Masselink and Bernards, 2000). Bop is expressed specifically in cardiac and muscle precursor cells and mediates chromatin modification as an HDAC-dependent repressor essential for cardiogenesis (Gottlieb et al., 2002). The MYND domain of Bop also interacts with muscle-specific transcription factor, nascent polypeptide-associated complex (sk-NAC) The PXLXP

motif of skNAC is required for interaction with MYND domain of m-Bop (Wang et al., 1998). The finding that the MYND domains of several proteins are involved in interactions with PXLXP motifs suggests that the same may be true of the MYND type domain of PHD2.

The fact that the N-terminal MYND domain of PHD2 has an inhibitory effect on the C terminal catalytic activity, and that many MYND domains are involved in protein-protein interactions suggests that the catalytic activity of PHD2 may be modulated by a cellular factor that interacts with the MYND domain of PHD2.

Acknowledgements

We thank Drs. Kyu-Won Kim (Seoul National University), Seoung-Eon Ryu (Korea Research Institute of Bioscience and Biotechnology) for providing pCMV/myc(3B)-VHL and GST-ODD. K-O. Choi, T. Lee and N. Lee contributed equally to this study.

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Regulation of PHD2 activity by MYND-Zn finger

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Footnotes

This work was supported by a grant (KRF-2002-041-C00207) from the Korean Research Foundation (to H. P.). K-O. C., T. L. and N. L. contributed equally to this work. Present address of N. L: Biologics Standardization Division, Biologics Evaluation Department, Korea Food & Drug Administration, 5 Nokbun-dong Eunpyung-Gu, Seoul, 122-704, Korea

Figure Legends

Fig. 1. HIF-1 α -prolyl-4-hydroxylation activity of PHD1, 2, 3. (A) HIF-1 α 20mer peptide (Biotin-DLDLEMLAPYIPMDDDFQL) was incubated with 15 μ l of each PHD-programmed rabbit reticulocyte lysate. All reactions were performed in the presence of 100 μ M ferrous chloride, 2 mM ascorbic acid, and 5 mM α -ketoglutarate at 30 $^{\circ}$ C. Labeled VHL was added with the treated bead-bound peptide, and the captured [35 S]-labeled VHL was visualized by SDS-PAGE and autoradiography. Ten percent (1 μ l) of the labeled VHL was loaded. As a negative control, 15 μ l of unprogrammed rabbit reticulocyte lysate was used, while hydroxylated peptide (Biotin-DLDLEMLA(P-OH)YIPMDDDFQL) served as a positive control. (B) Resin-bound GST-ODD was incubated with 15 μ l of each PHD-programmed rabbit reticulocyte lysate. All reactions were performed as mentioned above. The amount of each sample loaded was monitored by measuring GST-ODD stained with Coomassie blue. One representative of at least three independent experiments with similar results is shown. (C) The expression levels of the *in vitro* translated PHD1, 2 and 3 isoforms. [35 S]-labeled PHD isoforms were synthesized by *in vitro* transcription and translation. To estimate the amounts of the synthesized PHDs, 8 μ l of each programmed lysate was analyzed by 12% SDS-PAGE followed by autoradiography. One representative of at least three independent experiments with similar results is shown.

Fig. 2. Comparison of the activities of PHD2 deletion mutants (A) Structural analysis using ExPasy programs (<http://us.expasy.org>). The N-terminal region of PHD2 (amino acids 21 to 58) contains a MYND type zinc finger domain. The C-terminal region has the conserved catalytic domain (amino acids 294 to 392) of 2-oxoglutarate and Fe(II)-

dependent dioxygenases such as collagen prolyl-4 hydroxylase (amino acids 205 to 392). The consensus sequences among MYND-type zinc finger proteins, PHD2, MT8R, MTG8, DEAF, BS69, PDC2 are shown. The deletion mutants of PHD2 are shown. (B) Hydroxylation and VHL pull down activity of the PHD2 mutants. Each PHD2 deletion mutant was synthesized by *in vitro* transcription and translation using rabbit reticulocyte lysate. Resin-bound GST-ODD was incubated with 15 μ l of each PHD-programmed rabbit reticulocyte lysate. Prolyl-hydroxylation of HIF-1 α was measured by VHL-pull down as described in Methods. The amount of each sample loaded was monitored by measuring GST-ODD stained with Coomassie blue. (C) HIF-1 α 20mer peptide (Biotin-DLDLEMLAPYIPMDDDFQL) was incubated with 15 μ l of each PHD-programmed rabbit reticulocyte lysate. Prolyl-hydroxylation of HIF-1 α was measured by VHL-pull down as described in Methods. One representative of three independent experiments with similar results is shown. (D) The expression levels of the *in vitro* translated PHD2 mutants. [³⁵S]-labeled PHD2 mutants were synthesized by *in vitro* transcription and translation. To estimate the amounts of the synthesized PHDs, 8 μ l of each programmed lysate was analyzed by 12% SDS-PAGE followed by autoradiography. PHD2-184 (amino acids 184-418) was expressed as a his-tagged protein with an additional 40 amino acids between residue 418 of PHD2 and the histidine tag. One representative of three independent experiments with similar results is shown.

Fig. 3. Prolyl-hydroxylation activity of recombinant PHD2. (A) Expression of recombinant PHD2 in *E. coli*. Histidine-tagged full length PHD2 (PHD2-F, amino acids 1-426), and truncated PHD2 (PHD2-184, amino acids 184-419) were purified by Ni-affinity and gel-filtration chromatography. Elution fractions E6 to E8 (upper panel) and

E2 to E4 (lower panel) were used as PHD2-F protein and PHD2-184 protein, respectively. The purified proteins were analyzed on a SDS-PAGE and visualized by Coomassie blue staining. (B) Comparison of the activity of PHD2-F and PHD2-184. The indicated amount of PHD2-184 recombinant protein or PHD2-F recombinant protein was added to HIF-1 α 20mer peptide (7 μ g). The hydroxylation reaction was performed as described in Methods. The resin was washed and mixed with 10 μ l of [³⁵S]-labeled VHL, and VHL captured by hydroxylated HIF-1 α was resolved by SDS-PAGE and visualized by autoradiography. (C) MALDI-TOF spectra of biotinylated synthetic HIF-1 α peptide (556-575: biotin-DLDLEMLAPYIPMDDDFQLR), and HIF-1 α peptide containing hydroxyproline at position 564 (556-575: biotin-DLDLEMLA(P-OH)YIPMDDDFQL). The masses of the peptides are 2637.2 m/z and 2653.1 m/z , respectively. The lower panel shows a MALDI-TOF spectrum of the biotinylated HIF-1 α peptide after incubation with PHD2-184 protein (400 ng) as described in Methods. (D) Test for reversibility of PHD2 activity. 100 ng of PHD2-184 recombinant protein and S-100 fraction of HeLa cell extract (1 mg of microsomal proteins) that has the HIF-1 α -specific prolyl-hydroxylase activity (Masson *et al.*, 2001) were used as enzyme source. Hydroxylation was performed in normoxic condition for 30 min and the reaction mixtures were then incubated in anaerobic condition (0.1% O₂, 5% CO₂, 85% N₂, 10% H₂) for the indicated times. All reactions were performed in the presence of 100 μ M ferrous chloride, 2 mM ascorbic acid, and 5 mM α -ketoglutarate at 30°C. 10 μ l of labeled VHL was added with the treated bead-bound GST-ODD, and the captured [³⁵S]-labeled VHL was visualized by SDS-PAGE and autoradiography. Hydroxylation was performed in normoxic condition for 30 min and the reaction mixtures were then incubated in anaerobic condition (0.1% O₂, 5% CO₂, 85% N₂, 10% H₂) for the

indicated times. All reactions were performed in the presence of 100 μ M ferrous chloride, 2 mM ascorbic acid, and 5 mM α -ketoglutarate at 30 °C. 10 μ l of labeled VHL was added with the treated bead-bound GST-ODD, and the captured [³⁵S]-labeled VHL was visualized by SDS-PAGE and autoradiography.

Fig. 4. Effects of TPEN on PHD2 activities. (A) 50 ng of the recombinant PHD2-F or PHD2-184 was preincubated with 2 μ M, 5 μ M TPEN or 5 μ M TPEN plus 10 μ M ZnCl₂ for 10 minutes at room temperature. They were then added to biotin-HIF-1 α (556-575) peptide and incubated as described in methods. The Captured [³⁵S]-labeled VHL was visualized by SDS-PAGE, and autoradiography. One representative of at least two independent experiments with similar results is shown. (B) The indicated amount of *in vitro* translated PHD2-F, PHD2-60 or PHD2-184 was preincubated with 2 μ M, 5 μ M TPEN or 5 μ M TPEN plus 10 μ M ZnCl₂ for 10 minutes at room temperature. They were then added to biotin-HIF-1 α (556-575) peptide and incubated as described in methods. The Captured [³⁵S]-labeled VHL was visualized by SDS-PAGE, and autoradiography. (C) The recombinant PHD2-F protein (1 μ g) were preincubated with 2 μ M, 5 μ M TPEN or 5 μ M TPEN plus 10 μ M ZnCl₂ for 10 minutes at room temperature. They were then added to resin-bound GST-ODD and incubated in the presence of 100 μ M ferrous chloride, 2 mM vitamin C, 5 mM α -ketoglutarate for 90 minutes at 30°C. After washing with NETN buffer, 10 μ l [³⁵S]-labeled VHL was added and incubation continued at 4°C for 2 hours. Captured VHL was visualized by SDS-PAGE, and autoradiography. The amount of each sample loaded was monitored by measuring GST- ODD stained with Coomassie blue. One representative of two independent experiments with similar results is shown.

Fig. 5 Effect of the MYND domain on the stability of HIF-1 α . (A) HeLa cells (2×10^5) were transfected with the indicated amounts of pCMV-3xFlag, pCMV-3xFlag-PHD2-F or pCMV-3xFlag-PHD2-60 together with pcDNA3.1-HIF-1 α . 48 hours later the cells were harvested. Prior to harvest, some of the transfected cells were exposed to 5% O₂ for 4 hours. For the Western analysis with anti-HIF-1 α antibody, 6 μ g of protein from the hypoxic cells and 30 μ g from the normoxic cells were fractionated by SDS-PAGE. The Coomassie blue staining indicates the amounts of the proteins loaded. For the Western analysis with anti-Flag antibody, 30 μ g aliquots of protein were loaded for SDS-PAGE. One representative of three independent experiments with similar results is shown. (B) 0.7 μ g of pCMV-3xFlag, pCMV-3xFlag-PHD2-F or pCMV-3xFlag-PHD2-60 were transfected into HeLa cells (2×10^5) without pcDNA3.1-HIF-1 α . Prior to harvest, the transfected HeLa cells were exposed to partial hypoxia (5% oxygen) or anoxia for 4 hours. For Western analysis with anti-HIF-1 α antibody, 5 μ g aliquots of protein were loaded for SDS-PAGE. For Western analysis with anti-Flag antibody, 30 μ g aliquots were loaded. One representative of three independent experiments with similar results is shown.

Fig. 6. Effect of the MYND domain on transactivation of HIF-1 α . HeLa cells (7×10^4) were transfected with an HRE-driven luciferase reporter plasmid (100 ng), a β -galactosidase-encoding plasmid (pCHO110, 100 ng), an HIF-1 α -encoding plasmid (pcDNA3.1- HIF-1 α , 700ng) and with PHD2-F or PHD2-60, as indicated. Values are ratios of luciferase activities driven by the hypoxia-responsive elements and β -galactosidase activities. Data shown are the mean \pm S.D. of five independent

determinations.

Fig. 7. Schematic diagram for the functional domains of PHD2.

Fig. 1

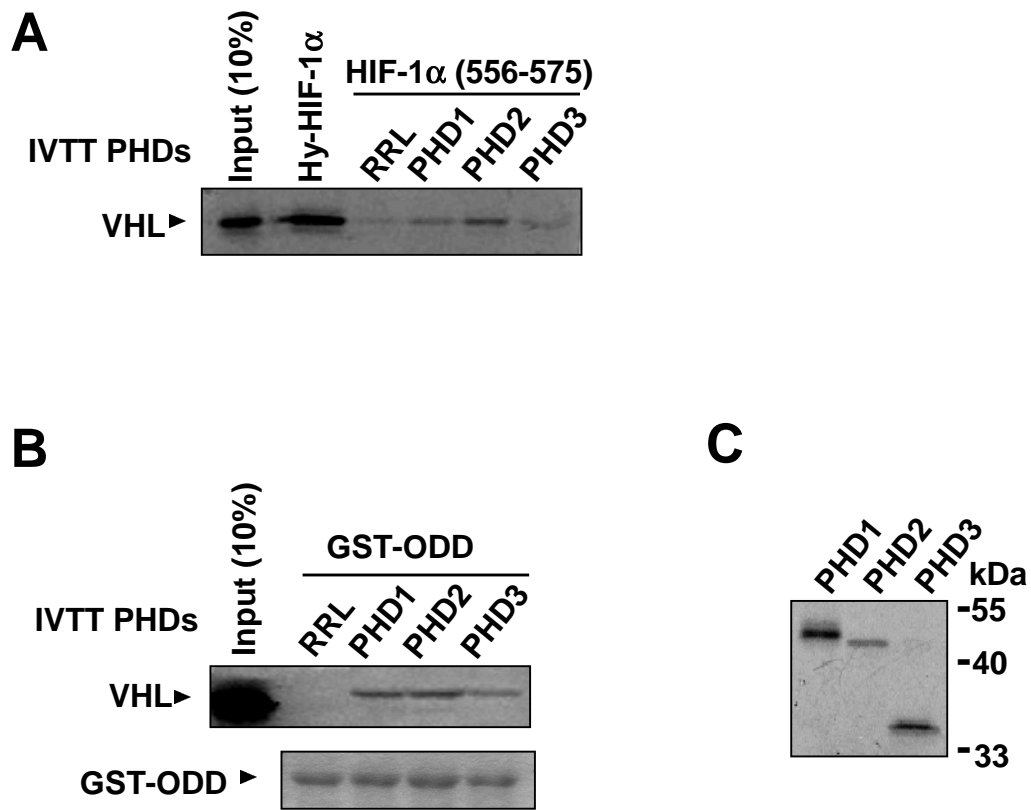


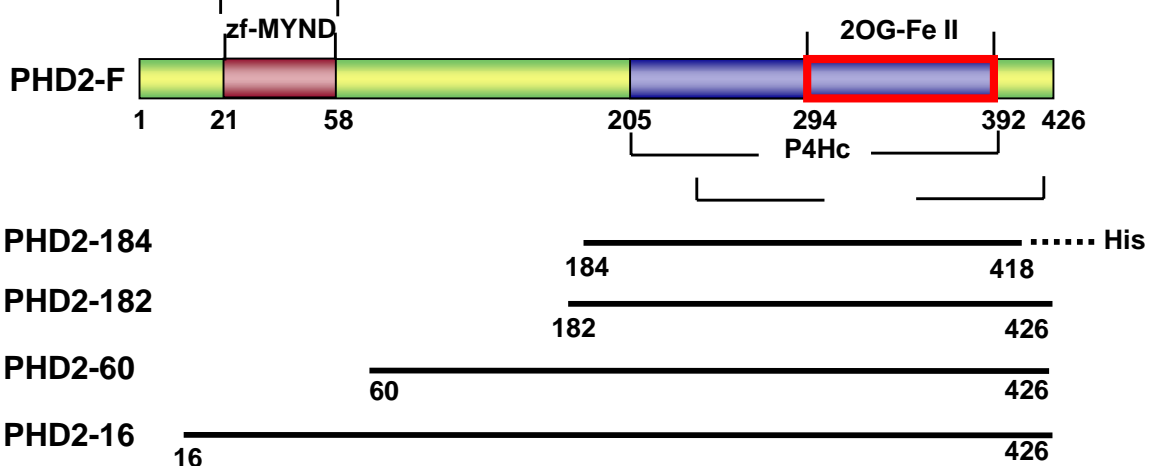
Fig. 2

A

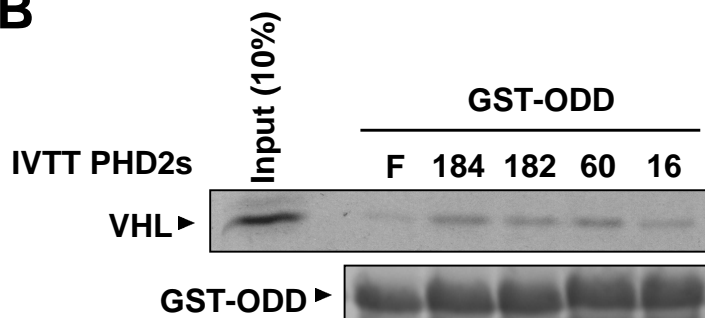
Consensus pattern

[CH]-X(2,4)-C-X(7,17)-C-X(0,2)-C-X(4)-[YFT]-C-X(3)-[CH]-X(6,9)-H-X(3,4)-C

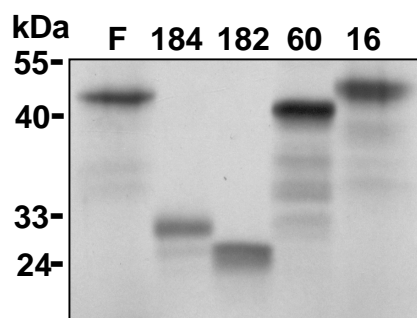
CELCGKMENL . . . LRCSRCRSSFYCCKEHQRQDWK . . . KHKLVC PHD2 (21-58)
CWNCGRKASE . . . - TCSGCNIARYCGSFCQHKDWE . . . RHHRLC MT8R (507-543)
CWNCGRKASE . . . - TCSGCNTARYCGSFCQHKDWE . . . KHHHIC MTG8 (515-551)
CVNCGREAMS . . . - ECTGCHKVNYCSTFCQRKDWK . . . DHQHIC DEAF (504-540)
CYNCEEEEAMY . . . - HC - - CWNTSYCSIKCQQEHWHa . . . EHKRTC BS69 (523-558)
CRVCGCLGP - . . . KTCSRCHKAYYCSKEHQTLDWRL . . . GHKQAC PDC2 (135-172)



B



D



C

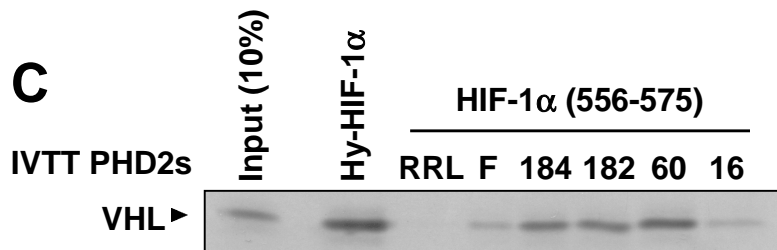
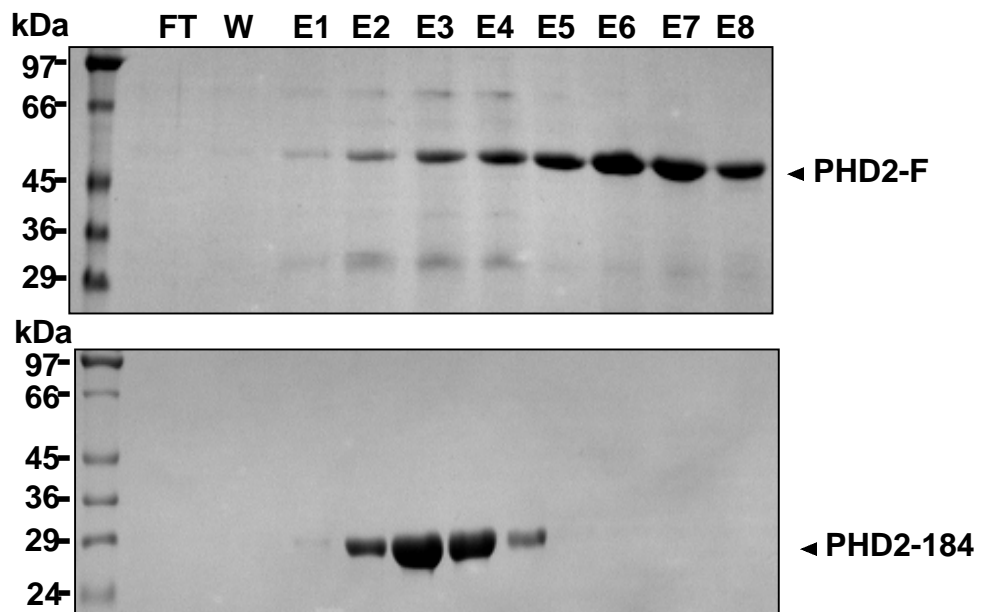


Fig. 3

A



B

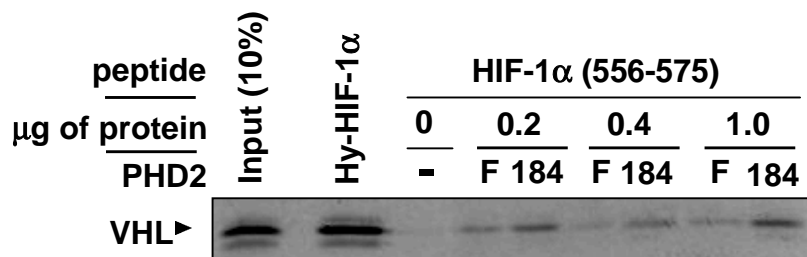


Fig. 3

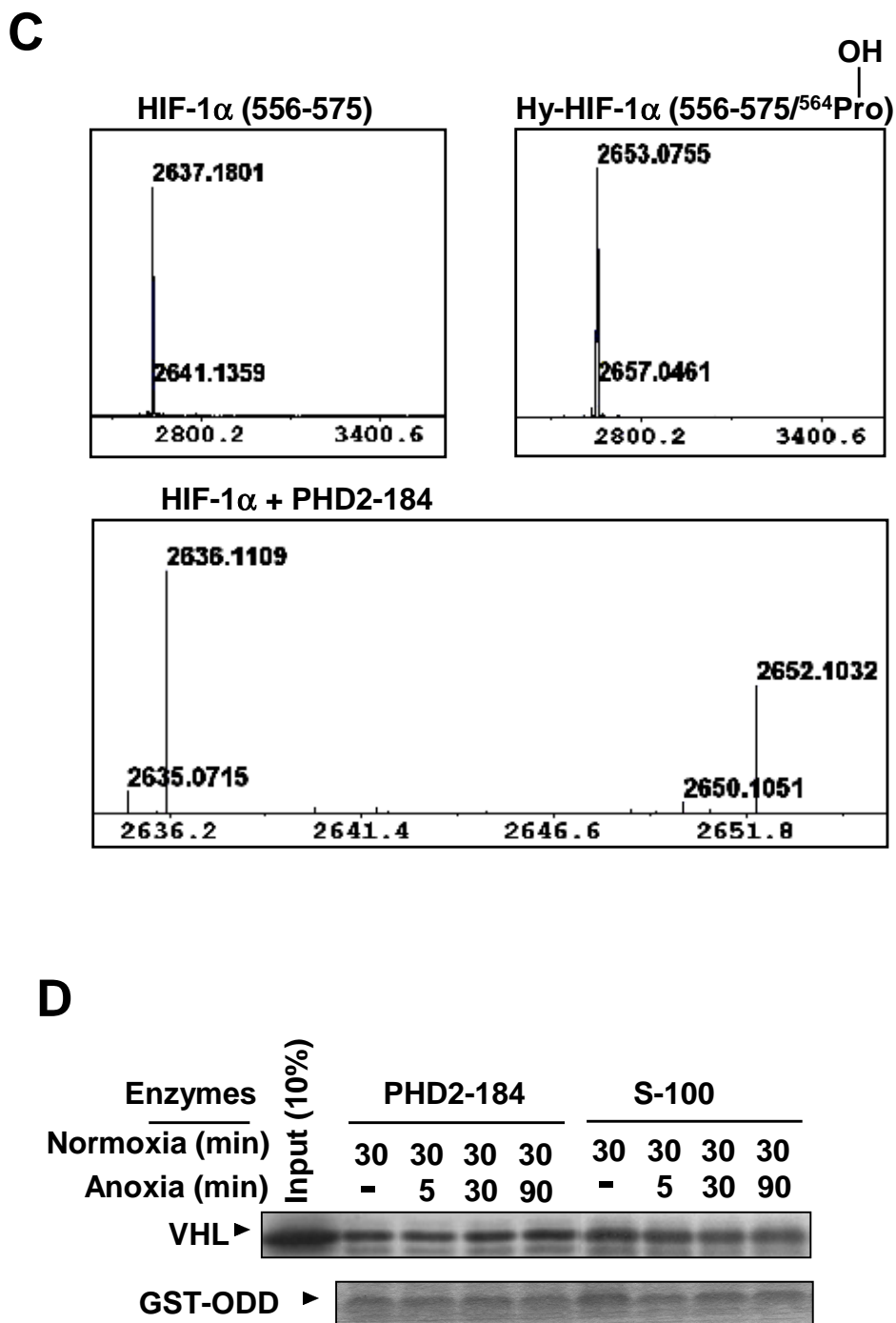


Fig. 4

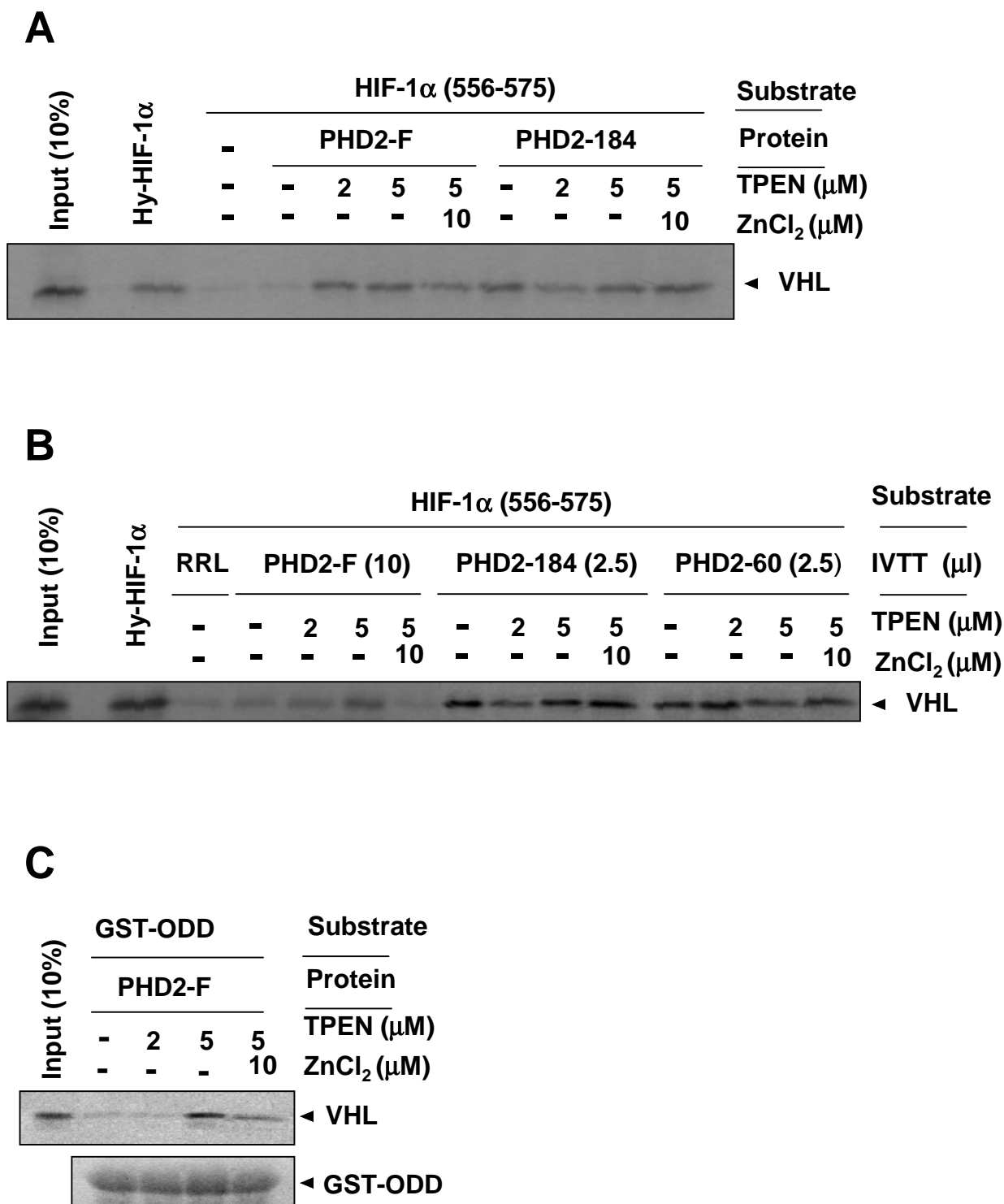


Fig. 5

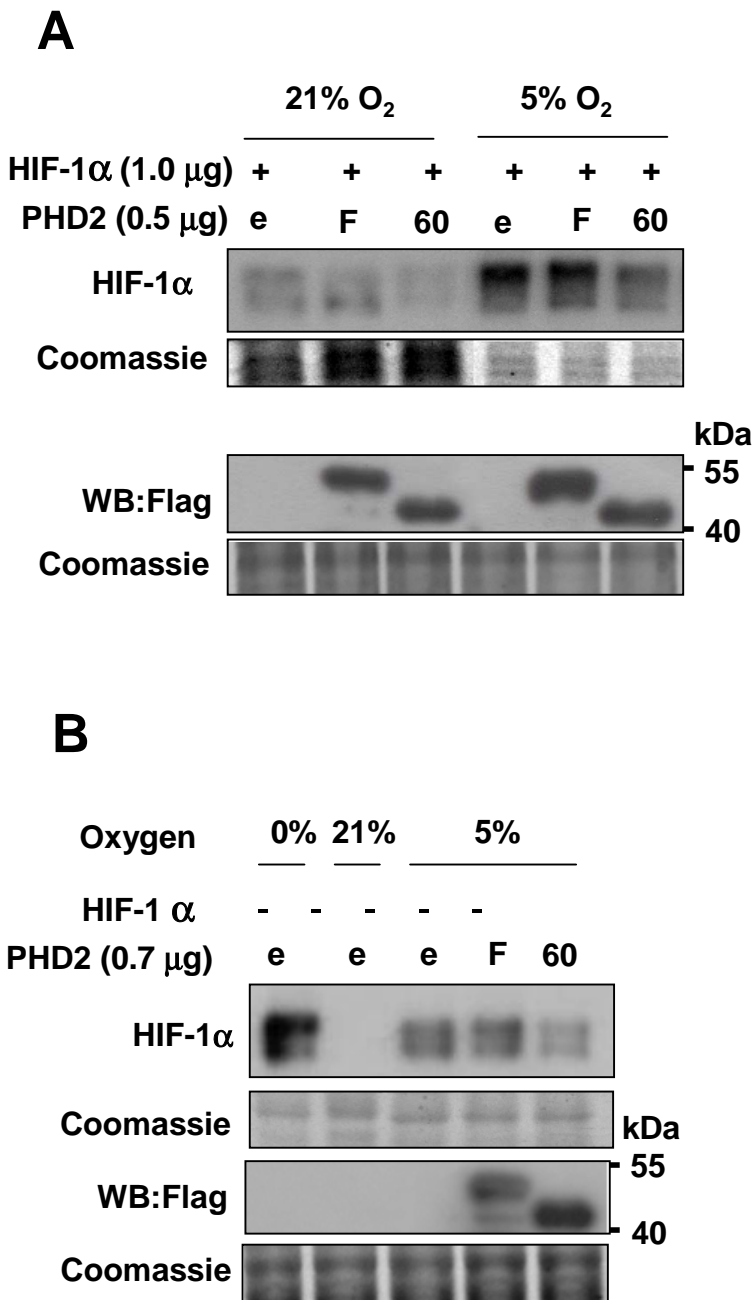


Fig. 6

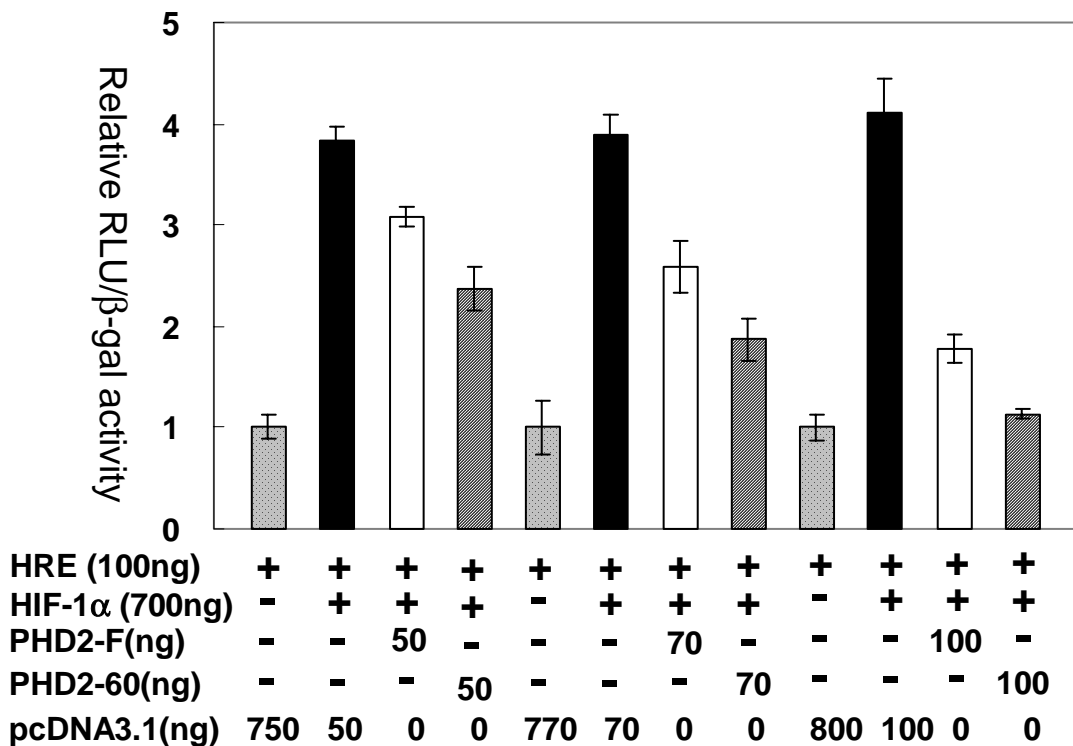


Fig. 7

