Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 337 (2005) 275–280
BBRC
www.elsevier.com/locate/ybbrc

A fluorescence polarization-based interaction assay for hypoxia-inducible factor prolyl hydroxylases ☆

Hyunju Cho [a], Hyunsung Park [b], Eun Gyeong Yang [a,*]

[a] *Life Sciences Division, Korea Institute of Science and Technology, Seoul, Republic of Korea*
[b] *Department of Life Science, University of Seoul, Seoul, Republic of Korea*

Received 5 September 2005
Available online 16 September 2005

Abstract

Oxygen-dependent ubiquitination and degradation of hypoxia-inducible factor 1α (HIF-1α) plays a central role in regulating transcriptional responses to hypoxia. This process requires hydroxylation of specific prolines in HIF-1α by HIF prolyl hydroxylase domain (PHD)-containing enzymes, leading to its specific interactions with von Hippel–Lindau protein-Elongin B–Elongin C (VBC). Here we describe a straightforward approach to apply these interactions to measure PHD activities. Employing fluorescently labeled HIF-1α peptides containing hydroxyproline, we developed a quantitative method based on fluorescence polarization for a systematic evaluation of binding of hydroxylated HIF-1α to recombinant VBC. The method was then successfully utilized for measuring the activity of the truncated, purified PHD2. The applicability of the assay was further demonstrated by examining effects of various cofactors and inhibitors for PHD2. The developed homogeneous assay would provide a convenient way of probing the biochemical properties of the HIF-1α–VBC interaction and PHDs, and of screening modulators for the interaction as well as the enzyme.

Keywords: Prolyl hydroxylase; Hydroxyproline; Hypoxia-inducible factor; von Hippel–Lindau protein; VBC; HIF-1α–VBC interaction; Fluorescence polarization

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional factor that regulates gene expression in mammalian development, physiology, and disease pathogenesis [1]. HIF-1 consists of an oxygen-sensitive HIF-1α subunit and a continuously expressed HIF-1β subunit. While rapidly degraded under normoxic conditions by the ubiquitin–proteasome system, HIF-1α is induced and stabilized in hypoxic conditions and functions as a master regulator of oxygen homeostasis [2]. HIF-1α proteolysis is mediated via hydroxylation of two highly conserved residues, Pro-402 and Pro-564, in human HIF-1α, which causes its direct and efficient interaction with von Hippel–Lindau protein (VHL) as a protein complex of VHL–Elongin B–Elongin C (VBC). Prolyl hydroxylation is catalyzed by three human prolyl hydroxylases (PHD1, PHD2, and PHD3), resulting in generation of 4-hydroxyproline [3–5]. These PHDs utilize iron and ascorbic acid as cofactors as well as oxygen and 2-oxoglutarate as substrates [6]. Among PHDs with distinct assigned functions, PHD2 is the critical oxygen sensor setting the low steady-state levels of HIF-1α in normoxia [7]. Interestingly, cobalt ion, *N*-oxyalyl amino acid derivatives, and iron chelators such as desferrioxamine (DFO) can mimic the effect of hypoxia on HIF-1α and modulate activities of PHDs [8–10]. Studies on molecular mechanisms of the HIF–VBC interaction and activities of PHDs have been commonly carried out by a tedious pull-down method involving immobilization of a HIF substrate on a Sepharose matrix, and its incubation with radiolabeled VBC expressed in reticulocyte lysate, followed by separation by SDS–PAGE

☆ *Abbreviations:* ACA, aminocaproic acid; DFO, desferrioxamine; EDHB, ethyl-3,4-dihydroxybenzoate; FP, fluorescence polarization; HIF, hypoxia-inducible factor; HyP, *trans*-4-hydroxyl-L-proline; IPTG, isopropyl β-D-1-thiogalactopyranoside; NOG, *N*-oxalylglycine; PHD, prolyl hydroxylase domain; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)ethylenediamine; VBC, von Hippel–Lindau protein-Elongin B–Elongin C; VHL, von Hippel–Lindau protein.

* Corresponding author. Fax: +82 2 958 5909.
E-mail address: eunyang@kist.re.kr (E.G. Yang).

0006-291X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2005.09.041

and detection by autoradiography. Very recently, more quantitative methods including surface plasmon resonance experiments [11] and an avidin microtiter plate-based immunoassay [12] have been developed for the binding kinetics and for the detection of hydroxylated HIF and inhibitors. In addition, a fluorescence-based assay using *o*-phenylenediamine [13] has been devised for 2-oxoglutarate-dependent oxygenases. While some of these methods present problems including lengthy analysis time and intricacy of the protocol, others require the use of radioactive reagents or expensive materials. Accordingly, we have developed an alternative homogeneous assay based on the HIF–VBC interaction to report the activity of HIF prolyl hydroxylases. This assay relies on the fluorescence polarization property of fluorescein-labeled HIF peptides that can be hydroxylated on the specific proline residues. The presence of hydroxyproline leads to binding of the peptides to VBC, resulting in an increase of fluorescence polarization values. The sensitivity and specificity of this quantitative interaction assay then enabled detection of PHD2 activity as well as evaluation of inhibitors for the enzyme. The developed assay system would serve as a convenient screening tool for inhibitors against PHDs as well as for modulators of the HIF-1α–VBC interactions.

Materials and methods

Materials. Ethyl-3,4-dihydroxybenzoate (EDHB), $CoCl_2$ hexahydrate, and DFO mesylate were purchased from Sigma–Aldrich (St. Louis, MO, USA). *N*-Oxalylglycine (NOG) was obtained from Axxora Life Sciences (San Diego, CA, USA) and *N*,*N*,*N*′,N′-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN) was from Calbiochem (Darmstadt, Germany). All other reagents were of the highest grade commercially available.

Preparation of peptides. Peptides containing amino acids 556–575 and 390–417 of human HIF-1α with or without a *trans*-4-hydroxyl-L-proline (HyP, Merck) substituted for Pro-564 and Pro-402, respectively, were synthesized. Fluorescein was conjugated to the peptides with the N-terminal insertion of an aminocaproic acid (ACA) linker. All the peptides were synthesized by AnyGen (KwangJu, Korea). The synthesized peptides were denoted as HyP564 (DLDLEALAHyPYIPADDDFQLR), F-P564 (FITC-ACA-DLDLEALAPYIPADDDFQLR), F-HyP564 (FITC-ACA-DLDLEALAHyPYIPADDDFQLR), F-P402 (FITC-ACA-LKKEPDA LTLLAPAAGDTIISLDFGSND), and F-HyP402 (FITC-ACA-LKK EPDALTLLAHyPAAGDTIISLDFGSND). Additionally, truncated peptides were prepared for N-segment (amino acids 561-568, ALAPY PA), HyN-segment (ALAHyPYIPA), and C-segment (amino acids 569–575, DDDFQLR).

Protein expression and purification. Plasmids for VHL (amino acids 54–213) and human Elongin B (amino acids 1–118) in pGEX-4T-1 (Amersham Biosciences) and for human Elongin C (amino acids 17–112) in pET29b (Novagen) kindly provided by Dr. Cheolju Lee (KIST, Korea) were co-expressed in *Escherichia coli* BL21(DE3). The truncated human PHD2 (amino acids 184–418) gene was subcloned into pGEX-4T-1 (Amersham Biosciences) and also expressed in the same *E. coli* strain. The recombinant proteins were induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG) at 18 °C for 15 h and purified using glutathione–Sepharose (Amersham Biosciences). The purified GST fusion proteins, GST-VBC and GST-PHD2, were confirmed by SDS–PAGE and quantified by BCA protein assay (Pierce).

Prolyl hydroxylation by PHD2. F-P564 peptide or F-P402 peptide at the final concentration of 1 μM was incubated with various amounts of recombinant GST-PHD2 in NETN buffer (20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P40, and 1 mM PMSF) containing cofactors such as 200 μM ascorbic acid and 20 μM α-ketoglutarate at room temperature. The reaction mixtures contained 5 mM α-ketoglutarate, 2 mM ascorbic acid, and 100 μM $FeCl_2$ as previously reported [14], unless stated otherwise. For stopping the reaction, the reactant was heated for 1 min at 95 °C, followed by addition of GST-VBC in EBC buffer (50 mM Tris, pH 8.0, 120 mM NaCl, and 0.5% Nonidet P40). For MALDI-TOF analyses to monitor hydroxylation reaction, α-cyano-4-hydroxycinnamic acid solution (Applied Biosystems) 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 (1:1 ratio) and mass spectrometric analyses were performed with a Voyager analyzer (Applied Biosystems).

Binding assays and calculations. Synthesized HIF-1α peptides or peptides treated with GST-PHD2 were mixed with GST-VBC in EBC buffer at room temperature, and fluorescence polarization values were measured immediately using an LS50B luminescence spectrometer (Perkin-Elmer). The final volume was 0.5 mL and the mixture contained 100 nM of fluorescein-labeled peptides. Data analyses were performed using KaleidaGraph software. The dissociation constants for the binding experiments and for the competition experiments, and the IC_{50} (or EC_{50}) values were determined according to the following equations, respectively:

$$FP = FP_0 + (FP_{\max} - FP_0) \times \left(\frac{([\mathrm{A}]_0 + [\mathrm{B}]_0 + K_\mathrm{d}) - \sqrt{([\mathrm{A}]_0 + [\mathrm{B}]_0 + K_\mathrm{d})^2 - 4[\mathrm{A}]_0[\mathrm{B}]_0}}{2[\mathrm{B}]_0} \right), \quad (1)$$

$$[C] = \left(\left(\frac{K_i(FP_{\max} - FP)}{K_\mathrm{d}(FP - FP_0)} \right) + 1 \right) \times \left([\mathrm{A}]_0 - K_\mathrm{d} \left(\frac{FP - FP_0}{FP_{\max} - FP} \right) - [\mathrm{B}]_0 \left(\frac{FP - FP_0}{FP_{\max} - FP_0} \right) \right), \quad (2)$$

$$FP = FP_0 + \left(\frac{(FP_{\max} - FP_0)}{\left(1 + 10^{(\log(FP) - \log(\mathrm{IC}_{50}))}\right)} \right), \quad (3)$$

where A, B, and C denote VBC, F-HyP564 or F-HyP402, and the unlabeled peptide, respectively, FP, FP_0, and $FP_{\max}$ are the fluorescence polarization values for the sample of interest, for the sample when the concentration of VBC is zero, and for the sample when the concentration of VBC is infinite, respectively, K_d and K_i denote the dissociation constant between VBC and the fluorescently labeled peptide, and the dissociation constant between VBC and the unlabeled peptide, respectively, IC_{50} is the concentration required for 50% inhibition, and $[\mathrm{A}]_0$ and $[\mathrm{B}]_0$ are the initial concentrations of VBC and F-HyP564 or F-HyP402, respectively.

Results and discussion

Fluorescence polarization-based measurements of the specific binding of hydroxyproline-containing HIF-1α peptides with VBC

Since binding of HIF to VBC is known to be highly dependent on the hydroxyproline residues of the HIF protein, we have explored conditions for assaying the enzyme activity through the HIF–VBC interaction analysis to provide an alternative to the laborious and often ambiguous equilibrium measurements performed via gel electrophoretic binding evaluations. Initially, fluorescein-labeled HIF-1α peptides having two conserved residues, Pro-564 and Pro-402, were designed and synthesized, and GST-VBC was expressed in *E. coli* and purified by affinity chromatography. Binding between the peptides and VBC was then examined by fluorescence polarization measurements for the requirement of hydroxyproline in these peptides in order to mimic

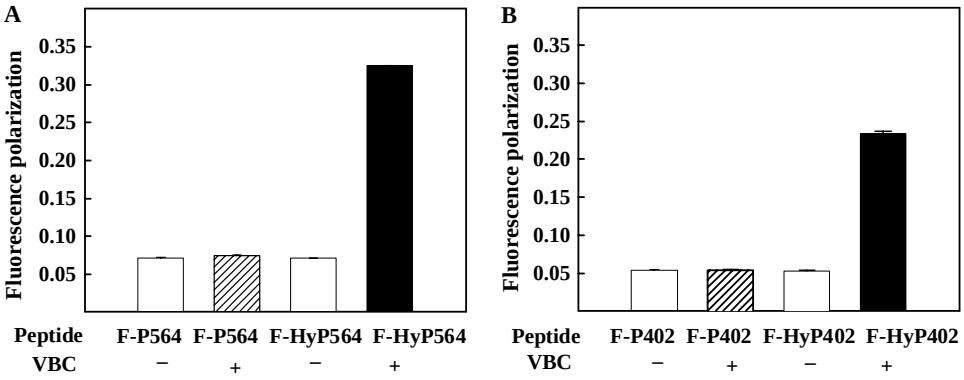

Fig. 1. Fluorescence polarization changes measured for the HIF-1α–VBC interaction using fluorescently labeled peptides. (A) Fluorescein-labeled peptides containing Pro-564 and (B) those containing Pro-402 were diluted in EBC buffer to the final concentration of 100 nM in the presence or absence of 800 nM GST-VBC. Each bar represents the mean of triplicates ±SD.

enzymatic hydroxylation. A dramatic increase in fluorescence polarization was observed for the hydroxylated peptide F-HyP564 upon addition of VBC, whereas the F-P564 peptide showed no difference in fluorescence polarization regardless of the presence of VBC (Fig. 1A). This increase indicated significant binding of the relatively small hydroxylated peptide to the relatively large VBC protein complex. In addition, F-HyP402 had a notable increase in the fluorescence polarization value when mixed with VBC, although relatively lower than for F-HyP564 (Fig. 1B). These results are consistent with the previous reports that HIF-1α binds VBC when the conserved prolines in HIF-1α are hydroxylated [3–5].

After establishing the hydroxyproline-specific interaction of the HIF-1α peptides with VBC, binding affinities were determined for the hydroxylated peptide–VBC mixtures with increasing VBC concentrations at a fixed peptide concentration of 100 nM by fluorescence polarization measurements. As expected, fluorescence polarization values of both F-HyP564 and F-HyP402 increased linearly with increasing concentrations of VBC and eventually approached a plateau (Fig. 2). Nonlinear regression analysis of the data was performed and the dissociation constants were determined to be 138.1 ± 16.6 nM for the F-HyP564 peptide and 337.8 ± 55.4 nM for the F-HyP402 peptide, similar to the previously obtained values by X-ray structural determination [11,15]. Therefore, the simple measurements of fluorescence polarization appear to be suited for quantitative analysis of the interaction between the fluorescein-labeled peptides and VBC.

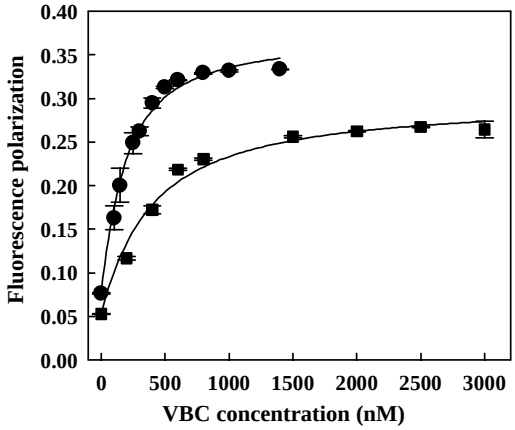

Fig. 2. Binding affinity measurements for the F-HyP564 (●) and F-HyP402 (■) peptides to VBC. The peptides in EBC buffer at the final concentrations of 100 nM were mixed with increasing concentrations of GST-VBC, and their fluorescence polarization changes were measured. The curves were fitted using KaleidaGraph program to obtain dissociation constants. Each point represents the average of triplicate assays ±SD.

Competitive assays against the HIF–VBC interaction

To further confirm that the binding of VBC to F-HyP564 depends on the hydroxylation of Pro-564, the competitive binding of the unlabeled hydroxylated peptide HyP564 was examined by adding varying concentrations of HyP564 to the F-HyP564-VBC mixtures. The fluorescence polarization value of the mixtures decreased as the HyP564 concentration increased gradually, eventually approaching the level close to the value of the unbound F-HyP564 peptide (Fig. 3). The dissociation constant between VBC and HyP564 was determined to be 3.7 ± 3.5 μM by nonlinear regression analysis. Then, the relative contribution of the F-HyP564 segments to its interaction with VBC was investigated by performing the competition experiments with truncated peptides. The fluorescence polarization values were measured for the mixtures of VBC and F-HyP564 with addition of HyN-segment, N-segment or C-segment at varying concentrations. As shown in Fig. 3, only the HyN-segment containing hydroxyproline inhibited the binding of VBC to F-HyP564 with a K_i of 20.1 ± 2.1 μM, while the non-hydroxylated N-segment and C-segment peptides did not inhibit the binding significantly up to 500 μM. Thus, we confirmed that the presence of hydroxyproline in the relatively small region of the N-terminal segment of HIF plays a crucial role in the HIF-1α–VBC binding, consistent with the previous report [15]. In addi-

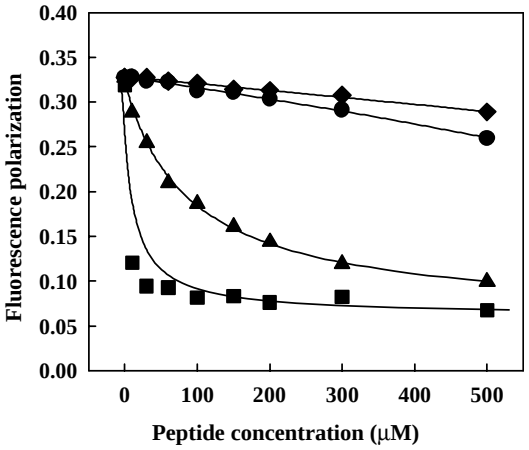

Fig. 3. Effects of various peptides on the HIF-1α–VBC interaction. HyP564 (■), N-segment (♦), HyN-segment (▲), and C-segment (●) were added to the mixture containing 100 nM F-HyP564 peptide and 500 nM GST-VBC and their competitive binding was assayed by measuring fluorescence polarization values. The curves were fitted using KaleidaGraph program to calculate inhibitory potency. Each point represents the average of triplicate assays ±SD.

tion, these experiments demonstrated that our developed method would serve as a convenient screening tool for inhibitors that block the HIF-1α–VBC interaction.

PHD2 activity measurements based on the HIF–VBC binding

In an attempt to utilize the assay described above for the detection of prolyl hydroxylase activity, we performed prolyl hydroxylation of the F-P564 peptide with recombinant PHD2, followed by fluorescence polarization measurements upon addition of VBC. As shown in Fig. 4A, the reacted F-P564 peptide exhibited a significant increase in fluorescence polarization similar to the synthesized F-HyP564, whereas there was no increase in the value of the untreated F-P564 peptide. However, fluorescence polarization change was minimal when the F-P402 peptide was reacted with the same amount of PHD2 and bound to VBC (Fig. 4B). The degree of F-P402 hydroxylation did not appear to increase by longer incubations at a higher enzyme concentration (data not shown), implicating inefficient hydroxylation of this peptide by PHD2. Consistent

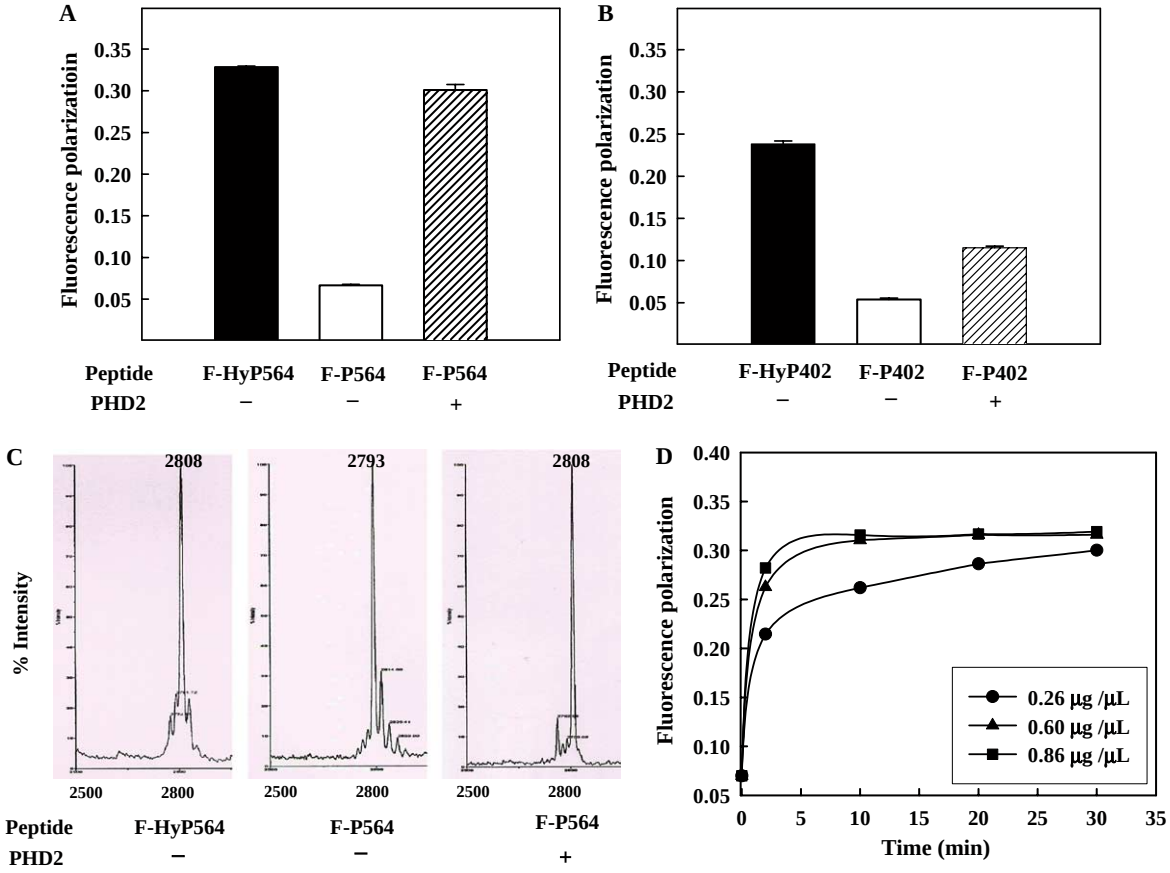

Fig. 4. Fluorescence polarization analyses for prolyl hydroxylation activity. (A) F-P564 and (B) F-P402 peptide substrates at 1 μM were incubated with or without 0.5 μg/μL recombinant PHD2 in NETN buffer containing 5 mM α-ketoglutarate, 2 mM ascorbic acid, and 100 μM $FeCl_2$. After the reactions were performed for 30 min at room temperature, VBC was added to a final concentration of 800 nM along with dilution of the peptide concentrations to 100 nM, followed by fluorescence polarization measurements. For positive controls, the fluorescence polarization values of F-HyP564 and F-HyP402 upon binding to VBC were measured and presented. Each bar represents the average of triplicate assays ±SD. (C) MALDI-TOF analysis of F-P564 after and before the reaction was performed and compared with the analysis of F-HyP564. (D) Time courses of prolyl hydroxylation of the F-P564 peptide substrate with 0.26, 0.60, and 0.86 μg/μL PHD2 were measured by fluorescence polarization changes upon binding to VBC.

with this binding assay, a very recent report has demonstrated that Pro-564 is hydroxylated prior to Pro-402, and mutation of Pro-564 dramatically reduces the Pro-402 hydroxylation [16]. Therefore, a short peptide containing Pro-402 is unlikely to serve as an appropriate substrate for PHD2.

To obtain direct evidence that the reaction product is hydroxylated by recombinant PHD2, MALDI-TOF analysis was performed for the reaction mixture along with the control peptides. While the untreated F-P564 peptide and the synthesized F-HyP564 yielded an atomic mass unit of 2793 and that of 2808, respectively, the peak of the F-P564 peptide sample treated with PHD2 in Fig. 4A had an atomic mass unit of 2808 (Fig. 4C). These analyses strongly confirmed that the peptide substrate was indeed hydroxylated almost completely by the enzyme during the incubation, which caused recruitment of VBC resulting in the increase of fluorescence polarization comparable to the synthesized F-HyP564 as in Fig. 4A. The appropriate incubation time and amount of the enzyme were then explored by monitoring the time course of the reaction with varying concentrations of PHD2. Analyses of the reaction mixtures by fluorescence polarization changes upon addition of VBC showed that the hydroxylation reached the maximum in 10 min for the use of 0.86 μg/μL of the enzyme, and took longer time for lower concentrations (Fig. 4D). Accordingly, the prolyl hydroxylation reaction was performed with 0.5 μg/μL PHD2 for 30 min for the rest of the experiments described below. These results collectively support the notion that the activity of PHDs could be easily detected by changes in the fluorescence polarization values of the HIF-1α peptide substrate.

Next, the effects of cofactors were evaluated for optimal hydroxylation conditions. As expected from its crucial role as a stoichiometic provider of dioxygen in prolyl hydroxylation [6], α-ketoglutarate activated PHD2 with an EC_{50} of ~3.5 μM in the presence of ascorbic acid and Fe^{2+} (Fig. 5A). In addition, the activity of PHD2 was inhibited by NOG, an analogue of α-ketoglutarate, with an IC_{50} value of 4.05 mM (Fig. 5B). On the other hand, little change was observed with increasing concentrations of ascorbic acid or $FeCl_2$ for our crudely purified recombinant PHD2 (Fig. 5A), in contrast to the previous reports [6,17–19]. These unexpected results might be partially due to the purity and quality of the enzyme preparation. Interestingly, PHDs expressed in insect cells and purified to near homogeneity have been recently found to retain the partial activities without added Fe^{2+}, suggesting the presence of firmly bound iron within the enzymes [20].

Since divalent metal ions are known to mimic hypoxia by inhibiting Fe^{2+}-dependent dioxygenases and/or VHL binding of HIF at the Pro-564 site [9,10,18,19], we investigated the inhibitory effect of Co^{2+}. When the prolyl hydroxylation reaction was performed in the absence of Fe^{2+}, gradual inhibition was observed with increasing concentrations of $CoCl_2$, but the extent of inhibition was far below expectation and PHD2 retained more than 60% activity even at 10 mM $CoCl_2$ (Fig. 5B). This ineffective inhibition by Co^{2+} is in accordance with the very recent report on poor inhibitory effects of metals on the activities of crude PHD preparations [20], suggesting that mechanisms other than the direct inhibition of PHD2 might contribute to the stabilization of HIF-1α by Co^{2+} in cells. We next tested the inhibitory effects of iron chelators including DFO, EDTA, and EDHB on the PHD2 activity. While EDHB ($IC_{50} = 2.21$ mM) and EDTA ($IC_{50} = 5.41$ mM) exerted inhibitory effects on the PHD2 activity, the enzyme inhibition by DFO was very marginal (Fig. 5B), despite its highest affinity for iron. Consistent with this surprising result, up to 1 mM DFO did not affect the activity of crude PHD2 [20]. Furthermore, a well-known zinc chelator TPEN blocked the recombinant PHD2 activity similarly

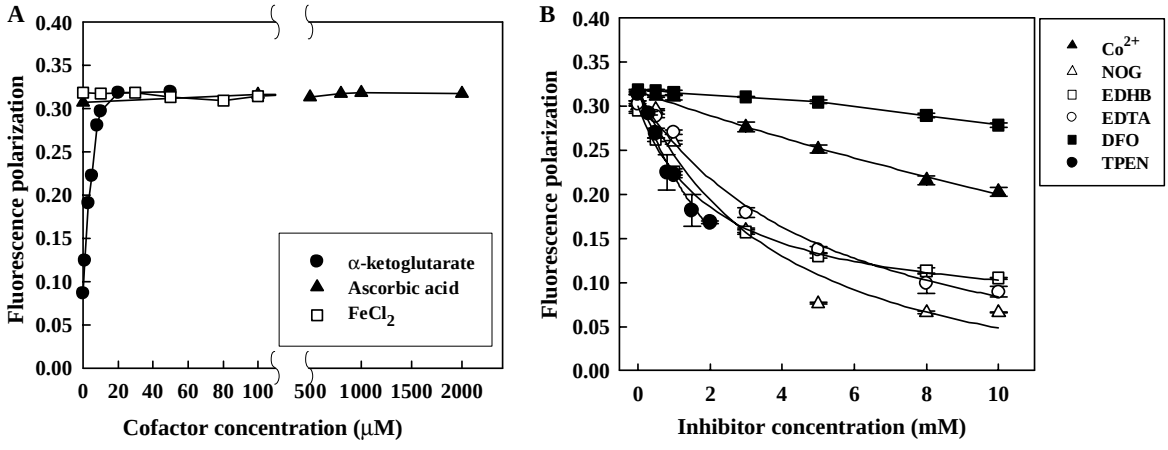

Fig. 5. Effects of cofactors and inhibitors on prolyl hydroxylation activity. 1 μM F-P564 was incubated with 0.5 μg/μL recombinant PHD2 in NETN buffer for 30 min at room temperature. VBC was then mixed with the reacted peptides and the mixtures containing 100 nM F-P564 and 800 nM VBC were measured for fluorescence polarization. (A) Dose–response curves for the PHD2 activation with α-ketoglutarate in the presence of 2 mM ascorbic acid and 100 μM $FeCl_2$ (●), ascorbic acid in the presence of 20 μM α-ketoglutarate and 100 μM $FeCl_2$ (▲), and $FeCl_2$ in the presence of 20 μM α-ketoglutarate and 200 μM ascorbic acid (□). (B) Dose–response curves for the PHD2 inhibition with $CoCl_2$ (▲), NOG (△), EDHB (□), EDTA (○), DFO (■), and TPEN (●). Each point represents the average of triplicate assays ±SD.

as EDHB, which could be speculated to be through iron chelation owing to its high affinities for heavy metals [21]. In separate experiments, all of these chemicals were found to have no effects on the HIF–VBC binding per se at their highest concentrations used in the enzyme inhibition studies (data not shown). These data implicate that the PHD2 may contain Fe^{2+} bound very tightly which presumably does not diffuse out of the enzyme easily.

In summary, we have presented a facile, sensitive, fluorescence polarization-based assay for the HIF–VBC binding and prolyl hydroxylation activity as well as for the inhibition studies. The easy applicability of the method would therefore enable us to characterize molecular actions mediated through hydroxylation of HIF-1α. Employing the developed assay, work aimed at dissecting molecular mechanisms of modulators for PHD isozymes is in progress.

Acknowledgments

The authors acknowledge Dr. Cheolju Lee for kindly providing the pGEX-4T-1/VHL-Elongin B plasmid and the pET29b/Elongin C plasmid and for helpful discussion on protein purification. The work was supported in part by the Functional Proteomics Center (21st Century Frontier Research and Development Program) and the KIST grant.

References

[1] G.L. Semenza, HIF-1 and mechanisms of hypoxia sensing, Curr. Opin. Cell Biol. 13 (2001) 167–171.
[2] G.L. Wang, B.H. Jiang, E.A. Rue, G.L. Semenza, Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension, Proc. Natl. Acad. Sci. USA 92 (1995) 5510–5514.
[3] F. Yu, S.B. white, Q. Zhao, F.S. Lee, HIF-1α binding to VHL is regulated by stimulus sensitive proline hydroxylation, Proc. Natl. Acad. Sci. USA 98 (2001) 9630–9635.
[4] N. Masson, C. Willam, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Independent function of two destruction domains in hypoxia-inducible factor-α chains activated by prolyl hydroxylation, EMBO J. (2001) 5197–5206.
[5] D.R. Mole, C.W. Pugh, P.J. Ratcliffe, P.H. Maxwell, Regulation of the HIF pathway: enzymatic hydroxylation of a conserved prolyl residue in hypoxia-inducible factor alpha subunits governs capture by the pVHL E3 ubiquitin ligase complex, Adv. Enzyme Regul. 42 (2002) 333–347.
[6] K.I. Kivirikko, J. Myllyharju, Prolyl 4-hydroxylases and their protein disulfide isomerase subunit, Matrix Biol. 16 (1998) 357–368.
[7] E. Berra, E. Benizri, A. Ginouvès, V. Volmat, D. Roux, J. Pouysségur, HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia, EMBO J. 22 (2003) 4082–4090.
[8] D.R. Mole, I. Schlemminger, L.A. McNeill, K.S. Hewitson, C.W. Pugh, P.J. Ratcliffe, C.J. Schofield, 2-Oxoglutarate analogue inhibitors of HIF prolyl hydroxylase, Bioorg. Med. Chem. Lett. 13 (2003) 2677–2680.
[9] Y. Yuan, G. Hilliard, T. Ferguson, D.E. Millhorn, Cobalt inhibits the interaction between hypoxia-inducible factor-α and von-Hippel–Lindau protein by direct binding to hypoxia-inducible factor-α, J. Biol. Chem. 278 (2003) 15911–15916.
[10] M. Ivan, K. Kondo, H. Yang, W. Kim, J. Valiando, M. Ohh, A. Salic, J.M. Asara, W.S. Lane, W.G. Kaelin, HIFα targeted for VHL-mediated destruction by proline hydroxylation: implications for O_2 sensing, Science 292 (2001) 464–468.
[11] W.-C. Hon, M.I. Wilson, K. Harlos, T.D. Claridege, C.J. Schofield, C.W. Pugh, P.H. Maxell, P.J. Ratcliffe, D.I. Stuart, E.Y. Jones, Structural basis for the recognition of hydroxyproline in HIF-1α by pVHL, Nature 417 (2002) 975–978.
[12] F. Oehme, W. Jonghaus, L. Narouz-Ott, J. Huetter, I. Flamme, A nonradioactive 96-well plate assay for the detection of hypoxia-inducible factor prolyl hydroxylase activity, Anal. Biochem. 330 (2004) 74–80.
[13] L.A. McNeill, L. Bethge, K.S. Hewiston, C.J. Schofield, A fluorescence-based assay for 2-oxoglutarate-dependent oxygenases, Anal. Biochem. 336 (2005) 125–131.
[14] M. Ivan, T. Haberberger, D.C. Gervasi, K.S. Michelson, V. Güzler, K. Kondo, H. Yang, I. Sorokina, R.C. Conaway, J.W. Conaway, W.G. Kaelin, Biochemical purification and pharmacological inhibition of a mammalian prolyl hydroxylase acting on hypoxia-inducible factor, Proc. Natl. Acad. Sci. USA 99 (2002) 13459–13464.
[15] J.H. Min, H. Yang, M. Ivan, F. Gertler, W.G. Kaelin, N.P. Pavletich, Structure of an HIF-1α-pVHL complex: hydroxyproline recognition in signaling, Science 296 (2002) 1886–1889.
[16] D.A. Chan, P.D. Sutphin, S.E. Yen, A.J. Giaccia, Coordinate regulation of the oxygen-dependent degradation domains of hypoxia-inducible factor 1 (alpha), Mol. Cell. Biol. 25 (2005) 6415–6426.
[17] H.J. Knowles, R.R. Raval, A.L. Harris, P.J. Ratcliffe, Effect of ascorbate on the activity of hypoxia-inducible factor in cancer cells, Cancer Res. 63 (2003) 1764–1768.
[18] A.C.R. Epstein, J.M. Gleadle, L.A. McNeill, K.S. Hewitson, J. O'Rouke, D.R. Mole, M. Mukherji, E. Metzen, M.I. Wilson, A. Dhanda, Y.-M. Tian, N. Masson, D.L. Hamilton, P. Jaakkola, R. Barstead, J. Hodgkin, P.H. Maxwell, C.W. Pugh, C.J. Schofield, P.J. Ratcliffe, *C.* elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation, Cell 107 (2001) 43–54.
[19] P. Jaakkola, D.R. Mole, Y.-M. Tian, M.I. Wilson, J. Gielbert, S.J. Gaskell, A.V. Kriegsheim, H.F. Hebestreit, M. Mukherji, C.J. Schofield, P.H. Maxwell, C.W. Pugh, P.J. Ratcliffe, Targeting of HIF-1α to the von Hippel–Lindau ubiquitlation complex by O2-regulated prolyl hydroxylation, Science 292 (2001) 468–472.
[20] M. Hirsila, P. Koivunen, L. Xu, T. Seeley, K.I. Kivirikko, J. Myllyharju, Effect of desferrioxamine and metals on the hydroxylases in the oxygen sensing pathway, FASEB J. 19 (2005) 1308–1310.
[21] P. Arslan, F. Di Virgilio, M. Beltrame, R.Y. Tsien, T. Pozzan, Cytosolic Ca^{2+} homeostasis in Ehrlich and Yoshida carcinomas, A new, membrane-permeant chelator of heavy metals reveals that ascites tumor cell lines have normal cytosolic Ca^{2+}, J. Biol. Chem. 260 (1985) 2719–2727.