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