Available online at www.sciencedirect.com
SCIENCE DIRECT®
Protein Expression and Purification 42 (2005) 295–304
Protein Expression & Purification
www.elsevier.com/locate/yprep

Cloning and characterization of the rat HIF-1α prolyl-4-hydroxylase-1 gene

Ronald R. Cobb [a,*], John McClary [a], Warren Manzana [a], Silke Finster [a], Brent Larsen [a], Eric Blasko [b], Jennifer Pearson [b], Sara Biancalana [c], Katalin Kauser [b], Peter Bringmann [a], David R. Light [d], Sabine Schirm [b]

[a] *Systems Biology, Berlex Biosciences, Richmond, CA 94806, USA*
[b] *Cardiovascular Department, Berlex Biosciences, Richmond, CA 94806, USA*
[c] *Biophysics Department, Berlex Biosciences, Richmond, CA 94806, USA*
[d] *Antibody Technology Group, Berlex Biosciences, Richmond, CA 94806, USA*

Received 25 January 2005, and in revised form 21 March 2005
Available online 30 April 2005

Abstract

Prolyl-4-hydroxylase domain-containing enzymes (PHDs) mediate the oxygen-dependent regulation of the heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1). Under normoxic conditions, one of the subunits of HIF-1, HIF-1α, is hydroxylated on specific proline residues to target HIF-1α for degradation by the ubiquitin–proteasome pathway. Under hypoxic conditions, the hydroxylation by the PHDs is attenuated by lack of the oxygen substrate, allowing HIF-1 to accumulate, translocate to the nucleus, and mediate HIF-mediated gene transcription. In several mammalian species including humans, three PHDs have been identified. We report here the cloning of a full-length rat cDNA that is highly homologous to the human and murine PHD-1 enzymes and encodes a protein that is 416 amino acids long. Both cDNA and protein are widely expressed in rat tissues and cell types. We demonstrate that purified and crude baculovirus-expressed rat PHD-1 exhibits HIF-1α specific prolyl hydroxylase activity with similar substrate affinities and is comparable to human PHD-1 protein.

Keywords: Hypoxia-inducible factor; Prolyl-4-hydroxylase; Rat

One of the critical cellular response mechanisms to stress is modulation of gene expression. Cells upregulate genes involved in angiogenesis, erythropoiesis, and glycolysis in response to reduced oxygen tension (reviewed in [1,2]). An important regulator of this response is the transcriptional activation of these genes by a common transcription factor, hypoxia-inducible actor-1 (HIF-1).[1] HIF-1 was identified as a heterodimeric transactivator composed of HIF-1α and the aryl hydrocarbon nuclear translocator (ARNT, also known as HIF-1β) that binds to sequences in the 3′-untranslated region of the erythropoietin gene [3,4]. While ARNT is constitutively expressed, HIF-1α accumulates only at low oxygen tension, resulting in an elevated nuclear expression of HIF-1 under conditions of hypoxia. Recently, the molecular mechanisms of induction of HIF-1 were elucidated (reviewed in [2,5]). Under normoxic conditions, HIF-1α is hydroxylated at specific proline residues (Pro-402 and Pro-564) residing in the oxygen-dependent degradation domain [6–9]. The hydroxylated prolines then bind the von Hippel–Lindau (VHL) complex [10–12], an E3 ubiquitin–protein ligase

* Corresponding author. Fax: +1 510 669 4245.
E-mail address: Ronald_Cobb@berlex.com (R.R. Cobb).

[1] *Abbreviations used:* HIF-1, hypoxia-inducible factor-1; ARNT, aryl hydrocarbon nuclear translocator; VHL, von Hippel–Lindau; PHD, prolyl-4-hydroxylase domain; h PHD-2, human PHD-2.

1046-5928/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.pep.2005.03.036

complex [13,14], resulting in the ubiquitination of HIF-1α [15–18] and subsequent degradation by the proteasome [19–21]. Under hypoxic conditions, the hydroxylation reaction is attenuated, allowing HIF-1α to escape degradation, form heterodimers with ARNT, and translocate to the nucleus. There HIF-1 associates with accessory transcription factors on hypoxia responsive elements, resulting in transcriptional stimulation of the target genes (reviewed in [2]).

The site-specific proline hydroxylation in mammalian cells is catalyzed by a family of enzymes referred to as prolyl-4-hydroxylase domain (PHD) containing proteins [22–24]. These proteins belong to a superfamily of 2-oxoglutarate and iron-dependent dioxygenases that catalyze a variety of hydroxylations [25]. The best-studied members of this family are the α-subunits of collagen-prolyl-4-hydroxylase. Only the major determinants for oxoglutarate and iron binding are conserved within the catalytic domains of the family members [26]. The PHDs are 2-oxoglutarate-dependent dioxygenases that use molecular oxygen [27] to hydroxylate the specific proline residues in HIF-1α [6–9].

The mammalian PHD proteins affect the accumulation of the transcription factor HIF-1 by destabilizing HIF-1α via proline hydroxylation (reviewed in [5]). Early descriptions of a rat isoform (rat SM20) indicated its involvement in growth regulation and apoptosis [28–30]. Also, studies in *Drosophila melanogaster* and *Caenorhabditis elegans* indicate a wider range of activities [31,32], unrelated to HIF-1 and manifested in an egg-laying defect and growth regulation, respectively. Interestingly, it is now evident that proline-hydroxylation may have a broader role as a cellular mechanism for targeting proteins for proteasomal degradation [33,34]. The identification of three mammalian PHD enzymes as compared to one in worms and insects has given rise to speculation about the potential for different biological functions for the different isozymes. The pre-requisite for a better understanding of the biological function is the characterization of the homologs and their specific inhibition or gene inactivation.

Three different PHD proteins (PHD-1, PHD-2, and PHD-3) have been identified in humans with homologs in other organisms [22,23]. In this study, we report the cloning of the cDNA from rat liver that encodes the rat ortholog of PHD-1. The cDNA encodes a protein with significant homology to previously reported mammalian PHD-1 proteins over its entire length. In addition, the catalytic domain of this rat PHD-1 is highly homologous to the rat PHD-2 and PHD-3 (SM20) proteins. We show that recombinant rat PHD-1 protein catalyzes the hydroxylation of an HIF-1α-specific peptide, and compare its enzymatic activity to that of the human PHD-1.

Materials and methods

Cloning of rat PHD-1 cDNA

The full-length rat PHD-1 cDNA was amplified from a rat liver cDNA library (Clontech, Palo Alto, CA). The PCR primers used for the amplification included sequences for an N-terminal Flag tag (forward primer: 5′-GCGGGATCCGCCACCATGGATTACAAGGAC GATGACGATAAGGGAGGCTCTTCAGACAGCC CGTGCCAGCCG CAGGCCC-3′; reverse primer: 5′-GCGGAATTCTTAGGTAGGTGTAGCTGGCTGT GACACGG-3′ PCRs were carried out using the Fail-Safe PCR kit (Epicenter Technologies, Madison, WI) following the instructions of the manufacturer. The amplified product was cloned into a TA vector using the TOPO TA kit of Invitrogen (Carlsbad, CA). Clones that gave appropriate sized restriction fragments upon digestion with *Bam*HI and *Eco*RI were subjected to double strand DNA sequencing using BigDye Terminator kits and the ABI3100 DNA Sequencer (Applied Biosystems, Foster City, CA). The positive clones were then digested with *Bam*HI and *Eco*RI and subsequently subcloned into the baculovirus transfer pBB4.5 that had been digested with the same enzymes.

Insect cell expression of rat PHD-1

Sf21 cells were grown in Grace's media (Invitrogen) supplemented with 10% fetal bovine serum. The baculovirus transfer vector (pBB4.5) was obtained from Invitrogen. Baculovirus containing the rat PHD-1 cDNA were generated using the Invitrogen Bac-N-Blue kit following the manufacturer's procedures. Recombinant baculovirus were expanded for protein expression which was then used for enzyme assay analyses. The recombinant protein was Flag-tagged via the PCR primers that were used to generate the amplified product. Recombinant baculovirus expression of rat PHD-1 was followed using Western blots of Sf21 cell pellets probed with anti-Flag antibodies (Sigma, St. Louis, MO).

Rat cell line culture

The H9C2, A10, and A7R5 cell lines were grown in DMEM with 10% FBS, glutamine, and penicillin/streptomycin. RFL-6 cells were grown in Ham's F12 with 10% FBS, glutamine, and pen/strep. RBL-2H3 cells were grown in EMEM with 10% FBS, glutamine, and penicillin/streptomycin.

Northern blot and Taqman analyses

Multiple tissue Northern blots were obtained from Clontech. The full-length rat cDNA in pBB4.5 was