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Role of the intracellular localization of HIF-prolyl hydroxylases

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ABSTRACT

Hypoxia-inducible factor-1 (HIF-1) is a major transcription factor regulating the response of tumor cells to hypoxia and is comprised of HIF-1α and Arnt (HIF-1β). In mammalian cells, HIF-1 protein levels are regulated by three HIF-prolyl hydroxylases, termed PHD1, PHD2 and PHD3. To assess whether intracellular localization of PHD1 and PHD2 affects the hypoxic response via HIF-1, we investigated the localization signal of PHDs. PHD1 possessed at least one nuclear localization signal (NLS), and PHD2 contained a region as essential for nuclear export in their N-terminal region. Treatment of cells with leptomycin B revealed that PHD2 was able to shuttle between the cytoplasm and the nucleus. Reporter assay indicated that differences in the intracellular distribution of PHD1 did not influence on HIF-1α activity. However, a PHD2 mutant lacking the region for nuclear export exhibited significantly reduced effect to HIF-1α activity compared to wild-type PHD2, suggesting that the regulation of the intracellular distribution of PHD2 is an effective pathway for the control of the hypoxic response.

1. Introduction

Hypoxia-inducible factors (HIFs) are the transcriptional regulators that play essential roles in the hypoxic response [1,2]. HIFs bind to hypoxia response element (HRE), located in the regulatory region of the target genes such as erythropoietin and vascular endothelial growth factor. HIFs are heterodimers formed by two subunits, the hypoxia-inducible factor-α (HIFα) and the constitutively expressed Arnt (HIF-1β) [3]. There are three HIFα proteins, HIF-1α, HLF (HIF-2α) and HIF-3α, reported previously [3–5]. Both HIF-1α and HLF translocate to the nucleus following stabilization in response to hypoxia, and finally form heterodimers (called as HIF-1 and HIF-2, respectively) with Arnt [6]. Thus, both HIF-1 and HIF-2 activate transcription of target genes bearing HRE in response to hypoxia.

In normoxia, rapid degradation of HIF-1α is mediated by the ubiquitin-dependent pathway [7]. The tumor suppressor von Hippel-Lindau gene product (pVHL) is involved in the proteasomal degradation of HIF-1α [8]. Furthermore, it was reported that hydroxylation of specific prolines in the oxygen-dependent degradation (ODD) domain of HIF-1α is required for the recognition of pVHL [9–11]. Subsequent studies revealed a family of three prolyl 4-hydroxylase-domain containing proteins (PHDs) 1, 2 and 3 as HIF-1α prolyl hydroxylases [12,13]. In hypoxia, decrease in hydroxylase activity of PHDs due to the short supply of oxygen results in the accumulation of HIF-1α and the induction of target gene expression controlled by HRE. Thus, PHDs are regarded as a cellular oxygen sensor.

Many studies have revealed functional differences among PHD1, PHD2 and PHD3 [14–16]. These three enzymes share the catalytic domain with sequence similarities and conserve amino acids for coordination to Fe (II) ion. Furthermore, K_m values of these PHDs for O_2 are comparable [14]. However, PHD2 has been shown to play a crucial role in the rapid degradation of HIF-1α in normoxia [17]. Conservation of the catalytic domain and similarity of the enzymatic activity between PHD1 and PHD2 suggest that the diversed N-terminal region is essential for their particular functions. It has been reported that PHD1 is present exclusively in the nucleus, while PHD2 is located mainly in the cytoplasm [18]. Thus, it would be possible that the localization gives rise to the functional diversity of these proteins. To evaluate whether the intracellular localization of PHD1 and PHD2 affects HIF-1α activity, we determined the localization signal in PHD1 and PHD2. Subsequently, we examined the effect of the intracellular localization of PHD1 and PHD2 on their activity.

2. Materials and methods

2.1. Cell culture and DNA transfection

COS-7 and Hep3B cells were obtained from Cell Resource Center for Biomedical Research (Institute of Development, Aging and Cancer, Tohoku University, Japan). COS-7, Hep3B and HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. DNA transfection into Hep3B cells in a 60 mm dish was carried out by the calcium phosphate method as described [19].

2.2. Fluorescence observation of cultured cells

COS-7 cells grown on the cover glass were transfected with 0.5 μg of plasmids expressing PHD variants fused to Citrine [20] using FuGENE6 transfection reagent (Roche), and incubated for 40 h before

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fixation in 4% paraformaldehyde in PBS. The fixed cells were incubated in 0.2 μg/ml of DAPI (4′,6′-diamidino-2-phenylindole) for 5 min. Imaging was performed with an Olympus BX50 fluorescence microscope and the Olympus DP70 digital camera. The fluorescent color of DAPI was changed from blue to red on a computer.

2.3. Construction of plasmids

A full-length PHD1 cDNA was subcloned into pBOS vector [21]. PHD1 was amplified by PCR and the fragment was inserted into HindIII/BamHI site of Citrine-C1 (pEYFP-C1 (Q69M), Clontech). Chimeric plasmids for Citrine-PHD1 deletion mutants were constructed as follows. A DNA fragment containing the partial region of PHD1 was amplified by PCR using appropriate primers and inserted into the HindIII/BamHI sites of Citrine-C1.

Mutant plasmids, Citrine-PHD1(K102A), Citrine-PHD1(R106A), Citrine-PHD1(R113A), Citrine-PHD1(R119A), Citrine-PHD1(R134A) with mutations in the NLS were constructed using QuikChange II site-directed mutagenesis kit (Stratagene).

A full-length PHD2 cDNA was subcloned into the EcoRV site of pBluescriptIISK+ vector (Stratagene) termed as pBS-hPHD2. PHD2 was excised with BamHI/HindIII and ligated into BglII/HindIII site of Citrine-C1, termed as Citrine-PHD2(1–426). A series of Citrine-PHD2 deletion mutants was constructed by the digestion of Citrine-PHD2 (1–426) with suitable restriction enzymes. All DNA constructions were validated by sequence analysis.

2.4. pVHL-HIF-1α binding assay

pVHL-HIF-1α binding assay was performed as described previously [19]. Cell extracts were prepared from HEK293T cells transiently transfected with the FLAG-tagged PHD expression plasmid. Samples were analyzed by SDS-PAGE and radioactivities were quantitated with BAS-1000 bioimaging system (Fujifilm Corp., Tokyo).

3. Results

3.1. PHD1 contains a nuclear localization signal

To investigate the intracellular localization of PHD1 and PHD2, Citrine (improved equivalent of EYFP)-fused PHD1 or PHD2 was expressed in COS-7 cells and observed the distribution with the fluorescent microscopy. The subcellular localization of Citrine-PHD1 or Citrine-PHD2 with the fluorescent microscopy was comparable to FLAG-tagged PHD1 or FLAG-tagged PHD2 with immunocytochemical detection, respectively (data not shown). The intracellular distribution of PHD1 and PHD2 is consistent with the previous results [18]. Subsequently we examined the intracellular localization of various deletion mutants of PHD1 fused with Citrine expressed in COS-7 cells to search the nuclear localization signal (NLS) (Fig. 1A). C-terminal region of PHD1 did not confer nuclear accumulation. Deletions in the N-terminal region showed that the region from amino acids 89 to 134 is sufficient for the NLS activity (Fig. 1A). However, the two divided regions, amino acids 89–112 and 113–134, were insufficient for the NLS. Therefore, we focused the role of basic amino acids in the region 89 to 134. Five basic amino acids selected from 89–134 were substituted by alanine (Fig. 1B). Four mutants (K102A, R106A, R113A, and R134A) were still localized in the nucleus. However, R119A mutant was distributed both in the nucleus and the cytoplasm indicating that the arginine 119 is important for the nuclear localization of PHD1. The NLS usually contains a cluster of basic

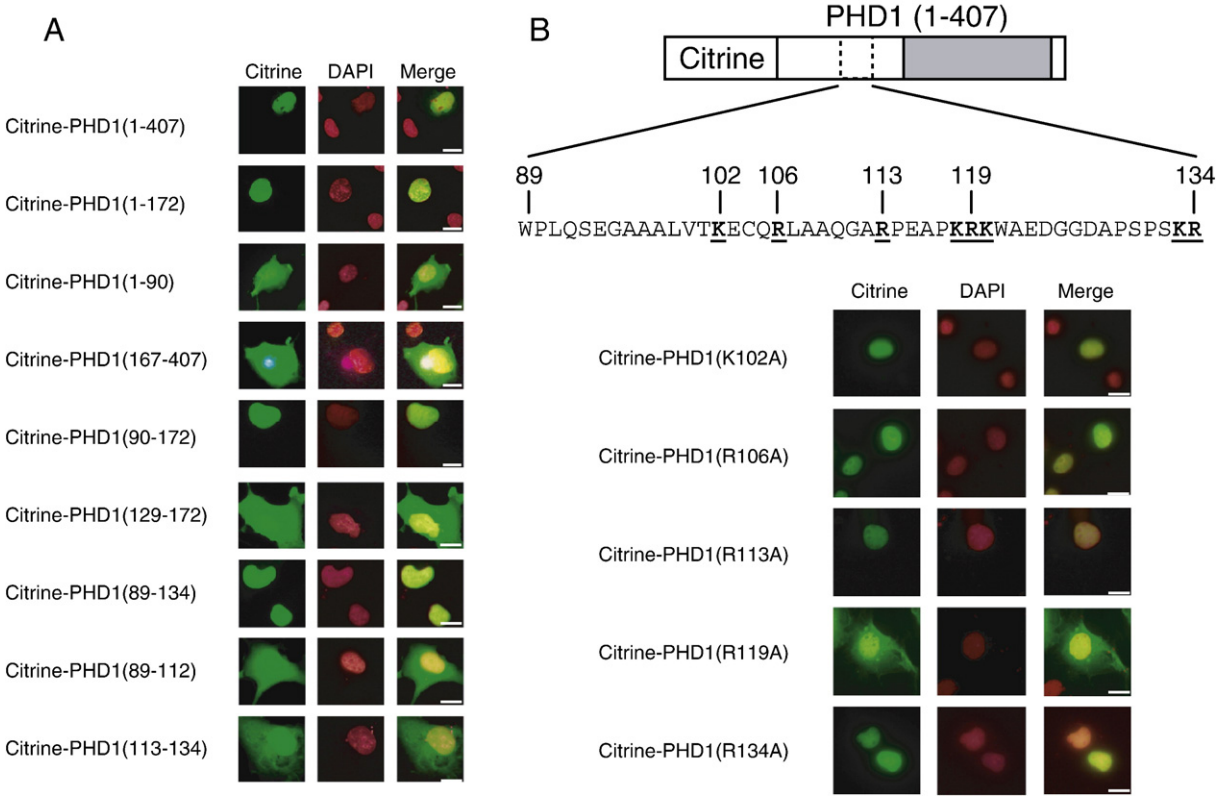

Fig. 1. Determination of the nuclear localization signal (NLS) for human PHD1. (A) Wild-type and various deletion mutants of human PHD1 were fused with Citrine, and positions of the deletions were shown. Dark box represents the catalytic domain of PHD1. Each chimeric protein was transiently expressed in COS-7 cells. Images of Citrine and DAPI fluorescence and their merged images were shown on the right. Scale bars represent 20 μm. (B) Mutational analysis of the NLS of human PHD1. Amino acid sequence from 89–134 of the PHD1 protein was shown at the top. Basic amino acids are underlined and 5 basic amino acids in the sequence were substituted with alanine, respectively. Images of the fluorescence of Citrine and DAPI and merged images were shown below.