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Regulation of HIF Prolyl Hydroxylases by Hypoxia-Inducible Factors

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Abstract Hypoxia and induction of hypoxia-inducible factors (HIF-1α and HIF-2α) is a hallmark of many tumors. Under normal oxygen tension HIF-alpha subunits are rapidly degraded through prolyl hydroxylase dependent interaction with the von Hippel-Lindau (VHL) tumor suppressor protein, a component of E3 ubuiquitin ligase complex. Using microarray analysis of VHL mutated and re-introduced cells, we found that one of the prolyl hydroxylases (PHD3) is coordinately expressed with known HIF target genes, while the other two family members (PHD1 and 2) did not respond to VHL. We further tested the regulation of these genes by HIF-1 and HIF-2 and found that siRNA targeted degradation of HIF-1α and HIF-2α results in decreased hypoxia-induced PHD3 expression. Ectopic overexpression of HIF-2α in two different cell lines provided a much better induction of PHD3 gene than HIF-1α. In contrast, we demonstrate that PHD2 is not affected by overexpression or downregulation of HIF-2α. However, induction of PHD2 by hypoxia has HIF-1-independent and -dependent components. Short-term hypoxia (4 h) results in induction of PHD2 independent of HIF-1, while PHD2 accumulation by prolonged hypoxia (16 h) was decreased by siRNA-mediated degradation of HIF-1α subunit. These data further advance our understanding of the differential role of HIF factors and putative feedback loop in HIF regulation. J. Cell. Biochem. 92: 491–501, 2004. Published 2004 Wiley-Liss, Inc.†

Key words: hypoxia; prolyl hydroxylases; HIF; siRNA; microarray

Cellular adaptation to changes in oxygen tension is an important process for a wide range of events including normal embryonic development and pathophysiology of ischemic and neoplastic disorders. In normal cells, oxygen concentration is maintained within a narrow range to ensure minimal risk of oxidative damage at high oxygen and hypoxic death at low oxygen. A decrease in the oxygen level results in the stabilization of hypoxia-inducible factors (HIFs) that activate transcription of genes encoding angiogenic factors (VEGF and FLT1), glucose transporters (Glut-1 and -3), and glycolytic enzymes involved in the production of ATP in the absence of O_2 [for review see Semenza, 1999; Wenger, 2002; Huang and Bunn, 2003]. Elevated levels of HIF-α proteins are found in the majority of solid tumors in the areas of profound hypoxia [Zhong et al., 1999]. These areas of hypoxia within the tumor are usually associated with high resistance to chemotherapy, high number of necrotic cells and poor patient survival [Hockel et al., 1996; Brown and Giaccia, 1998].

Expression of the hypoxia-inducible factors is tightly regulated in cells under normal oxygen tension. There are two subunits of HIF transcription factors (HIF-α and HIF-β) where HIF-α is a regulatory protein and HIF-β is constitutively expressed [Huang et al., 1996]. When the two subunits form heterodimer, they interact with the consensus hypoxia response elements (HRE) in their target genes through

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the basic helix-loop-helix domains [Semenza et al., 1991; Maxwell et al., 1993; Nagao et al., 1996]. The HIF-α subunit has two (N- and C-terminal) transactivation domains (TAD), both implicated in the oxygen-dependent regulation of the protein. Under normal oxygen tension the N-terminal TAD is hydroxylated at two critical proline residues by the members of conserved prolyl hydroxylase protein family, PHD-1, -2, and -3 (also known as EGLN 2, 1, and 3 or HPH-3, -2, and -1, respectively) [reviewed in Bruick, 2003]. This proline hydroxylation modification is required for the binding of the von Hippel-Lindau (VHL) tumor suppressor protein. VHL in turn recruits an E3 ubiquitin–ligase complex that targets HIF-α for proteasomal degradation. Oxygen is a rate-limiting co-factor of PHD enzymes. At low oxygen the prolyl hydroxylases are unable to modify HIF-α, no VHL interaction occurs and as a result, HIF-α subunits are stabilized under hypoxic conditions [Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Yu et al., 2001].

Another critical modification of HIF-α involves hydroxylation of an asparagine residue in the C-terminal TAD by the factor inhibiting HIF-1 (FIH-1) [Mahon et al., 2001]. Asparagine hydroxylation negatively regulates transcriptional activity of HIF by preventing its interaction with p300 and CBP transactivators [Lando et al., 2002a,b].

Besides conditions of low oxygen, the p53 tumor suppressor gene and Hsp90 control HIF-1α degradation [Ravi et al., 2000; Isaacs et al., 2002]. HIFs are also induced by a number of oncogenes like AKT, Src, and oncogenic Ras [reviewed in Goonewardene et al., 2002; Semenza, 2003]. In this case, the upregulation of HIF-α takes place under normal oxygen conditions and the physiological role of HIF stimulation is not clear.

In this study, we set out to identify novel VHL and hypoxia regulated genes by microarray. We present here the comprehensive list of genes whose transcription was changed in the presence of VHL either hypoxia-dependent or -independent. Among the genes regulated coordinately with the known HIF targets, we identified prolyl hydroxylase 3 (PHD3), which is involved in HIF degradation. No difference in PHD2 was found, although previously it has been shown that both PHD3 and 2 were induced by hypoxia [Epstein et al., 2001]. We further studied regulation of these genes by HIF-1 and HIF-2 and found that knock-down of HIF-1α or HIF-2α subunits by siRNA duplexes results in reduced hypoxic induction of PHD3. Overexpression of HIF-2α increased the amount of endogenous PHD3 transcript as did overexpression of HIF-1α but to a much lesser extent. On the contrary, PHD2 induction by hypoxia was not regulated by HIF-2 and required HIF-1 only for long-term hypoxia. These data suggest functional diversity of PHD family members and provide further evidence that induction of HIF-α proteins in vivo is under tight regulation by a negative feedback mechanism.

MATERIALS AND METHODS

Cell Lines

786-O renal clear cell carcinoma, Hep3B hepatocellular carcinoma and U2OS osteosarcoma cells were cultured in DMEM supplemented with 10% fetal calf serum. For hypoxic conditions cells were split the day before experiment and sealed in a hypoxia chamber equilibrated with 0.5% O_2, 5% CO_2, and 94.5% nitrogen and incubated at 37°C for indicated period of time.

786-O cells studied by microarray were originally provided by Dr. W.G. Kaelin, Jr. and were the same used in our previous study [Maranchie et al., 2002]. The 786-O cells overexpressing wild-type or mutant VHL used for quantitative RT-PCR were obtained by cloning of the PCR amplified VHL into retroviral vector RetroTet-ART [Rossi et al., 1998]. The mutated forms of VHL (a gift from Dr. David Chen) were processed in a similar way. 293 Phoenix cells were transiently transfected with the VHL constructs and 48 h later the conditioned media was used to infect 786-O cells. All puromycin resistant cells were pooled and subsequently cultured in 2 μg/ml of puromycin.

Plasmids

HIF-1αP564A and HIF-2αP531A cloned in pcDNA3 expression vector were a gift from Dr. W.G. Kaelin, Jr. (Harvard Medical School, Boston, MA). HIF-1α with deletion of ODD domain, AA401-603, (HIF-1α ΔODD) and HIF-1α P402A, P564A cloned in pcDNA3 vector were a gift from Dr. Eric Huang (National Cancer Institute, Bethesda, MD).

Microarray Analysis

Total RNA was purified using Trizol Reagent (Life Technologies, Carlsbad, CA). Total RNA (5 μg) was labeled, hybridized, and processed according to the Affymetrix Technical Manual. All experiments were repeated twice. For the analysis, total signal intensities for each chip were scaled to the same target value of 500. Signal log ratios (one-step Tukey's Biweight estimate of logarithmic ratios of probe pair signals) for the gene expression were determined using Affymetrix Microarray Suite version 5 (MAS5) software by comparison to the vector control samples under normoxic conditions. Each array of an experimental condition was compared with each of the baseline arrays. Additional filters were applied based on MAS5 Detection and Change calls to eliminate unreliable ratio values. The genes absent in all samples ($P < 0.05$) were removed. In four pairwise comparisons for each experimental condition, we required 3 out of 4 "Change" calls were "Increased" or "Decreased" (change $P < 0.0025$ or $P > 0.9975$). Cut-off 2 was applied for the final gene selection. Using this approach for microarray analysis, we routinely confirm more than 80% of genes by quantitative RT-PCR. For the table in supplementary materials, the Signal log ratios for pairwise comparisons were averaged.

Quantitative Real-Time RT-PCR

RNA was isolated using Trizol Reagent (Life Technologies). Total RNA (1 μg) was reverse transcribed in 50 μl reaction and 5 μl of cDNA was then used for PCR reaction according to Applied Biosystems technical manual. Gene expression of PHD1, 2, 3 and VEGF were quantified relative to the expression level of β-actin. Probes for PHD family members and β-actin were purchased from Applied Biosystems, Inc. (Foster City, CA) The VEGF probe and primers were described in 19. The difference in threshold number of cycles between the gene of interest and β-actin was then expressed relative to the standard chosen for each experiment (=1 in the graphs) and converted into real fold difference.

Northern Blotting

RNA was purified from cells using Trizol Reagent (Life Technologies) and 20 μg were fractionated in 1.2% agarose-formaldehyde gel and transferred to nitrocellulose membrane. The membrane was probed with the full-length PHD3 cDNA, produced with *Not*I digestion of clone BC1646_D12 (OriGene Technologies, Inc.) or fragment of VCAM1 obtained by *Eco*RI digestion of clone BC017276. Purified DNA fragment was ^{32}P-labeled using the Random Primer DNA Labeling Kit (Life Technologies, Inc.) and hybridized at 68°C in Quik-Hyb solution (Stratagene, San Diego, CA) according to the manufacturer's instructions. The membranes were scanned with Phosphoimager Typhoon 8600 (Molecular Dynamics).

Western Blotting

To verify the overexpression of HIF-α constructs in U2OS (Fig. 3D), cells were lysed in RIPA buffer with addition of 1 mM sodium orthovanadate, protease inhibitor mix (Boehringer Mannheim, Indianapolis, IN) and 1 mM PMSF. Protein (50 μg) were fractionated by SDS–PAGE and transferred to PVDF membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was probed with 1 μg/ml anti-HIF-1α antibody (Transduction Laboratories, San Diego, CA) or HIF-2α antibody (Santa Cruz, CA) for 2 h, followed by washing and incubation with HRP-conjugated secondary antibody (Jackson Lab, Bar Harbor, MN) and developed using ECL^{TM} reagents (Amersham Biosciences, Piscataway, NJ).

To validate the effect of siRNA on the levels of endogenous HIF-1α and HIF-2α (Fig. 4 and 5) nuclear extracts were prepared [Andrews and Faller, 1991] and 25 μg of protein were analyzed as described above.

Cell Transfections

U2OS or Hep3B cells were split at density 2×10^5 cells per 60 mm plate and transfected next day with 6 μl of Fugene 6 Reagent (Roche Diagnostics Corporation) and a total of 4 μg DNA according to manufacturer's instruction. The amount of DNA in the transfection mixture was balanced with empty vector. Cells were analyzed 48 h after transfection.

siRNA Experiments

siRNA oligonucleotides were designed using Qiagen developed algorithm and duplexes were synthesized and purified by Xeragon, Inc. A 70% confluent cells on 100-mm plates were transfected with siRNA duplexes at final concentration of 60 nM in complete growth media

with 20 μl of Mirus TransIT-TKO Transfection Reagent (Mirus, Inc., Houston, TX). After 24 h, cells were either placed in hypoxia for indicated period of time or left untreated. Initially four different oligonucleotides were screened using quantitative RT-PCR and the two best were used for the experiments. The sequences for the HIF-1α gene were: HIF1_1: 5′-r(CUGGACACAGUGUGUUUGA)d(TT)-3′ and HIF1_2: 5′-r(CUGAUGACCAGCAACUUGA)d(TT)-3′. For the HIF-2α, the sequences were: HIF2_2: 5′-r(GCUCUUCGCCAUGGACACA)d(TT)-3′ and HIF2_4: 5′-r(GCGACAGCUGGAGUAUGAA)-d(TT)-3′. As a control we used Scramble II oligonucleotide from Dharmacon, Inc. (Lafayette, CO).

RESULTS

Identification of VHL Regulated Genes

It has been shown previously that cells with a mutated *vhl* tumor suppressor gene express high levels of hypoxia-inducible factors. After re-introduction of the *vhl* gene by ectopic expression, the HIF-α protein level is downregulated. However, when VHL expressing cells are placed in hypoxic conditions, VHL loses its ability to target HIF-α proteins for degradation resulting in stimulation of HIF transcriptional activity and expression of HIF downstream target genes. We used the well characterized renal clear cell carcinoma cell line 786-O with both alleles of *vhl* gene mutated and the same cell line transfected with *vhl* gene to identify additional VHL-dependent genes. Although these cells were previously studied by microarray approach [Wykoff et al., 2000] the availability of chips with larger gene representation makes this approach rather fruitful. We utilized HG-U133A oligonucleoitide array chip (Affymetrix, Inc.) consisting of more than 22,500 unique genes and ESTs. Both cell lines, transfected with either vector alone or wild type VHL, were analyzed under normoxic conditions or subjected to 16 hours incubation in the atmosphere containing 0.5% oxygen. In total there were 107 genes induced and 134 genes suppressed more than twofold by re-introduction of *vhl* gene. The complete list of genes can be found in the Supplementary material. Hierarchical clustering of outlier genes is shown in Figure 1A. Based on the pattern of gene expression in presence of VHL and hypoxia all genes fall into four clusters (Fig. 1B). Four genes from different clusters were validated by either Northern blot (Fig. 1C) or quantitative real-time RT-PCR (Fig. 1D) and show good agreement with microarray data. Overall this set of genes should be valuable as a starting point for further understanding of HIF and VHL functions.

In this study, we decided to focus on the regulation of prolyl hydroxases by hypoxia-inducible factors. We identified PHD3 among the genes in cluster 1. Microarray data show that two other family members (PHD1 and 2) were expressed at significant levels but did not change in response to VHL. We first checked the reproducibility of the microarray data in a different set of cells. We utilized the retroviral promoter driven overexpression of *vhl* gene in 786-O cells. Although the vector has been designed for tetracycline inducible expression of target genes the leakage of the promoter provided low but close to physiological levels of VHL expression. Cells transfected with either empty vector or vector with the wild type VHL were untreated or subjected to 16 h of hypoxia and PHD3 mRNA was analyzed by Northern blotting (Fig. 1C). Two non-functional VHL mutants were also included in the study, however, only under normal oxygen concentration. Consistent with the microarray data, we observed a suppression of PHD3 expression with wild type but not mutant VHL. Cells expressing wild type VHL under hypoxia were not able to suppress PHD3 transcription.

Effect of Wild-Type or Mutated VHL on PHD 1, 2, and 3

The expression patterns of three PHD family members were analyzed in the same set of cell lines by quantitative real-time RT-PCR (Fig. 2). Similar to results from microarray analysis, PHD1 and 2 did not respond to VHL, while PHD3 was downregulated by wild-type VHL but not by mutant forms of VHL. This observation raises an interesting question: whether PHD3 is regulated by VHL through both HIF-1 and HIF-2. Of note, 786-O cell line does not express HIF-1α protein and hypoxia-dependent gene regulation is presumably mediated by HIF-2 protein. Besides, like others we found that both PHD2 and 3 (but not PHD1) were induced by hypoxia in many tumor cell lines [Epstein et al., 2001; Cioffi et al., 2003; Metzen et al., 2003].

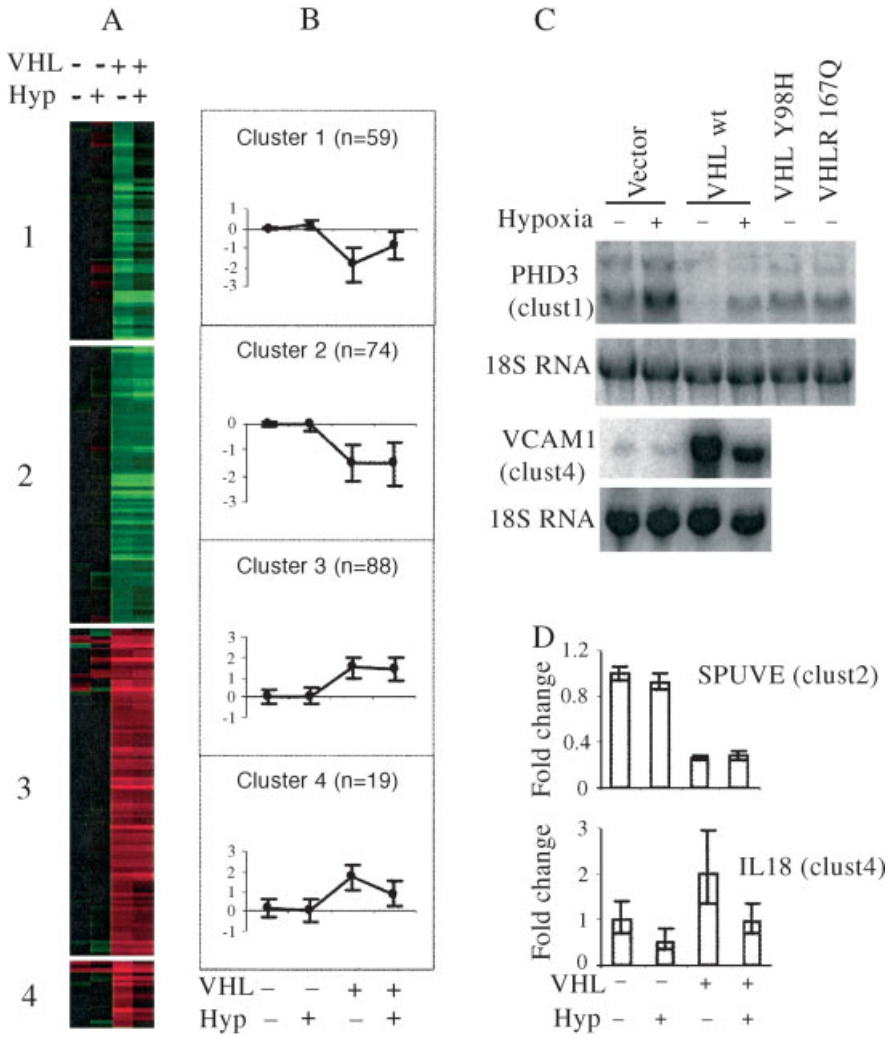

Fig. 1. Microarray analysis of genes differentially expressed in VHL re-introduced 786-O cells. **A**: Hierarchical clustering of 241 genes more than twofold up- (red color) or down-regulated (green color) by VHL. **B**: Four major clusters of genes with different pattern of expression regulated by VHL and hypoxia. Average signal log ratios (Y-axis) for all genes within each cluster are plotted for each experimental condition (X-axis). **C**: Northern blot or (**D**) quantitative real-time RT-PCR validation for several genes.

It is possible that we do not see VHL regulation of PHD2 in 786-O cell line because HIF-1α protein is absent.

Effect of HIF-1α or HIF-2α Overexpression on Regulation of PHD2 and 3

To answer these questions, we first overexpressed increasing amounts of HIF-1α or HIF-2α in Hep3B cell line under normal oxygen concentration. To increase the stability of HIFα subunits they both carried mutation in Proline 564 (HIF-1α) or Proline 531 (HIF-2α). It was clear from previous publications and our results that PHD1 is not regulated by either VHL, or HIF, or hypoxia, therefore we focused on PHD2 and 3 in our next experiments. We first studied the effect of either hypoxia-inducible factor on PHD3 expression by quantitative real-time RT-PCR. In Hep3B cell line, we found that increasing amounts of HIF-2αP531A results in profound stimulation of PHD3 transcription. The same amount of HIF-1αP564A provided very little induction of PHD3 (Fig. 3A). In order to clarify whether HIF-1α is capable of transcriptional activation of PHD3, we transfected the same cells with three other HIF-1α expression vectors (Fig. 3B). PHD3 was not induced by ectopic expression of wild-type HIF-1α, or by HIF-1α with a deletion of the oxygen-dependent degradation domain, which is transcriptionally

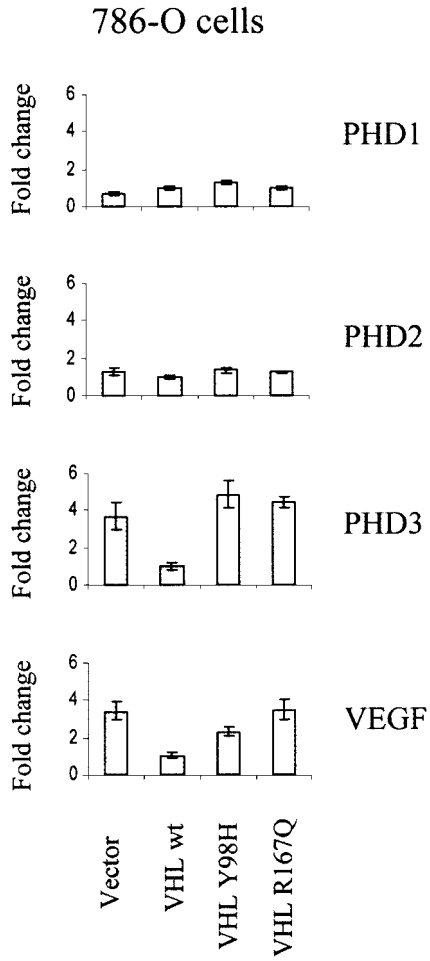

Fig. 2. PHD1, 2, and 3 regulation by VHL. Quantitative real-time RT-PCR was performed with the parental 786-O cells or the same cells expressing wild-type or mutant VHL under normoxic conditions. VEGF was used as a positive control. For each gene, the values in wild-type VHL expressing cells were set up at 1 and other experiments were calculated as fold increase relative to that value. All experiments were performed in triplicate and repeated at least twice. Results of one experiment are shown. The data represent means ± SE.

active in normoxia [Huang et al., 1998]. HIF-1α with mutated Proline 402 and Proline 564 (HIF-1αPP) induced expression of the endogenous PHD3 by 2.5-fold. A similar pattern was observed in U2OS cells where only HIF-1αPP activated transcription of PHD3 gene, though again to less extent than HIF-2α (Fig. 3C). All recombinant HIF-producing vectors show similar levels of HIF-alpha proteins in U2OS cells (Fig. 3D). Therefore, it is not clear at this moment why HIF-1αPP has higher transcriptional activity compared to other HIF-1α expression vectors used in this study. This effect is not specific for the PHD3 because transcription of another known HIF-1 target gene, *Glut1*, was also activated mainly by HIF-1αPP (data not shown). Transfection of different HIF-1α constructs or HIF-2α failed to induce transcription of endogenous PHD2 (data not shown).

Effect of siRNA-Mediated Degradation of HIF-1α and HIF-2α on PHD2 and 3 Inductions by Hypoxia

We further studied the role of HIF-1 or HIF-2 in hypoxic activation of PHD2 and PHD3 genes by using siRNA approach. Hep3B cell line was selected for these experiments due to robust induction of endogenous HIF-1α and HIF-2α proteins (data not shown). As a negative control we used Scramble oligoribonucleotide duplex showing no significant homology to any known human gene. To avoid the possibility of unspecific effects for any particular siRNA, we selected two different duplexes for either HIF-1α or HIF-2α. Twenty-four hours after transfection, the effect of siRNA on HIF-1α or HIF-2α expression was tested by quantitative real-time RT-PCR (Figs. 4A and 5A) and by Western blotting (Figs. 4B and 5B). We found no change in HIF-2α expression in cells transfected with HIF-1α siRNA and vice versa (Fig. 4A,B and Fig. 5A,B). Stabilization of hypoxia-inducible factors occurs very early after reduction of oxygen. Usually, maximal levels of HIF proteins are observed after 3 h of hypoxia. Therefore, we suggest that direct HIF target genes are affected by siRNA after short period of hypoxia (4 h), while long-term hypoxia (16 h), may provide us with additional information about secondary events mediated by HIFs. We found that knock-down of HIF-1α results in significant downregulation of PHD3 after 4 h and after 16 h of hypoxia (Fig. 4C,D). Similarly, two known HIF-1 target genes, VEGF and Glut1, were also suppressed at both time-points after hypoxia (data not shown). PHD2 induction by hypoxia at 4 h was not changed with targeted degradation of HIF-1α (Fig. 4C). However, at 16 h of hypoxia both HIF-1α siRNA duplexes showed statistically significant ($P < 0.05$) downregulation of PHD2 compared to Scramble transfected cells (Fig. 4D). These data suggest that PHD2 accumulation by hypoxia depends on different mechanisms. The response to short-term hypoxia is HIF-1 independent but prolonged exposure to hypoxia requires HIF-1 for PHD2 induction.

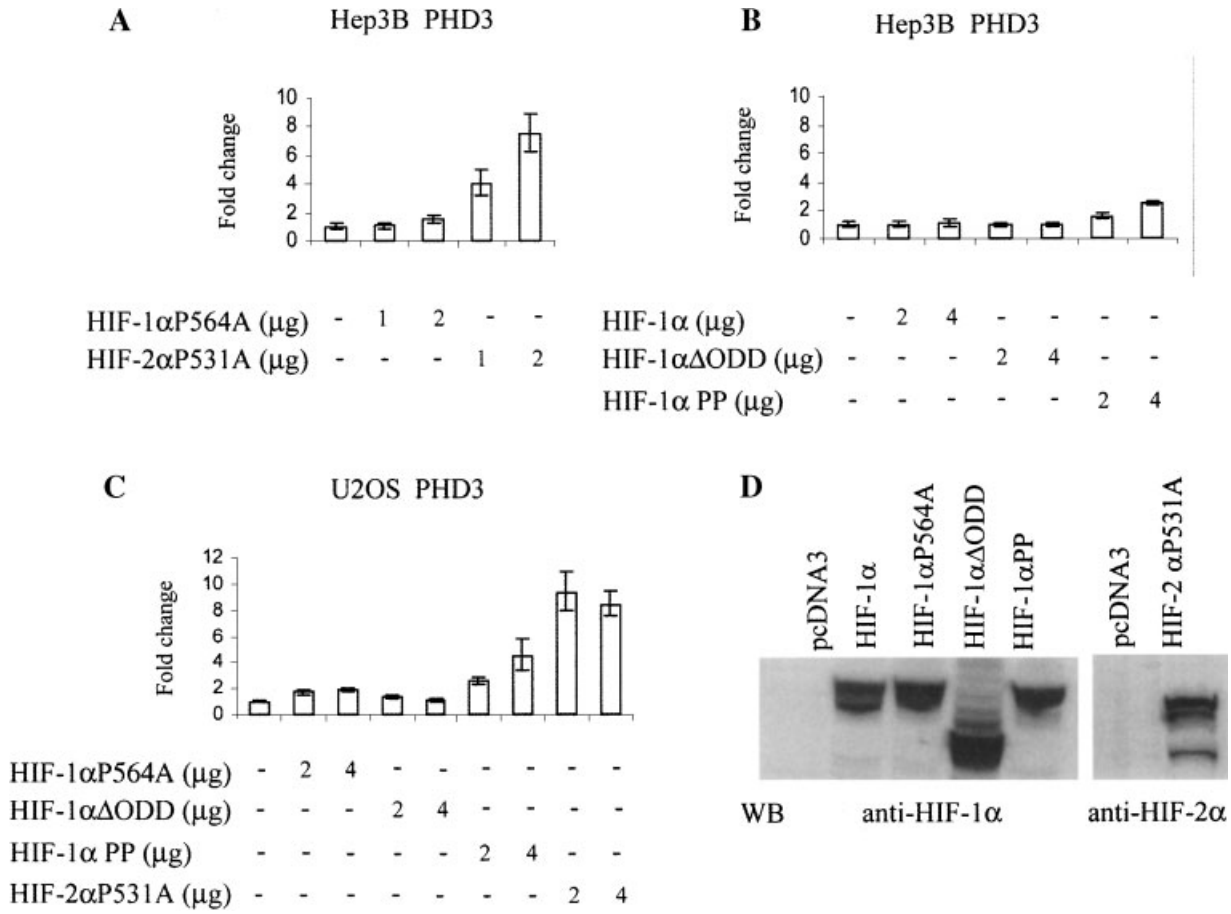

Fig. 3. Induction of endogenous PHD3 gene by forced overexpression of HIF-1α or HIF-2α. Quantitative real-time RT-PCR was performed 48 h after transfection of Hep3B (**A** and **B**) or U2OS (**C**) cells with increasing amounts of HIF-1α or HIF-2α expression vectors. The expression of PHD3 in vector only transfected cells was set as 1 and other values were calculated relative to that value. Similar levels of HIF-1α and HIF-2α expression were confirmed in U2OS cells by Western blotting (**D**).

Knock-down of HIF-2α reduced hypoxic induction of PHD3, but not PHD2, at both timepoints (Fig. 5C,D) which correlates well with induction of PHD3, but not PHD2, by forced expression of HIF-2α in two cell lines described above (Fig. 3 and data not shown). Lack of PHD2 regulation by HIF-2 may be the reason why PHD2 expression in 786-O cells is not decreased by VHL in our microarray study or quantitative RT-PCR (Fig. 2). HIF-1α subunit is lost in 786-O cells and effect of VHL and hypoxia is mediated mainly by HIF-2α. Overall these data indicate that although PHD2 and 3 both respond to low oxygen tension, the mechanism of their transactivation is different. While PHD3 is probably a direct target of both HIF-1 and HIF-2, the PHD2 in hypoxia is regulated by a mechanism requiring HIF-1 in chronic but not transient hypoxia.

DISCUSSION

Both HIF-1 and HIF-2 transcription factors play a critical role in tumor adaptation to hypoxia through activation of genes involved in angiogenesis, anaerobic glycolisis, and adaptation to acidic pH. Modification of these factors by prolyl hydroxylation followed by VHL-mediated degradation may provide the critical tool in manipulating HIFs stability. Three members of HIF prolyl hydroxylases belong to the iron- and 2-oxoglutarate dependent dioxygenase family with oxygen being a rate-limiting factor in the catalysis of substrates hydroxylation [Bruick and McKnight, 2001; Epstein et al., 2001; Hirsila et al., 2003]. Despite the similarity between the family members, they are regulated differently. In the majority of tumor cell lines PHD1 and 2 are predominantly expressed under normoxic

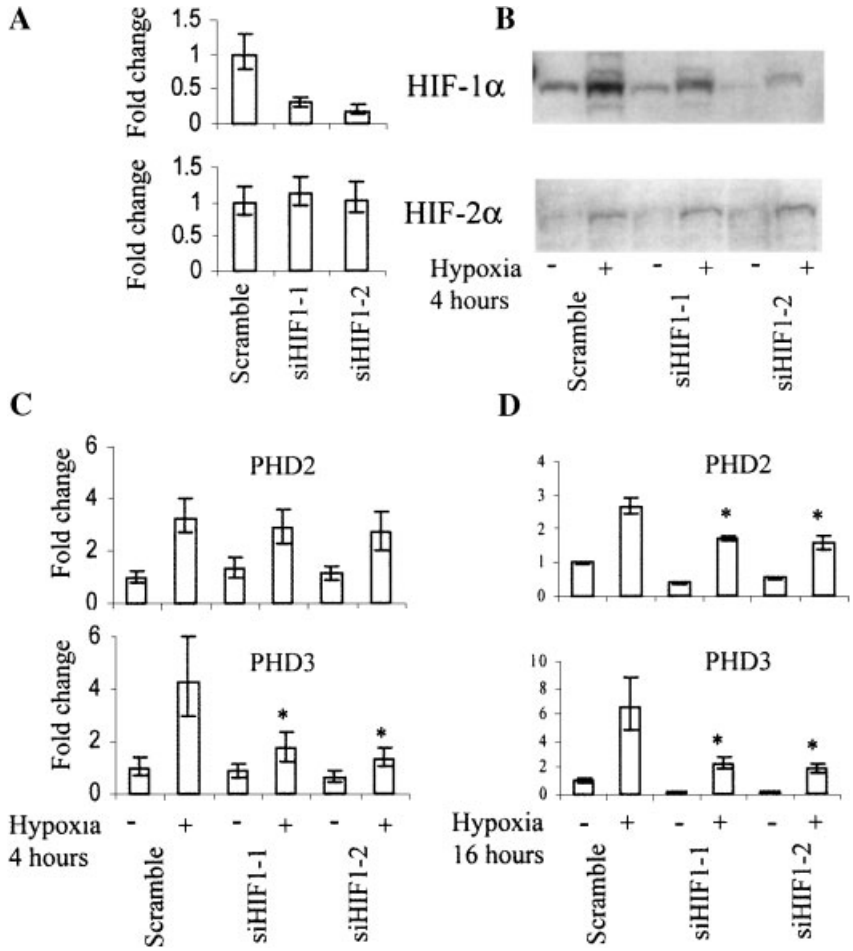

Fig. 4. Effect of targeted degradation of HIF-1α on hypoxic induction of PHD2 and PHD3. Two siRNA duplexes targeted HIF-1α were transfected into Hep3B cell line and amounts of HIF-1α or HIF-2α mRNA (**A**) or protein (**B**) was analyzed 24 h later. Scramble duplex was used as a negative control. **C**: Hep3B cells transfected with siRNA duplexes were placed under hypoxia for 4 h (C) or 16 h (**D**) or left untreated. The amount of PHD2 and PHD3 transcripts was analyzed by quantitative real-time RT-PCR. In the graphs, all values are expressed relative to the Scramble duplex under normoxic conditions. All experiments were done in triplicate and repeated at least twice. Student *t*-tests show statistically significant difference ($P < 0.05$) for hypoxic induction of PHDs between Scramble transfected cells and siRNA duplexes transfected cells for the samples labeled with *.

conditions [del Peso et al., 2003 and unpublished communication]. However, in normal mice, the members of this family show certain tissue specificity with the highest levels of PHD3 in heart and PHD2 being the only form expressed in testis [Lieb et al., 2002]. Silencing of PHD2, but not PHD1 or 3, by siRNA was sufficient for stabilization of HIF-1α in normoxia [Berra et al., 2003] implying that PHD2 is the major oxygen sensor responsible for low steady-state levels of HIF-1α in normoxia. PHD2 was biochemically purified as a major form of proline hydroxylase [Ivan et al., 2002] and shown to have the highest specific activity toward the Pro 564 hydroxylation site in HIF-1α [Huang et al., 2002]. Furthermore, the introduction of oncogenic form of Ras and v-Src resulted in the stabilization of HIF-1α in normoxia and the loss of Pro 564 hydroxylation probably due to inhibition of prolyl hydroxylase [Chan et al., 2002].

Our study provides a direct comparison of the effect of HIF-1 and HIF-2 functional inactivation on the transcription of two hypoxia-inducible PHD genes. Using siRNA targeted specific degradation of either HIF-1α or HIF-2α, we found that at least in hepatocellular carcinoma cells, PHD3 is upregulated by both transcription factors. These results are in agreement with recently published study showing that cell lines defective in HIF pathway components

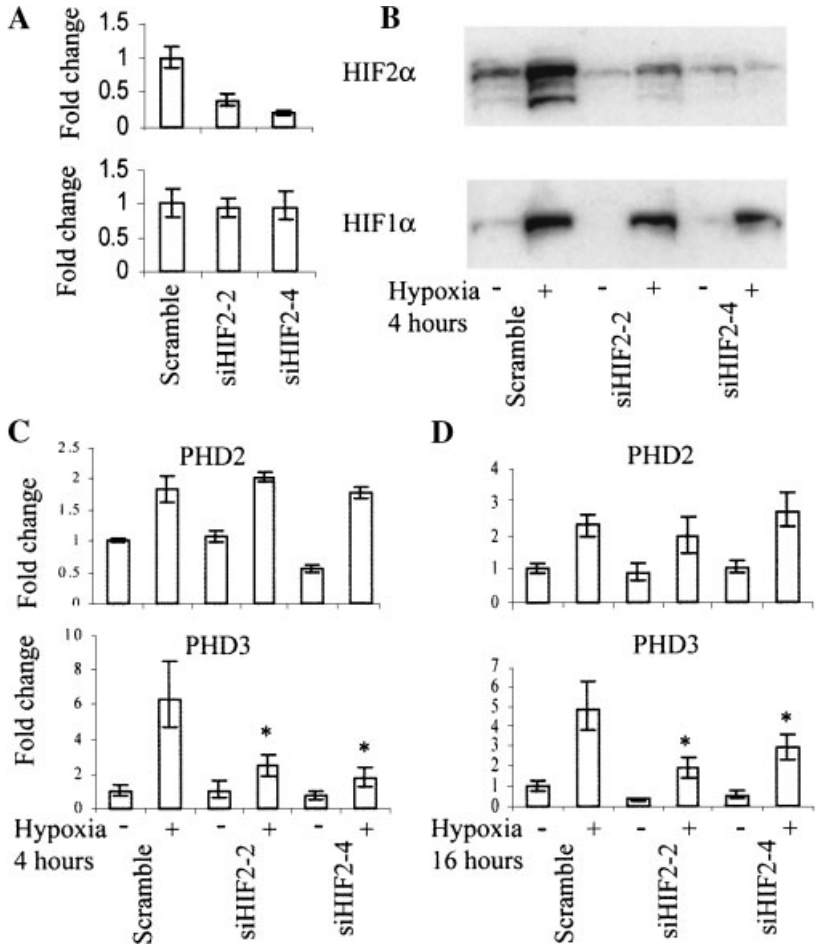

Fig. 5. siRNA targeted knock-down of HIF-2α results in decrease in PHD3, but not PHD2, induction by hypoxia. The experiments were done as described in Figure 4.

have compromised induction of PHD3 [del Peso et al., 2003]. Our experiments demonstrate that siRNA-mediated degradation of either HIF-1α or HIF-2α mRNA has very similar effect on attenuation of hypoxic induction of PHD3. However, overexpression of HIF-2α in two different cell lines provides much better activation of PHD3 than overexpression of HIF-1α. One of the possibilities is that despite the redundancy in HIF-1 and HIF-2 function, they may be activated by different mechanism. While overexpression of HIF-2α under normoxic conditions is sufficient to induce transcription, HIF-1α may require further cooperation with other factors stimulated by hypoxia. A cooperation of several transcription factors, such as AP-1, CREB-1/ATF-1, and HNF-4, with HIF-1 has been described [Norris and Millhorn, 1995; Damert et al., 1997; Ebert and Bunn, 1998; Kimura et al., 2000], however, it is not yet clear whether HIF-2 utilizes the same mechanism.

For the first time, we show that overexpression of HIF-1α or HIF-2α in two different cell lines does not induce PHD2 transcription under normoxia. Similarly, siRNA-mediated inactivation of HIF-2α in Hep3B cells demonstrated no effect on hypoxic induction PHD2. However, we found that stimulation of PHD2 by prolonged, but not short-term hypoxia, depends on intact HIF-1α subunit. This finding indicates that PHD2 accumulation has biphasic response to hypoxia when initially it is activated by factor(s) independent of HIF-1 but under chronic hypoxia the elevated levels of PHD2 require HIF-1 or factors, downstream of HIF-1, or cooperating with HIF-1. Failure of ectopically expressed HIF-1α to induce PHD2 under normoxic conditions (Fig. 4) also implies that some other factor(s) induced by hypoxia may be required. Similar observation has been described for the hypoxic induction of c-Jun. Mouse embryo fibroblasts with targeted deletion of

HIF-1α were not able to accumulate c-Jun mRNA and phosphorylation under chronic hypoxia, however, early response of c-Jun to hypoxia was HIF-1-independent [Laderoute et al., 2002].

In conclusion, we have demonstrated that both HIF-1 and HIF-2 are involved in feedback regulation of their activity through either rapid induction of PHD3 or sustained induction of PHD2 through HIF-1 only. Further insight into HIF-mediated gene regulation in tumor microenvironment with areas of chronic hypoxia is important for understanding malignant progression and for development of tools to manipulate HIF-mediated adaptation of tumors to hypoxic conditions.

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