Intracellular localisation of human HIF-1α hydroxylases: implications for oxygen sensing

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Summary

Hypoxia-inducible factor1 (HIF-1) is an essential transcription factor for cellular adaptation to decreased oxygen availability. In normoxia the oxygen-sensitive α-subunit of HIF-1 is hydroxylated on Pro564 and Pro402 and thus targeted for proteasomal degradation. Three human oxygen-dependent HIF-1α prolyl hydroxylases (PHD1, PHD2, and PHD3) function as oxygen sensors in vivo. Furthermore, the asparagine hydroxylase FIH-1 (factor inhibiting HIF) has been found to hydroxylate Asp803 of the HIF-1 C-terminal transactivation domain, which results in the decreased ability of HIF-1 to bind to the transcriptional coactivator p300/CBP. We have fused these enzymes to the N-terminus of fluorescent proteins and transiently transfected the fusion proteins into human osteosarcoma cells (U2OS). Three-dimensional 2-photon confocal fluorescence microscopy showed that PHD1 was exclusively present in the nucleus, PHD2 and FIH-1 were mainly located in the cytoplasm and PHD3 was homogeneously distributed in cytoplasm and nucleus. Hypoxia did not influence the localisation of any enzyme under investigation. In contrast to FIH-1, each PHD inhibited nuclear HIF-1α accumulation in hypoxia. All hydroxylases suppressed activation of a cotransfected hypoxia-responsive luciferase reporter gene. Endogenous PHD2mRNA and PHD3mRNA were hypoxia-inducible, whereas expression of PHD1mRNA and FIH-1mRNA was oxygen independent. We propose that PHDs and FIH-1 form an oxygen sensor cascade of distinct subcellular localisation.

Key words: Hypoxia, Oxygen sensing, Hypoxia inducible factor, Hydroxylase

Introduction

The transcription factor hypoxia-inducible factor 1 (HIF-1) plays a central role in adaptation processes to reduced cellular oxygen availability (Semenza, 1999; Wenger, 2000; Wenger, 2002). HIF-1 is composed of two subunits, which are both basic helix-loop-helix PAS proteins (Wang et al., 1995). Although the β-subunit is a constitutive nuclear protein, the α-subunit is rarely detectable in normoxia but strikingly induced in hypoxia in all cell types examined so far. HIF-1α bears two nuclear translocation signals (Kallio et al., 1998), and thus most of the protein is found in the nucleus. It is still under discussion whether the translocation process is hypoxia inducible (Ruas et al., 2002) or not (Groulx and Lee, 2002; Hofer et al., 2001). In the nucleus HIF-1α dimerises with its partner protein HIF-1β, which is also termed the aryl hydrocarbon receptor nuclear translocator (ARNT). The α/β heterodimer binds to hypoxia-responsive elements (HRE) in regulatory regions of an array of hypoxia-inducible genes (Semenza, 1998). The target genes of HIF-1 include those encoding the glycoprotein hormone erythropoietin, which leads to the production of red blood cells, the angiogenic factor vascular endothelial growth factor (VEGF), enzymes generating vasoactive substances, for example, inducible nitric oxide synthase, and virtually all glycolytic enzymes. Thus the induction of HIF-1α is central for the adaptation of cells, tissues and the whole organism to hypoxia.

In normoxia HIF-1α is bound by the von-Hippel-Lindau gene product (pVHL) (Hon et al., 2002; Maxwell et al., 1999; Min et al., 2002), which is the substrate-recognising component of an E3 ubiquitin ligase complex (Cockman et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). HIF-1α is then polyubiquitynated and almost instantaneously degraded by the proteasome (Huang et al., 1998; Salceda and Caro, 1997). Complete inactivation of pVHL in cultured cells leads to accumulation of HIF-1α in normoxia, suggesting that oxygen-dependent degradation of HIF-1α is exclusively controlled by pVHL. The existence of an oxygen- and pVHL-independent HIF-1α degradation pathway has been reported very recently (Isaacs et al., 2002).

In the presence of oxygen, binding of HIF-1α to pVHL is induced by hydroxylation of two proline residues of HIF-1α, P564 and P402 (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Shortly after this demonstration, a family of enzymes capable of hydroxylating HIF-1α subunits was identified (Epstein et al., 2001). The first enzyme shown to have this activity was the *Caenorhabditis elegans* protein

EGL-9. By database search a group of human enzymes was found that were termed prolyl hydroxylase domain containing proteins 1, 2, and 3 (PHD1, PHD2, and PHD3). Another group of investigators have independently identified the same enzymes calling them HPH1, HPH2 and HPH3, where HPH stands for HIF prolyl hydroxylases (Bruick and McKnight, 2001). The existence of a fourth HIF-1α prolyl hydroxylase has been postulated very recently (Oehme et al., 2002). On a more theoretical basis PHD1, PHD2 and PHD3 were shown to belong to the same gene family as EGL-9 before a function had been assigned to them. Therefore they were also named EGLN1, EGLN2 and EGLN3 (Taylor, 2001).

Very recently oxygen-dependent hydroxylation of Asp803 in the C-terminus of human HIF-1α has been demonstrated to inhibit the function of the C-terminal transactivation domain (C-TAD) (Lando et al., 2002a). A protein that was termed the factor inhibiting HIF (FIH-1) was isolated (Mahon et al., 2001) and subsequently shown to abrogate the interaction between HIF-1α and the transcriptional coactivator p300/CBP (Hewitson et al., 2002; Lando et al., 2002b). The existence of FIH-1 and the PHDs suggests that in the presence of oxygen HIF-1 target gene expression is tightly regulated by two separate mechanisms: prolyl hydroxylases initiate the degradation of HIF-1α and the asparagine hydroxylase FIH-1 inactivates the C-terminal transactivation domain of HIF-1α.

As PHD1, PHD2, PHD3 and FIH-1 hydroxylate HIF-1α in an oxygen-dependent manner they have been postulated to function as oxygen sensors in vivo. Here we set out to determine the intracellular localisation of these enzymes. To this end we expressed the hydroxylases fused to enhanced green fluorescent protein (EGFP) in cultured human osteosarcoma cells and assessed their effect on the hypoxic induction of endogenous HIF-1α by immunofluorescence. We also tested the effect of a transient transfection of PHDs or FIH-1 on an HRE-driven luciferase reporter gene. Furthermore, we checked for expression of endogenous PHD mRNAs and their responses to hypoxia and the hypoxia mimics desferrioxamine and cobalt ions.

Materials and Methods

Plasmids and IVTT

Expression vectors for human prolyl hydroxylases PHD1-pcDNA3, PHD2-pcDNA3, PHD3-pcDNA3 (Epstein et al., 2001) and PHD-PK (versions that bear a nine amino-acid C-terminal paramyxoviral PK-tag) were generously provided by J. M. Gleadle and P. J. Ratcliffe (University of Oxford, Oxford, UK). The PHD1 coding sequence was amplified by PCR using *Pfu* polymerase (Promega, Mannheim, Germany), a T7 promoter primer and a reverse oligonucleotide 5′-TCCCCGCGGGGTGGGCGTAGGCGGCTGTG-3′ replacing the endogenous stop codon with a *Sac*II restriction site. The PCR product was digested with *Hin*dIII and *Sac*II (New England Biolabs, Frankfurt/M, Germany) and ligated into pEGFP-N1 (Clontech, Heidelberg, Germany). PHD3-EGFP-N1 was produced in the same way, the reverse oligomer in this case was 5′-TCCCCGCGGGT-CTTCAGTGAGGGCAGATTC-3′. PHD2-EGFP-N1 was generated in two steps. First we excised an N-terminal restriction fragment from PHD2-pcDNA3 making use of the *Hin*dIII restriction site in the pcDNA3 multiple cloning site and an internal *Xho*I site and ligated this fragment into pBluescript KS+ (Stratagene, Amsterdam, Netherlands). Next we amplified the 3′ end of the PHD2 coding sequence using oligonucleotides 5′-CTCATCGCTGTTCCAGGAG-AAG-3′ and 5′-CGCGGTACCGCGAAGACGTCTTTACCGACCG-3′ as primers. The latter one carries a *Kpn*I site. After digestion with *Xho*I and *Kpn*I we ligated the PCR product into 5′PHD2-pBluescript. The full-length PHD2 could now be released using *Hin*dIII/*Kpn*I and be ligated into pEGFP-N1. All PHD-EGFP fusion vectors were transformed into Dam-deficient *E. coli* JM110 (Stratagene). After plasmid isolation PHD1-EGFP- and PHD2-EGFP-coding sequences were excised using *Hin*dIII/*Xba*I. PHD3-EGFP was released by *Hin*dIII and *Not*I digestion. Each PHD-EGFP fusion was then ligated into pcDNA3. In vitro transcription/translation reactions (IVTT) were performed in T7 TNT reticulocyte lysate (Promega) following the supplier's instructions. ^{35}S-labelling of the IVTT products and SDS-PAGE showed protein species of the expected molecular weight. The FIH-1 coding sequence was PCR amplified from a cDNA sample prepared from U2OS cells using the forward oligo 5′-CCCAAGCTTGCGGAGATGGCGGCGACAGCGGCG-3′ and the reverse oligo 5′-GGAATTCCTAGTTGTATCGGCCCTTGATC-3′. The PCR product was digested with *Hin*dIII and *Eco*RI and ligated into pcDNA3. A second PCR product was generated using the same 5′ oligo and a 3′ oligo eliminating the stop codon and containing a *Sac*II restriction site. This product was ligated into pEGFP-N1. Interestingly both FIH-1 PCR products contained a nucleotide substitution that led to replacement of Pro41 by Ala. All expression vectors were verified by DNA sequencing.

Cell culture and transient transfections

The human osteosarcoma cells (U2OS) were a kind gift from J. M. Gleadle and P. J. Ratcliffe. U2OS and Hep3B cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, Karlsruhe, Germany) supplemented with 10% FCS (Gibco), 50 IU/ml penicillin and 50 μg/ml streptomycin sulfate (Sigma). Cells were grown in six-well dishes for reporter gene assays and RNA preparation or on coverslips in 24-well dishes for microscopical analysis. Transient transfections were performed as described previously (Masson et al., 2001). In brief, for each well to be transfected 3 μl Fugene 6 (Roche, Mannheim, Germany) per μg plasmid DNA were suspended in 100 μl DMEM/well. After 5 minutes at room temperature plasmid DNA was added. The mixture was incubated for another 30 minutes at room temperature and then added to the cell culture medium. Cells were incubated for 24 hours and then subjected to the experimental conditions. Hypoxic incubations were done in an atmosphere of 1% or 3% O_2 in a hypoxia workstation (Ruskinn Technology, Leeds, UK).

Fluorescence microscopy

U2OS cells were grown on coverslips to 50% density and transfected with the EGFP- or PK-tagged PHD1, PHD2, PHD3 and FIH-1 as described above. Cells were incubated for 4 hours in normoxia (20% oxygen) or in hypoxia (1% oxygen) and fixed by ice cold methanol/acetone (1:1) for 5 minutes. For indirect immunofluorescence, cells were blocked with 3% BSA in PBS and incubated with a monoclonal mouse anti-HIF-1α antibody (1:50, Transduction Laboratories) or with a monoclonal mouse anti-PK antibody (1:200, Serotec). Cells were washed in PBS prior to an incubation with an Alexa-568-conjugated goat anti-mouse IgG secondary antibody (1:400, Molecular Probes, Göttingen, Germany). Coverslips were mounted on slides with Mowiol (Calbiochem, Bad Soden, Germany).

Images of the immunostained cells were captured using a Nikon E1000 microscope (Nikon, Germany) with Apochromat ×60 oil immersion objective lens, equipped with an Optronics digital charge-coupled device (CCD) camera (Visitron Systems, Pucheim, Germany). The images were assessed by using EZ2000 software (Coord, Netherlands). Fluorescence intensities were visualised by using a false color table shading from black (lowest), through blue, green, yellow, orange, red and purple to white (highest intensity).

Two-photon confocal laser scanning microscopy

PHDs fused to EGFP were three dimensionally recorded using two-photon confocal laser scanning microscopy (2PCLSM) as described previously (Bestvater et al., 2002). The two photon pulses were provided by a mode-locked Ti: Sapphire laser [Coherent Mira 900 F; excitation at 720-910 nm, pulse interval/repetition rate: 13.2 nseconds, 110 fsecond pulses and a high power (5 W, 532 nm) laser (Verdi, Coherent, Darmstadt); 40 mW at 560 nm]. The light was guided through a grading dispersion compensator (GDC) to a scan unit (PCM2000, Nikon, Germany) mounted on an inverted fluorescence microscope (TE300, Nikon). The x,y,z image stacks (512×512×64 pixels) were obtained by optical sectioning with a 60× water objective (Nikon, Plan Apochromat DIC H; NA 1.2). 850 nm for excitation was applied for GFP excitation giving an emission peak at 520 nm. Fluorescence intensities recorded by a photo multiplier were digitised and visualised by the EZ 2000 software (Version 2.1.4, Coord Automatisering, Netherlands). The signal-to-noise ratio was determined and the images were deconvoluted by the Huygens System software (Version 2.2.1, Scientific Volume Imaging; PS Hilversum, Netherlands) on a Silicon Graphics Octane workstation using the maximum likelihood estimation (MLE) method. The data were reconstructed with the Application Visualisation System (AVS Waltham, Mass., USA). Calculation of isosurfaces was performed on a Unix-based Octane workstation (Silicon Graphics, Mountain View) on the basis of the application visualisation system using a marching cube algorithm (AVS-Express, Waltham) as described above.

In vitro protein interaction assay

The hydroxylase activity of the PHD-EGFP fusion proteins was demonstrated in a GalDBD-HIF-1α549-582–pVHL in vitro interaction assay as described previously (Jaakkola et al., 2001). In brief, we produced GalDBD-HIF-1α549-582, PHD, PHD-EGFP fusion proteins and ^{35}S-labelled pVHL in a T7-coupled rabbit reticulocyte lysate in vitro transcription/translation system (Promega) as recommended by the manufacturer. GalDBD-HIF-1α549-582 was purified with GalDBD-antibodies conjugated to agarose beads (Santa Cruz Biotechnology, Heidelberg, Germany). 5 μl GalDBD-HIF-1α549-582 IVTT solution was then incubated for 30 minutes with either 5 μl unprogrammed reticulocyte lysate or PHD or PHD-EGFP fusion protein IVTT solution in the presence of 1 mM ascorbate, 1 mM α-ketoglutarate and 20 μM $FeCl_2$. The beads were washed several times before 5 μl of ^{35}S-pVHL IVTT solution was added. The mixture was incubated overnight at 4°C on an end-over-end rotator. Unbound ^{35}S-pVHL was removed by washing, the beads were boiled in SDS-PAGE loading buffer and the proteins were separated on a 15% SDS-polyacrylamide gel. ^{35}S-pVHL was detected by autoradiography.

Reporter gene assays

Cells were grown in six-well dishes and transfected as detailed above. For each well, 250 μg of a plasmid containing six copies of a hypoxia-responsive element from the PGK promoter in front of a firefly luciferase gene (HRE-luc, a kind gift of C. Pugh, University of Oxford, Oxford, UK), 250 μg of an SV40 promoted β-galactosidase plasmid (Promega) and 500 μg of a PHD-pcDNA3 construct were used. After 24 hours the cells were given fresh medium and incubated in the normoxic (20% O_2) or in the hypoxic (1% O_2) atmosphere for 24 hours. Cells were harvested and luciferase assays were done with a commercially available assay kit (Promega) following the supplier's instructions. β-galactosidase activity was measured exactly as published previously (O'Rourke et al., 1999). HRE luciferase values were divided by β-galactosidase values to correct for variations in transfection efficiency. All transfections were done in three separate wells, data are given as mean plus standard deviation.

Reverse transcription and quantitative real time PCR

Total RNA (1 μg) was reverse transcribed with oligo (dT) and M-MLV Reverse Transcriptase (Promega). Gene expression of human PHD1, PHD2, PHD3 and FIH-1 was quantified using the qPCR™ Mastermix for SYBR Green I (Eurogentec, Belgium) and the GeneAmp®5700 sequence Detection System (PE Biosystems, Foster City, CA). The PCR reactions were set up in a final volume of 25 μl per 0.5 μl cDNA, 1× reaction buffer with SYBR Green I, 10 pmol forward (F) and 10 pmol reverse primer (R). The primer sets used for PHD1 were (F) 5′-GGCGATCCCGCCGCGC-3′ and (R) 5′-CCTGGGTAACACGCC-3′, for PHD2 (F) 5′-GCACGACACCGGGAAGTT-3′ and (R) 5′-CCAGCTTCCCGTTACAGT-3′, for PHD3 (F) 5′-GGCCATCAGC-TTCCTCCTG-3′ and (R) 5′-GGTGATGCAGCGACCATCA-3′, for FIH-1 (F) 5′-ACAGTGCCAGCACCCACAA-3′ and (R) 5′-GCCCA-CAGTGTCATTGAGCG-3′, for the house keeping gene 60S acidic ribosomal protein (F) 5′-ACGAGGTGTGCAAGGAGGGC-3′ and (R) 5′-GCAAGTCGTCTCCCATCTGC-3′. Agarose gel electrophoresis confirmed the specificity of the amplification product. Ten-fold dilutions of purified PCR products (High Pure PCR Product Purification Kit, Roche) starting at 1 pg to 0.1 fg were used as

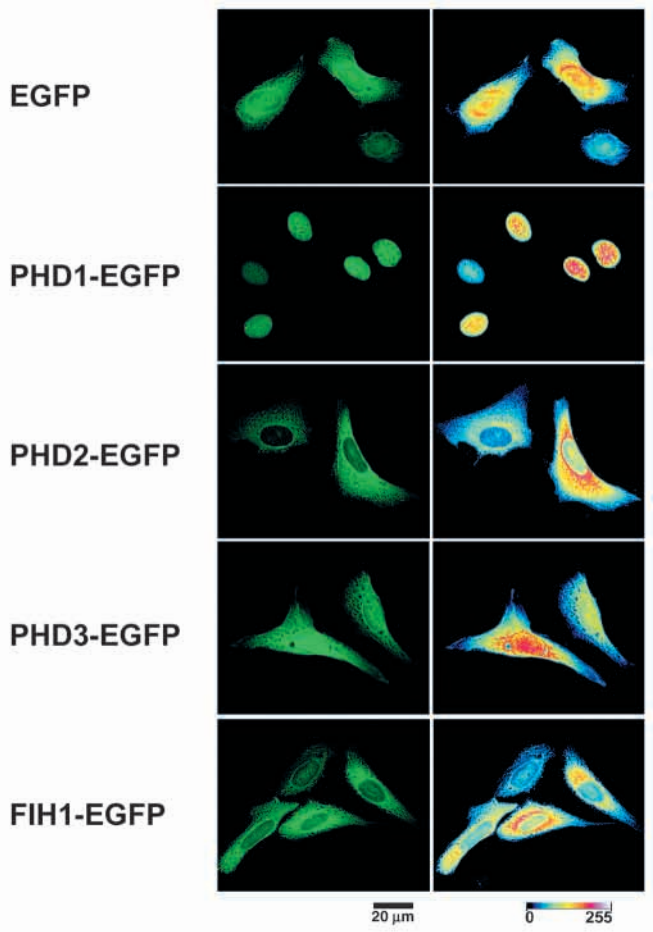

Fig. 1. Control EGFP or HIF-1 hydroxylase. EGFP fusion proteins transiently expressed in subconfluent human U2OS osteosarcoma cells. Cells were transfected with pEGFP-N1 or with plasmids encoding PHD-EGFP or FIH-1-EGFP. Cells were fixed with cold 4% paraformaldehyde/PBS for 10 minutes. Left column, EGFP fluorescence microscopy; right column, fluorescence intensities visualised using a false colour table shading from black (lowest intensity), through blue, green, yellow, orange, red and purple to white (highest intensity).

standards. Amplification conditions were set to 10 minutes at 95°C followed 30 PCR cycles [15 seconds at 95°C, 1 minutes at 60°C (PHD1, 2 and FIH-1) or 62°C (PHD3 and ribosomal protein)]. The quantity of cDNA used in each reaction was normalised to the ribosomal protein cDNA and expressed as cDNA sample/cDNA ribosomal protein.

Results

Subcellular localisation of HIF-1α hydroxylases

We produced chimeric proteins composed of one of the HIF-1α prolyl hydroxylases or FIH-1 fused in frame to the N-terminus of EGFP. The fusion proteins were then transiently expressed in human U2OS osteosarcoma cells. Intracellular localisation was analysed by fluorescence microscopy as well as by three-dimensional two-photon confocal laser scanning microscopy (2PCLSM). We found that the hydroxylases have a distinct pattern of subcellular localisation. PHD1 was detectable exclusively in the nucleus, whereas the majority of PHD2 and FIH-1 was found in the cytoplasm. The latter result is of interest because PHD2 and FIH-1 have a calculated molecular weight of 46 kDa and 40.3 kDa, respectively. As molecules of up to 60 kDa usually have free access to the nuclear compartment (Quimby and Corbett, 2001), PHD2 and FIH-1 would be expected to be present in cytoplasm and nucleus. This finding suggests that PHD2 and FIH-1 are actively excluded from the nucleus. PHD3 was distributed more evenly in the cytoplasm and nucleus (Figs 1, 2). We also analysed the localisation of the prolyl hydroxylases carrying a nine amino-acid C-terminal paramyxoviral PK tag instead of EGFP. Because of its low molecular weight and its C-terminal position, this tag was not expected to influence subcellular localisation. Importantly the tagged proteins showed exactly the same intracellular distribution as the EGFP fusion proteins (data not shown). Expression of PHD3 frequently resulted in deposition of perinuclear aggregates that did not colocalise with any subcellular compartment. These aggregates were not enclosed by a cellular membrane, as demonstrated by electron microscopy (data not shown), and were substantially reduced when we used the PK-tagged PHD3-construct followed by immunohistochemistry with a PK antibody. These data indicate that the aggregates were artefacts caused by cytoplasmic deposition of overexpressed PHD3.

Effect of hydroxylase expression on endogenous HIF-1α

We confirmed by in vitro transcription/translation in rabbit reticulocyte lysate that all HIF-1α prolyl hydroxylase EGFP fusions could be expressed as single bands of the expected molecular weight (Fig. 3A). Next we demonstrated that the prolyl hydroxylase fusion proteins can modify HIF-1α at Pro564, enabling pVHL binding. To this end we set up an in vitro protein interaction assay as described previously (Jaakkola et al., 2001). In a control experiment we excluded the possibility that GalDBD or the agarose beads bind to pVHL non-specifically before or after treatment with HIF-1α prolyl hydroxylases (data not shown). We then treated a GalDBD-ODD fusion protein bound to agarose beads with the hydroxylases or the hydroxylase EGFP fusion protein and analysed pVHL-binding activity by incubation with ^{35}S-labelled pVHL followed by autoradiography.

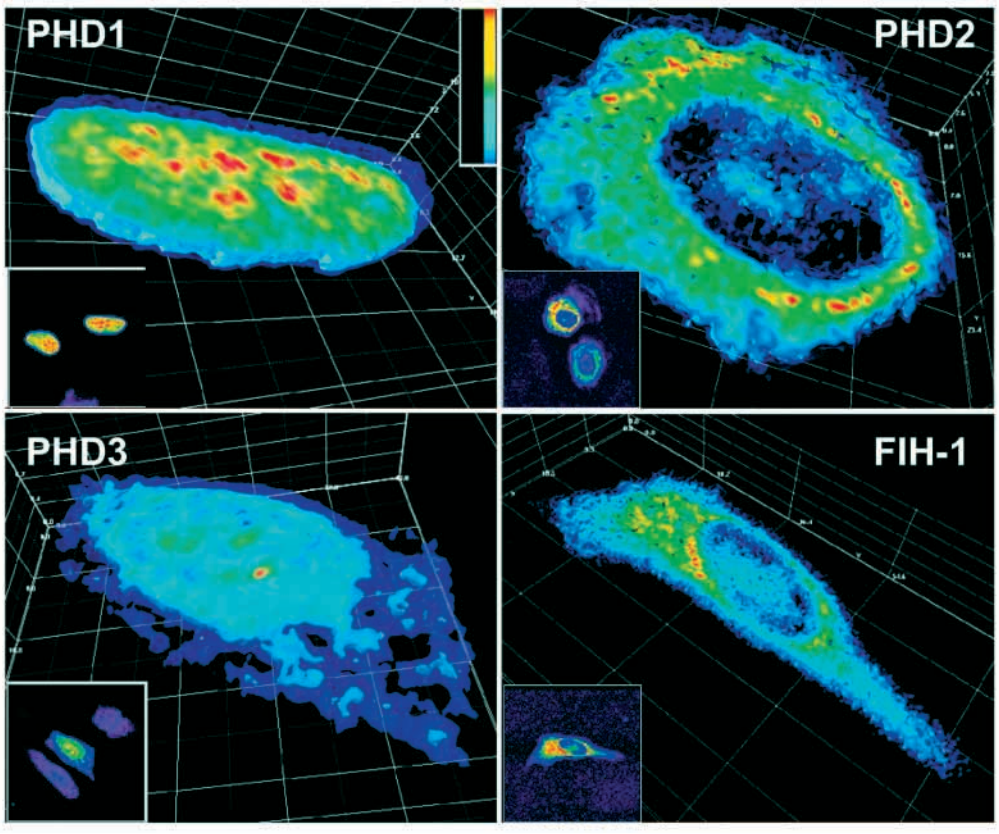

Fig. 2. Three-dimensional two-photon confocal laser scanning microscopy of PHD1, PHD2, PHD3 and FIH-1. Different EGFP fluorescence intensities of single cells were visualised in false colours as indicated by the color table (right column). Therefore, up to 64 optical slices through the transfected cells were recovered by two-photon confocal laser scanning microscopy. After reconstruction of the optical slices, the distribution of the EGFP fluorescence within a single cell was visualised in three dimensions. A cut through the cell reveals the inside distribution. An overlay of all optical slices is shown in the inserts.

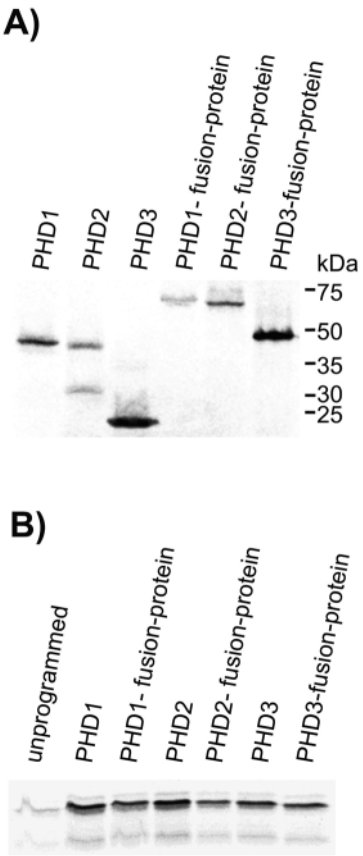

Fig. 3. In vitro characterisation of PHD-EGFP fusion proteins. (A) In vitro transcription/ translation products detected by 8% SDS-PAGE and autoradiography. (B) An in vitro protein interaction assay. After treatment of an immobilised GalDBD- HIF-1α 549-582 fusion protein with unprogrammed reticulocyte lysate or PHD or PHD-EGFP fusion protein, ^{35}S-labelled pVHL was added. Two pVHL isoforms (24 kDa and 19 kDa) were detected because translation was initiated at two distinct ATG codons in VHL. The ability to capture pVHL indicates hydroxylation of HIF-1α Pro564. Note that reticulocyte lysate has a low but significant hydroxylating activity.

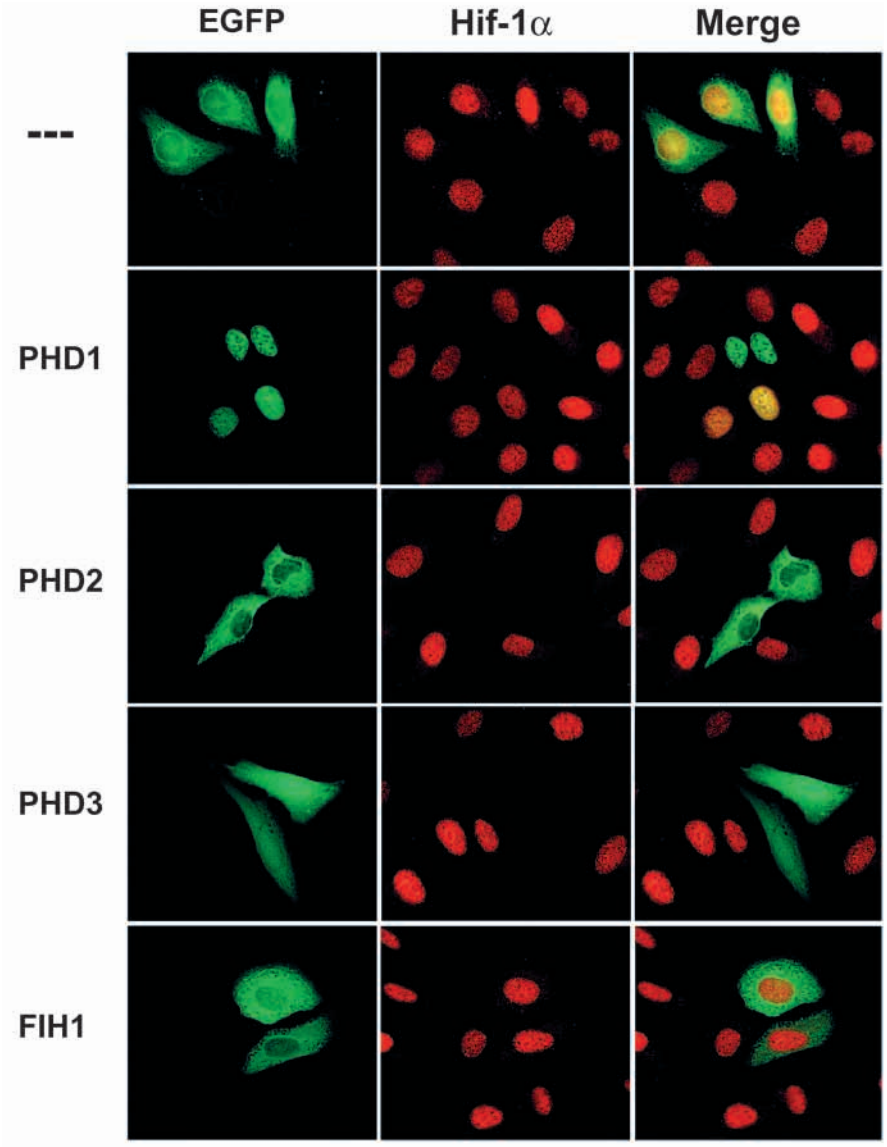

Fig. 4. Effect of transiently expressed PHD fusion or FIH-1 fusion proteins on the nuclear accumulation of endogenous HIF-1α under hypoxia. PHD-EGFP fusion or FIH-1-EGFP fusion protein or control EGFP was transiently expressed in U2OS cells. The cell cultures were placed in a hypoxia workstation (1% O2) for 4 hours. Cells were then fixed in ice-cold methanol/acetone and immunostained for endogenous HIF-1α as detailed in Materials and Methods. The left column shows hydroxylase EGFP-fusion proteins, the middle column indirect immunofluorescence of endogenous HIF-1α and the right column (Merge) an overlay of both columns.

Although the activity of the fusion proteins appeared to be somewhat reduced compared to the native enzymes, the fusion proteins have clearly retained HIF-1α hydroxylase activity (Fig. 3B). We then expressed the PHD-EGFP fusions in U2OS cells and assessed the nuclear accumulation of endogenous HIF-1α in response to hypoxia by immunohistochemistry. We found that each of the PHDs reduced HIF-1α induction significantly (Fig. 4). Interestingly cells transfected with PHD1 showed a somewhat heterogeneous appearance with some cells still staining positive for HIF-1α after hypoxic incubation. PHD2 and PHD3 transfection virtually eliminated the HIF-1α accumulation. As expected, overexpression of FIH-1 did not result in a reduction of hypoxic HIF-1α levels, as this enzyme is not involved in the HIF-1α degradation pathway. Taken together these results strongly suggest that degradation of HIF-1α can be initiated by prolyl hydroxylation in the nucleus as well as in the cytoplasm even under hypoxic conditions.

Effect of transient overexpression of HIF-1α hydroxylases on HRE-luciferase expression

To confirm that all enzymes under investigation have a

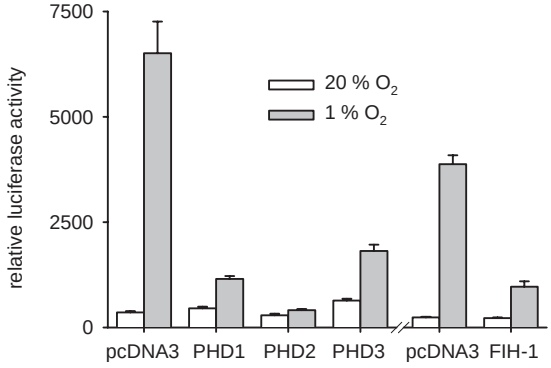

Fig. 5. Effect of the transient overexpression of HIF-1α hydroxylases on HRE-luciferase expression. U2OS cells were cotransfected with the indicated HIF-1α hydroxylase construct and a hypoxia-inducible firefly luciferase gene. Luciferase values of three separate wells were divided by β-galactosidase values to correct for variations of transfection efficiency and are given as means plus standard deviation.

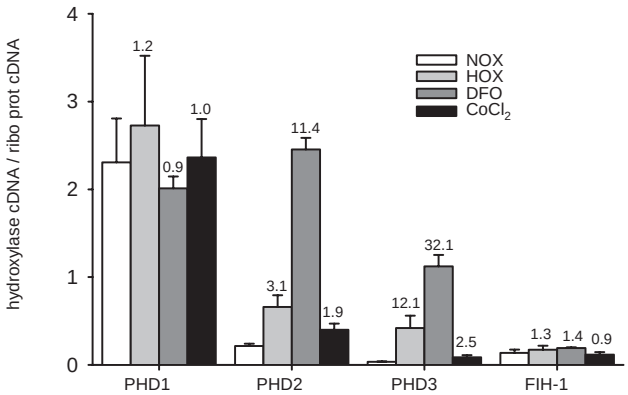

Fig. 6. HIF-1α hydroxylase expression in normoxia and responses to hypoxia, desferrioxamine (150 μM) or cobalt chloride (100 μM) treatment. U2OS total mRNA was reverse transcribed and subjected to quantitative PCR as detailed in Materials and Methods. Numbers above the bars indicate fold induction compared to normoxia. Results are given as means of four separate samples plus standard deviation.

significant impact on hypoxia-induced gene expression we transiently expressed the hydroxylases in U2OS cells together with an HRE-driven luciferase reporter gene and incubated the cells overnight in an atmosphere of 1% oxygen. Each of the PHDs nearly abrogated hypoxic induction of the reporter gene construct (Fig. 5). Repetition of the assay in U2OS and Hep3B cells indicated that there was no reproducible, statistically significant difference between PHD1, PHD2 and PHD3 with respect to the inhibition of HRE-luciferase induction. FIH-1 had the same effect although it does not act by pVHL binding and subsequent degradation but by hydroxylation of Asp803, which impairs the recruitment of the transcriptional co-activator p300/CBP.

Effect of hypoxia on the expression of endogenous PHDmRNA and FIH-1mRNA

In HeLa cells, PHD2mRNA and PHD3mRNA are hardly detectable in normoxia but are induced by hypoxia when PHD1 transcription is independent of ambient oxygen concentration (Epstein et al., 2001). In U2OS cells, expression of the PHDs and their response to hypoxia has not been investigated so far. Quantitative RT-PCR revealed that the transcripts of PHD1, PHD2 and FIH-1 are easily detectable in normoxia, whereas PHD3 transcripts were very close to the detection limit. As in HeLa cells PHD2mRNA and PHD3mRNA showed significant upregulation in U2OS cells in response to hypoxia. Desferrioxamine (150 μM) induced the transcription of the two prolyl hydroxylases PHD2 and PHD3 more efficiently than hypoxia or cobalt ions. PHD1 and FIH-1, however, were expressed constitutively (Fig. 6).

Discussion

The transcription factor HIF-1 is a central mediator of cellular responses to hypoxia. Very recently enzymatic prolyl hydroxylation has been found to control the stability of the oxygen-sensitive α-subunit (Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). The hydroxylation reaction is catalysed by specific enzymes PHD1, PHD2 and PHD3 (Epstein et al., 2001). Hydroxylated HIF-1α binds to the von Hippel Lindau protein, which targets it for proteasomal degradation by polyubiquitynation. In the presence of oxygen a second hydroxylation reaction inactivates the C-terminal transactivation domain of HIF-1α: if the amino-acid residue Asp803 is hydroxylated by the asparagine hydroxylase FIH-1, recruitment of the transcriptional coactivator p300/CBP is inhibited (Lando et al., 2002a; Hewitson et al., 2002). In the present study we show that human HIF-1α hydroxylases have a distinct pattern of intracellular localisation. PHD1 is localised exclusively to the cell nucleus, the majority of PHD2 is expressed in the cytoplasm and PHD3 is partly present in the cytoplasm and partly in the nucleus. The asparagine hydroxylase FIH-1 is mainly located in the cytoplasm; a small but significant portion, however, is also found in the nucleus. While our manuscript was in preparation the intracellular localisation of PHD1, PHD2, PHD3 in COS1 cells has been reported (Huang et al., 2002). The data for PHD1 and PHD2 are in agreement with ours: PHD3 was predominant in the cytoplasm. Interestingly the authors have shown that all PHDs reduce the normoxic expression of an HA-tagged, transfected HIF-1α protein. Our experiments prove that PHD1, PHD2 and PHD3 also abrogate the hypoxic induction of endogenous HIF-1α. This effect was most pronounced for PHD2 and PHD3. Although PHD1 clearly reduced the number of cells staining positive for HIF-1α, some cells showed nuclear colocalisation of overexpressed PHD1 and endogenous HIF-1α. From our experiments it is not entirely clear whether the conditions were less than ideal for PHD1 or whether PHD1 is not as efficient as the other HIF-1α prolyl hydroxylases. Our results are in line with a report (Huang et al., 2002) that demonstrates that PHD2 has a higher specific activity toward HIF-1α than to PHD1. It has been reported (Groulx and Lee, 2002) that the degradation of HIF-1α is initiated by strictly nuclear prolyl hydroxylation and subsequent export of HIF-1α-pVHL complexes. From our data it appears unlikely that PHD1 is the dominant enzyme in HIF-1α degradation. This would suggest that the nuclear portions of PHD2 and PHD3 are the active biological oxygen sensors in vivo.

Interestingly, PHD1 is induced by estrogen and stimulates cell proliferation (Seth et al., 2002). It is possible that regulation of HIF-1α is not the primary function of PHD1. From a more general perspective it seems that HIF prolyl hydroxylases have widespread effects other than the regulation of HIF-1α. *C. elegans* lacking the prototype HIF hydroxylase EGL-9 show an egg-laying defect (Trent et al., 1983) and are resistant to an otherwise paralytic *Pseudomonas aeruginosa* toxin (Darby et al., 1999). A rat PHD homologue named SM-20 takes part in survival/apoptosis decisions in sympathetic neurons (Lipscomb et al., 2001). It is unclear at present whether these phenotypes are related to loss of HIF-1 regulation or whether they are caused by the loss of hydroxylation of other PHD substrates. Certainly characterisation of further PHD substrates will be important for defining new roles for prolyl hydroxylation.

It was observed several years ago that the expression of C-TAD fusion proteins is not oxygen dependent, whereas its transactivating function is (Ema et al., 1999; Pugh et al., 1997). This function was later found to be hydroxylation sensitive (Sang et al., 2002). In our experiments FIH-1 did not prevent HIF-1α stabilisation, whereas it markedly inhibited activation of an HRE-driven luciferase reporter gene. Our results confirm that FIH-1 is not involved in the degradation pathway; instead it inactivates the C-terminal transactivation domain of HIF-1α. Together with our finding that FIH-1mRNA is easily detectable in normoxia it seems likely that FIH-1 has an important function in cellular oxygen sensing.

Two out of three HIF-1α prolyl hydroxylases are hypoxia inducible in U2OS cells (Fig. 6) and in Hep3B cells (data not shown). PHD2 and PHD3 showed an upregulation of mRNA expression after hypoxia or incubation with the hypoxia mimics, desferrioxamine and cobalt ions. PHD1 was expressed constitutively, confirming results reported previously for HeLa cells (Epstein et al., 2001). The FIH-1mRNA expression could not be stimulated by hypoxic incubation. Our data may suggest that PHD2 and PHD3 tightly regulate the HIF level in hypoxia to avoid excessive nuclear HIF-1α accumulation. Prior to the characterisation of the HIF-1α prolyl hydroxylases it had been demonstrated that the half-life of HIF-1α after reoxygenation depends on the duration of the hypoxic incubation before reoxygenation: a longer hypoxic period shortens the half-life of HIF-1α (Berra et al., 2001). This finding, together with our data, suggests another function for PHD2 and PHD3: during hypoxia, PHD2 and PHD3 are induced while the actual HIF-1α turnover is low because oxygen supply is limiting. In the case of reoxygenation the half-life of HIF-1α would then be extremely short because of a high PHD capacity within the cells.

In conclusion we propose a model in which HIF-1α can be hydroxylated in the cytoplasm as well as in the cell nucleus. Because of their distinct intracellular localisation the HIF-1α prolyl hydroxylases PHD1, PHD2, PHD3 and the asparagine hydroxylase FIH-1 form a cascade of oxygen sensors that tightly control the expression of HIF-1 target genes.

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