Genetic Analysis of the Role of the Asparaginyl Hydroxylase Factor Inhibiting Hypoxia-inducible Factor (HIF) in Regulating HIF Transcriptional Target Genes*

Received for publication, June 16, 2004, and in revised form, July 27, 2004 Published, JBC Papers in Press, August 9, 2004 DOI 10.1074/jbc.M406713200

Ineke P. Stolze‡§, Ya-Min Tian‡, Rebecca J. Appelhoff‡‡, Helen Turley‡, Charles C. Wykoff***, Jonathan M. Gleadle‡, and Peter J. Ratcliffe‡ ‡‡
From ‡‡‡The Henry Wellcome Building of Genomic Medicine, University of Oxford, Roosevelt Drive, Headington, Oxford OX3 7BN, the Cancer Research United Kingdom (CRUK) Tumour Pathology Group, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, John Radcliffe Hospital, Oxford OX3 9DU, and the **CRUK Molecular Oncology Group, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that directs a broad range of cellular responses to hypoxia. Recent studies have defined a set of 2-oxoglutarate and Fe(II)-dependent dioxygenases that modify HIF-α subunits by prolyl and asparaginyl hydroxylation. These processes potentially provide a dual system of control, down-regulating both HIF-α stability and transcriptional activity. Although genetic analyses in both primitive organisms and mammalian cells have demonstrated a critical role for the prolyl hydroxylase pathway in the regulation of HIF, analogous studies have not been performed on the HIF asparaginyl hydroxylase pathway, and its role in directing the expression of endogenous HIF transcriptional targets has not yet been clearly defined. Here we demonstrate, using small interfering RNA-mediated FIH suppression and controlled overexpression by a doxycycline-inducible system, that alterations in FIH expression in both directions have reciprocal effects on the expression of a range of HIF target genes. These effects were observed in normoxic and severely hypoxic cells but not anoxic cells. Evidence for FIH activity in severely hypoxic cells contrasted with results for the prolyl hydroxylase PHD2, suggesting that these enzymes display different oxygen dependence in vivo, with PHD2 requiring higher levels of oxygen for biological activity. Our results demonstrate an important physiological role for FIH in regulating HIF-dependent target genes over a wide range of oxygen tensions and indicate that inhibition of FIH has the potential to augment HIF target gene expression even in severe hypoxia.

Exposure of mammalian cells to hypoxia activates a conserved transcriptional response pathway mediated by hypoxia-inducible factor (HIF).1 These adaptive responses include up-regulation of target genes involved in angiogenesis, erythropoiesis, matrix metabolism, and glycolysis, as well as vasomotor control (1, 2). HIF is a heterodimeric transcription factor composed of two basic-helix-loop-helix proteins of the PAS (PER-ARNT-SIM) family, a hypoxia-inducible α subunit and a constitutively expressed β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT) (1, 2).

Recent analyses of the mechanisms that control the activity of HIF have uncovered a series of Fe(II) and 2-oxoglutarate-dependent dioxygenases that catalyze oxygen-dependent hydroxylation of specific residues in HIF-α subunits (3–6). Under normoxic conditions, von Hippel-Lindau tumor suppressor protein (pVHL) targets HIF-α subunits for proteasomal degradation following prolyl hydroxylation at two independent sites (Pro-402 and Pro-564 in human HIF-1α) within the oxygen-dependent degradation domain (7–10) by three HIF prolyl hydroxylase isoforms (PHD1–3) (3, 4).

In addition, the transcriptional activity of HIF is modulated through asparaginyl hydroxylation (11) by another dioxygenase, first identified as a factor inhibiting HIF-1 (FIH-1) (12). In the presence of oxygen, FIH hydroxylates a conserved asparagine residue within the C-terminal transactivation domains of two HIF-α isoforms: Asn-803 in human HIF-1α and Asn-851 in human HIF-2α (5, 6). This modification at the β-carbon of the asparaginyl residue (13) prevents interaction of the HIF-α C-terminal transactivation domain with the CH-1 domain of the transcriptional co-activator p300 (6, 11, 14, 15), potentially reducing the transcriptional activity of HIF-α subunits that have escaped degradation.

Although forced expression of FIH reduces hypoxia-responsive reporter gene expression in transiently transfected cells (12, 16, 17), analysis of the physiological effects on gene expression is to date only limited, and the role of endogenous FIH in directing the native HIF transcriptional response remains uncertain. To address this, we first characterized FIH protein levels in different human cell culture lines and used these data to guide functional studies with siRNA-based knockdown and controlled overexpression of FIH in target cells. We show that FIH protein is widely expressed, that it has important nonredundant effects on the expression of a range of HIF transcriptional targets, and that biologically significant FIH activity is observed in vivo even in severe hypoxia.

GLUT1, glucose transporter 1; LDH-A, lactate dehydrogenase A; PHD, prolyl hydroxylase domain; RPA, ribonuclease protection assay; siRNA, small interfering RNA; U6 SnRNA, U6 small nuclear RNA; VEGF, vascular endothelial growth factor; WT, wild type.

This paper is available on line at http://www.jbc.org 42719
FIH Regulates HIF Target Gene Expression in Vivo

EXPERIMENTAL PROCEDURES

Cell Culture—Cell lines were obtained from ATCC (American Type Culture Collection) (Manassas, VA), ECACC (European Collection of Animal Cell Culture), or National Institutes of Health, NCl cultures according to their recommendations. Stable transfecants were cultured in Dulbecco's modified Eagle's medium supplemented with 10% Tet system-approved fetal bovine serum (Clontech), penicillin G (50 IU/ml), streptomycin (50 μg/ml) (Invitrogen), 1-glutamine (2 mM) (Sigma), blasticidin S (5 μg/ml) (Invitrogen) and maintained in hygroscopic medium supplemented with Roche Applied Science) or G418 (1 mg/ml Geneticin, Invitrogen). Hypoxic/anoxic incubations of cells were performed in an Invivo2 400 hypoxic work station (Ruskinn Technologies).

Plasmids and Stable Transfectants—The human fih gene was amplified by PCR from Image clone 4138066 and subcloned as a SacII/Ascl fragment into pcDNA3 (Invitrogen)/fih/pcDNA3. fih/pUHD10 was constructed by cloning a SacII/NotI fragment from fih/pDNA3 into a pUH10-3MCs vector bearing a modified polylinker (18). To express a catalytically inactive form of FIH, plasmid D201Afih/pET28a(+) was constructed using the QuikChange site-directed mutagenesis kit (Stratagene) and fih/pET28a(+) as template (5), both vectors being kindly provided by K. S. Hewitson. D201Afih/pUHD10 was generated by inserting a BsrGI/NsiI fragment containing the D201A mutation from D201Afih/pET28a(+) into fih/pUHD10. Full-length hIFA (19) with a C-terminal PK tag was subcloned as a NotI/NcoI fragment into pUH10-3MCs (hIFA-1/PgpKuHd). Phd2/pUHD10 was constructed by subcloning full-length phd2 as a HindIII/XbaI fragment from phd2/pDNA3 (3) into a modified pUH10-3MCs vector. Phd2-hhp containing the hygromycin resistance gene was obtained from ATCC, pcDNA3.1(+) (G418B) containing the neomycin resistance gene was from Invitrogen.

Stable transfecants were generated from U-2OS bearing the reverse tetracycline response transactivator (18) and a tetKRAB silencer construct (20) by transfection with the pUHD10 vector alone, fih/pUHD10, D201Afih/pUHD10, or phd2/pUHD10 in combination with pBS2-hhp (ratio 1:1) or hIFA-1/PgpKuHd in combination with pcDNA3.1(+) (G418B) using FuGENE 6 (Roche Applied Science). 48 h after transfection, cells were separated onto 10-cm dishes and treated with hygromycin (200 μg/ml) or neomycin (1 mg/ml, G418) for 2-3 weeks. Independent clones were picked and tested for doxycycline-inducible gene expression.

FIH Antibody Production and Characterization—Full-length human FIH was expressed in Escherichia coli BL21 (DE3) cells and purified as described (5). Two BALB/c mice were immunized with 50 μg of the purified immunogen in complete Freund's adjuvant followed by four further immunizations in phosphate-buffered saline at 10-day intervals until appropriate reactivity was observed with immunoblots of fih/pDNA3-transfected COS cell extract. Fusion of mouse spleenocytes with myeloma cells was performed using standard techniques. Hybridomas secreting hybridoma supernatant or mouse anti-human FIH-1 (BD Transduction Laboratories, clone 54), mouse anti-β-tubulin I monoclonal antibody SAP.4G5 (Sigma), mouse anti-human CA9 antibody M75 kindly provided by Silvia Pastorekova (26), polyclonal anti-mouse GLUT1 antibody (GT-11A) (Alpha Diagnostic), mouse anti-human RNA polymerase II (A-10) (Santa Cruz Biotechnology), rabbit anti-PHDI, mouse anti-PHD2, and mouse anti-PHDS (27).

Immunofluorescence Microscopy—Cells grown on lysine-coated glass slides (polylysine; BDH, Poole, UK) were washed in ice-cold phosphate-buffered saline and fixed in methanol/acetone (1:1) at −20 °C for 10 min and air-dried. Slides were blocked with 3% bovine serum albumin/phosphate-buffered saline for 10 min followed by incubation with MoAb162C hybridoma supernatant or mouse anti-human FIH-1 (BD Transduction Laboratories, clone 54, 1:50) for 2 h. After washing in phosphate-buffered saline, cells were incubated for 1 h with Alexa Fluor 488 goat anti-mouse IgG (1:400; Molecular Probes), and nuclei were stained with propidium iodide/Vectashield (Vector Laboratories). Immunofluorescence-stained cells were analyzed using an MRC-1024 laser scanning confocal imaging system (Bio-Rad) with ×60 oil immersion objective lens. Images were captured and assessed using Bio-Rad LaserSharp2000 software.

RESULTS

Expression of FIH in Human Tissue Culture Cell Lines—Immunoblot analysis of whole cell extracts using MoAb162C revealed FIH protein to be widely expressed at similar levels across a wide range of cell types (Fig. 1A). Since levels of HIF prolyl hydroxylases are modulated by hypoxia through the action of HIF (23, 28, 29), we tested whether FIH protein levels are altered by hypoxia. Whole cell extracts from HeLa, Hep3B, U-2OS, MCF-7, and PC3 were prepared following parallel normoxic and hypoxic (0.2% oxygen) incubations for 16 h (Fig. 1B). FIH protein expression was unaffected by hypoxia, whereas as expected, HIF-1α was strongly induced. Similarly, exposure of Hep3B and U-2OS cells to cobaltous ions (CoCl2, 150 μM), the iron chelator desferroxamine mesylate (100 μM), and dimethyl-oxalylglycine (1 mM), a competitive inhibitor of 2-oxoglutarate-dependent dioxygenases, for 6 h had no effect on FIH protein levels despite the striking induction of HIF-1α (data not shown). Immunohistochemical analysis of U-2OS cells using MoAb162C revealed a predominant cytoplasmic location for FIH that was not altered when cells were incubated in 0.2% oxygen for 6 h (Fig. 1C). Similar results were obtained by immunoblot analysis of nuclear and cytoplasmic extracts from U-2OS, Hep3B, and HeLa cells exposed to 21 and 0.2% oxygen for 6 h (Fig. 1D and data not shown). Taken together, these experiments demonstrate that FIH is widely expressed and thus potentially available for regulation of HIF activity across a broad range of cells and culture conditions.

Silencing of FIH by RNA Interference Up-regulates HIF-dependent Target Gene Expression in Normoxia—To define the role of FIH in regulating endogenous HIF-dependent target gene expression, we used an siRNA approach to suppress FIH expression using two independent double-stranded oligonu-
cleotide sequences. To control for nonspecific effects of the siRNA transfection, two irrelevant siRNA duplexes were used, and as a further control, in certain experiments, mock transfections were performed in which cells were exposed to the transfection reagent alone. Following preliminary experiments to optimize dose and transfection protocol, experiments were performed using three applications of the siRNA spaced by 24 h. Specific silencing of FIH in U-2OS cells was documented by immunofluorescence microscopy and immunoblotting (Fig. 2A). Successful silencing reciprocally confirmed that the immunoreactive signals detected by MoAb162C in immunofluorescence and immunoblot analysis correspond to FIH.

To evaluate whether endogenous FIH exerts tonic inhibitory effects on HIF target gene expression in normoxic cells (21% oxygen), we transfected HeLa, Hep3B, and U-2OS cells with FIH-specific or irrelevant control siRNA duplexes (Fig. 2B). In all three cell lines, very substantial suppression of FIH was achieved. The low levels of HIF-1α protein in these normoxic cells were unaffected by FIH suppression. In contrast, HIF transcriptional target gene expression was clearly induced. The highly inducible HIF target CA9 was up-regulated by FIH suppression in all cells, whereas GLUT1 was up-regulated in both HeLa and U-2OS but remained below the limit of detection in Hep3B cells. This suggested that the low levels of HIF-1α in normoxic cells are potentially active and that this transcriptional activity is normally suppressed by FIH. Because of the importance of PHD2 in regulating HIF-1α in normoxia (23), we sought to compare the effects of FIH sup-
pression on HIF target genes with those of PHD2 siRNA (P2)-transfected U-2OS cells. As shown in Fig. 2B (right panel), the effect of FIH suppression on CA9 was relatively small when compared with the up-regulation of CA9 in PHD2 siRNA-transfected cells. Interestingly, the difference between GLUT1 induction in FIH and PHD2 knock-down cells was less striking, which might indicate that FIH has gene-specific effects or other targets that might be responsible for differential effects.

Both CA9 and GLUT1 are dominantly responsive to HIF-1α in the cells under study (30). Therefore, to confirm that the observed up-regulation of GLUT1 and CA9 expression is mediated by the HIF system, U-2OS cells were co-transfected with FIH-specific or control siRNA either in the absence (additional irrelevant control) or in the presence of specific HIF-1α-targeting siRNA duplexes. As shown in Fig. 2C, lanes 1–4, HIF-1α was effectively suppressed, and this abolished up-regulation of GLUT1 and CA9 protein expression by FIH knock-down. To further confirm that the effects of FIH suppression were mediated by HIF, we tested the effects of FIH RNAi on HIF target gene expression in a U-2OS stable transfectant (c29) that inducibly overexpresses HIF-1α using a doxycycline-dependent “Tet-on” expression system (Fig. 2C, lanes 5–8). Following induction of HIF-1α by doxycycline treatment of normoxic c29 cells, both GLUT1 and CA9 were moderately up-regulated, with the siRNA-based suppression of FIH resulting in striking further induction. Taken together, these results demonstrate that endogenous FIH exerts a tonic inhibitory effect on the expression of at least two HIF target genes under standard tissue culture conditions and that this effect is substantial even in normoxic cells expressing very low steady-state levels of HIF-1α.

**FIH Modulates HIF Activity in Vivo over a Range of Oxygen Tensions**—We next investigated the effects of FIH suppression on the induction of HIF target gene expression by hypoxia. To analyze effects on progressive induction of the HIF transcriptional response by graded hypoxia, Hep3B and U-2OS cells were transfected with FIH-specific or control siRNA duplexes and then incubated for 16 h in hypoxic atmospheres ranging from 7.5% oxygen down to 1% oxygen. Whole cell extracts were analyzed by immunoblotting for CA9 and GLUT1, and results are shown in Fig. 3A. Although induction by FIH suppression was modest in relation to the total amplitude of induction by hypoxia, CA9 and GLUT1 protein levels were clearly up-regulated by FIH suppression over the entire range of oxygen tensions from 21 to 1% oxygen. These results suggested that biologically significant FIH activity persists even in quite severe hypoxia. However, since transfections with siRNA were performed in normoxia prior to the 16-h hypoxic exposure, we considered it possible that the observed changes in target gene expression might simply reflect early changes in FIH, preceding hypoxic equilibration. To analyze this and also to determine the effects on additional HIF target genes, we therefore performed further experiments in which cells were equilibrated in hypoxia prior to the application of siRNA. In these experiments, U-2OS cells were exposed to 1% oxygen for 4 h prior to a transfection protocol involving three applications of siRNA spaced over a 48-h period followed by harvest after a total of 72 h in hypoxia. Total RNA was prepared, and mRNAs encoding CA9, GLUT1, LDH-A, and VEGF were analyzed by RNase protection (Fig. 3B). Up-regulation of these HIF target genes was clearly observed following FIH suppression, effects being somewhat variable and greater on CA9, GLUT1, and LDH-A mRNAs than on VEGF mRNA. These results in pre-equilibrated cells confirm that endogenous FIH is also able to limit transcriptional effects of HIF even at low (1%) oxygen availability.

**Modest Increase in FIH Expression Down-regulates HIF Target Gene Expression in Severe Hypoxia**—To further explore the role of FIH in regulation of HIF target gene expression, we next generated stably transfected U-2OS cells capable of generating modest controlled increments in FIH expression using a doxycycline-dependent expression system. Using this system, we ascertained that FIH expression could be enhanced even in cells pre-equilibrated with oxygen atmospheres substantially below 1% oxygen, whereas in such severe hypoxia, siRNA transfections over 72 h were not well tolerated. Preliminary doxycycline titration experiments were performed to derive conditions under which hypoxia pre-equilibrated cells could be induced to enhance FIH protein expression in the region of 5-fold (Fig. 4A). Fig. 4B (upper panel) shows the effects on GLUT1 mRNA expression. Interestingly, basal levels of GLUT1 mRNA in normoxic cells were further suppressed by these increases in FIH (Fig. 4B, lanes 1 and 2). Down-regulation of GLUT1 mRNA was particularly striking under moderate hypoxia (2% oxygen) but still clearly evident in severe hypoxia (0.5 and 0.2% oxygen). To determine whether integrity of the FIH catalytic site was necessary for these effects, we proceeded to construct further stable transfectants that express a mutant FIH in which the critical Asp-201 at the catalytic site is replaced by Ala (D201A) (6). Doxycycline titrations were again performed so as to match expression levels in transfecteds indubitably expressing either the wild-type (WT) or the mutant FIH (D201A) (4A), and effects on HIF target gene expression were assayed in cells pre-equilibrated in severe hypoxia (0.2% oxygen). The results of a typical experiment are shown in Fig. 4C. To exclude nonspecific effects of doxycycline, we included U-2OS cells stably transfected with the vector alone and exposed these cells to the same final doxycycline

---

2 R. R. Raval and P. J. Ratcliffe, unpublished observation.
FIH Regulates HIF Target Gene Expression in Vivo

DISCUSSION

Altogether, four members of the 2-oxoglutarate-dependent dioxygenase superfamily (the asparaginyl hydroxylase FIH and three prolyl hydroxylases, PHD1–3) have been demonstrated to hydroxylate HIF-α sequences in vitro and to down-regulate hypoxia-responsive reporter gene activity in forced expression studies (31). These findings have raised important physiological questions as to the contribution of individual enzymes in the regulatory circuitry of HIF. To date, genetic studies that address these questions have focused on the prolyl hydroxylases, with a recent study highlighting an important role for PHD2 in determination of the level of HIF-1α and HIF transcriptional activity in normoxic cells (23). In other work, we have demonstrated that under the appropriate conditions, all concentration as transfectant expressing wild-type FIH. Despite comparable levels of overexpression, wild-type but not mutant D201A FIH suppressed mRNAs for the HIF targets GLUT1, LDH-A, and VEGF (left panel). To further analyze the oxygen dependence of the suppressive effect of FIH, we equilibrated wild-type FIH transfectant in anoxia attained using an hydrogen/palladium catalyst system prior to induction with doxycycline (0.3 μg/ml) for 16 h. As shown in Fig. 4D, anoxia prevented the suppressive effect of FIH on the expression of GLUT1 mRNA.

Finally, we sought to compare the suppressive effects of FIH overexpression at low oxygen tensions with those of the HIF prolyl hydroxylase PHD2 using a similar strategy, employing a stable doxycycline-dependent Tet-on PHD2 transfectant in the same (U-2OS) cell background. Induction of PHD2 readily reduced HIF target gene expression at 2% oxygen (Fig. 4B, lower panel). In contrast with FIH, effects were barely discernable at 0.5% oxygen and absent in 0.2% oxygen. This contrast with PHD2 was observed even in experiments performed at 0.2% oxygen in which induced expression levels of PHD2 were significantly in excess of those for FIH (Fig. 4C, right panel), demonstrating the existence of different oxygen-dependent characteristics for these enzymes in vivo.

FIH Modulates PHD2 and PHD3 Protein Expression—Since the prolyl hydroxylases phd2 and -3 are HIF target genes (23, 28, 29), we also sought to determine the effects of FIH siRNA on PHD protein expression. Immunoblot analysis with specific antibodies against PHD (1–3) showed hypoxic induction of PHD2 and PHD3 levels in HeLa and U-2OS cells (Fig. 5A). Hypoxia-induced PHD3 levels were strikingly further up-regulated in HeLa and U-2OS cells by FIH siRNA, whereas PHD2 levels were only slightly increased in U-2OS cells and not affected under these conditions in HeLa cells. In contrast, the nonhypoxia-inducible PHD1 protein was not altered by FIH siRNA treatment. Interestingly, in hypoxic cells, these changes were accompanied by a significant decrease in HIF-1α protein levels (Fig. 5, A and B), suggesting that FIH activity might indirectly affect the stability of HIF-1α by modulating PHD2 and -3 protein expression.

Fig. 4. Effect of enhanced FIH expression on HIF target gene expression in normoxia and hypoxia. A, immunoblot analysis of FIH showing response to doxycycline in hypoxia (0.2% oxygen) pre-equilibrated U-2OS transfectants either expressing WT FIH or expressing mutant (D201A) FIH. B, RPA for GLUT1 mRNA in doxycycline-inducible U-2OS stable transfectants expressing WT FIH (upper panel) or PHD2 (lower panel). Cells were equilibrated for 8 h at 21, 2, 0.5, and 0.2% oxygen prior to incubation either in the absence (−) or in the presence (+) of doxycycline (0.2 μg/ml for WT FIH transfectant; 0.05 μg/ml for PHD2 transfectant) for a further 16 h. C, RPA of total RNA from vector alone (VA), WT FIH, mutant (D201A), and PHD2-expressing transfectants equilibrated for 8 h in 0.2% oxygen prior to stimulation with (+) or without (−) Dox (0.2 μg/ml Dox for vector alone, WT FIH, PHD2 transfectants; 0.05 μg/ml Dox for D201A mutant transfectant) for an additional 16 h. Analysis for HIF transcriptional targets GLUT1, LDH-A, and VEGF was performed; FIH and PHD2 is shown in the presence of Dox (0.3 μg/ml) (without −) or with (+) Dox) for 16 h. Analyses for GLUT1 and FIH are shown. Lc (loading control) indicates signal from U6 SnRNA.
three human PHD enzymes contribute to the regulation of HIF (27). The current work focuses on the asparaginyl hydroxylase FIH and shows that this enzyme also contributes importantly to control of the native HIF transcriptional response in both normoxic and hypoxic cells. FIH was found to be widely expressed, consistent with a general role in the regulation of HIF and with previously reported EST sequence analyses of HIF transcripts (12). The predominant cytoplasmic location of the endogenous protein is consistent with previous studies of the location of overexpressed tagged protein (16) and a recent report on localization of the endogenous protein in HEK293T cells (32). In hypoxia, FIH remained cytoplasmic and did not co-localize to the nucleus with HIF-1α, suggesting that modification by asparaginyl hydroxylation most probably occurs in the cytoplasm prior to nuclear entry of HIF-1α.

Experimental maneuvers that altered FIH expression in both directions had clear reciprocal effects on HIF target gene expression. Somewhat surprisingly, these effects were clearly observed both at high and at low oxygen concentrations, even when cells had been pre-equilibrated at a given oxygen concentration so that the effects of manipulation of FIH levels could be clearly assigned to the activity of FIH at that oxygen concentration. In normoxic cells, siRNA-based suppression of FIH induced GLUT1 expression, whereas FIH overexpression reduced GLUT1 expression. This indicates not only that FIH action is important in restricting the normoxic HIF transcriptional response but also that this action is incomplete even in the presence of oxygen, so that the response could be further suppressed by enhancing FIH expression. Equally, in severe hypoxia, we found similar reciprocal effects of changes in FIH expression on HIF target gene expression, clearly indicating that FIH activity persisted at low oxygen availability. These effects were observed even in cells pre-equilibrated at the lowest oxygen tensions in which we could reliably achieve siRNA-based suppression of FIH (~1% oxygen). They were also observed in overexpression studies of FIH using cells pre-equilibrated in an atmosphere of ~0.2% oxygen, although not anoxia.

In contrast, PHD2 overexpression appeared less active in severe hypoxia, and even high amplitude overexpression in cells pre-equilibrated at 0.2% oxygen did not suppress HIF activity, indicating that these enzymes display different oxygen dependence in vivo. In vitro kinetic studies using recombinant FIH have demonstrated an apparent Km for oxygen for FIH in the region of 90 μM (33), which is somewhat lower than the values of 230–250 μM defined for the HIF prolyl hydroxylases (34), although still high in relation to oxygen concentrations measured in normal tissues and cell monolayers in culture. Because enzyme and substrate concentrations are orders of magnitude lower in vivo than in these in vitro assays and pericellular and intracellular oxygen gradients exist that are difficult to assay, these studies are not easy to relate to in vivo assessments of enzyme activity. However, it should be noted that our evidence for FIH activity in vivo under conditions of low oxygen availability is not in conflict with in vitro analyses of HIF hydroxylase activities, which also demonstrate that enzyme activity remains detectable even at low oxygen concentrations (33). Our analysis also suggests that the enzymes behave differently at low oxygen tensions in vivo, with FIH manifesting a distinctly lower oxygen threshold for discernable activity than PHD2.

Because FIH binding to HIF-α C-terminal activation domains could in theory directly compete with p300 without the need for hydroxylation or FIH could have as yet unknown effects on HIF target gene expression, it is possible that some of the observed effects reflect nonenzymatic processes. To address this, we assessed the action of an inactivating mutation at the catalytic site of FIH (D201A) and found that, consistent with previous experiments involving uncontrolled expression following transient transfection (6), the inactive mutant did not suppress HIF activity. This finding, together with the absence of co-localization to the nucleus (Fig. 1, C and D) and the loss of the suppressive effect of FIH under anoxic conditions (Fig. 4D), is consistent with the FIH action being enzymatic rather than directly blocking HIF-α C-terminal activation domain interactions with p300.

Taken together, our results clearly indicate that FIH contributes to the regulation of the endogenous HIF transcriptional response over oxygen concentrations that encompass both the physiological and pathophysiological range. Effects were, however, modest in relation to the full dynamic range of HIF activation in hypoxia, with effects of FIH suppression being substantially less than those of PHD2 suppression in normoxic cells. This suggests that they might contribute to “fine-tuning”

---

3 J. Myllyharju, personal communication
of the response. In addition to regulating the hypoxic response directly, such tuning might also occur by cross-talk between the asparaginylation and prolyl hydroxylation systems since FIH activity can adjust the expression levels of PHD2 and -3 in hypoxia and thus the prolyl hydroxylation activity available for limiting the hypoxic response or promoting HIF degradation upon reoxygenation. Despite this potential for FIH to promote HIF stability by limiting PHD expression levels, our studies revealed an overall inhibitory effect for FIH on the activity of HIF under all circumstances tested. Substantial effects on HIF target genes involved in angiogenesis and glucose metabolism suggest that pharmacological inhibition of FIH could be of potential therapeutic benefit, augmenting the HIF response in ischemic/hypoxic disease.

Acknowledgments—We thank Kirsty S. Hewitson for kindly providing the purified FIH immunogen and D201A/fh/pET28a (+) plasmid; Kevin C. Gatter, Adrian L. Harris, Christopher W. Pugh, and Christopher J. Schofield for advice and discussion; Andrew Jefferson for help with image processing; Muhammad Sohail for help with the design of FIH-specific siRNA duplexes; Norma Masson for critical review of the manuscript; and Silvia Pastorekova for antibody M75 against CA9.

REFERENCES

1. Weng, R. H. (2002) FASEB J. 16, 1151–1162
2. Semenza, G. L. (2003) Nat Rev. Cancer 3, 721–732
3. Epstein, A. C. R., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O’Rourke, J., Mole, D. R., Mukherji, M., Metzen, E., Wilson, M. I., Dhand, A., Tian, Y.-M., Masson, N., Hamilton, D. L., Jaakkola, P., Barstead, R., Hodgkin, J., Maxwell, P. H., Pugh, C. W., Schofield, C. J., and Ratcliffe, P. J. (2001) EMBO J. 20, 5197–5206
4. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9630–9635
5. Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y.-M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M., Ratcliffe, P. J., Pugh, C. W., and Schofield, C. J. (2002) J. Biol. Chem. 277, 26351–26355
6. Lande, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruck, R. K. (2002) Genes Dev. 16, 1466–1471
7. Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S., and Kaelin, W. G., Jr. (2001) Science 292, 464–468
8. Jaakkola, P., Mole, D. R., Tian, Y.-M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A. v., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
9. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9630–9635
10. Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Cancer Res. 61, 4941–4949
11. Freedman, S. J., Sun, Z.-Y. J., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G., and Eck, M. J. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 5367–5372
12. Mahon, P. C., Hirota, K., and Semenza, G. L. (2001) Science 292, 468–472
13. McNeill, L. A., Hewitson, K. S., Claridge, T. D., Seibel, J. F., Horsfall, L. E., and Schofield, C. J. (2002) Biochem. (Tokyo) 367, 571–575
14. Metzen, T., Bencherif-Pfannschmidt, U., Stengel, P., Marxsen, J. H., Stolze, I., Klinger, M., Huang, W. Q., Wotzlaw, C., Hellwig-Burgel, T., Jelkmann, W., Acker, H., and Fandrey, J. (2002) J. Cell Sci. 116, 1311–1326
15. Park, S.-k., Dadak, A. M., Haase, V. H., Fontana, L., Garcia, J. A., and Johnson, R. S. (2003) Mol. Cell. Biol. 23, 4959–4971
16. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Science 268, 1766–1769
17. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9630–9635
18. Wiesener, M. S., Turley, H., Allen, W. E., William, C., Eckardt, K.-U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (1998) Blood 92, 2260–2268
19. Wiesener, M. S., Rice, R., Moore, J. W., Ratcliffe, P. J., and Harris, A. L. (2003) Cancer Res. 63, 6130–6134
20. Sowers, R. H., Raval, R. R., Moore, J. W., Ratcliffe, P. J., and Harris, A. L. (2003) Cancer Res. 63, 6130–6134
21. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschel, T. (2001) Nature 411, 494–498
22. Sower, H. M., Raval, R. R., Moore, J. W., Ratcliffe, P. J., and Harris, A. L. (2003) Cancer Res. 63, 6130–6134
23. Berra, E., Benzi, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. (2003) EMBO J. 22, 4982–4990
24. Maxwell, P. H., Wiesener, M. S., Chang, G.-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999) Nature 399, 271–275
25. Wiesener, M. S., Turley, H., Allen, W. E., William, C., Eckardt, K.-U., Talks, K. L., Wood, S. M., Gatter, K. C., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Maxwell, P. H. (2001) Science 295, 2771–2775
26. Wiesener, M. S., Rice, R., Moore, J. W., Ratcliffe, P. J., and Harris, A. L. (2000) Cancer Res. 60, 7075–7083
27. Pastorekova, S., Zavadova, Z., Kostal, M., Babuskaova, O., and Zavadova, J. (1992) Virology 187, 620–629
28. Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) J. Biol. Chem. 279, 38146–38150
29. Appelhoff, R. J., Tian, Y.-M., Raval, R. R., Turley, H., Harris, A. L., Pugh, C. W., Ratcliffe, P. J., and Gleadle, J. M. (2004) J. Biol. Chem. 279, 38146–38150
30. Marxsen, J. H., Stengel, P., Doege, K., Heikkinen, P., Jokilehto, T., Wagner, T., Jelkmann, W., Jaakkola, P., and Metzen, E. (2004) Biochem. J. 381, 521–527
31. del Peso, L., Castellanos, M. C., Temes, E., Martin-Puig, S., Cuevas, Y., Olmos, G., and Landazuri, M. O. (2003) J. Biol. Chem. 278, 48690–48695
32. von der Hundsburg, K. G. (2003) Proc. Natl. Acad. Sci. U. S. A. 97, 9082–9087
33. Schofield, C. J., and Ratcliffe, P. J. (2004) Nat. Rev. Mol. Cell. Biol. 5, 343–354
34. Linke, S., Stojkoski, C., Kewley, R. J., Booker, G. W., Whitelaw, M. L., and Peet, D. J. (2004) J. Biol. Chem. 279, 14391–14397
35. Koivunen, P., Hirsilä, M., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2003) J. Biol. Chem. 278, 9989–9994
36. Hirsilä, M., Koivunen, P., Gunzler, V., Kivirikko, K. I., and Myllyharju, J. (2003) J. Biol. Chem. 278, 30772–30780