Hypoxia-inducible factor (HIF) is a transcriptional regulator that plays a key role in many aspects of oxygen homeostasis. The heterodimeric HIF complex is regulated by proteolysis of its α-subunits, following oxygen-dependent hydroxylation of specific prolyl residues. Although three HIF prolyl hydroxylases, PHD1, PHD2, and PHD3, have been identified that have the potential to catalyze this reaction, the contribution of each isoform to the physiological regulation of HIF remains uncertain. Here we show, using suppression by small interfering RNA that each of the three PHD isoforms contributes in a non-redundant manner to the regulation of both HIF-1α and HIF-2α subunits and that the contribution of each PHD under particular culture conditions is strongly dependent on the abundance of the enzyme. Thus in different cell types, isoform-specific patterns of PHD induction by hypoxia and estrogen alter both the relative abundance of the PHDs and their relative contribution to the regulation of HIF. In addition, the PHDs manifest specificity for different prolyl hydroxylation sites within each HIF-α subunit, and a degree of selectively between HIF-1α and HIF-2α isoforms, indicating that differential PHD inhibition has the potential to selectively alter the characteristics of HIF activation.

The response of cells to hypoxia is characterized by specific alterations in the expression of a large number of genes, many of which are regulated directly or indirectly by hypoxia-inducible factor (HIF).1 A transcriptional regulator that plays a key role in many aspects of oxygen homeostasis (1–3). HIF binds to hypoxia response elements (HREs) in DNA cis-acting sequences as an α/β heterodimer. Regulation by oxygen is mediated by the α-subunits (HIF-1α, HIF-2α, and HIF-3α), and recently it has been shown that control by oxygen involves novel signal pathways mediated by enzymatic hydroxylation of specific amino acid residues in these proteins. Two distinct processes have so far been identified. Trans-4-Hydroxylation of specific proline residues (Pro-402 and Pro-564 in human HIF-1α ODD (oxygen-dependent degradation domain)) (4–7) promotes interactions with the von Hippel-Lindau ubiquitin ligase complex that targets HIF-α subunits for ubiquitin-mediated proteolysis (8, 9), whereas β-hydroxylation of an asparagine residue (Asn-803 in human HIF-1α) down-regulates transactivation by preventing association of the HIF-α subunit C-terminal activation domain with the co-activator p300/CBP (10). Both prolyl and asparaginyl hydroxylation of HIF-α are catalyzed by enzymes belonging to the Fe(II)/2-oxoglutarate-dependent dioxygenase superfamily (11–14). These enzymes have an absolute requirement for molecular oxygen as co-substrate, and activity is reduced in hypoxia (12, 15–17), allowing non-hydroxylated HIF-α subunits to escape destruction, recruit the p300/CBP co-activator, and form a transcriptionally active complex. Thus the characterization of these HIF hydroxylases provides an important focus for analyses of cellular responses to hypoxia.

To date a single HIF asparaginyl hydroxylase, factor inhibiting HIF (FIH) has been identified (13, 14, 18), whereas the mammalian genome encodes three closely related proteins that have HIF prolyl hydroxylase activity. These proteins, termed prolyl hydroxylase domains PHD1, PHD2, and PHD3 (12), (equivalent to HPH3, HPH2, and HPH1 (11), or EGLN2, EGLN1, and EGLN3, respectively (19)) appear to have arisen by gene duplication and are represented by a single gene in Caenorhabditis elegans and Drosophila melanogaster. Whereas genetic studies in these organisms have demonstrated the critical function of the single C. elegans HIF prolyl hydroxylase, Eg9l, and its Drosophila homolog, Fatig, in the regulation of the stability of HIF-α (11, 12, 20), the existence of three closely related PHD proteins in mammalian cells raises important questions as to whether and in what way each of the three enzymes contributes to the regulation of HIF in higher organisms. Initial analysis has established that all have the ability to hydroxylate HIF-α polypeptides in vitro (11, 12) and that all can suppress HRE-mediated reporter gene activity when over-expressed in cells (21, 22). However, the precise function of each PHD isoform in vivo and indeed whether HIF is the physiological substrate of all three enzymes, are important unanswered questions. Using siRNA techniques, Berra and colleagues (23) have shown a dominant role for PHD2 in con-
trolling the levels of HIF-1α in normoxia in a range of cell types. Little or no effect was observed with siRNAs for PHD1 and PHD3, raising questions as to the function of these enzymes. However, given the absence of precise knowledge of protein abundance, it is unclear whether such results reflect variation in the efficacy of the siRNA interventions at the protein level, dominant expression of PHD2 in the cells analyzed, or a greater specificity of PHD2 action on HIF-1α. Because enzymes of this type are non-equilibrium enzymes, i.e. they do not catalyze the reverse reaction, it would be predicted that enzyme abundance will be an important determinant of the rate of substrate hydroxylation and hence, potentially, an important determinant of any role played in the regulation of HIF. Analysis of mRNA expression for the PHDs has suggested the existence of tissue-specific expression patterns (16, 24–29). However, it is not yet clear to what extent this predicts the abundance of the protein product.

Given the central role of the HIF system in cellular physiology, and in ischemic and neoplastic disease, we undertook a comprehensive analysis of the expression and function of the PHD proteins and have examined the effects of siRNA-mediated suppression of individual isoforms in a wide range of cells and culture conditions. We show that there is substantial variation in the expression of the PHD isoforms in different cells but that all these enzymes contribute to the regulation of HIF, with the effects of siRNA-mediated suppression of individual enzymes being influenced strongly by the level of expressed PHD protein. Differential induction and cell type-specific PHD expression patterns are shown to alter the relative importance of PHD isoforms in setting levels of HIF-α proteins under different conditions. Furthermore, the actions of the PHDs on different HIF-α isoforms were not equivalent, with PHD2 having relatively more influence on HIF-1α than HIF-2α and PHD3 having relatively more influence on HIF-2α than HIF-1α. Our findings thus establish a role for all of the PHD isoforms in the regulation of HIF but show differential effects that have the potential to shape the physiological characteristics of the system.

EXPERIMENTAL PROCEDURES

Cell Culture—The human cell lines studied were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/mL streptomycin except for BT-474, OVCAR-3, 833K, and SuSa, which were cultured in RPMI 1640 and A549 in Ham’s F-12, both with identical supplements. Hypoxic conditions were achieved in an Invivo 2/400 hypoxic chamber (Biotronex).

RNase Protection Assays—Total RNA was extracted in RNXol B (Biogenesis, Bournemouth, UK) and dissolved in hybridization buffer (80% formamide, 40 mM PIPES, 400 mM sodium chloride, and 1 mM EDTA (pH 8.0)). 45 μg of total RNA was assayed with U6 small nuclear RNA serving as an internal control as previously described (30). The PHD riboprobe templates have been described previously (12).-glycosylation and S-transferase fusions in BL21(DE3) cells was high but predominantly insoluble. The proteins were partially purified by solubilization in 2–8 M urea Triton X-100/5 mM dithiothreitol/ PBS/pH 7.5 and dialysis against 1% Triton X-100/PBS. Both fusion proteins precipitated after dialysis and were suspended in Triton/PBS for mouse immunization.

To create an anti-PHD1 polyclonal antibody, two rabbits were immunized with 200 μg of protein in Complete Freund’s adjuvant (Difco, Detroit, MI), followed by four boosts at 4-week intervals with 100 μg of protein in Incomplete Freund’s adjuvant. Test bleeds were examined by immunoblot analysis of COS7 cell extract transfected with pcDNA3 PHD1. The PHD1 antisera was affinity-purified by H. H. Sera-Lab (Loughborough, UK) to generate polyclonal antibody P1-1.1a.

Balb/c mice were immunized with 50 μg of protein of each PHD immunogen in Complete Freund’s adjuvant followed by three further immunizations of 50 μg of protein in PBS at 10-day intervals. Fusion of mouse splenocytes with myeloma cells was performed using standard techniques. Hybridoma supernatants were screened by enzyme-linked immunosorbent assays against the fusion proteins and fusion tags alone and immunolabeling of COS7 cells, which were transfected with corresponding PHD plasmids. Positive clones were confirmed by immunoblot analysis of COS7 cell extracts of PHD transfectants and are designated mAb P1-112, mAb P2-76a, and mAb P3-188.

To create an anti-PHD2 monoclonal antibody against HIF-1α (clone-54, H72320, BD Transduction Laboratories), HIF-2α (clone-190b (31), carbonic anhydrase-IX (M75 (32)), and BNIP3 (Ana48; Sigma). Horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibodies (DAKO) were used with the ECL Plus system (Amersham Biosciences) to visualize immunoreactive bands.

siRNA Preparation—To screen for effective siRNAs, candidate siRNA sequences for each PHD enzyme were selected based upon criteria in Refs. 33 and 34 and prepared in vitro transcription using the Silencer™ siRNA construction kit (Ambion). Effective siRNA sequences were subsequently chemically synthesized with two deoxythymidine nucleotide 3’ overhang and PAGE-purified (Ambion). siRNA strands were annealed in a buffer consisting of 30 mM HEPES (pH 7.4), 100 mM potassium acetate, and 2 mM magnesium acetate by heating to 90 °C for 1 min, followed by incubation for 1 h at 37 °C. The two siRNAs targeting PHD1 siRNA (GenBank accession NM_022073) were designed to correspond to nucleotides 835–855 and 1471–1489; those to PHD2 (GenBank™ accession number NM_022073) correspond to nucleotides 3921–4077 and 4077–4192; those to PHD3 (GenBank™ accession number NM_022073) correspond to nucleotides 901–929 and 4077–4192; and those to PHD3 (GenBank™ accession number NM_022073) correspond to nucleotides 901–929 and 4077–4192. Control siRNAs were the Silencer™ negative control siRNA (Ambion), a sequence provided by the manufacturers that has no significant homology to any mammalian gene, and a previously described siRNA (23) that targets nucleotides 2558–2578 of the D. melanogaster HIF-1α homolog, Sima (GenBank™ accession number U43090).

siRNA Transfections—Cells were seeded at 30% confluence in antibiotic-free medium 24 h prior to transfection. Oligofectamine reagent (Invitrogen) was used to transfect cells with 20 nM siRNA duplex twice according to the manufacturer’s protocol. siRNA transfection in each experiment was ascertained by immunoblotting.

Transient Transfections and Reporter Gene Assays—MCPS cells were seeded in 12-well dishes at 1.5 × 10^5 cells per dish and incubated overnight. The cells were then co-transfected using FuGene6 transfection reagent (Roche Applied Science) with plasmids expressing a Gal4-tetracycline responsive luciferase reporter (pCMV/VP16) (35), and pcDNA3 PHD1, PHD2, or PHD3 (12). The HIF-α domains encoded by the Gal4/HIF/V- 16 plasmids were as follows: pGal/HIF-1αNODD/VP16 (amino acids 344–553), pGal/HIF-1αCDD/VP16 (amino acids 554–698), pGal/HIF-2αNODD/VP16 (amino acids 517–617), and pGal/HIF-2αCDD/VP16 (amino acids 517–617). CAT promoter–tetracycline repressor–lacZ reporter construct (pCMV/CAT) was used to measure transfection efficiency. After 24 h, 1 μg of each plasmid was transfected into cells seeded in 12-well dishes at 1.5 × 10^5 cells per dish and incubated overnight. Cell lysates were then prepared and analyzed by luciferase assay (36).

The dose of transfected plasmid encoding the PHD enzymes was ad-

Downloaded from http://www.jbc.org/ by guest on February 9, 2020
JUSTIFIED IN THE RANGE 2–10 ng IN PRELIMINARY EXPERIMENTS SO AS TO ACHIEVE APPROXIMATELY EQUIMOLAR CONCENTRATIONS OF THE THREE ENZYMES IN THE CELL LYSATES (SEE FIG. 8C). TRANSFECTIONS WERE PERFORMED IN TRIPlicate. AFTER 24 h, CELL EXTRACTS WERE PREPARED IN PASSIVE LYSATE BUFFER (Promega) AND ANALYZED FOR β-GALACTOSIDASE AND LUCIFERASE ACTIVITIES AS PREVIOUSLY DESCRIBED (35). THE SAME CELL EXTRACTS WERE USED TO EXAMINE EXPRESSION LEVELS OF THE PHD PROTEINS BY IMMUNOBLOTTING.

RESULTS

PHD Isoforms Manifest Distinct Patterns of Cellular Expression—To guide the functional analysis of PHD proteins in the regulation of HIF, we first undertook a detailed study of PHD expression in a range of cell lines. In the first instance, expression was defined at the mRNA level using RNase protection assays. Following reports of PHD mRNA regulation by hypoxia in certain cell lines (12, 22, 27, 36), all cells were studied in normoxic culture and after exposure to hypoxia (0.5% oxygen) for 16 h. Fig. 1 shows results for cells derived from a variety of human tissues: BxPC-3 (pancreatic carcinoma); PC-3 (prostate carcinoma); MCF7, HS-587T, MDA-435, T47D, and ZR-75–1 (breast carcinoma); U-2 OS (osteosarcoma); OVCAR-3 (ovarian carcinoma); A549 (lung carcinoma); HT1080 (rhabdomyosarcoma); and JAR (choriocarcinoma). Major variations in expression were observed both across the panel of cells and even within those derived from a single (breast carcinoma) tumor type (Fig. 1). In normoxic cells both PHD1 mRNA and PHD2 mRNA were widely expressed but at varying levels; expression of PHD3 mRNA was also variable and in normoxia was often at, or below, the detection threshold for these assays. Individual PHD isoforms, however, showed generally consistent patterns of regulation by hypoxia. Whereas levels of PHD1 mRNA were unchanged or significantly decreased by hypoxia (ZR75–1, OVCAR-3, and JAR), levels of PHD2 and PHD3 were increased by hypoxia, induction being particularly striking for PHD3 mRNA.

To define expression at the protein level we next produced a panel of specific antibodies. The antibodies detected the PHD proteins generated by IVTT or in cell extracts at a mobility consistent with their predicted molecular masses (PHD1, 43.6 kDa; PHD2, 46.0 kDa; and PHD3, 27.3 kDa) (Fig. 2A). Equimolar amounts of proteins radiolabeled with [35S]methionine were generated by IVTT in reticulocyte lysate, and saturating concentrations of antibody were used to estimate the sensitivity and specificity of detection (Fig. 2A). This indicated that detection of PHD1 with polyclonal antibody P1-1.1a and PHD3 with mAb P3-188a had the highest (and essentially equivalent) sensitivity, whereas PHD2 was clearly detected with mAb P2-76a, but at lower sensitivity. No cross-reactivity was observed. Having established the relative sensitivity of the antibodies to equimolar amounts of IVTT-generated PHD proteins we used similarly generated proteins to calibrate immunoblots and estimate the relative abundance of PHD proteins in cell extracts (Fig. 2C).

Fig. 2B shows representative immunoblots for PHD proteins in a range of cells. As was observed for the respective mRNAs, both PHD2 and PHD3 proteins were induced by hypoxia, with PHD3 induction being particularly striking in certain cells. Although these patterns of protein expression generally paralleled patterns of mRNA expression there were also important differences. In particular relative PHD1 protein levels were substantially lower than predicted by relative PHD1 mRNA levels and were barely detectable in many cell lines. As other
work had highlighted the expression of PHD1 mRNA in breast carcinoma BT-474 cells (37), and in the testis (29), we also examined BT-474 cells, and two other cell lines (833K and SuSa) of testicular origin (Fig. 2, B and C). PHD1 protein levels were again modest, but in the BT-474 and SuSa cell lines expression of PHD1 was somewhat higher than in the other lines examined.

Overall, therefore, both PHD1 and PHD3 levels were relatively low across a wide range of normoxic cells so that PHD2 was clearly the most abundant enzyme in normoxic culture in all cells where detection thresholds permitted quantification. Even cells (e.g. A549 and H1T1080) expressing relatively low levels of PHD2 mRNA showed substantial levels of PHD2 protein (data not shown). In hypoxia, the more striking induction of PHD3 than PHD2 altered these proportions substantially in some cells (e.g. BT-474 and MCF7) but not others (MDA-435, in which PHD3 remained undetectable). As with PHD1 mRNA, PHD1 protein was not induced by hypoxia. Interestingly, however, PHD1 was observed as a doublet in cell extracts, and the faster migrating species was reduced by hypoxic exposure of cells. Both species were observed with different antibodies and were down-regulated by siRNAs directed against PHD1 transcripts (see below) indicating that they represent specific species derived from PHD1 mRNA.

**PHD Suppression by siRNA**—To analyze the role of the different PHD isoforms in the regulation of the HIF system we sought to reduce specifically the expression of individual PHDs (both in isolation and in combination) using siRNA. Following preliminary experiments to optimize dose and transfection protocol (Fig. 3A), experiments were performed using an oligonucleotide concentration of 20 nM and two separate transfections 24 h apart, unless otherwise stated. Fig. 3B illustrates the extent and specificity of suppression; individual PHD protein levels were strikingly and specifically reduced by their targeting oligonucleotides, but not unrelated control oligonucleotides, or oligonucleotides directed against other PHD isoforms.

To test the effects of specific suppression of PHD proteins on HIF, we first applied these siRNAs to three cell lines (U-2 OS, MCF7, and Hep3B) that expressed differing amounts of each PHD protein in normoxic culture. Effects on HIF-1α protein levels and on expression of the HIF-1α target genes, carbonic anhydrase IX (CA-IX) (38), and BNIP3, an apoptotic regulator (39), were assayed by immunoblotting (Fig. 3C). Both HIF-1α protein and target gene expression were induced by PHD2 suppression in all three cell lines, the effect being specific and similar in magnitude with two different siRNAs directed against PHD2. Despite more than 70% suppression of PHD1 and PHD3, no effect on HIF-1α or target gene expression was observed (Fig. 3C). These results therefore confirm the importance of PHD2 in setting normoxic levels of HIF-1α (23). However, because, even in these cells, PHD2 was by far the most abundantly expressed enzyme under standard normoxic culture conditions, this leaves open the questions as to whether and in what way PHD1 and PHD3 contribute to the regulation of HIF.

**PHD Isoforms 1–3 Contribute to the Regulation of HIF-1α under Different Conditions**—To define the potential contribution of PHD1 and PHD3 to HIF regulation, we next sought to test the effects of inactivation under conditions where these proteins contribute to a greater proportion of total PHD protein expression. We therefore studied BT-474 cells in which PHD1 protein expression was relatively high under basal conditions (see Fig. 2B), and in which induction of PHD1 mRNA by estrogen stimulation had been reported (37). Following estrogen stimulation PHD1, but not PHD2 or PHD3, protein level was further induced ~2.5-fold (Fig. 4A). To assess the role of different PHD isoforms in the regulation of HIF under these conditions we undertook siRNA transfections using each PHD siRNA alone or in combination with one or both of the other PHD siRNAs.

Under basal conditions siRNA for PHD2 had a smaller, but still discernable effect on induction of HIF-1α, and PHD1 siRNA was found to have a clearly discernable effect that was similar to that of PHD2 siRNA (Fig. 4B, upper panels). These effects were more striking on estrogen-stimulated cells, which appeared to express somewhat higher levels of HIF-1α even without PHD inhibition (Fig. 4B, lower panels). In keeping with the lower contribution of PHD3 to total PHD expression, PHD3 siRNA had little effect in isolation. However, comparison of combined siRNA directed against PHD1, PHD2, and PHD3 versus PHD1 and 2 suggested that, when PHD1 and 2 levels were lowered to a sufficient extent, PHD3 also made a non-redundant contribution to the regulation of HIF-1α (Fig. 4B).

To test this possibility further we sought to utilize the greater induction of PHD3 than PHD2 by hypoxia to analyze the effects of PHD3 suppression under conditions in which PHD3 contributed more to total PHD protein levels. MCF7 cells were therefore transfected with siRNAs directed against PHD1, PHD2, or PHD3 and then exposed to hypoxia (0.5% oxygen) for 4 h and then re-oxygenated by exposure to 21%
bodies against PHD1, PHD2, and PHD3.

estradiol for 48 h. Whole cell extracts were immunoblotted with antibodies against PHD1, PHD2, and PHD3. B, effect of PHD siRNA on HIF-1α protein level. BT-474 cells were transfected three times on consecutive days with the indicated combinations of PHD-directed siRNAs. Individual siRNAs were used at concentration of 15 nM, whether applied individually or in combination. Cells were incubated for 48 h in the absence (−) or presence (+) of 20 ng/ml estradiol before harvest. Whole cell extracts were immunoblotted with antibodies against HIF-1α and PHD1.

oxygen. Extracts were prepared from normoxic cells, cells exposed to 4-h hypoxia, and cells after 5-, 10-, 20-, and 40-min re-oxygenation. To permit accurate comparison, each test for siRNA intervention was undertaken in parallel with a mock transfection, and samples were analyzed in parallel on the same immunoblot. Under these conditions, siRNA-mediated suppression of either PHD3 or PHD2 consistently reduced the rate of decline of HIF-1α following re-oxygenation, although in each case HIF-1α levels ultimately approached baseline (Fig. 5A). Similar effects were observed following 18 h of hypoxic exposure and 20 min re-oxygenation where semi-quantitative assessment of HIF-1α immunoreactivity revealed that levels were 5.4-fold greater following siRNA targeting of PHD3 than mock transfected cells (n = 5) compared with 3.5-fold greater than mock transfected cells for PHD2 siRNA. Similar results were also obtained with BxPC3 cells. To confirm the specificity of these effects, a comparison was made with MDA-435 cells, which do not express PHD3. In keeping with this, no effect of siRNA directed against PHD3 was observed (compare panels A and B, Fig. 5). Finally, we tested the effects of combined PHD2 and PHD3 suppression by siRNA in MCF7 cells, which revealed much more striking prolongation of HIF-1α immunoreactivity following re-oxygenation than was observed with suppression of either alone (see Fig. 5C). Taken together these findings indicate that all three PHD proteins play a significant role in the regulation of HIF-1α that is dependent to a substantial extent on their relative abundance under the conditions of analysis.

**Differential Effects of PHDs on HIF-1α and HIF-2α**—To further understand the role of the PHD enzymes in the regulation of HIF, we compared effects of siRNAs targeting PHD proteins on the two best characterized HIF-α isoforms, HIF-1α and HIF-2α. Somewhat surprisingly, given their highly conserved sites of prolyl hydroxylation, effects of PHD protein suppression by siRNA on the two HIF-α isoforms were not equivalent.

In normoxic MCF7 cells, the main differences were that, compared with effects on HIF-1α, suppression of PHD2 alone had a smaller action on the induction of HIF-2α levels (Fig. 6, A and B) and that suppression of PHD1 and PHD3 either singly or in different combinations with other PHDs had greater effects on HIF-2α than on HIF-1α (Fig. 6A), suggesting that despite relatively low levels under these conditions they contributed somewhat more to the regulation of HIF-2α than HIF-1α.

Because PHD3 is more strikingly induced by hypoxia than PHD2 and levels of the two isoforms are more comparable in hypoxic cells, we next sought to compare effects of PHD protein suppression on HIF-1α versus HIF-2α under hypoxic conditions. In MCF7 cells siRNA-mediated suppression of PHD3 alone was found to have substantial effects both on the level of HIF-2α induced by hypoxia (Fig. 6B) and on its decay following re-oxygenation (Fig. 6C). These effects were clearly greater than any effects of isolated suppression of PHD3 on HIF-1α (Fig. 6B, and compare Figs. 6C with 5A).

These results suggest that PHD3 retains significant activity under hypoxic conditions and that the enzyme is important in limiting physiological activation of HIF (particularly HIF-2α) in hypoxia. To explore this further additional experiments were performed in which cells were equilibrated in hypoxia (1.5% oxygen) prior to siRNA transfection (Fig. 7A). HIF-α levels were found to be induced maximally early after hypoxic exposure and then to decline significantly; the effect was more marked for HIF-2α than for HIF-1α. Suppression of PHD3 by siRNA had a marked effect in preventing this decline in HIF-2α that contrasted with minimal effects on HIF-1α, where neither PHD3-directed siRNA nor other individual siRNAs were able to augment induction by hypoxia (Fig. 7A). To test whether
combined PHD activity was in any way limiting HIF-1α induction under these conditions we performed further experiments using PHD siRNAs in combination. These experiments demonstrated a modest increment in the induction of HIF-1α in hypoxic cells with combinations of siRNAs targeting both PHD2 and PHD3 (Fig. 7B), again indicating that these enzymes retain activity in hypoxia that limits the activation of HIF. Finally, we wished to compare results in cells expressing different levels of PHD2 and PHD3. We therefore compared BxPC3 cells, which (like MCF7 cells) have a relatively high ratio of PHD3/PHD2 in hypoxia with Hep3B cells in which the ratio PHD3/PHD2 remains low. The bias toward PHD3 exerting a greater effect on HIF-2α than HIF-1α was again observed. However, in keeping with differences in PHD expression pattern, suppression of PHD3 had more substantial effects on HIF-2α in BxPC3 cells than in Hep3B cells.

Action of PHD Proteins on Isolated HIF-α Degradation Domains—Both HIF-1α and HIF-2α contain two sites of prolyl hydroxylation (the N-terminal oxygen-dependent degradation domain (NODD) and C-terminal oxygen-dependent degradation domain (CODD)), each of which can operate independently to mediate von Hippel Lindau-dependent proteolysis (6). In vitro studies have suggested that PHDs have different specificity for the sites of hydroxylation within HIF-α polypeptides (12, 16). To explore whether this might contribute to the differential action of the PHDs on HIF-1α and HIF-2α observed in vivo, we performed transfection studies to test the action of PHDs on isolated NODD and CODD sequences in vitro. MCF7 cells were transfected with plasmids expressing the relevant NODD or CODD from HIF-1α or HIF-2α as part of a Gal4/VP16 fusion protein. Clear differences were observed (Fig. 8, A and B). In particular PHD3 was found to be the most effective enzyme at suppressing CODD-bearing fusions, and the least effective (essentially inactive) in suppressing NODD-bearing fusions from either source. These results are therefore consistent with in vitro studies indicating that PHD3 hydroxylates CODD but not NODD sequences (12, 16). Comparison of HIF-1α CODD- versus HIF-2α CODD-bearing fusions revealed similar relative activities of the PHD proteins indicating that action at this site was unlikely to explain any differential effects on HIF-2α than HIF-1α in the above studies. In contrast substantial differences were observed between fusions bearing NODD sequences from HIF-1α but not HIF-2α.

**Fig. 6. Comparative effects of PHD suppression on the regulation of HIF-1α and HIF-2α.** Immunoblots showing HIF-1α and HIF-2α levels in MCF7 cells after transfection with the indicated PHD siRNAs. A, effects on HIF-1α and HIF-2α levels in normoxic cells. B, effects in hypoxic cells (16 h, 0.5% oxygen). C, effects during re-oxygenation. After transfection cells were incubated in normoxia (N) or 0.5% oxygen (H) for 18 h then harvested, or re-exposed to 21% oxygen for the indicated time periods (minutes) prior to harvest. To allow precise determination of the effects of siRNA intervention, each test for siRNA transfection was undertaken in parallel with a mock transfection (left panels), and samples were analyzed in parallel. siRNA transfections were performed twice on consecutive days using 20 nM of each siRNA, again indicating that these enzymes retain activity in hypoxia that limits the activation of HIF.

**Fig. 7. Effects of PHD siRNA on HIF-1α and HIF-2α levels in sustained hypoxia.** Immunoblots of whole cell extracts from MCF7 cells (A and B) or BxPC3 and Hep3B cells (C). For experiments in hypoxia, cultures were equilibrated in 1.5% oxygen for 4 h before undergoing two transfections with siRNA on consecutive days within the hypoxia workstation. The oxygen atmosphere was maintained, and cells were harvested after the indicated total duration (hours) of hypoxic exposure. Each siRNA was used at 20 nM whether applied individually or in combination. Anti-PHD immunoblots are shown (B, lower panels) to illustrate efficacy and specificity of siRNAs under these conditions.

HIF Regulation by Prolyl Hydroxylases

38463
substantially more effective on the HIF-1α NODD than the HIF-2α NODD, and second, the HIF-1α NODD was much more effective in reducing the activity of the Gal4 fusion protein. Overall, these results therefore confirm the existence of PHD substrate specificity as suggested by in vitro analyses and suggest that differences between the HIF-1α/H9251 NODD rather than CODD sequences may underlie differential effects on HIF-1α versus HIF-2α.

**DISCUSSION**

In vitro and forced expression studies have indicated that each of three closely related prolyl hydroxylases (PHD1–3) have the potential to hydroxylate HIF-α subunits (11, 12, 21, 22). However, genetic studies to date have defined a key non-redundant role for a single isoform (PHD2) in the regulation of HIF-1α (23), leaving open questions as to whether and to what extent the other isoforms contribute to control of the HIF system. In keeping with studies that have shown substantial tissue-specific variation in mRNA expression of the PHD enzymes (16, 24–29) we observed significant differences in protein abundance in cell types from different tissues. Using an siRNA approach guided by assessment of PHD protein abundance, we have shown that all PHD proteins contribute in a non-redundant manner to regulation of the HIF system. The effect of suppression of each individual PHD by siRNA was well correlated with the cellular abundance of the relevant enzyme. This result is in keeping with HIF hydroxylation being a non-equilibrium reaction in which the rate of hydroxylation may be anticipated to be directly related to the abundance of active enzyme. Notably, significant effects on the HIF system were observed even when the impact of suppression of a single PHD on the total level of PHD protein was only modest, in keeping with hydroxylation being a regulatory (as opposed to permissive) step in HIF-α proteolysis.

Our results confirm and explain the previously reported dominant effect of PHD2 suppression in raising levels of HIF-1α in normoxic cells (23), because in most cells PHD2 is substantially the most abundant HIF prolyl hydroxylase under these conditions. Importantly, however, they also demonstrate that both PHD1 and PHD3 contribute to the regulation of the system and that, at least for PHD3, the contribution may be as great or greater than that of PHD2 under appropriate conditions. Thus PHD3 was very strikingly induced by hypoxia in certain cells, and under these conditions suppression of PHD3 by siRNA had major effects on the HIF system, substantially...
prolonging the half-life of HIF-α subunits when hypoxic cells were re-oxygenated, and maintaining induction under continuous hypoxia.

Although overall we found that all three PHD proteins contributed to the regulation of both HIF-1α and HIF-2α, a significant bias was observed so that PHD3 appeared to contribute more substantially to the regulation of HIF-2α than HIF-1α. Depending on the relative levels of PHD2 and PHD3 expression, we observed either that the induction of HIF-2α was affected less than HIF-1α by suppression of PHD2 alone, or that PHD3 suppression alone led to induction of HIF-2α but not HIF-1α. Because the effects of suppression by siRNA are so dependent on relative expression levels of the PHD proteins under a given set of conditions, we performed further analyses of the effects of modest overexpression of comparable amounts of each PHD enzyme on the activity of Gal4/HIF-ODD/VP16 fusions. These experiments showed that the ability of each enzyme to suppress the activity of fusions containing the CODD prolyl hydroxylation site was closely similar between HIF-1α and HIF-2α and in the order PHD3 > PHD2 > PHD1. Results for the NODD prolyl hydroxylation site were quite different; PHD3 was inactive, thus confirming the results of previous in vitro analyses (12, 16). Two further effects were observed in these experiments that might contribute to the bias of PHD2 to HIF-1α regulation and PHD3 to HIF-2α regulation. First, the HIF-2α NODD appeared relatively ineffective in suppressing chimeric gene activity, so that this gene might be more dependent on the PHD3-CODD interaction. Second, PHD2 appeared to be more effective on the HIF-1α NODD versus the HIF-2α NODD.

Whatever the explanation, it is interesting that relative selectivity has also recently been reported for the regulation of PHD isoforms by HIF-α isoforms, in that PHD2 was found to be specifically induced by HIF-1α, whereas PHD3 was responsive to HIF-2α as well as HIF-1α (40). The possibility that induction of PHD expression by HIF may serve to limit the response to hypoxia implies that the enzymes themselves retain at least some activity at low oxygen tensions. The marked effect of PHD3 siRNA on HIF-2α levels in cells equilibrated in hypoxia prior to siRNA intervention clearly supports this and is also compatible with in vitro assays showing significant activity at low oxygen tensions despite a high apparent \( K_m \) for oxygen for these enzymes. Interestingly, in vitro assays have demonstrated similar oxygen dependence for all three PHD enzymes with high values for the apparent \( K_m \) for oxygen, in the region of 230–250 μM, being reported (12, 16). Taken together the results predict that the rate of HIF hydroxylation is likely to be oxygen-dependent over a wide range of oxygen concentrations spanning pathological and physiological hypoxia.

Given that all PHD isoforms contribute to the regulation of HIF, their differing cell specific and inducible behavior presumably allow flexibility in the regulation of the HIF response to hypoxia. The findings also predict that relatively specific pharmacological inhibition of a particular PHD enzyme could have the potential for selective modulation of the HIF response that would be useful in therapeutic application. For instance, PHD2 inhibition might be predicted to activate the HIF response broadly across a range of cell types under resting conditions. In contrast specific inhibition of PHD3 might be predicted to selectively augment the response to hypoxia in certain tissues that express high levels of the enzyme. Whether this will be useful in activating the HIF system as a therapeutic approach to ischemic/hypoxic diseases remains unclear, but genetic studies in whole organisms should now be of value in addressing these questions.

Acknowledgments—Thanks to all members of the Ratcliffie Lab and Christopher Schofield for helpful discussions, to Kevin Gatter for the production of antibodies to Kirsty Hewitson for providing the PHD1 protein, to Mahbubeh Sohail for help with the design of siRNA sequences, and to Silvia Pastorekova for antibody M75 against CA-IX.

REFERENCES

1. Brucc, R. K. (2003) Genes Dev. 17, 2614–2623
2. Semenza, G. L. (2003) Nat. Rev. Cancer 3, 721–732
3. Huang, L. E., and Bunn, H. F. (2003) J. Biol. Chem. 278, 18575–19578
4. Jurek, A., Pole, M. R., Thomas, Y. M., Wilson, M. I., Geibel, J., Gadzell, S. J., Kriegshein, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) Science 292, 468–472
5. Ivan, M., Kondo, K., Yang, Y., Kim, W., Valando, J., Ohh, M., Sale, A., Asara, J. M., Lane, W. S., and Kaelin, W. G. (2001) Science 292, 464–468
6. Masson, N., William, C., Maxwell, P. H., Pugh, C. W., and Ratcliffe, P. J. (2001) J. Cell Biol. 153, 259–266
7. Yu, F., White, S. B., Zhao, Q., and Lee, F. S. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9630–9635
8. 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
9. Kaelin, W. G. (2002) Nat. Rev. Cancer 2, 673–682
10. Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) Science 295, 858–861
11. Brucc, R. K., and McNight, S. L. (2001) Science 294, 1337–1340
12. Kaelin, W. G., Gorman, J. J., and Christopher Schofield for helpful discussions, to Kevin Gatter for

HIF Regulation by Prolyl Hydroxylases

3846
Differential Function of the Prolyl Hydroxylases PHD1, PHD2, and PHD3 in the Regulation of Hypoxia-inducible Factor
Rebecca J. Appelhoff, Ya-Min Tian, Raju R. Raval, Helen Turley, Adrian L. Harris, Christopher W. Pugh, Peter J. Ratcliffe and Jonathan M. Gleadle

J. Biol. Chem. 2004, 279:38458-38465.
doi: 10.1074/jbc.M406026200 originally published online July 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M406026200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 20 of which can be accessed free at http://www.jbc.org/content/279/37/38458.full.html#ref-list-1