Cell-specific Regulation of Hypoxia-inducible Factor (HIF)-1α and HIF-2α Stabilization and Transactivation in a Graded Oxygen Environment*

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The hypoxia-inducible factor (HIF)-1α and HIF-2α are closely related, key transcriptional regulators of the hypoxic response, countering a low oxygen situation with the up-regulation of target genes associated with numerous processes, including vascularization and glycolysis. This involves a dual mechanism of control through both stabilization and transactivation, regulated via prolyl and asparaginyl hydroxylation. Despite high similarity with respect to protein sequence and activation pathway, a growing number of physiological and mechanistic differences between HIF-1α and HIF-2α are being reported. To further characterize this nonredundancy, the stabilization of endogenous proteins and regulation of the transactivation domains were compared in a graded oxygen environment across a series of cell lines. Although generally similar results were found, interesting and specific differences between the HIF-α proteins were observed within certain cell lines, such as rat adrenal PC12s, emphasizing the cell-specific nature of HIF-α regulation. We characterize a conserved amino acid substitution between HIF-1α and HIF-2α that contributes to the intrinsically higher FIH-1-mediated asparaginyl hydroxylation of HIF-1α and, hence, lower HIF-1α activity. In addition, our data demonstrate that the different cell lines can be classified into two distinct groups: those in which stabilization and transactivation proceed in conjunction (HeLa, 293T, and COS-1) and those cells in which HIF-α is stabilized prior to transactivation (PC12, HepG2, and CACO2). Interestingly, the initial stabilization of HIF-α prior to transactivation up-regulation predicted from in vitro derived hydroxylation data is only true for a subset of cells.

In a state of hypoxia, where the demand for oxygen exceeds supply, physiological responses are mounted, including increasing the capacity for blood to carry oxygen to tissues and facilitating the production of ATP by anaerobic glycolysis. The hypoxia-inducible factors (HIFs) are key transcriptional regulators of this genomic response to hypoxia in essentially all mammalian cells. HIF is composed of an oxygen-regulated HIF-α subunit (HIF-1α or HIF-2α) and the ubiquitous aryl hydrocarbon receptor nuclear translocator (or HIF-1β) partner protein. HIF-α protein turnover in normoxia is very rapid due to the inhibitory action of the HIF-α prolyl hydroxylases (PHDs). These oxygen-dependent enzymes hydroxylate two conserved prolyl residues within a central oxygen-dependent degradation domain of the HIF-α proteins, promoting binding of the Von Hippel-Lindau protein, ubiquitylation, and proteasomal degradation (1–3). Any HIF-α escaping this normoxic degradation is also subject to hydroxylation of a conserved asparagine within the C-terminal transactivation domain (CAD) that represses activity via abrogation of CBP/p300 recruitment (4, 5). Under hypoxic conditions, the activity of the prolyl and asparaginyl hydroxylases is inhibited, and the HIF-α proteins are stabilized and transcriptionally activated, leading to potent induction of target genes.

Despite high conservation in overall amino acid sequence, domain structure, and hypoxia-dependent mechanisms of activation, HIF-1α and HIF-2α have distinct physiological roles. This is demonstrated by deficient mouse phenotypes and immunohistochemical analyses of whole rodents, where hypoxia induces either HIF-1α or HIF-2α stabilization in distinct cell populations, even within the same tissue (6–8). Multiple examples of tissue-specific differences in regulation have also been reported. For example, HIF-1α is induced by more moderate hypoxia in the brain for a longer period of time, compared with the liver or kidney, which require more severe hypoxia to stabilize HIF-1α in a more transient manner (6). Similarly, HIF-2α is strongly induced by hypoxia in the liver, but only weakly in the brain and kidney, although in each organ HIF-2α protein was maintained over a longer time course than HIF-1α (7). These observations demonstrate physiological specificity between HIF-1α and HIF-2α, as do a growing number of differences noted with respect to nonhypoxic stimuli, target genes, and interaction partners, which may underlie differences in deficient mouse phenotypes and tumorigenicity. This has been recently demonstrated in renal clear cell carcinoma cells, in

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4 The abbreviations used are: HIF, hypoxia-inducible factor; hHIF, human HIF; PHD, HIF prolyl hydroxylase; CAD, C-terminal transactivation domain; DBD, DNA binding domain; DP, dipyridyl; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein.
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### EXPERIMENTAL PROCEDURES

**Plasmid Construction**—HRE-luciferase (22), GalRE-luciferase (23), and control Renilla luciferase (Promega) reporter plasmids have been described previously, as have hHIF-1α 727–826, mouse HIF-2α 774–874 (4), MBP-FIH, GalDBD-hHIF-1α 737–826, and pET32-hHIF-1α 737–826 (16). hHIF-2α 781–870 was amplified by PCR and cloned BamHI/XbaI into similarly digested pGal-O and pET-32a expression vectors to generate GalDBD-hHIF-2α 781–870 and pET-32a-hHIF-2α 781–870. hHIF-1α P564A and hHIF-2α P531A-pEFBOS vectors were described previously (4) and were used to also generate HIF-1α P402A/HIF-2α P431A and HIF-1α V804A/HIF-2α A848V mutants by site-directed mutagenesis PCR.

**Cell Culture and Reporter Assays**—HEK293T, HeLa, COS-1, and HepG2 cells were routinely grown at 37 °C, 5% CO₂ in Dulbecco’s modified Eagle’s medium (Invitrogen) with 10% fetal calf serum. PC12 cells were grown as above in RPMI (Invitrogen) supplemented with 10% fetal calf serum.

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luciferase system (Promega) according to the manufacturer’s instructions, using RLTK as a transfection control.

**Hypoxic Incubation**—Hypoxic environments were created via pumping air at specific O₂ concentrations with 5% CO₂ into valved air-tight containers and incubated as described. O.5% O₂ was created using anaerobic sachets (Oxoid), and O₂ levels were verified by an oxygen sensor (Teledyne AX300I).

**Western Blotting**—Preparation of whole cell extracts was performed as previously described (24), with the exception of in Fig. 1, where to avoid normoxic degradation after hypoxia, samples were prepared by rapid suspension of the adherent cells in SDS sample buffer and then immediately boiled prior to SDS-PAGE and Western blotting. Western blotting was performed using commercial HIF-1α, HIF-2α (Novus), GalDBD (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and tubulin (Abcam) antibodies, and our previously described polyclonal FIH-1 (16).

**Hydroxylation Assays**—In vitro asparaginyl hydroxylation assays were performed using purified maltose-binding protein-tagged FIH-1 and thioredoxin-His6-HIFα substrate at 10, 40, and 80 μM as previously described (16).

**RESULTS**

In order to directly compare the activities of HIF-1α and HIF-2α and to test the relative thresholds of HIF-α stabilization and transactivation, a varied panel of six cell lines was chosen, with four of human origin (293T, HeLa, HepG2, and CACO-2) in addition to cells derived from monkeys (COS-1) and rats (PC12), to facilitate the analysis of cell-specific HIF-α regulatory effects. Levels of endogenous HIF-1α and HIF-2α protein were analyzed by Western blotting, and transcriptional activity of the CAD was analyzed in the same cells under similar conditions using CADs fused to the heterologous Gal4 DNA-binding domain (Gal-DBD) in a reporter gene assay. Cells were transfected for 6 h and then incubated for 14 h in an atmosphere containing 20% (normoxia), 10%, 5%, 2%, or less than 1% oxygen. In addition, cells at normoxia were also treated for 14 h with a 1 mM concentration of the hypoxia mimetic dipyridyl (DP).

Endogenous HIF-1α protein was induced by hypoxia in all cell lines analyzed, with maximal protein induction at <1% O₂. Hypoxic induction of endogenous HIF-1α has been previously demonstrated in response to 4–24 h of exposure to hypoxia in many of these cell lines, although only HIF-1α protein induction in HeLa cells has been analyzed in any detail in graded O₂ environments (10). In agreement with Jiang et al. (10), HIF-1α protein in the HeLa cells was barely detectable at ≥5% O₂ but was up-regulated at 2% O₂ and further induced by <1% O₂ or DP (Fig. 1A). Importantly, significant differences in induction of HIF-1α protein were observed between cell types. For example, in 293T cells (Fig. 1B), HIF-1α protein is detectable in 5% O₂ and strongly expressed in 2% O₂, yet PC12 and CACO-2 cells require much more severe hypoxia (<1% O₂) for significant stabilization (Fig. 1, D and E). These differences may reflect different sensitivities of the cell to hypoxia or simply be a consequence of different metabolic rates and hence absolute oxygen levels within different cell types.

In the majority of cell lines, stabilization of HIF-2α paralleled that of HIF-1α, with maximal protein induction at <1% O₂. The one major exception was in PC12 cells, where a 4-h hypoxic incubation in <1% O₂, although sufficient to strongly stabilize HIF-1α, was not sufficient to stabilize detectable levels of HIF-2α (Fig. 1D). However, extending the incubation to 16 h
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led to a robust induction of HIF-2α protein. This difference in the kinetics of induction of HIF-2α compared with HIF-1α is also observed to a lesser extent in HeLa cells but not any of the other cell types. Also in contrast to the other cell lines, DP only weakly stabilized HIF-2α in PC12 and HeLa cells compared with severe hypoxia (Fig. 1, D and A). The functionality of DP in these cells is demonstrated by strong up-regulation of HIF-1α protein and CAD induction in reporter assays (see below). These data suggest that the mechanism of HIF-2α degradation in PC12 and to a lesser extent HeLa cells is distinct from HIF-1α and the other cell types analyzed. The absence of detectable HIF-2α protein in 293T cells (Fig. 1B) also agrees with a previous report (11). Thus, overall, HIF-1α and HIF-2α stabilization occur under similar O2 levels, and differences are cell-specific rather than general in nature.

The carotid body protects against hypoxia by measuring arterial oxygen tension and regulating such processes as breathing and heart rate (via catecholamine secretion) accordingly. PC12 cells are derived from a rat pheochromocytoma and are commonly used to investigate oxygen sensing as they share a number of characteristics with those of the type I carotid body. It was therefore of interest that PC12 cells displayed a unique property of differential HIF-α protein induction kinetics (Fig. 1D). To analyze this further, a hypoxic time course was performed that confirmed maximal HIF-1α protein stabilization not simply attributable to FIH-1 protein levels, since these did not vary widely between cell lines, and the cells with the highest level of FIH-1 expression, 293T and HepG2 cells, represented different classes of CAD activity (Fig. 2C). This cell-specific difference is also largely in contrast to protein stabilization, where most cell lines displayed very similar profiles of HIF-1α and HIF-2α, being stabilized at O2 levels of between 5 and 2% and reaching maximal up-regulation at <1% O2. Similar to the regulation of the CAD, more severe levels of hypoxia were required to stabilize the HIF-α proteins in PC12 and CACO-2 cells. In contrast, HepG2 cells strongly stabilized HIF-α at oxygen levels of between 5 and 2% yet clearly still required <1% oxygen for CAD function.

Thus, these results demonstrate that in many cells oxygen-dependent stabilization and transactivation are not linked, and the profile of one can vary without the profile of the other changing. It also demonstrates that as oxygen levels decrease, protein stabilization does not necessarily precede transactivation, but is dependent upon cellular context.

Interestingly, consistent differences were observed in normoxic or basal CAD activity. For all cell lines, the HIF-2α CAD had higher normoxic activity than HIF-1α, with the exception of HeLa cells. These differences varied from around 2-fold in COS-1 cells, up to at least 10-fold in HepG2 cells (Fig. 1F), and do not appear to reflect a difference in CAD expression levels as determined by α-GalDBD Western blotting (Fig. 1, A, B, and D, within 2 h, yet full HIF-2α induction required 8–16 h (Fig. 2A). This confirms an obvious difference in kinetics and not just lower levels of HIF-2α compared with the other cell types. Furthermore, treatment with desferroxamine, another hypoxia-mimicking iron chelator, also only stabilized HIF-1α but not HIF-2α (Fig. 2B). This further demonstrates that in PC12 cells, the pathways by which hypoxia-mimicking chemicals and physiological hypoxia regulate the HIF-α proteins differ between HIF-1α and HIF-2α.

Analyses of transfected CADs in the same cell types showed no obvious differences in O2-dependent regulation of HIF-1α and HIF-2α; the CADs from both proteins were activated in a similar manner by graded hypoxia within a given cell line. However, the different cell lines are separable into two distinct groups based upon CAD activation: those in which the HIF-α CADs are progressively activated from 10% down to <1% O2 (HeLa, 293T, COS-1) and those in which the CADs only activated in severe hypoxia (<1% O2) (PC12, CACO-2, and HepG2). This difference between cell lines in hypoxia-induced transactivation is
lower panels). Due to transfection efficiencies, the GalDBD CADs were only visualized in HeLa, 293T, and PC12 cells. These results agree with the higher normoxic activity of full-length HIF-2α/H9251 commonly reported (4, 13) and the reduced in vitro hydroxylation by FIH-1 of the HIF-2α/CAD compared with HIF-1α/H9251 (12). Thus, although in the majority of cell lines the relative oxygen levels at which their CADs become activated are similar for the two HIFs, the normoxic activities of HIF-1α/H9251 and HIF-2α/H9251 vary depending upon cell line and may contribute to their differential functions.

**HIF-1α and HIF-2α CADs Display the Same Stability within Cells**—The higher normoxic reporter activity of HIF-2α when compared with HIF-1α is consistent with previous studies and true of both full-length proteins and isolated CADs (7, 13, 14). Although there is no consistent difference in the expression level of these proteins, a difference in protein turnover could contribute to hydroxylation efficiency and subsequently to CAD activity. Therefore, to determine whether differences in normoxic activity correlated with differential protein stability, transfected 293T and PC12 cells were treated for up to 4 h with the protein translation inhibitor cycloheximide, and decreasing HIF-α/CAD protein levels were monitored by α-GalDBD Western blot (Fig. 3). Results indicate that the stabilities of HIF-1α and HIF-2α fusion proteins are similar in both cell lines, yielding an approximate half-life of 70 min, despite discrepancies between the starting levels of the HIF-α CADs, particularly in 293T cells (Fig. 3A, bottom). These results indicate that since there is no obvious difference in the stability or amount of the HIF-α CADs, the inherent differences between HIF-1α and HIF-2α activity are mostly attributable to intrinsic differences in CAD activity.

Interestingly, the finding that the HIF-α CADs are still relatively labile proteins, despite the absence of recognized oxygen-dependent degradation domains, also implies the existence of instability elements in the CAD that may be responsible for or contribute to the rapid turnover of the full-length HIF-α proteins that still occurs in hypoxia (15).

**Specific Determinants of Higher Normoxic HIF-2α CAD Activity**—A mechanism for increased normoxic HIF-2α CAD activity compared with HIF-1α is suggested by previously published in vitro hydroxylation assays, which demonstrate that HIF-1α CAD-derived peptides are hydroxylated more efficiently than HIF-2α (12), thereby predicting higher HIF-2α normoxic activity due to greater CBP/p300 binding. Many residues within the CAD are highly conserved between HIF-1α and HIF-2α and between species, due to the requirement for FIH-1 and CBP/p300 interactions (Fig. 4). However, with the exception of HIF-1α V802 (HIF-2α V846), many conserved residues flanking the hydroxylated asparagine show a high tolerance to point mutation (16). The HIF-1α V802A mutant exhibits a 4-fold lower $V_{\text{max}}$ than wild type without altering the $K_{\text{m}}$, indicating a reduction in catalysis without affecting FIH-1/HIF-α binding. This inefficiency of FIH-1 hydroxylation correlates with constitutive activity of GalDBD-hHIF-1α V802A 737–826 in cells without changes in protein level.

In HIF-1α, the backbone carbonyl of Val802 forms an intramolecular hydrogen bond with the NH of Ala804, which is completely conserved in all HIF-1α orthologs (17). Of particular interest, however, is that this alanine is replaced by a fully conserved valine in all HIF-2α orthologs (Fig. 4). This observation of a conserved difference led to the hypothesis that the residue in this position might contribute to the intrinsically less efficient hydroxylation of the HIF-2α CAD by FIH-1 and con-
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FIGURE 1—continued

sequentially its higher normoxic activity as reported previously and noted again in Fig. 1.

To test the importance of this conserved amino acid substitution on FIH-1-mediated asparaginyl hydroxylation, wild type HIF-1α CAD and a V804A mutant (or A848V in HIF-2α) were expressed, purified, and tested as FIH-1 substrates using in vitro hydroxylation assays. In agreement with reported data (12), HIF-1α was hydroxylated more efficiently than HIF-2α, with a greater $V_{\text{max}}$ (Fig. 5), despite a stronger FIH-1/HIF-2α interaction (data not shown). In support of our hypothesis, the single amino acid substitution to make the HIF-2α CAD more like HIF-1α was sufficient to increase the HIF-2α V848A $V_{\text{max}}$ to levels similar to wild type HIF-1α, whereas the equivalent HIF-1α A804V mutant displayed a decreased $V_{\text{max}}$ to or below the levels observed for wild type HIF-2α (Fig. 5). Importantly, the $K_m$ values were not significantly altered, indicating that these changes are due to an effect on catalysis, as opposed to FIH-1/HIF-α binding. These results confirm the important contribution of this single amino acid to the differential hydroxylation efficiency by FIH-1 observed in vitro.

In order to verify these in vitro results in a cellular context, the same HIF-1α and HIF-2α mutants used in the previous hydroxylation assays were cloned as GalDBD fusions and transfected into 293T and HepG2 cells, and activity was determined in normoxia and hypoxia. 293T and HepG2 cell lines were chosen, since these also represent the two classes of cells defined by HIF-α CAD activation that either occurs in a gradual manner starting at higher O2 levels (10% O2, 293T cells) or in a rapidly responsive manner only in severe hypoxia (<1% O2, HepG2 cells) (Fig. 1, B and F). Despite this difference in graded O2 responsiveness, both 293T (Fig. 6A) and HepG2 (Fig. 6C) cells display similar Gal-responsive luciferase reporter results in normoxia or severe hypoxia (<1% O2). When comparing wild type CADs, HIF-2α displays higher normoxic activity than HIF-1α in both cell types. As predicted by in vitro hydroxylation assays, the HIF-1α A804V mutant displayed greatly increased activity compared with wild type HIF-1α in both normoxia and hypoxia (Fig. 6, A and C), independently of protein levels (Fig. 6B, lane 2 versus lane 3), once again conferring HIF-2α-like activity on the HIF-1α CAD. However, the fact that the HIF-1α A804V mutant is still less active than wild type HIF-2α suggests that although this single residue contributes to the differential activities of the HIF-1α versus HIF-2α CADs, this residue alone is not solely responsible for the increased activity of the HIF-2α CAD within cells. The HIF-2α V848A mutant had only a slightly reduced activity compared with wild type HIF-2α, further supporting the importance of other determinants in differentiating the normoxic activities of the HIF CADs in a cellular context.

Finally, to establish the effect of these HIF-1α and HIF-2α substitution mutations in the context of full-length human proteins, induction of an HRE-luciferase reporter gene was assessed in 293T and HepG2 cells. Transfection of wild type HIF-1α and HIF-2α strongly increased reporter activity, with HIF-2α again being more active than HIF-1α at normoxia (Fig. 7, A and C), although less so in the context of full-length protein than the CADs in isolation. Full-length HIF-1α and HIF-2α are subject to prolyl hydroxylation-dependent regulation, mediated by the oxygen-dependent degradation domain. To separate the effects of transactivation from stabilization in the context of full-length protein, the two prolines targeted for hydroxylation were mutated to alanines in both HIF-1α (P402A/P564A) and HIF-2α (P405A/P531A). As expected, transfection of the HIF-1α and HIF-2α proline mutants further increased normoxic reporter gene activation (Fig. 7, A and C). In agreement with previous CAD reporter assays (Fig. 6), the HIF-1α A804V mutation significantly increased normoxic HIF-1α activity to near HIF-2α levels. Little effect was observable at hypoxia due to activity already being maximal (data not shown). The increase in activity was, however, to a lesser extent than with
the CAD in isolation, presumably due to the influence of factors such as the N-terminal transactivation domain. These results were not due to changes in protein levels between double proline and double proline/valine-alanine mutants (Fig. 7B) and further support a role for Ala804 in rendering HIF-1α/HIF-1β less active than HIF-2α/HIF-2β at normoxia in the context of full-length protein. Also in agreement with Fig. 5, in the context of full-length protein, the corresponding HIF-1α/HIF-1β-like, HIF-2α/V848A mutation had little impact on HIF-2α function, although small and reproducible decreases were noted as previous data would predict. This is also true with transfection of 10-fold lower DNA amounts, ensuring that the effects seen are not attributable to the reporter system being at saturating levels.

**DISCUSSION**

Despite mounting evidence of the nonredundancy between the HIF-α proteins (reviewed in Refs. 9 and 18), both HIF-1α and HIF-2α displayed similar properties with regard to both hypoxia-dependent stabilization and transactivation when compared across a number of cell lines, derived from both different tissues and species. One exception to this, however, is the requirement for longer term hypoxia (16 versus 4 h) for the stabilization of HIF-2α, but not HIF-1α, in PC12 and HeLa cells. Similarly, in PC12 and HeLa cells, and in contrast to the other cell lines examined, DP is only able to poorly stabilize HIF-2α, despite strongly stabilizing HIF-1α and up-regulating the transactivation of both HIF-α proteins. As reported with mouse embryonic fibroblasts (13), this implicates cell line-specific mechanisms of HIF-2α stabilization.

HIF-2α regulation in PC12 and HeLa cells aside, what are apparent in these experiments are not large differences in the intrinsic hypoxia-dependent stabilization or transactivation of HIF-1α and HIF-2α, but rather more significant differences in HIF regulation between cell lines. This is particularly true with respect to transactivation regulation, where both HIF-1α and HIF-2α CAD responses can be separated into two groups of cells: those in which the HIF-α CADs are progressively activated from 10% to <1% O2 (293T, HeLa, and COS-1) and those in which the CADs are rapidly activated but require more severe hypoxia (<1% O2) (HepG2, CACO-2, and PC12). This is in contrast to protein stabilization by hypoxia, where all cell lines stabilized endogenous HIF-1α and HIF-2α after 16 h of exposure to O2 concentrations of between 5 and 2%, reaching maximal up-regulation at <1% O2. It is important to note that these are atmospheric oxygen concentrations and give little indication of absolute oxygen concentrations within these cells, except that they decreased as atmospheric levels decreased. However, since all of the cells were grown and treated under standardized conditions, including culture volume and confluence, the only consistent differences between samples of the same cell types were the levels of atmospheric oxygen and hence the relative, graded levels of cellular oxygen.

It is likely that reporter gene assays reveal more subtle changes than Western blots, since reporter assays are able to quantitatively detect basal activity levels, whereas Western blots require a higher threshold for detection. Hence, it is difficult to conclude with certainty in cells in which the CADs are partially activated even by mild hypoxia that FIH-1 is being inactivated at higher O2 concentrations than the PHDs. However, in HeLa cells, for example, in 5% oxygen the CAD activity has increased 3-fold and has reached more than 35% of its full activity. If the PHDs were inactivated to a similar extent, a significant increase in detectable protein would be expected in the Western blot, but this is not evident. Therefore, it is apparent that inactivation of FIH-1, and hence CAD activation, is occurring if not prior to then at least in conjunction with protein stabilization. This is in contrast to in vitro data, where the $K_m$ values for oxygen for
FIH-1 versus the PHDs (90 and 250 mM, respectively) suggest that as oxygen levels decrease, the PHDs would be inactivated before FIH-1, and hence the HIF-α/HIF-β proteins would be stabilized before the CAD is activated. Hence, in HeLa, 293T, and COS-1 cells (Fig. 1, A–C) the model of PHD inactivation preceding FIH-1 is not supported by cell-based experiments. In contrast, however, cells that require severe hypoxia for CAD activation (PC12, CACO2, and HepG2) (Fig. 1, D–F) behave in a manner consistent with reported O2 K_m values, stabilizing the HIF-α/HIF-βs at higher O2 concentrations than those at which the CADs are activated. This is particularly true of HepG2 cells, where both HIF-1α and HIF-2α are strongly stabilized by 2% O2 yet require <1% O2 for CAD induction (Fig. 1F). This would presumably lead to an increased pool of partially active protein due to N-TAD or residual CAD activity.

It is important to note that CAD activity and stabilization, both hydroxylation-mediated processes, are not directly linked, with...
the relative oxygen sensitivity of one regulated independently of the oxygen sensitivity of the other. For example, the stabilization profile of HIF-1α protein with varying oxygen levels in HeLa and HepG2 cells is similar, whereas the profile of CAD activity within the same cells under identical conditions differs greatly (Fig. 1, A and F). This clearly demonstrates that the oxygen sensitivity of CAD activity, mediated by FIH-1, can be regulated within cells independently of protein stabilization, mediated by the PHDs.

These results also indicate that additional factors are involved in sensing and responding to cellular hypoxia and that the cellular response to hypoxia mediated by HIF may not be determined exclusively by the $K_m$ for oxygen of the HIF hydroxylases or that the $K_m$ values may be altered. Such factors may include the differential expression or modification of various HIF-α regulators, the hydroxylases themselves, levels of essential cofactors, such as 2-oxoglutarate, or various recently identified factors, including OS-9, which mediates the interaction between HIF-α and the PHDs (19), the HIF-α deubiquitylating factor VDU-2 (20), and Tid-1, which enhances the HIF-VHL interaction and thereby destabilizes HIF-α (21).

As reported in whole rodents exposed to varying degrees of hypoxia (6, 7), there are also differences in the inherent O2 responsiveness of different cells with respect to HIF stability. PC12 and Caco2 cells, for example, require <1% O2 for strong HIF-1α and HIF-2α protein stabilization (Fig. 1, D and E), in contrast to HeLa, 293T, COS-1, and HepG2 cells in which the HIF-α proteins are strongly induced at higher O2 levels (between 5 and 2% O2) (Fig. 1, A–C and F). Thus, for both cell lines and tissues, there exists variable O2 sensitivity, where the hypoxic state of a given O2 concentration is determined in a cell-specific manner. The mechanism by which cells set a given O2 concentration as normoxia remains uncertain and may provide an explanation for conflicting data relating to the role of mitochondria and reactive oxygen species in O2 sensing. In addition, differences in rates of metabolism and oxygen consumption are also likely to have a significant impact.

As previously observed (11, 13, 14), in the context of both the CAD and full-length protein, HIF-2α displays higher normoxic activity, attributable to the more efficient FIH-1-mediated hydroxylation of HIF-1α compared with HIF-2α, first reported...
It is known that despite high sequence identity between HIF-1α/H9251 and HIF-2α/H9251, single alanine point mutations are tolerated at many sites, with the exception of a single valine (HIF-1α Val802/HIF-2α Val846) (16). Being adjacent to the site of hydroxylation, this fully conserved valine sits within the small hydroxylation catalytic pocket and the backbone carbonyl forms a direct intramolecular hydrogen bond with the Ala804 NH that is fully conserved in all HIF-1α orthologs (17). The fact that this alanine is replaced with a fully conserved, bulkier valine residue in HIF-2α, however, suggested the identity of this amino acid could be an important determinant of asparaginyl hydroxylation and contribute to the more efficient hydroxylation of HIF-1α.

The swapping of this residue between HIF-1α/H9251 and HIF-2α/H9251 is indeed sufficient to swap hydroxylation efficiency of the HIF-1α and HIF-2α peptides by FIH-1, altering the $V_{\text{max}}$ of the reaction without changing the $K_m$ for FIH-1/HIF-α binding. Once established in vitro, both Gal- and HRE-responsive luciferase reporter genes showed the HIF-2α-like HIF-1α A804V mutation significantly increased activity as anticipated, independently of protein levels, although this single amino acid mutation alone was not sufficient to fully replicate HIF-2α activity. The corresponding HIF-2α V848A mutation, however, had a smaller effect, implicating additional features of the HIF-2α CAD that contribute to its high normoxic activity. In addition, another HIF-1α to HIF-2α mutation was constructed and tested (HIF-1α S797R), since this is the only other fully conserved substitution within the major FIH-1 binding site of HIF-1α 795–806 (17). Mutagenesis of this residue, however, did not affect HIF-α activation (data not shown). To further characterize the determinants of specificity in HIF-α CAD activity, additional amino acid substitutions or domain fragment swap chimeras may be utilized, especially since no fewer than 4 separate, fully conserved amino acid substitutions are illustrated in the alignment of 25 HIF-α CAD amino acids in Fig. 4.

In conclusion, therefore, by comparing HIF-1α and HIF-2α stabilization and transactivation in multiple cells, we have identified a number of interesting differences, such as the dipyridyl unresponsiveness and slower induction kinetics of HIF-2α (versus HIF-1α) stabilization in PC12 and HeLa (but not other) cells. We also demonstrate that cells may fall into one of two
classes in relation to CAD responsiveness to decreasing oxygen levels. Interestingly, one of these classes (293T, HeLa, and COS-1 cells) up-regulates CAD activity prior to (or at the very least in concordance with) protein stability, contradicting in vitro derived predictions of stabilization preceding transactivation, based upon oxygen affinities of the regulatory hydroxylases. We have confirmed previous observations that the HIF-1α CAD is hydroxylated more efficiently than HIF-2α, conferring increased HIF-2α basal (normoxic) activity. From this observation, we have gone on to demonstrate the importance of a conserved amino acid substitution immediately C-terminal to the hydroxylated asparagine, as a determinant of this difference in FIH-1-mediated hydroxylation. This is likely to represent a fundamental biochemical difference between HIF-1α and HIF-2α that contributes to their different roles and properties within cells.

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