Substrate Requirements of the Oxygen-sensing Asparaginyl Hydroxylase Factor-inhibiting Hypoxia-inducible Factor*  

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The hypoxia-inducible factor α subunits 1 and 2 (HIF-1α and HIF-2α) are subjected to oxygen-dependent asparaginyl hydroxylation, a modification that represses the carboxyl-terminal transactivation domain (CAD) at normoxia by preventing recruitment of the p300/cAMP-response element-binding protein coactivators. This hydroxylation is performed by the novel asparaginyl hydroxylase, factor-inhibiting HIF-1* (FIH-1), of which HIF-1α and HIF-2α are the only reported substrates. Here we investigated the substrate requirements of FIH-1 by characterizing its subcellular localization and by examining amino acids within the HIF-1α substrate for their importance in recognition and catalysis by FIH-1. Using immunohistochemistry, we showed that both endogenous and transfected FIH-1 are primarily confined to the cytoplasm and remain there under normoxia as following treatment with the hypomimetic, dipyridyl. Individual alanine mutations of seven conserved amino acids flanking the hydroxylated asparagine in HIF-1α revealed the importance of the valine (Val-802) adjacent to the targeted asparagine. The HIF-1α CAD V802A mutant exhibited a 4-fold lower V_max in enzyme assays, whereas all other mutants were hydroxylated as efficiently as the wild type HIF-1α CAD. Furthermore, in cell-based assays the transcriptional activity of V802A was constitutive, suggesting negligible normoxic hydroxylation in HEK293T cells, whereas the wild type and other mutants were repressed under normoxia. Molecular modeling of the HIF-1α CAD V802A in complex with FIH-1 predicted an alteration in asparagine positioning compared with the wild type HIF-1α CAD, providing an explanation for the impaired catalysis observed and confirming the importance of Val-802 in asparaginyl hydroxylation by FIH-1.

The ability of all human cells to sense and rapidly respond to changes in oxygen concentrations, both at the cellular level and systemically, is crucial for survival and is a significant factor in many major diseases (1). The hypoxia-inducible factors (HIFs) are transcription factors central to the genomic response to hypoxia, as they directly up-regulate numerous genes involved in increased oxygen delivery and metabolic adaptation to decreased oxygen availability. Recent studies have demonstrated that the HIF prolyl hydroxylases (PHDs), together with the HIF asparaginyl hydroxylase factor-inhibiting HIF (FIH-1), act as cellular oxygen sensors, directly regulating the activity of the HIFs in response to changing oxygen levels.

The HIFs are heterodimers that comprise two members of the bHLH/PAS (basic helix-loop-helix/Per-Arnt-Sim homology) superfamily of proteins: the HIF-α subunits and their ubiquitous partner Arnt (also known as HIF-1β) (1). Unlike Arnt, the HIF-α subunits are regulated by hypoxia, both at the level of transactivation and protein stability. Regulation is provided by the PHDs and FIH-1, both of which are members of the 2-oxoglutamate (2-OG)-dependent dioxygenase superfamily of hydroxylases. Specifically, under normoxic conditions, two conserved proline residues within the central oxygen-dependent degradation domains of the HIF-α proteins are hydroxylated by the PHDs. This promotes binding of the von Hippel Lindau tumor suppressor protein (pVHL), part of an ubiquitin ligase complex, resulting in polyubiquitylation and rapid degradation (2–5). Similarly, a conserved asparagine residue in the carboxyl-terminal transactivation domain (CAD) of the HIF-α proteins is hydroxylated at normoxia by FIH-1, preventing the recruitment of the p300/CBP transcriptional coactivators leading to transcriptional repression (6–9). Under hypoxia, the oxygen-dependent hydroxylases are not active, and the unmodified HIF-α proteins avoid ubiquitylation and degradation, heterodimerize with Arnt, recruit the p300/CBP coactivators, and up-regulate transcription of target genes. As such, both FIH-1 and the PHDs act as sensors of cellular oxygen tension (4, 5, 8–10).

FIH-1 was originally identified as a hypoxia-dependent regulator of HIF (6) and was subsequently shown to be a novel 2-OG-dependent asparaginyl hydroxylase capable of hydroxylating the HIF-α CADs (8, 9). Analyses of FIH-1 have demonstrated that, although related, it is distinct from the other well characterized class of asparaginyl hydroxylases, the asparyl-β-hydroxylases (BAHs), responsible for the hydroxylation of both aspartate and asparagine residues in the epidermal growth factor domains of numerous proteins (11). Similar to

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1 The abbreviations used are: HIF, hypoxia-inducible factor; FIH-1, factor-inhibiting HIF; DP, dipyridyl; MD, molecular dynamics; PHD, HIF prolyl hydroxylase; CAD, carboxyl-terminal transactivation domain; Trx-6H, thioredoxin-6-histidine; MBP, maltose-binding protein; 2-OG, 2-oxoglutarate; CBP, cAMP-response element-binding protein; BAH, asparyl-β-hydroxylase; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
FIH-1, the BAHs are also Fe(II)- and 2-OG-dependent dioxygenases, but they specifically hydroxylate the aspartic acid/asparagine residue (bold) within the sequence CXXD/N/XXXX-(P/Y)XXCXC, which is distinct from the sequence surrounding the targeted asparagine residue in the HIF-α CADs (12). By comparison, it has been difficult to define a consensus hydroxylation sequence for FIH-1 based on sequence conservation of the HIF-α CAD substrates around the targeted asparagine, as many of these amino acids are also essential for the interaction with the p300/CBP coactivators (13, 14). The BAHs and FIH-1 are also expected to localize to different subcellular compartments given that the known FIH-1 substrates, the HIF-α s, are nuclear and cytoplasmic. Finally, the x-ray crystal structure of FIH-1 has confirmed the adoption of a double-stranded β-helix enzyme core (predicted from a primary sequence analysis and characteristic of members of the 2-OG-dependent dioxygenase superfamily) but has revealed unusual 2-OG binding and distinct structural variations from other family members (17–19).

In this study, we describe experiments characterizing the functional substrate requirements of FIH-1. Immunohistochemistry was used to determine the subcellular localization of FIH-1, demonstrating predominantly cytoplasmic expression. Attempts to identify a substrate recognition sequence using alanine scanning site-directed mutagenesis of the HIF-1α CAD substrate identified the valine residue at position 802, adjacent to the hydroxylated asparagine, as essential for efficient hydroxylation. Molecular modeling based on the x-ray crystal structure of the FIH-1-HIF-1α CAD complex supported these results, implicating the valine residue in optimal positioning of the asparagine residue in the HIF-

**EXPERIMENTAL PROCEDURES**

**Plasmids**—pET-32a HIF-1α (737–826) was constructed by PCR amplification from human HIF-1α cDNA and cloned into pET-32a (Novagen) using BamHI/Sall to generate a thioredoxin-6-histidine (Trx-6H) amino-terminal fusion. This construct was used as a template for mutagenesis (QuikChange, Stratagene) to generate the following mutants using the oligonucleotides described: Y798A, 5′-CCACGCTGACTCTGCGAGATTGTAAGTCGACACTC-3′; D799A, 5′-CTGACAGCTTATGCGATGCGAATTGAAATGCTCCTATACAAGG-3′; E801A, 5′-CAGTTATGATGGTCGAGTTAAGGCAGC-3′; V802A, 5′-GTTATGATGGTGAAGGACGTAGATGCCTCCTATACAG-3′; N803A, 5′-GGTTATGATTGGAAGTGGCGCCGCTCTATACAAGGCACG-3′; P805A, 5′-GGTGAGATTAAGTCTGCTATACGGGCCAG-3′, and G808A, 5′-GCTGCTTATAAGCTTCTAGGAGCTACCTACTGCG-3′. All constructs were verified by sequencing. pMBP-hFIH-1 and hFIH-1 cDNAs were described previously (7). The pGal-HIF-1α (737–826) constructs were generated by insertion of an oligonucleotide linker (upper, 5′-CATGGAGTGTCGACTAGCCATGTGAACTTATAC-3′; lower, 5′-GTCGACTCATGACCGTCTGCGTCTAGTCTGCTAAGAGCAGTACGAGCAACG-3′, and G808A, 5′-GCTGCTTATAAGCTTCTAGGAGCTACCTACTGCG-3′) into the NcoI site of the corresponding pET-32a construct, permitting excision by SalI followed by in-frame ligation into pET-32a (Invitrogen) using BamHI/SalI to generate a thioredoxin-6-histidine (Trx-6H) fusion. This construct was used as a template for alanine scanning site-directed mutagenesis (QuikChange, Stratagene) to generate the following mutants: Y798A, 5′-CCACGCTGACTCTGCGAGATTGTAAGTCGACACTC-3′; D799A, 5′-CTGACAGCTTATGCGATGCGAATTGAAATGCTCCTATACAAGG-3′; E801A, 5′-CAGTTATGATGGTCGAGTTAAGGCAGC-3′; V802A, 5′-GTTATGATGGTGAAGGACGTAGATGCCTCCTATACAG-3′; N803A, 5′-GGTTATGATTGGAAGTGGCGCCGCTCTATACAAGGCACG-3′; P805A, 5′-GGTGAGATTAAGTCTGCTATACGGGCCAG-3′, and G808A, 5′-GCTGCTTATAAGCTTCTAGGAGCTACCTACTGCG-3′. All constructs were verified by sequencing. pMBP-hFIH-1 and hFIH-1 cDNAs were described previously (7).

**Transfections**—All transfections used HEK293T cells maintained in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum, and DNA transfections were performed using LipofectAMINE 2000 according to the manufacturer’s instructions (Invitrogen).

**Immunofluorescence**—HEK293T cells plated on glass coverslips in 6-well trays were transfected with 5 μg of FIH-1 pcDNA3.1/V5-His or were left untransfected. The cells were then left untreated or were treated with 100 μM dipiridyld (DP) for 16 h. The cells were then fixed, probed, mounted, and visualized as described previously (21) using a rabbit polyclonal anti-FIH-1 primary antibody generated to full-length MBP-FIH-1 (1/400 in 5% skim milk) and fluorescein isothiocyanate-conjugated sheep anti-rabbit secondary antibody (Silenus, 1/1000 in 1% skim milk).

**FIG. 1. Immunohistochemistry showing nuclear localization of FIH-1. A, anti-FIH-1 immunoblot using whole cell extracts from untransfected HEK293T cells (U) or HEK293T cells transiently transfected with FIH-1 pcDNA3.1/V5-His (T). Asterisk indicates background band. Representative of five independent experiments. B and C, immunohistochemistry of untransfected (B) or FIH-1/V5-His-transfected HEK293T cells (C) grown for 16 h at normoxia on coverslips fixed with formaldehyde and probed with preimmune serum (PI) or a polyclonal primary anti-FIH-1 antibody (FIH). Microscopy was performed after labeling cells with a secondary antibody conjugated to fluorescein isothiocyanate (left panels), and nuclei were visualized by staining with Hoechst 33258 (right panels). Untransfected (D) or FIH-1/V5-His-transfected HEK293T cells (E) were treated for 16 h with 100 μM DP before analysis as described in B and C above.

**CO2 Capture Assays**—Assays were adapted from Zhang et al. (22). Specifically, 40-μl reactions containing 3 mg/ml bovine serum albumin, 40 μM [1-13C]2-OG (PerkinElmer Life Sciences, specific activity 14 nCi/mmol), 4 mM ascorbate, 1.5 mM FeSO4, 0.5 mM dithiothreitol, 112 mM NaCl, 30 mM Tris, pH 7.0, purified MBP-FIH-1, and purified TX-6H-HIF-1α (737–826) substrate at the indicated concentrations were incubated for 60 min at 37 °C in 1.5-ml sealed polypropylene tubes with Ca(OH)2-soaked filters suspended above the reactions. Pilot experiments demonstrated, that under these conditions, the hydroxylation rate was linear, and cofactors (ascorbate, Fe2+, and 2-OG) were at saturating concentrations. Upon reaction completion, filters were dried, soaked with scintillation fluid (Opti-scint Hisafe, Wallac), and captured 13C was quantitated by scintillation counting. All samples were examined in triplicate, and data were expressed as mean ± S.E. (n = 3). Kinetic curves were performed such that substrate consumption was <10% or, if between 10 and 40%, substrate concentrations were corrected accordingly. PRISM software was employed to fit data to hyperbolic curves and calculate K_m and V_max.

**Protein Expression and Purification**—MBP-FIH-1 and all Trx-6-HIF-1α (737–826) wild type and alanine-mutant proteins were expressed and purified as described previously (7) and were visualized by SDS-PAGE (Tris/glycine) followed by Coomassie staining. Protein concentrations were determined using calculated extinction coefficients and measurement of absorbance at 280 nm.

**Reporter Assays**—Dual luciferase reporter assays were carried out in
24-well trays using HEK293T cells (~8 × 10⁵ cells/well). The cells were transiently transfected with 100 ng of pEG1B-Luc, 10 ng of pRL-TK, and 100 ng of pGalO (empty vector) or pGal-HIF-1α-(737–826) (wild type or alanine-mutant) and were then left untreated (normoxia) or were treated with either hypoxia (using AnaeroGen sachets, OXOID) or 100 μM DP for 14 h as described previously (7). Luciferase activity was examined using the Dual Luciferase Assay kit (Promega) according to the manufacturer’s instructions.

Immunoblot—Anti-HIF-1α immunoblots were performed using whole cell extracts (as described previously (7)) taken from untransfected HEK293T cells or cells transiently transfected in 10-cm dishes (~3 × 10⁶ cells/dish) with 7 μg of pcDNA3.1/V5-HIS using Lipo-fectAMINE 2000 according to the manufacturer’s instructions for 14 h. Equivalent amounts of protein (as quantitated by the Bradford assay (Bio-Rad)) were separated by SDS-PAGE (Tris/Tricine) transferred to nitrocellulose membranes, and blocked for 1 h using 10% milk, phosphate-buffered saline, and 0.1% Tween 20. The membranes were incubated with anti-HIF-1 rabbit polyclonal antibody at 1:500 dilution in 5% milk, phosphate-buffered saline, and 0.1% Tween 20 overnight, washed three times for 10 min in phosphate-buffered saline, 0.1% Tween 20, incubated with secondary antibody, and developed as described previously by Lando et al. (7). Anti-human HIF-1α immunoblots were performed using HEK293T cells transiently transfected in 6-well trays (~5 × 10⁵ cells/well) with 2.5 μg of pGalO (empty vector) or with pGal-HIF-1α-(737–826) (wild type or alanine-mutant) and were left untreated (normoxia) or were treated with either hypoxia or DP for 14 h as described above. Whole cell extracts were prepared, and equivalent amounts of protein as quantitated by the Bradford assay (Bio-Rad) were separated by SDS-PAGE (Tris/Tricine) and examined by anti-HIF-1α immunoblotting as described previously (7).

Molecular Modeling—The x-ray crystal structure of FIH-1 complexed with HIF-1α-CAD (Protein Data Bank code 1H2L) was used to construct a V802A mutant model by truncating Val-802 of HIF-1α. Missing amino acids were reconstructed with SYBYL v6.9 (23) and its BIOPOLYMER module. Hydrogen atoms and partial charges for 2-oxoglutarate were calculated with SYBYL, whereas van der Waals, bond, angle, dihedral, and improper dihedral parameters were constructed with XPLOR v3.3 (24). All other missing hydrogen atoms from the crystal structure were added with X-PLOR v3.851 (25). All residues, with the exception of His-199 and His-279 of FIH-1, were modeled in their charged state. The bonds between iron and coordinated groups were modeled explicitly. Molecular dynamics (MD) simulations were carried out using NAMD v2.5b1 (26) utilizing the CHARMm (27) force field with explicit hydrogen atoms. Initially, 30,000 steps of energy minimization were performed on reconstructed residues and hydrogen atoms. The structure was solvated using SOLVATE v1.2 as employed in VMD v1.8 (28), and an 18-Å radius around Asn-803 of HIF-1α was used in 1-ns MD simulations with a time step of 1 femtosecond (fs). Simulations were carried out at 300 K with a dielectric constant (ε) of 1. A switch function on van der Waals forces was applied to interactions exceeding 8 Å, and truncation occurred at 12 Å. Residences of the nonbonded interactions were done every 20 time steps. Wild type and V802A mutant MD simulations were conducted with five different starting seeds.

RESULTS

Subcellular Localization of FIH-1—One important substrate requirement for any enzyme is colocalization. The only known substrates for FIH-1 are HIF-1α and HIF-2α. These proteins are reported to be cytoplasmic under normoxia, but upon activation by hypoxia, they translocate to the nucleus to partner with Arnt and activate transcription (29). We used a polyclonal antibody generated to full-length FIH-1, demonstrated to be specific for endogenous and transiently transfected FIH-1 in HEK293T cells (Fig. 1A), in immunohistochemistry experiments to determine the subcellular localization of endogenous FIH-1. Compared with the relatively weak dispersed staining observed throughout the cell with the preimmune control, the FIH-1 antibody produced a pattern of predominantly cytoplasmic staining with little if any staining visible in the nucleus (Fig. 1B), indicating that endogenous FIH-1 is essentially confined to the cytoplasm. To confirm the specificity of staining, immunohistochemistry was repeated on cells transiently transfected with FIH-1. As with endogenous FIH-1, overexpression produced a similar but more intense cytoplasmic pattern of staining (Fig. 1C).

Experiments were also designed to investigate whether FIH-1 translocates to the nucleus along with the HIF-α substrates under hypoxia when this oxygen-dependent enzyme would be inactive. Because of the rapid reversibility of hypoxic activation upon reoxygenation and the inherent difficulties in performing immunohistochemistry under hypoxic conditions, the hypoxia mimetic DP was used rather than hypoxia. The results observed with DP treatment were essentially the same as observed at normoxia, with endogenous and overexpressed FIH-1 staining still predominantly cytoplasmic (Fig. 1, D and E). This suggests that FIH-1 does not translocate to the nucleus with HIF-α substrates under hypoxic conditions.

Amino Acid Substrate Requirements for Asparaginyl Hydroxylation by FIH-1 in Vitro—We sought to determine the primary amino acid sequence determinants required for asparaginyl hydroxylation by FIH-1 in vitro. The targeted asparagine residue in CADs of HIF-1α and HIF-2α is conserved throughout many species, from Xenopus through to humans, as are many of the surrounding amino acids. This conserved region of the CADs is also involved in a direct interaction with the p300/CPB class of coactivators (13, 14). To determine the individual amino acids of the HIF-1α CAD required for recognition by FIH-1, we performed alanine scanning mutations of eight conserved residues, including the targeted asparagine (Fig. 2), and assessed the ability of FIH-1 to hydroxylate these mutants in vitro.

Trx-6H-HIF-1α CAD fusion proteins containing the carboxy-terminal 90 amino acids of human HIF-1α and MBP-FIH-1 fusion proteins were expressed in Escherichia coli and affinity-purified to near homogeneity for use in [¹⁴C]CO₂ capture assays (Fig. 3A). Initial experiments demonstrated that the wild type HIF-1α CAD was an effective substrate with a specific Km (40 μM) and Vmax (82 nmol/min/mg) that reported previously for bacterially expressed FIH-1 (9). A recent study using FIH-1 expressed in insect cells also reported a similar Km but a 10-fold greater Vmax (10). The assays were repeated with each
FIG. 3. Asn hydroxylation of HIF-1α-(737–826) by MBP-FIH-1 is tolerant to alanine mutation at all positions examined except Val-802 and Asn-803. A, Trx-6H-HIF-1α-(737–826) wild type and alanine-mutant proteins were purified to near homogeneity from E. coli using nickel affinity chromatography, separated by SDS-PAGE (12.5% Tris-glycine), and visualized by Coomassie staining. MBP-FIH-1 was purified to near homogeneity from E. coli using amylose affinity chromatography, separated by SDS-PAGE (10% Tris-glycine), and visualized by Coomassie staining. Molecular mass markers are shown in kilodaltons (kDa). Asterisks represent flow-through from nickel affinity chromatography. B, the effect of alanine mutation on hydroxylation rate was evaluated by in vitro [14C]CO2 capture assay. 150 μM purified Trx-6H-HIF-1α-(737–826) wild type or mutant protein substrate was incubated with 200 nM purified MBP-FIH-1 for 60 min at 37 °C in the presence of cofactors (ascorbate,
of the eight point mutants at substrate concentrations of 150 μM, well above the $K_m$ (Fig. 3B). As expected, mutation of Asn-803 to Ala resulted in no detectable enzyme activity, even though there are three other Asn residues within the last 90 amino acids of HIF-1α and an additional four Asn residues within the Trx-6H fusion partner sequence. Hence, this confirms absolute specificity for Asn-803 and shows that no uncoupled decarboxylation of 2-OG occurred under these conditions. Mutation of the Val-802 to Ala, adjacent to the targeted Asn, resulted in a 4–5-fold decrease in hydroxylation activity, suggesting that this valine residue is essential for optimal hydroxylation. All of the other Ala point mutants were hydroxylated as efficiently as the wild type HIF-1α substrate despite the Ala substitutions being less conserved than the substitution at Val-802, including charged (Asp-799, Glu-801) and large hydrophobic residues (Tyr-798, Pro-805). These experiments were repeated at substrate concentrations of 30 μM, which is close to the observed $K_m$ of 40 μM, with similar results (data not shown). Collectively these data indicate that with the exception of Val-802, individually these other conserved residues are not essential determinants of substrate recognition by FIH-1 in vitro.

A more detailed kinetic analysis of the V802A mutant demonstrated a similar $K_m$ to the wild type protein (52 μM compared with 40 μM, respectively) but a 4-fold lower $V_{max}$ (Fig. 3C). This suggests that mutation of the Val-802 is likely to have a major effect on the catalysis of hydroxylation without significantly affecting the binding of substrate to FIH-1.

**Amino Acid Substrate Requirements for Asparaginyl Hydroxylation in Mammalian Cells**—To investigate the importance of these conserved amino acids in regulating the activity of the HIF-1α CAD by asparaginyl hydroxylation in the context of mammalian cells, we used Gal4-DNA-binding domain fusions of the HIF-1α CADs in cell-based reporter assays. Either the wild type HIF-1α CAD or each of the alanine point mutants were transiently transfected into HEK293T cells along with a Gal4-responsive reporter gene, and activity was measured under normoxia and hypoxia (<0.1% O2) or the hypoxia mimetic Fe$^{2+}$, and [14C]2-OG, and liberated [14C]CO$_2$ was quantitated by scintillation counting. Data are expressed as -fold activity compared with wild type, presented as mean ± S.E. (n = 3), and are representative of three independent experiments. C, alanine mutation of Val-802 in HIF-1α (737–826) causes a 4-fold reduction in the $V_{max}$ of FIH-1. The hydroxylation rate was evaluated by in vitro [14C]CO$_2$ capture assay as in B using 0–190 μM Trx-6H-HIF-1α (737–826) wild type or V802A substrate with 100 or 200 μM MBP-FIH-1, respectively. Liberated [14C] was quantitated using scintillation counting and expressed as $V_c$ (nmol/min/mg). Values for $K_m$ (wild type, 40 μM; and V802A, 52 μM) and $V_{max}$ (wild type, 82 nmol/min/mg; V802A, 19 nmol/min/mg) were calculated by PRISM software using nonlinear regression to fit data to hyperbolic equations ($r^2$ wild type = 0.98; $r^2$ V802A = 0.91). Data are also displayed as a double reciprocal plot (inset). Results are presented as mean ± S.E. (n = 3) and are representative of four independent experiments. [S] = substrate concentration (μM), and V = hydroxylation rate (nmol/min/mg).
Fig. 5. Schematic representation of the active site of FIH-1 complexed with HIF-1α during molecular dynamics. A, the wild type FIH-1-HIF-1α complex showing an association between Val-802 and Trp-296, with Asn-803 oriented proximally to the iron. B, the V802A mutant FIH-1-HIF-1α complex showing disassociation of the Ala-802 and Trp-296 interaction and coordination of Asn-803 to the iron. Secondary structure of FIH-1 is shown in cyan; secondary structure of HIF-1α is shown in red. Iron, green; carbon, cyan; oxygen, red; nitrogen, blue; hydrogen, white. The figure was generated with the molecular graphics program VMD v1.8.

DP (Fig. 4). As shown previously (8), the activity of the wild type HIF-1α CAD was induced at least 12-fold by hypoxia or DP, whereas the N803A mutant was constitutively active because it could not be hydroxylated and therefore interacted constitutively with p300/CBP (Fig. 4, A and B). Immunoblots of whole cell extracts showed that, with the exception of the G805A mutant, all proteins were expressed at similar levels, and there were no significant changes in protein levels between normoxia and hypoxia (or DP, data not shown), confirming that differences in reporter activity are a reflection of altered transcriptional activation potential (Fig. 4C).

The activity of the V802A mutant in reporter assays closely reflected the in vitro hydroxylation results with essentially constitutive activity at normoxia. If anything, this result suggests that the inability of the V802A mutant to be hydroxylated efficiently in vitro is amplified in a cellular context. As expected, the Y798A, C800A, and to a lesser extent, E801A mutants displayed similar levels of induction to the wild type protein, although they displayed lower overall activity, suggesting that the efficient hydroxylation observed in vitro was representative of normoxic hydroxylation within cells. Supporting these results, other studies have also reported that equivalent C800A mutants behave like wild type HIF-α-CAD in cell-based reporter assays (30, 31). Two of the mutants, D799A and P805A, showed very little activity, in agreement with results reported previously using a similar P805A mutant (8). The complete lack of activity with the G805A mutant can be partially explained by its very low level of expression compared with the other mutants (Fig. 4C), which may reflect a poorly folded and/or unstable protein. Taken together, these results reflect those obtained in vitro with FIH-1 and confirm the importance of Val-802 as a crucial determinant of asparaginyl hydroxylase substrate recognition in vivo.

Molecular Modeling of HIF-1α CAD-V802A-FIH-1 Complex—The x-ray crystal structure of FIH-1 complexed with HIF-1α CAD shows that the Val-802 is buried deep within the interface between these two proteins (18). Furthermore, a hydrogen bond between Val-802 and Ala-804 appears to be crucial in positioning the Asn-803 side chain within the catalytic site to enable hydroxylation of the β-carbon. The Val-802 is also responsible for a significant shift in Trp-296 of FIH-1 upon binding the HIF-1α CAD. Therefore, it is not surprising that mutation of this integral Val-802 residue to Ala has a significant effect on catalysis. To further investigate the influence of the V802A mutation on the structure of the HIF-1α CAD-FIH-1 complex, we used MD of the V802A mutant compared with the wild type protein based on the published structure of this complex (18).

Considerable differences were observed between the results of MD simulations of the wild type and V802A mutant (Fig. 5). In the wild type situation, the interface between the ring system of Trp-296 of FIH-1 and Val-802 of HIF-1α observed in the published structure was maintained. As a result, Asn-803 of HIF-1α was fixed in a position proximal to the iron. A water molecule from the surrounding environment filled the remaining coordination site on the iron (Fig. 5A). In contrast, substitution with an alanine at position 802 led to a local structural rearrangement, including loss of side chain contacts between Ala-802 and Trp-296 and new contacts between Asn-803 and the iron. No significant rearrangements were observed around the iron center of other coordinated groups during either set of simulations.

These predicted structural rearrangements confirm the importance of Val-802 for catalysis by FIH-1, given that the altered position of the β-carbon (the hydroxy acceptor) in the V802A mutant renders it relatively inaccessible to hydroxylation according to the currently accepted model. Subsequently, the association between the Asn-803 amide oxygen and the iron is predicted to competitively inhibit the binding of dioxygen.

DISCUSSION

These results demonstrate for the first time that endogenous FIH-1 is expressed predominantly in the cytoplasm in HEK293T cells. This cytoplasmic localization is in agreement with that reported for over-expressed FIH-1 fused to enhanced green fluorescent protein in osteosarcoma cells under normoxic and hypoxic conditions (32). Previous studies have demonstrated that HIF-1α is localized cytoplasmically at normoxia prior to degradation (29), hence the colocalization of FIH-1 with the HIF-1α subunits would enable rapid hydroxylation and transcriptional repression immediately after translation. The cytoplasmic localization of FIH-1 under simulated hypoxia, however, indicates that upon reoxygenation, active nuclear HIF-1α would not be rapidly repressed by FIH-1-dependent hydroxylation unless it was exported to the cytoplasm. This implies that PHDs play the major role in mediating the rapid down-regulation of HIF-α activity upon reoxygenation by targeting the protein for protosomal degradation. Furthermore, the reported nuclear localization of PHD-1 and PHD-3 and the hypoxic induction of PHD-3 (5, 32, 33) support the key role of the PHDs rather than FIH-1 in rapidly down-regulating HIF activity following reoxygenation.

The alanine mutation experiments with the HIF-1α CAD demonstrate that, with the exception of Val-802, individually the other conserved residues surrounding Asn-803 are not essential for efficient hydroxylation in vitro or in cell-based assays. These results are similar to those observed with the PHDs, where mutagenesis experiments targeting nine amino acids within the HIF-1α oxygen-dependent degradation domains demonstrated that the only obligatory residue for prolyl
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hydroxylation was the targeted proline (34). They also further demonstrate the significant differences between the HIF-α and HIF-β proteins, in which hydroxylation of the HIF-α proteins is not observed in vitro. The analysis of other residues less proximal to Asn-803 and combinatorial mutants. Ultimately, the results presented in this study and other data define substrate specificity should facilitate the identification of other potential FIH-1 substrates using bioinformatic approaches and aid in the design of therapeutics to regulate FIH-1 and consequently HIF activity.

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