Hypoxia-inducible Factor (HIF) Asparagine Hydroxylase Is Identical to Factor Inhibiting HIF (FIH) and Is Related to the Cupin Structural Family*

Activity of the hypoxia-inducible factor (HIF) complex is controlled by oxygen-dependent hydroxylation of prolyl and asparaginyl residues. Hydroxylation of specific prolyl residues by 2-oxoglutarate (2-OG)-dependent oxygenases mediates ubiquitinylation and proteasomal destruction of HIF-α. Hydroxylation of an asparagine residue in the C-terminal transactivation domain (CAD) of HIF-α abrogates interaction with p300, preventing transcriptional activation. Yeast two-hybrid assays recently identified factor inhibiting HIF (FIH) as a protein that associates with the CAD region of HIF-α. Since FIH contains certain motifs present in iron- and 2-OG-dependent oxygenases we investigated whether FIH was the HIF asparaginyl hydroxylase. Assays using recombinant FIH and HIF-α fragments revealed that FIH is the enzyme that hydroxylates the CAD asparagine residue, that the activity is directly inhibited by cobalt(II) and limited by hypoxia, and that the oxygen in the alcohol of the hydroxyasparagine residue is directly derived from dioxygen. Sequence analyses involving FIH link the mechanism by which these stimuli suppress HIF-α degradation and activate the transcriptional cascade.

Preceding analyses of the HIF-α CAD have indicated that, with proteolysis, the action of hypoxia is clearly mimicked by cobaltous ions and iron chelators, suggesting the operation of a related regulatory process. However, sequence similarities between the HIF-α CAD and the prolyl-containing motifs in the oxygen-dependent degradation domain are not apparent. Insight into this paradox has been provided by mass spectrometric and mutational analyses of the HIF-α CAD that demonstrate regulatory hydroxylation of a specific asparaginyl residue (Asn-803 in HIF-1α) (14). In the presence of oxygen, hydroxylation at this site prevents interaction with the p300 CH1 domain, whereas in hypoxia suppression of the modification allows interaction with p300 and transcriptional activation. Consistent with this model, NMR studies of the human HIF-1α CAD complexed to CH1 domain demonstrated that the unmodified Asn-803 is buried at the interface between the proteins (15, 16). The dependence of the asparaginyl-modifying activity on iron and inhibition by a 2-OG analogue implied that the HIF asparaginyl hydroxylase was a member of the same oxygenase family as the PHD isozymes, although the enzyme was not identified. Recent studies have identified factor inhibiting HIF (FIH) as a protein interacting with the CAD of HIF and which is in-
volved in preventing the transcriptional activation role of CAD (17). Here we demonstrate that FIH is the oxygenase catalyzing hydroxylation of Asn-803 in HIF-1α and report on the characterization of recombinant enzyme. Our findings provide further evidence for the importance of this class of enzyme in mediating cellular responses to oxygen availability. They also establish an evolutionarily relevant structural relationship that links the Fe(II)-dependent family of 2-OG and related oxygenases with the cupin superfamily.

**EXPERIMENTAL PROCEDURES**

**Cloning, Expression, and Purification of fih/FIH**—The fih gene was PCR-amplified from Image clone 4138066 and subcloned as a NdeI/BamHI fragment into the pET28a(+) vector (Novagen) to generate an N-terminal His₆-tagged FIH fusion protein. Primers used were: forward, 5′-ggaggactctatagcagacagacagcggg-3′; reverse, 5′-ggaggactctatagctatagcagacagcggg-3′. Following amplification the integrity of fih was confirmed by DNA sequencing. The fih/pET28a(+) construct was transferred into Escherichia coli BL21(DE3) and grown at 37 °C in 2TY medium containing 30 µg/ml kanamycin and was induced with isopropyl-β-D-thiogalactoside. The N-terminal His₆-tagged FIH fusion protein was purified using nickel affinity chromatography (Novagen), and the N-terminal His₆ tag was removed from the HIF-1α polypeptide using a thrombin. Size exclusion chromatography (Superdex 75) yielded FIH of >95% purity by SDS-PAGE analysis. Electrospray ionization MS revealed that the mass of the isolated FIH was consistent with that expected from its predicted amino acid sequence (observed, 40,556 Da; calculated, 40,567 Da).

**Cloning, Expression, and Purification of HIF-1α Fragments**—The desired segments of human HIF-1α were cloned directly as StuI/AseI fragments from pcDNA3 vectors (12) into a modified version of pGEX-6P-1 (Amersham Biosciences) to generate N-terminal glutathione S-transferase (GST)-tagged fusion proteins. The constructs were transformed into E. coli BL21(DE3) and grown at 37 °C in 2TY medium containing 100 µg/ml ampicillin and were induced with isopropyl-β-D-thiogalactoside. The GST-tagged fusion proteins were purified using glutathione-Sepharose™ 4B resin (Amersham Biosciences). The GST tag was removed from the HIF-1α fragments when required by PreScission™ Protease treatment. Size exclusion chromatography (Superdex 75) yielded >95% pure protein by SDS-PAGE analysis. His₆-tagged sections of HIF-1α were prepared as described in Ref. 8.

**Mutagenesis Studies of HIF-1α(775–826)—** Mutant GST-HIF-1α-(775–826) His₆-polypeptides were produced from the wild-type construct using the QuickChange system (Stratagene). The primers for each of the asparagine substitutions were: N803A: forward, 5′-gttagttgaagtgtgctgtcetataacaggg-3′; reverse, 5′-gttagttgaagtgtgctgtcetataacaggg-3′; N803Q: forward, 5′-gttagttgaagtgtgctgtcetataacaggg-3′; reverse, 5′-gttagttgaagtgtgctgtcetataacaggg-3′; N803E: forward, 5′-gttagttgaagtgtgctgtcetataacaggg-3′; reverse, 5′-gttagttgaagtgtgctgtcetataacaggg-3′. Bold codons indicate mutations. Mutations were confirmed by DNA sequencing.

**FIH Assays**—Assays for decarboxylation of 2-OG were performed using radiolabeled 2-OG (PerkinElmer Life Sciences) as reported in Ref. 8. Incubations contained ascorbate, dithiothreitol, catalase, 2-OG, FIH, and substrate in 50 mM Tris/HCl, pH 7.4 and were carried out at 37 °C typically for 20 min. HPLC separations were achieved on a Phenomenex Jupiter C4 (15 cm × 4.6 mm) column by using a linear gradient of CH₃CN in 0.1% trifluoroacetic acid. For inhibition and metal dependence assays, GST-HIF-1α-(775–826) was used as substrate at 30 µM and with iron(II) ammonium sulfate, cobalt(II) chloride, zinc(II) chloride, and N-aceotolylglycine at final concentrations of 0, 2, 10, 40, 80, and 200 µM. Assays under an atmosphere of 18O₂ were performed as reported previously (18).

**GST Pull-down Assays**—To examine for effects of FIH on HIF-1α binding to p300, the GST fusion protein expressing amino acids of HIF-1α-(775–826) and the N803A mutant were exposed to recombinant FIH under conditions designed to promote the hydroxylation. N-Oxalacetate was added to 5 mM when required. Glutathione-Sepharose beads were added for 40 min at 25 °C and then washed with 50 mM Tris/HCl, pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, and 1 mM dithiothreitol. The beads bearing the treated GST-HIF fusion proteins were mixed with 35S-labeled CH1-p300 (pCMX-GALA-CH1 encoding amino acids 300–528 of p300) produced in rabbit reticulocyte lysate. Binding was performed for 1 h at 25 °C, and the beads were then washed in the same buffer. The bound proteins were eluted in SDS running buffer and analyzed by SDS-PAGE and autoradiography.

**RESULTS AND DISCUSSION**

**FIH Is a HIF-α CAD Hydroxylase**—Preliminary analysis of the predicted FIH amino acid sequence in light of the sequences for 2-OG oxygenases of known structure and function (19–21) suggested that FIH contained the conserved HX(D/E) . . . H “facial triad” of residues that binds the Fe(II) cofactor. Given the reported interaction of FIH with the CAD of HIF-1α and its ability to down-regulate transactivation we considered that FIH might function as the Asn-803 hydroxylase.

N-terminally His₆-tagged FIH was therefore produced in E. coli BL21(DE3) and purified to >95% purity. HIF-1α polypeptides encompassing all three identified sites of hydroxylation were then examined as putative substrates for purified recombinant FIH (Table I, entries 1–6) using an assay that monitors decarboxylation of 2-OG. The results clearly indicate that there was no FIH-mediated hydroxylation of those HIF fragments previously shown to be substrates for the PHD isoforms, thereby implying that FIH is not a HIF prolyl hydroxylase (Table I, entries 4–6). In contrast, CO₂ production was strikingly stimulated in the presence of HIF-1α fragments containing the CAD (Table I, entries 1–3). Both GST-fused and free forms of a polypeptide encompassing the human HIF-1α CAD (residues 775–826) were observed to cause significant stimulation of FIH-mediated 2-OG turnover. A high ratio (>10:1) of prime substrate coupled:uncoupled 2-OG turnover was observed in the case of the free 775–826 substrate, and preliminary kinetic parameters for this peptide obtained using the 2-OG turnover assay were K_M(peptide) = 10 µM, K_M(n2-OG) = 10 µM, and V_max = 0.3 µmol/min/mg.

Since the 2-OG turnover assay does not directly measure oxidation of the prime substrate, unequivocal demonstration of FIH-mediated hydroxylation was sought from mass spectrometric analyses. Using HIF-1α-(775–826) peptide as a substrate under normoxic conditions, an increase in mass of 16 Da was observed after HPLC isolation of product (Fig. 1a, panels a and b). The results demonstrate that FIH is a 2-OG-dependent hydroxylase that modifies the HIF-1α CAD. To test whether this activity regulates the interaction with the p300 co-activator, GST-fused HIF-1α CAD was exposed to purified recombinant FIH under conditions that support decarboxylation of 2-OG, purified, and assayed for the ability to interact with 35S-labeled CH1 by GST pull-down assay. Fig. 1b shows that...
polypeptides. Mutation of Asn-803 in GST-HIF-1α introduces mutations at this site and performed further 2-OG decarboxylation by HIF-1α/H9251 domain of p300. The autoradiograph shows the capture of 35S-labeled peptide isolated from reaction under 18O2 atmosphere, mass increase of 826 Da; observed mass (MH +) = 6694.7 Da; panel b, peptide isolated from reaction under normoxic (16O2) conditions, mass increase = 16.5 Da; panel c, peptide isolated from reaction under 18O2 atmosphere, mass increase = 18.3 Da. B, FIH modulates HIF-1α CAD binding to the CH1 domain of p300. The autoradiograph shows the capture of 35S-labeled CH1 by HIF-1α CAD that had or had not been preincubated with recombinant FIH. Preincubation with FIH strikingly reduced the ability of HIF-1α CAD to capture CH1 (compare lanes 1 and 2). Inclusion of N-oxaloylglycine in the FIH/HIF-1α CAD reaction step inhibited the effect of FIH. Binding of CH1 to the N803A mutant HIF-1α CAD that is constitutively active was unaffected by preincubation with FIH (lanes 4 and 5).

pretreatment with FIH greatly reduced the ability of the HIF-1α CAD polypeptide to interact with CH1 (compare lanes 1 and 2).

Following previous MS/MS assignment of Asn-803 as the modified residue in the HIF-1α CAD (14), we constructed a series of mutations at this site and performed further 2-OG decarboxylation and interaction assays using the mutant polypeptides. Mutation of Asn-803 in GST-HIF-1α-(775–826) to alanine abolished all activity in the 2-OG decarboxylation assay (Table I, entries 8–10) and prevented modulation of CH1 binding in the interaction assay (Fig 1b, lanes 4 and 5). Mutation to glutamine and glutamate also abolished activity, while an Asp-803 mutant still supported some 2-OG turnover but only at a maximum of 7% of the analogous Asn-803 substrate (Table I, entry 7).

Taken together these results demonstrate that FIH is the dioxygen-requiring Asn-803 hydroxylase that controls HIF-α C-terminal transactivation by regulating the interaction with the CH1 domain of p300. The clear preference of FIH for an asparaginyl rather than an aspartyl residue as substrate contrasts with the previously reported human Asp/Asn hydroxylase that catalyzes hydroxylation at the β-carbon of both aspartyl and asparaginyl residues (22). This observation raises the possibility that FIH-mediated hydroxylation does not occur at the β-carbon of Asn-803 but at another atom. Oxidation of either the α-carbon or the carbonyl oxygen of the primary amide seems unlikely given the relative lack of activity of the Asp-803 and other mutants and likely instability of the putative products. It is possible that nitrogen of the primary amide is hydroxylated to give a hydroxamic acid (CH2CONHOH). The available NMR data suggest that like β-hydroxylation, such a modification would be disruptive to the interaction between CAD and p300 (15, 16).

Further Characterization of FIH Activity—To explore the mechanism of FIH and its relationship to the characteristics of in vivo HIF activation, in vitro analyses of FIH using the purified recombinant protein were performed. Analyses revealed that the purified recombinant FIH contained ~1 metal ion per protein, consistent with the operation of a mono-iron catalytic site.2 FIH was inhibited by N-oxaloylglycine, a known inhibitor of the PHD isozymes and other 2-OG oxygenases, both in bioassays of CH1 capture by HIF-1α CAD (Fig 1b, compare lanes 2 and 3) and in vitro kinetic assays (IC50, 25 μM). Cobalt(II) also inhibited recombinant FIH activity (IC50, 10 μM), explaining the ability of these substances to regulate HIF-1α CAD activity (13). Interestingly addition of exogenous iron(II) to the purified FIH/HIF-1α CAD assay produced no further increase in activity, implying relatively tight metal binding by FIH. This may reflect the ability of the hydroxylated asparagine product to chelate iron. Because of the relationship of FIH to the zinc(II)-binding proteins from the cupin family (see below) we also tested the action of zinc(II) and found similar inhibition to that with cobalt(II) (IC50, 10 μM). Although zinc(II) does not induce a HIF transcriptional response, recent studies have demonstrated that it does stabilize HIF-1α but blocks transactivation by inducing alternative splicing to a shortened form that lacks the CAD (23). Although the physiological relevance of these findings is still unclear, it therefore seems likely that zinc(II) inhibits both FIH and the PHD enzymes.

The requirement of FIH for dioxygen as a co-substrate indicates that, like the PHD isozymes, FIH may act as a cellular oxygen sensor. Indeed, when assayed under conditions of graded reduction of atmospheric oxygen (8), FIH demonstrated large and progressive reductions in HIF-1α CAD activity. Unlike PHD1 and procollagen prolyl hydroxylase (18, 24). These results contrast with similar incorporation experiments with microbial 2-OG oxygenases where incorporation of oxygen from water as well as dioxygen is observed. The exchange process has been proposed to occur via binding of water to a pentacoordinate ferryl species (Fe(IV)=O ↔ Fe(III)–O–) (25). In the case of FIH and PHD1, it may be that the presence of a large peptide substrate at the active site blocks access of water, thereby preventing exchange.

Biological and Structural Implications—The involvement of an additional member of the 2-OG oxygenase superfamily, distinct from the PHD isozymes, in the regulation of HIF transactivation raises several biological issues. First it defines another link between the availability of dioxygen and HIF activity...
that may help shape the physiological characteristics of the transcriptional response to hypoxia. Second it provides an explanation for the characteristic action of cobalt in mimicking the effect of hypoxia on both the isolated degradation and activation domains of HIF-α subunits. Third it provides a further target for the development of therapeutic agents that augment HIF activity in ischemia/hypoxic disease. Fourth the reported interactions of FIH with both histone deacetylases and pVHL (17) suggest that these proteins may be involved in additional oxygen-regulated processes that affect the HIF transcriptional response or other pathways.

Our findings also raise interesting structural and evolutionary issues with respect to the 2-OG oxygenases and related enzymes. Sequence comparisons reveal that FIH, like the PHD isozymes, utilizes a 2-His-1-carboxylate facial triad formed from a conserved HX(D/E)...H motif as found in other 2-OG
oxygenases (19). Together with crystallographic insights and secondary structure predictions, the analyses imply that FIH possesses the jellyroll β-sheet core (double-stranded β-helix) common to other 2-OG oxygenases. They also reveal that FIH possesses a ~40-residue insert between β-strands 4 and 5 of the eight-stranded jellyroll motif (Fig. 2a) and suggest that the structure of the FIH will be significantly different from the PHD isoyzmes, which are closely related to one another.

Although kinetic analyses clearly demonstrate a requirement of FIH for 2-OG, an unusual feature of FIH concerns the identity of residues involved in binding the 5-carboxylate of 2-OG. They also reveal that FIH possesses a common to other 2-OG oxygenases. They also reveal that FIH (CAS1), and the cupin superfamily, exemplified by phosphomannose isomerase (PMI) that, together with the pres-

Mahon et al. (17) used BLAST searches to identify proteins with regions of sequence similarity to FIH. One of these regions (GenBank™ accession number AF168362.1) is a JmjC homology region, present in the jumonji transcription factors, which have been identified as members of the cupin structural superfamily (26). The identification of FIH as an Fe(II)-dependent oxygenase inhibited by Zn(II) prompted us to compare crystal structures from the 2-OG and cupin superfamilies. These analyses reveal a striking similarity between the cores of the 2-OG oxygenases, exemplified by clavamid acid synthase (CAS1), and the cupin superfamily, exemplified by phosphomannose isomerase (PMI) that, together with the presence of conserved motifs, suggests the 2-OG oxygenases belong to the cupin superfamily (Fig. 2b). Further, the HXX...H motif is well established within the cupin superfamily (e.g. in quercetin 2,3-dioxygenase, Ref. 28) and is modified to a QXH...H motif in the case of Type II PMI. Interestingly, the JmjC transcription factors have been implicated in cell growth and heart development (29, 30) and possess a conserved HX(D/E)...H motif (26, 31) as in the 2-OG oxygenases, suggesting that, like FIH and the PHD isoyzmes, they might be iron oxygenases involved in the regulation of transcription.

3 Research Collaboratory for Structural Bioinformatics Protein Data Bank code 1DRY.

4 Research Collaboratory for Structural Bioinformatics Protein Data Bank code 1PMI.

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Kirsty S. Hewitson, Luke A. McNeill, Madeline V. Riordan, Ya-Min Tian, Alex N. Bullock, Richard W. Welford, Jonathan M. Elkins, Neil J. Oldham, Shoumo Bhattacharya, Jonathan M. Gleadle, Peter J. Ratcliffe, Christopher W. Pugh and Christopher J. Schofield

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