Disruption of dimerization and substrate phosphorylation inhibit factor inhibiting hypoxia-inducible factor (FIH) activity

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HIF (hypoxia-inducible factor) is an $\alpha\beta$ transcription factor that modulates the hypoxic response in many animals. The cellular abundance and activity of HIF- α are regulated by its post-translational hydroxylation. The hydroxylation of HIF is catalysed by PHD (prolyl hydroxylase domain) enzymes and FIH (factor-inhibiting HIF), all of which are 2-oxoglutarate- and Fe(II)-dependent dioxygenases. FIH hydroxylates a conserved asparagine residue in HIF- α (Asn-803), which blocks the binding of HIF to the transcriptional co-activator p300, preventing transcription of hypoxia-regulated genes under normoxic conditions. In the present paper, we report studies on possible mechanisms for the regulation of FIH activity. Recently solved crystal structures of FIH indicate that it is homodimeric. Site-directed mutants of FIH at residues Leu-340 and Ile-344, designed to disrupt dimerization, were generated in order to examine the importance of the dimeric state in determining FIH activity. A single point mutant, L340R (Leu-340 $\rightarrow$ Arg), was shown to be predominantly

monomeric and to have lost catalytic activity as measured by assays monitoring 2-oxoglutarate turnover and asparagine hydroxylation. In contrast, the I344R (Ile-344 $\rightarrow$ Arg) mutant was predominantly dimeric and catalytically active. The results imply that the homodimeric form of FIH is required for productive substrate binding. The structural data also revealed a hydrophobic interaction formed between FIH and a conserved leucine residue (Leu-795) on the HIF substrate, which is close to the dimer interface. A recent report has revealed that phosphorylation of Thr-796, which is adjacent to Leu-795, enhances the transcriptional response in hypoxia. Consistent with this, we show that phosphorylation of Thr-796 prevents the hydroxylation of Asn-803 by FIH.

Key words: dimerization, factor inhibiting hypoxia-inducible factor (FIH), hypoxia, oxygen sensing, oxygenase, phosphorylation.

INTRODUCTION

The hypoxic response in animals involves the expression of an array of genes, including those for erythropoietin and VEGF (vascular endothelial growth factor) [1–3], and is mediated by a specific $\alpha\beta$ -heterodimeric transcription factor, HIF (hypoxia-inducible factor), the α -subunit of which accumulates under hypoxic conditions [4]. Since the genes involved in the hypoxic response include those involved in angiogenesis, control of the hypoxic response is of interest from the perspectives of developing new therapies for both cancer and cardiovascular disease.

HIF is involved in the development of tumours associated with defects in the von Hippel–Lindau tumour suppressor protein, pVHL [5]. Both HIF subunits belong to the basic helix–loop–helix PAS [Per–ARNT (aryl-hydrocarbon nuclear translocator)–Sim] protein family [6], and the HIF- $\alpha\beta$ dimer promotes transcription by binding to hypoxia-response elements upstream of target genes. Three forms of the HIF- α subunit are known. The HIF-1 α and HIF-2 α isoforms are closely related (approx. 30 % identity), but HIF-3 α is significantly different and is expressed as a number of alternatively spliced variants [7]. HIF- β , a nuclear protein also known as ARNT, is believed to exist as a single isoform, and its levels are not oxygen-regulated.

Recent studies have shown that both the cellular concentration and activity of HIF- α molecules are regulated by post-translational hydroxylation [8–13]. Under hypoxic conditions, cytoplasmic HIF- α translocates into the nucleus and binds HIF- β , forming the active heterodimer, which then acts in conjunction with nuclear co-activators, including p300. Under normoxic conditions, two different, but related, dioxygenases, PHD (prolyl hydroxylase domain) enzymes and FIH (factor inhibiting HIF) inhibit hypoxic responses by mediating the post-translational hydroxylation of HIF- α (Scheme 1). Sequence alignments [13,14] and X-ray crystallography of FIH [15–17] have been used to show that the PHD isoforms and FIH are members of the family of Fe(II)- and 2OG (2-oxoglutarate)-dependent dioxygenase enzymes [18].

Both HIF-1 α and HIF-2 α contain a central ODDD (oxygen-dependent degradation domain). Pro-402 and Pro-564 (in human HIF-1 α) are situated in two subdomains of the ODDD. Hydroxylation of these residues, catalysed by PHD enzymes, enables binding of HIF- α to the VBC (pVHL–elongin B/C) complex, which recruits an E3 ubiquitin ligase, mediating ubiquitination of HIF- α and its subsequent proteasomal destruction (Scheme 1). Each proline residue forms part of a conserved LXXLAP motif [19–21], and modification of either can independently promote

Abbreviations used: ARNT, aryl-hydrocarbon nuclear translocator; CAD, C-terminal transactivation domain; CBP, cAMP-response-element-binding-protein-binding protein; CITED2, CBP/p300-interacting transactivator with ED-rich tail; ESI, electrospray ionization; FIH, factor inhibiting hypoxia-inducible factor; GST, glutathione S-transferase; HIF, hypoxia-inducible factor; IPTG, isopropyl β -D-thiogalactoside; ODDD, oxygen-dependent degradation domain; 2OG, 2-oxoglutarate; PHD, prolyl hydroxylase domain; pVHL, von Hippel–Lindau tumour-suppressor protein.

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Scheme 1 Role of HIF hydroxylases in the hypoxic response

degradation [22]. The binding of hydroxylated HIF- α to pVHL relies upon two hydrogen bonds formed by the alcohol of the *trans*-4-hydroxylated proline residue in HIF-1 α to the side chains of serine and histidine residues in pVHL [23–26].

Hydroxylation by FIH at the pro-*S* position of the β -carbon of a conserved asparagine (Asn-803 in HIF-1 α) residue in the CAD (C-terminal activation domain) of HIF prevents binding of HIF to the CH1 domain of p300, a nuclear co-activator protein involved in transcription (Scheme 1) [10,11,27,28]. The introduced hydroxy group is thought to disrupt the interaction between HIF- α and p300. Sequence analyses suggested that FIH is one of a discrete subfamily of oxygenases that are involved in transcription [10,27]. Structural analyses of FIH have shown it to be homodimeric (Figure 1A), with each monomer containing the double-stranded β -helix core typical of the 2OG oxygenase superfamily [15]. One structure also revealed that two substrate fragments can bind simultaneously to the FIH homodimer; it is unknown if this is the case with the full-size HIF- α , a protein of 96 kDa. Mutagenesis studies suggest that Val-802, adjacent to the hydroxylated asparagine (Asn-803), is important for substrate recognition by FIH [29]. The interface between the C-terminal α -helices in the FIH dimer is predominantly hydrophobic, and Dann et al. [16] have reported that deletion of the two C-terminal α -helices of FIH prevents binding to a fragment of HIF-2 α . We hypothesized that the introduction of hydrophilic residues at

this site would disrupt the dimer interface (Figure 1B), resulting in a monomeric FIH mutant with altered catalytic activity. We report a single point mutation that disrupts FIH dimerization, and generates a catalytically inactive form of the enzyme, but is capable of binding substrate.

The crystal structure of FIH in complex with fragments of HIF [15] revealed that HIF interacts with FIH predominantly at two sites. Recent studies by Koivunen et al. [30] have also shown that the length of the HIF-1 α fragment used as a substrate affects the rate of 2OG turnover, since a 35-residue peptide of HIF-1 α (788–822) stimulated 2OG turnover by FIH up to 25 times more than shorter peptides [30]. Sequence alignments of HIF-1 α and HIF-2 α reveal that residues interacting with site 1 on FIH have the highest conservation (Figure 2). These residues include the strictly conserved Leu-795 and Thr-796 residues (in human HIF-1 α). It has been proposed that, in addition to hydroxylation of Asn-803, phosphorylation at Thr-796 in the HIF- α CAD region is involved in controlling the hypoxic response [31]. In contrast with the hydroxylation of Asn-803, NMR studies on the complex formed between CAD and p300 do not identify any obvious interactions that may be disrupted by phosphorylation of Thr-796 [32,33]. We therefore used a synthetic 19-residue peptide, corresponding to residues 788–806 of HIF-1 α , phosphorylated at Thr-796, to examine the effect of phosphorylation on the activity of FIH.

Figure 1 Positions of Leu-340 and Ile-344 at the dimer interface

(A) FIH dimer. The two FIH subunits are in red and blue. (B) Close-up of the dimer interface showing the two interlocking C-terminal α -helices. The close proximity of the side chains from Leu-340 suggests that they are involved in maintaining the dimer interface.

EXPERIMENTAL

Generation of mutant genes

Mutations were introduced into the FIH gene in the *fiH*/pET28a construct using the QuikChange mutagenesis method (Stratagene). Primers used were: L340R (Leu-340 $\rightarrow$ Arg): forward, 5'-GTGGGGCCCTTGAGGAACACAATGATCAAGGGC-3'; reverse, 5'-CTTGATCATTGTGTTTCCTCAAGGGCCCCACCTC-3'; I344R (Ile-344 $\rightarrow$ Arg): forward, 5'-CCCTTGTTGAA-CACAATGAGGAAGGGCCGATACAAC-3'; reverse, 5'-TAGTTGTATCGGCCCTTCCTCATTGTGTTCAACAAGGG-3'; L340R/I344R: forward, 5'-GAGGTGGGGCCCTTGAGGAACACAATGAGGAAGGGCCG-3'; reverse, 5'-CTAGTTGTATCGGCCCTTCCTCATTGTGTTTCCTCAAGGGCC-3'; FIH₁₋₃₃₁: forward, 5'-GAGGCCTTGGGGTAGCCACAAGAGGTCGGG-3'; reverse, 5'-CACCTCTTGTGGCTACCCCAAGGCCTCTCCAAG-3'. All mutations were confirmed by DNA sequencing.

Protein expression

Expression of wild-type and mutant FIH in *Escherichia coli* BL21(DE3) cells grown in 2TY medium [1.6% (w/v) tryptone/1% (w/v) yeast extract/0.5% (w/v) NaCl] supplemented with 30 μ g/ml kanamycin was induced by IPTG (isopropyl β -D-thiogalactoside) as described previously [10]. Wild-type and mutant FIH was purified on nickel-affinity resin (Novagen), followed by cleavage of the His₆ tag with thrombin and subsequent size-exclusion chromatography on S75 Sephadex resin as described in [10]. SDS/PAGE analysis showed that all proteins were purified to greater than 95% purity. The *hif-1 α* -(786–826)/pGEX-6P-1 DNA construct was transformed into *E. coli* BL21(DE3) and expressed using IPTG induction as described in [10]. Purification was on glutathione–Sephadex 4B resin (Amersham Biosciences), followed by desalting into 50 mM Tris/HCl, pH 7.5, on a PD10 column (Amersham Biosciences).

Figure 2 Sequence alignment of HIF- α and related proteins showing the conservation of residues that bind to site 1 of FIH-1

Grey arrows indicate the strictly conserved Leu-795 and Thr-796. gi number 40849965, HIF-1 α (*Ctenopharyngodon idella*); gi number 32469773, HIF-1 α (*Oncorhynchus mykiss*); gi number 27696866, HIF-1 α (*Xenopus laevis*); gi number 25392208, HIF-1 α (*Gallus gallus*); gi number 4504385, HIF-1 α (*Homo sapiens*); gi number 38374005, HIF-1 α (*Canis familiaris*); gi number 31095610, HIF-1 α (*Oryctolagus cuniculus*); gi number 40254439, endothelial PAS (Per-ARNT-Sim domain protein) domain protein 1 (*H. sapiens*); gi number 27807411, endothelial PAS domain protein 1 (*Bos taurus*); gi number 2895756, HIF-1 α related factor (*Mus musculus*); gi number 12831205, endothelial PAS domain protein 1 (*Rattus norvegicus*); gi number 5051910, endothelial PAS domain protein 1 (*G. gallus*); gi number 6644408, HIF-2 α (*Coturnix coturnix*); gi number 19716307, HIF-2 α (*Fundulus heteroclitus*).

CD

CD spectra of wild-type and mutant FIH samples (25 μ M) were obtained using a Jasco J720 spectropolarimeter with 1 mm path-length quartz cuvettes.

Native gel electrophoresis

Native gel electrophoresis was conducted using linear 12.5% polyacrylamide gels. Approx. 10 μ g of each protein sample was loaded and run at 100 V at 4 $^{\circ}$ C.

MS

ESI (electrospray ionization) mass spectra of wild-type and mutant FIH (10 μ M) were obtained on a VG Bio-Q quadrupole instrument with a cone voltage of 50 V. Native MS was carried out on a Waters Q-ToF Micro mass spectrometer in 5 mM ammonium acetate.

Molecular mass determination

Analytical gel filtration was carried out on a 30 ml Superdex S75 column (Amersham Biosciences), calibrated using Blue Dextran (Sigma), isopenicillin N synthase [34] (prepared in house), carbonic anhydrase (Sigma), and cytochrome *c* (Sigma).

Enzyme assays

Enzymic activity was measured using a method based on that used to measure 14 CO $_2$ release by α -oxoisocaproate oxygenase [35]. Standard assay conditions consisted of a total volume of 100 μ l, 50 mM Tris/HCl, pH 7.5, 4 mM ascorbate, 2 mM dithiothreitol, 80 μ M 2OG (2.5% [14 C]), 80 μ M (NH $_4$) $_2$ SO $_4$ · FeSO $_4$ · 6H $_2$ O, 0.48 mg/ml catalase, 0.3 μ M FIH and 100 μ M substrate. In the case of the FIH mutant assays, this was GST (glutathione S-transferase)–HIF-1 α -(786–826), whereas for the assay of phosphorylated substrate a synthetic 19-residue peptide corresponding to residues 788–806 and phosphorylated at Thr-796 (Institute of Biomolecular Sciences, Southampton, U.K.) was used. Assays were performed in triplicate unless otherwise stated. Values quoted are the means $\pm$ S.D.

Substrate hydroxylation was analysed by HPLC–MS. Incubations were performed as described for the activity assay above, except that 2.5% [14 C]2OG was replaced with unlabelled 2OG, and GST–HIF-1 α -(786–826) with a synthetic 19-residue peptide corresponding to residues 788–806. After 30 min of incubation, the reaction mixtures were injected directly on to a Jupiter C4 HPLC column (15 cm $\times$ 4.6 mm), and separated using a linear gradient of acetonitrile in 0.05% methanoic (formic) acid. The flow from the column was analysed on a Micromass ZMD quadrupole mass spectrometer.

Surface area calculations

The difference in buried surface area of site 1 with and without helix α 12 (332–349) and then without α 11 and α 12 (303–349) was calculated using the FIH–CAD crystal complex (Protein Data Bank code 1H2L) [15]. The program GRASP [36] was used to calculate buried surface area between substrate and enzyme. Residues were simply deleted from the co-ordinate file to represent appropriate truncation mutants. Substrate residues 795–806 were used to represent binding site 1.

RESULTS

Production and characterization of mutant proteins

Analysis of the crystal structure of FIH complexed with iron(II), 2OG and a CAD fragment (Figure 1A) led to the proposal that mutation of either Leu-340 or Ile-344 to a hydrophilic residue might, by virtue of the 2-fold symmetry of the interface, be expected to introduce unfavourable interactions (Figure 1B). Arginine was chosen to replace the hydrophobic leucine and isoleucine residues, since it was hoped that its bulky, positively charged side chain would disrupt the hydrophobic interactions between the two FIH subunits. We created a C-terminal-truncation mutant of FIH by deleting the C-terminal α -helix of FIH (α 12, Gly-331–Leu-349). Successful mutagenesis of the FIH gene allowed production and purification of mutant proteins using the methods described for wild-type FIH [10]. SDS/PAGE and ESI–MS analyses were consistent with the predicted masses for the L340R, I344R and L340R/I344R mutants (Table 1). Analytical gel filtration on Superdex 75 showed that I344R elutes predominantly in the same volume as wild-type FIH, suggesting a dimeric enzyme, whereas L340R, L340R/I344R and FIH $_{1-331}$ eluted predominantly as monomeric species (Figure 3). The traces indicated that, under the assay conditions, there may be a very small (<5%) amount of monomeric enzyme in the case of the I344R mutant which is not seen in wild-type FIH. Likewise, the L340R mutant appeared to exhibit some dimeric properties by gel filtration. However, this was also observed for the truncated mutant, FIH $_{1-331}$, which exists predominantly as a monomer by native PAGE analysis. It is unlikely that gross alterations in the folding of the protein can account for these differences, since the CD spectra of the L340R, I344R, L340R/I344R mutants were similar to the wild-type (results not shown). Like wild-type FIH [15], the I344R mutant behaved as a dimer by native PAGE analysis (see the Supplementary Figure at <http://www.BiochemJ.org/bj/383/bj3830429add.htm>), but the L340R and L340R/I344R mutants gave a smeared appearance, perhaps reflecting monomer–dimer equilibria under these conditions.

Activity assays

Assays detecting the decarboxylation of the 2OG co-substrate revealed a correlation between the quaternary structure and catalytic activity of the enzyme (see Figure 5 and Table 1). The

Table 1 Summary of the characterization analyses for the mutant FIH proteins

calc., calculated; n.d., not determined due to precipitation of protein in acid required for ESI-MS; n.a., not analysed.

Mutant	Apparent molecular mass			Predominant oligomeric state		Activity	
	SDS/PAGE (kDa)	Native PAGE (kDa)	ESI-MS (Da) (calc.)	Size exclusion	Native ESI-MS	¹⁴ CO ₂ assay (% of wild-type)	Hydroxylation assay
Wild-type	41	80	40 566 (40 566)	Dimer	Dimer	100 (20 in the absence of CAD)	Yes
L340R	41	Smear	40 608 (40 609)	Monomer	Monomer	20	No
I344R	41	80	40 608 (40 609)	Dimer	Dimer	100	Yes
L340R/I344R	41	Smear	40 653 (40 652)	Monomer	n.a.	24	No
FIH ₁₋₃₃₁	38	38	n.d.	Monomer	n.a.	16	No

Figure 3 Quantitative gel filtration of FIH mutants

Superdex S75 column calibrated using Blue Dextran (2 MDa), cytochrome *c* (12.4 kDa), carbonic anhydrase (28 kDa) and isopenicillin N synthase (37 kDa). L340R/I344R eluted, as shown for L340R FIH. mAu, milli-absorbance units.

dimeric mutant, I344R, displayed similar activity to wild-type FIH in the decarboxylation assay, but the predominant monomeric mutants showed greatly reduced turnover, barely above that exhibited when the assay was performed in the absence of enzyme. The catalytic hydroxylation activity of the I344R mutant was confirmed using a 19-residue synthetic peptide, corresponding to HIF-1 α -(788–806), as a substrate followed by HPLC/MS analysis. No hydroxylation activity was observed for the other mutants (Table 1). Comparison of the activity of FIH towards a synthetic 19-residue peptide with and without a phosphate group on Thr-796, revealed that hydroxylation of the peptide was not observed, and evolution of CO₂ from 2OG was not stimulated.

Native ESI-MS

Under mild ESI conditions, the native structures of proteins can be substantially retained and analysed by MS [37]. In the present study, wild-type, L340R and I344R forms of FIH were examined. The dimeric form of both the wild-type and I344R FIH was clearly observed (Figures 4a and b). It was noted, however, that the charge states of wild-type and I344R FIH differed, with the I344R mutant becoming increasingly charged during the ionization procedure. This may reflect the change from a neutral to a basic residue in

I344R, and that wild-type FIH retained native structure under a higher cone voltage than the mutant (up to 200 V for wild-type, but only 100 V for I344R FIH, see the Supplementary Figure at <http://www.BiochemJ.org/bj/383/bj3830429add.htm>), indicating that wild-type FIH may possess a more stable fold than the I344R mutant. In addition, I344R FIH showed some evidence of a partially unfolded monomer/dimer species which is absent in the spectrum of the wild-type shown here, but appeared in a wild-type sample stored at –20 °C for several months (results not shown). The native mass spectrum for the L340R mutant showed no evidence for the presence of the dimer at 60 V (Figure 4c), and lowering the cone voltage further did not provide any evidence for the dimer. This analysis therefore supports the finding that the single point mutation L340R significantly blocks FIH dimerization *in vitro*.

Similar mass spectrometric conditions were also used to study the binding of the 19-residue synthetic CAD peptide to the wild-type and mutant FIH proteins. At a CAD/enzyme ratio of 3:1, the analyses implied that dimeric wild-type and I344R FIH bound two CAD peptides, whereas monomeric L340R FIH bound one peptide at a cone voltage of 60 V (Figures 4d, e and f). Despite its catalytic inactivity, monomeric L340R FIH, however, maintained the binding of a single CAD fragment up to 100 V.

DISCUSSION

Oligomerization is well established as a mechanism for controlling enzyme activity. For example, hormone-sensitive lipase, the mammalian enzyme responsible for liberating fatty acids from adipose tissue has been shown to become 40 times more active as a dimer than as a monomer [38], and pro-caspase 8, a member of the pro-apoptotic cascade of proteases only becomes active as a homodimer, presumably as a defence against aberrant apoptosis [39]. Active 2OG oxygenases are known to occur in monomeric [40] and oligomeric [41,42] forms, and it has been shown that wild-type FIH is predominantly dimeric both by X-ray crystallographic and solution analyses [15].

Dann et al. [16] have reported that a truncated FIH mutant (FIH₁₋₃₀₂), in which the two C-terminal α -helices were deleted, did not interact with a HIF-2 α -(774–874) peptide fragment using a pull-down assay. This approach, involving the removal of both the C-terminal helices (α 11 and α 12) decreases the calculated buried surface area at site 1 buried by the CAD fragment from 1642 Å² (1 Å = 0.1 nm) to 1244 Å². The removal of only helix α 12 (the FIH₁₋₃₃₁ mutant) gave no decrease in the interacting surface area and yet still resulted in the disruption of dimerization. Dimerization is also substantially hindered, if not prevented, by a single point mutation, L340R, designed to disrupt symmetry-related hydrophobic interactions at the dimer interface. This latter

Figure 4 Native ESI-MS spectra of wild-type, I344R and L340R FIH (a–c), and in the presence of 3-fold excess CAD (d–f)

All spectra run with a cone voltage of 60 V. Numbers refer to the charge states of the FIH, asterisks indicate species with CAD bound and m indicates a monomeric species.

Figure 5 Graphs showing activity of FIH as measured by the 2OG turnover assay

Comparison of wild-type and mutant enzyme in the presence (black bars) and absence (grey bars) of prime substrate GST-HIF-1 α -(786–826).

mutant was inactive both in terms of hydroxylation activity and 2OG turnover assays (Figure 5 and Table 1). In contrast, the I344R mutant was, like wild-type FIH, fully active and predominantly dimeric. The native mass spectrometric analyses extended the results obtained by gel filtration and electrophoresis. Significant binding of one molecule of synthetic HIF CAD-(788–806) to the dimeric form of wild-type FIH was observed by MS. Since some monomeric FIH was detected, it was not possible from the present work alone to conclude that substrate binds preferentially to the dimeric form. However, in the case of the active I344R mutant under the same assay conditions (substrate/enzyme, 3:1), both dimeric and monomeric forms were detected, but, for this mutant, only binding of one substrate molecule to the dimer was observed.

The results with the I344R mutant thus imply that, although it is fully active under the standard assay conditions, the mutation has weakened the dimer and that binding of the substrate, at least with this mutant, occurs preferentially to the dimeric form. With the catalytically inactive L340R, MS indicated that the mutant was not substantially dimeric, but that the monomeric form was able to bind substrate.

Taken together, these results imply that the dimeric form of FIH is the active form under standard assay conditions, demonstrate that the L340R mutation significantly hinders dimerization leading to an inactive enzyme, and may be useful in the design of FIH inhibitors that block dimer formation and/or productive substrate binding at the dimer interface. The results, albeit obtained with a fragment of the natural substrate and with the L340R mutant, also demonstrate that the monomeric form of FIH can bind substrate. Analysis of the FIH crystal structure complexed with substrate [HIF-1 α -(786–826)] reveals that Leu-340 and Ile-344 do not interact directly with the substrate. Sequence alignments of FIH from several species show that Leu-340 is more conserved than Ile-344 (Figure 6). Leu-340 is buried deep in the hydrophobic dimer interface with no access to solvent, but, conversely, Ile-344 is in a location that is solvent-accessible, rationalizing why dimer formation still occurs in the case of the I344R mutant. It cannot be ruled out that the modification of the side chain in the I344R mutant leads to a binding interaction that is not present in the wild-type structure, perhaps involving the methylene groups of the arginine side chains, but, given the large dimer interface (3210 Å²), this seems unlikely.

The most N-terminal residue of the HIF fragment observed in the crystal structure of the FIH–substrate complex is Leu-795, which projects into a hydrophobic pocket at the FIH dimer–HIF interface (Figure 7). NMR studies on complexes of HIF-1 α with p300/CBP (cAMP-response-element-binding-protein-binding protein) show the same residue of HIF, Leu-795, is buried in

Figure 6 Sequence alignment of FIH-1 from *Homo sapiens* (gi number 14043456) and its known close homologues from *Rattus norvegicus* (gi number 34863451), *Xenopus tropicalis* (gi number 38181679) and *Danio rerio* (gi number 27882525)

The locations of C-terminal helices $\alpha 11$ and $\alpha 12$ are indicated above the sequence. Grey arrowheads under the sequence indicate Leu-340 and Ile-344. Strict conservation is observed for Leu-340, while Ile-344 is not fully conserved, consistent with the results observed for the FIH mutants L340R and I344R (see text).

Figure 7 Surface representation of the FIH dimer

Hydrophobic residues of the surface in both molecules of the dimer are coloured yellow, while the remaining residues are coloured green and blue in their respective molecules. The dimer interface is primarily hydrophobic. The HIF CAD fragment is shown in stick representation with different carbon atom colouring (salmon and white) for each molecule. The inset shows the hydrophobic pocket formed at the dimer interface in which the HIF Leu-795 is buried. The Figure was created using the PyMOL Molecular Graphics System (<http://www.pymol.org>).

a pocket of the CH1 domain of the p300/CBP complex [32,33]. Thus Leu-795 probably plays an important role both in productive binding of HIF to FIH and in binding to p300/CBP. Furthermore, studies of the HIF-1 α negative regulator, CITED2 (CBP/p300-interacting transactivator with ED-rich tail), complexed with CH1 p300/CBP, has revealed that the same CH1 pocket is occupied by a conserved leucine residue from CITED2 [43].

Given that our mutagenesis studies at the dimerization interface of FIH have indicated that modification of the enzyme–substrate interactions at a site relatively distant from the active-site iron could deleteriously affect catalysis, we were interested in the effect that phosphorylation of Thr-796 of HIF-1 α , adjacent to this

hydrophobic interaction, would have on FIH activity. In contrast with its non-phosphorylated analogue [10], a synthetic HIF-1 α -(788–806) fragment, phosphorylated at Thr-796 did not become hydroxylated by FIH, nor did it stimulate 2OG turnover. Full kinetic analysis on the post-translational modification of HIF has been hampered by the lack of procedures for producing soluble, intact HIF proteins. Consequently, most kinetic experiments are performed using either synthetic HIF peptides or recombinant HIF fragments. In the case of FIH substrates, Koivunen et al. [30] reported recently that HIF-1 α -(788–806) stimulates 2OG turnover by FIH to a lesser extent than a 35-residue peptide [HIF-1 α -(788–822)], and that the 35-mer binds FIH with a K_m of 100 μ M.

Scheme 2 Potential roles for phosphorylation in the control of the HIF-1 α transactivation

Preliminary analyses indicated that the K_m of a synthetic 19-mer peptide HIF-1 α -(788–806) is approx. 400 μ M (L. A. McNeill, unpublished work). Consequently, any effect of phospho-HIF-1 α -(788–806) on FIH activity would also be predicted to occur using longer substrates [e.g. HIF-1 α -(788–822)]. Modelling studies of a HIF CAD peptide phosphorylated at Thr-796 complexed to FIH suggests that phosphorylation will disrupt the binding interaction between FIH and HIF- α . The conservation of Thr-796 (Figure 2), suggests that phosphorylation of HIF-1 α and HIF-2 α CAD could also be a regulatory mechanism in other species. Although the NMR structural complexes of p300/CBP CH1 domain and a HIF-1 α CAD fragment do not show significant interactions involving Thr-796, it is possible that phosphorylation may mediate other protein interactions in the transcriptional complex.

HIF-1 α is phosphorylated at several, possibly multiple, sites [44], but the relevant kinases have yet to be identified. PKC ζ (protein kinase C ζ) has been shown to modulate the interaction between HIF-1 α and p300, but a direct role for it in HIF- α phosphorylation has not been demonstrated [45]. Phosphorylation at Thr-796 in HIF-1 α increases the affinity of the interaction between HIF-1 α and the transcriptional co-activator CBP/p300, and may be necessary for transcriptional activation [31]. Since phosphorylation of other transactivation domains has been described, its occurrence as part of the HIF- α regulatory mechanism is not itself surprising. Although inhibition of FIH activity by substrate phosphorylation may promote the binding of HIF- α to CBP/p300, phosphorylation promotes binding in an oxygen-independent manner [31]. At this stage, interpretation of the

results should be regarded as preliminary, not least because most of the available data derive from studies utilizing fragments rather than full-length HIF- α . However, it seems likely that both hydroxylation and phosphorylation of the HIF- α CAD are both part of the regulation of HIF activity. One possibility is that phosphorylation/dephosphorylation represent a way of reversibly regulating the transactivation activity of CAD, whereas hydroxylation of Asn-803 irreversibly deactivates the CAD (Scheme 2). From a mechanistic perspective, this is reasonable since the 2OG oxygenases catalyse an essentially irreversible oxidation, whereas phosphorylation may be reversed by the action of phosphatases.

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