FIH-1: a novel protein that interacts with HIF-1α and VHL to mediate repression of HIF-1 transcriptional activity

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Hypoxia-inducible factor 1 (HIF-1) is a master regulator of oxygen homeostasis that controls angiogenesis, erythropoiesis, and glycolysis via transcriptional activation of target genes under hypoxic conditions. O2-dependent binding of the von Hippel-Lindau (VHL) tumor suppressor protein targets the HIF-1α subunit for ubiquitination and proteasomal degradation. The activity of the HIF-1α transactivation domains is also O2 regulated by a previously undefined mechanism. Here, we report the identification of factor inhibiting HIF-1 (FIH-1), a protein that binds to HIF-1α and inhibits its transactivation function. In addition, we demonstrate that FIH-1 binds to VHL and that VHL also functions as a transcriptional corepressor that inhibits HIF-1α transactivation function by recruiting histone deacetylases. Involvement of VHL in association with FIH-1 provides a unifying mechanism for the modulation of HIF-1α protein stabilization and transcriptional activation in response to changes in cellular O2 concentration.

[Key Words: Corepressor; histone deacetylase; hypoxia; transactivation]

Received July 2, 2001; revised version accepted August 21, 2001.
Figure 1. Identification of FIH-1 by yeast two-hybrid screen. (A) Structure of HIF-1α and the bait and prey proteins. HIF-1α contains basic helix–loop–helix (bHLH) and PAS domains that are required for dimerization and DNA binding, a proline/serine/threonine-rich protein stabilization domain (PSTD), two transactivation domains (TAD-N and TAD-C), and an inhibitory domain (ID) that negatively regulates transactivation domain function under nonhypoxic conditions. For two-hybrid screening, the bait vector encoded a chimeric protein consisting of the DNA-binding domain from the yeast GAL4 transcription factor (GAL4 DBD) fused to residues 576–826 of HIF-1α. The prey vectors encoded the GAL4 transactivation domain (GAL4 TAD) fused to residues encoded by human brain cDNAs, one of which encoded FIH-1. (B) Amino acid sequence of FIH-1 and identification of related protein sequences. TBLASTN searches of GenBank databases were performed using the human FIH-1 sequence (top line). For each GenBank entry only those sequences showing significant similarity to FIH-1 are shown.
TAD-C (residues 786–826), which bind coactivators including CBP, p300, SRC-1, and TIF-2 (Arany et al. 1996; Ema et al. 1999; Carrero et al. 2000). Residues 576–785 comprise an inhibitory domain the deletion of which increases transactivation domain function especially under nonhypoxic conditions (Jiang et al. 1997). Transactivation domain function is negatively regulated by O₂ independently of protein stability. Exposure of cells to hypoxia, cobalt chloride, or the iron chelator desferrioxamine induces both HIF-1α protein stabilization and transactivation (Jiang et al. 1997; Pugh et al. 1997), suggesting the existence of a common molecular mechanism.

We have utilized the yeast two-hybrid assay to screen for proteins that interact with HIF-1α and modulate its biological activity. Here, we report the identification and characterization of FIH-1 (factor inhibiting HIF-1), a negative regulator of HIF-1α transactivation domain function. We demonstrate that FIH interacts with HIF-1α as well as with VHL and that both FIH-1 and VHL inhibit HIF-1α transactivation domain function. The involvement of VHL provides a unifying mechanism for the O₂-dependent regulation of HIF-1α protein stability and transcriptional activity.

**Results**

**Identification of FIH-1 in a screen for HIF-1α-interacting proteins**

To perform the two-hybrid assay (Fields and Song 1989), his³ yeast strain Y190 was cotransformed with a bait vector, encoding the GAL4 DNA-binding domain expressed in-frame with HIF-1α residues 576–826 (comprising the inhibitory domain and TAD-C) and prey vectors allowing expression of human brain cDNA sequences in-frame with the GAL4 transactivation domain (Fig. 1A). Yeast transformants (2 × 10⁶) were screened for histidine auxotrophy and α-galactosidase (α-gal) expression. The yeast colony demonstrating the strongest α-gal activity was subjected to additional positive and negative screens (as described in Materials and Methods), and both histidine auxotrophy and α-gal activity were found to be dependent on the combined presence of bait and prey vectors.

Nucleotide sequence analysis revealed that the prey vector contained a 2.98-kb human cDNA encoding a 349-amino-acid protein that was subsequently designated FIH-1 (GenBank accession no. AF395830). A BLASTN search revealed high sequence similarity to the human genomic DNA sequence in GenBank (see below) was in agreement with the FIH-1 cDNA sequence. EST data indicate that FIH-1 is expressed in multiple cell types derived from adrenal, bladder, brain [adult and fetal], breast, colon, embryo, foreskin, germ cell, heart, kidney, lung, lymph, marrow, muscle, nerve, ovary, parathyroid, prostate, skin, testis, tonsil, and uterus.

**Interaction of FIH-1 with HIF-1α in vitro**

³⁵S-labeled HIF-1α was synthesized in vitro and tested for its interaction with bacterially expressed glutathione-S-transferase (GST) or GST fused to FIH-1. GST pull-down assays revealed that HIF-1α bound to GST–FIH-1 but not to GST alone (Fig. 2A). Next, FIH-1 was synthesized in vitro and tested for its interaction with GST alone or fused to HIF-1α sequences spanning the entire protein. Amino acids 531–826 of HIF-1α interacted with FIH-1, whereas residues 1–329 and 429–608 did not (Fig. 2B). Further analysis of GST fusion proteins containing sequences from the C terminus of HIF-1α revealed that deletions that eliminated all but residues 757–826 did not affect binding to FIH-1 [Fig. 2C]. HIF-1α residues 786–826 (TAD-C) did not interact with FIH-1, nor did residues 531–575 [TAD-N], whereas residues 576–784 (inhibitory domain) interacted with FIH-1 but with reduced efficiency compared with residues 531–826 (Fig. 2D). These data indicate that sequences within the inhibitory domain of HIF-1α were required for the interaction with FIH-1 but that optimal binding also required TAD-C.

**FIH-1 inhibits transcriptional activation mediated by HIF-1α in human cells**

Human embryonic kidney 293 cells were cotransfected with reporter plasmid p2.1, which contains an SV40 promoter-luciferase transcription unit downstream of a 68-bp hypoxia-response element that mediates HIF-1-dependent gene transcription (Semenza et al. 1996). As demonstrated previously, reporter gene expression was markedly increased in cells exposed to 1% O₂ relative to cells exposed to 20% O₂ (Fig. 3A), reflecting hypoxia-induced HIF-1α stabilization and transactivation. Cotransfection of an expression vector encoding FIH-1 resulted in a dose-dependent inhibition of reporter gene expression at 1% O₂. Transcription at 20% O₂ was un-
were incubated with 35S-labeled in vitro-translated FIH-1, captured on glutathione–Sepharose beads, and analyzed as described above. (0) GST only.

effected, as expected because HIF-1α is not detectable in nonhypoxic 293 cells. Similar results were obtained when Hep3B human hepatoblastoma cells were analyzed (Fig. 3B). Transfection of Hep3B cells with an expression vector in which the FIH-1 cDNA was inserted in the antisense orientation resulted in increased reporter gene expression at 1% O2 (Fig. 3C) and similar results were observed in 293 cells (data not shown). Reporter gene expression was unaffected by co-transfection of the antisense FIH-1 expression vector in 293 cells exposed to 20% O2 as expected because HIF-1α is not present (Fig. 3D). However, forced expression of HIF-1α at 20% O2 mediated increased p2.1 expression that was dramatically potentiated by cotransfection of the antisense FIH-1 expression vector. These data suggest that endogenous FIH-1 inhibits the transcriptional activity of HIF-1α in nonhypoxic cells.

To test this hypothesis, Hep3B cells were co-transfected with reporter plasmid pG5ElbLuc, containing five GAL4-binding sites upstream of an adenovirus E1b promoter-luciferase transcription unit, and an expression vector encoding the GAL4 DNA-binding domain alone (Gal0) or in frame with HIF-1α residues 531–826 (GalA). In contrast to HIF-1α, GalA is constitutively expressed (Jiang et al. 1997) because it does not contain the entire domain (residues 429–608) required for O2-dependent ubiquitination and degradation (Huang et al. 1998; Sutter et al. 2000). As shown previously, the HIF-1α sequences in GalA resulted in greatly increased reporter gene transactivation, especially under hypoxic conditions (Fig. 3E).

Cotransfection of the FIH-1 expression vector markedly inhibited GalA-mediated transactivation under both nonhypoxic and hypoxic conditions, whereas it had no effect on basal transcription mediated by Gal0.

**Figure 2.** Localization of HIF-1α residues interacting with FIH-1. [A] GST and GST–FIH-1 fusion proteins were expressed in E. coli, purified, incubated with 35S-labeled in vitro-translated HIF-1α, captured on glutathione–Sepharose beads, and analyzed by SDS-PAGE and autoradiography. (B–D) GST-fusion proteins, containing the indicated HIF-1α residues at their C terminus were incubated with 35S-labeled in vitro-translated FIH-1, captured on glutathione–Sepharose beads, and analyzed as described above. (0) GST only.

**FIH-1 interacts with VHL in vitro**

Because FIH-1 and VHL are both negative regulators of HIF-1α, we investigated whether they interact with one another. 35S-labeled VHL bound to a GST–HIF-1α(429–608) fusion protein that had previously been incubated with reticulocyte lysate to induce proline hydroxylation (Fig. 4A) as expected on the basis of the known interaction of VHL with residues 556–574 of HIF-1α (Ivan et al. 2001; Jaakkola et al. 2001). 35S-labeled FIH-1 bound to GST–HIF-1α(429–608) in the presence of VHL (Fig. 4A, lane 2). However, in the absence of VHL, no FIH-1 binding was observed (lane 3), as shown in Figure 2B. Similarly, FIH-1 bound to GST–HIF-1α(757–826), whereas VHL bound in the presence but not in the absence of FIH-1 (lanes 4–6).

To demonstrate binding directly and to localize the region of FIH-1 that interacted with VHL and with HIF-1α, 35S-labeled FIH-1 that was either full length [1–349) or lacking N-terminal residues [126–349] was synthesized and tested for interaction with FLAG-tagged VHL or GST–HIF-1α[531–826]. FIH-1[1–349] bound to FLAG-VHL or GST–HIF-1α[531–826], whereas FIH-1[126–349] bound only to GST–HIF-1α[531–826] (Fig. 4B). These data indicate that VHL and HIF-1α interact with FIH-1 at distinct sites, with the VHL-binding site located N-terminal to the HIF-1α-binding site.

C-terminal truncations of VHL were also tested for interaction with GST–FIH-1 or reticulocyte lysate-treated GST–HIF-1α(429–608). Whereas VHL residues 1–155 were sufficient for binding to FIH-1, residues 1–213 were required for efficient binding to HIF-1α (Fig. 4C). These studies indicate the presence of distinct binding sites that allow the simultaneous interaction of FIH-1, HIF-1α, and VHL.

Finally, we analyzed the interaction of VHL with GST fusion proteins, containing different regions of HIF-1α, that were either untreated or preincubated with reticulocyte lysate. The binding of VHL to GST–HIF-1α[531–575] was strictly dependent on preincubation with reticulocyte lysate (Fig. 4D). VHL did not bind to GST–HIF-1α[786–826] or GST–HIF-1α[757–826] in the presence or absence of lysate. Remarkably, significant binding of VHL to GST–HIF-1α[531–826] was detected in the absence of lysate. These results suggest that VHL may interact with other regions of HIF-1α or with other
Interaction of FIH-1 and HIF-1α in human cells

The results of the GST-pulldown and GAL4-transactivation studies suggest that FIH-1 interacts with HIF-1α in human cells. To test this hypothesis directly, 293 cells were cotransfected with expression vectors encoding FIH-1 tagged with hemagglutinin (HA) epitope, FLAG–VHL, and HIF-1α. Aliquots of whole cell lysates were analyzed for expression of the proteins directly or following immunoprecipitation of HA–FIH-1 and HIF-1α (Fig. 5). Exposure of cells to 1% O2 did not alter the interaction of HIF-1α and HIF-1α-binding proteins [e.g., FIH-1] by a mechanism that does not require lysate-dependent hydroxylation.

Figure 3. Effect of FIH-1 on HIF-1-mediated reporter gene transcription. Human 293 (A,D) or Hep3B (B,C) cells were cotransfected with pSV-Renilla, a reporter gene containing the SV40 promoter and Renilla luciferase-coding sequences, p2.1, a reporter gene containing a 68-bp hypoxia-response element upstream of the SV40-promoter and firefly luciferase-coding sequences, and the indicated amount of expression vector containing FIH-1 cDNA [inserted in either the sense or antisense (AS) orientation], HIF-1α cDNA, or empty vector (EV). For each expression vector, the amount (in nanograms) of plasmid DNA transfected is indicated. (E) Hep3B cells were co-transfected with pSV-Renilla, reporter pG5ElbLuc, containing five GAL4-binding sites upstream of an adenovirus Elb promoter and firefly luciferase coding sequences, expression vector encoding the GAL4 DNA-binding domain alone (Gal0) or fused to HIF-1α residues 531–826 (GalA), and EV or vector encoding FIH-1. In each panel, cells were exposed to 20% [open bars] or 1% [closed bars] O2 for 16 h and the ratio of firefly:Renilla luciferase activity was determined. The results were normalized to those for cells transfected with EV and exposed to 20% O2 [relative luciferase activity]. The mean and standard deviation based on 3–9 independent transfections are shown.
FIH-1, consistent with the ability of overexpressed FIH-1 to inhibit HIF-1α transactivation domain function at both 20% and 1% O2 [Fig. 3]. The proteasome inhibitor MG132 increased HIF-1α expression and the recovery of HIF-1α in anti-HA immunoprecipitates [Fig. 5]. These results demonstrate the interaction of FIH-1 and HIF-1α in human cells. However, because the interaction was detected in cells overexpressing these proteins, no conclusion can be drawn as to whether this interaction is regulated by the cellular O2 concentration. HIF-1α protein expression was not regulated, and no interaction of VHL with FIH-1 or HIF-1α could be demonstrated under these conditions [data not shown], suggesting that VHL expression may have been limiting.

Inhibition of HIF-1α transactivation domain function by VHL and FIH-1 in human cells

We analyzed the effect of FIH-1, VHL, or both proteins on transactivation mediated by GAL4–HIF-1α fusion proteins that contained binding sites for FIH-1, VHL, both proteins, or neither protein. Hep3B cells were first cotransfected with reporter pG5ElbLuc and expression vector pGaA, which encodes HIF-1α[531–826] and thus includes the binding sites for both VHL and FIH-1. Forced expression of VHL mediated a dramatic inhibition of reporter gene transactivation by GaA in both nonhypoxic and hypoxic cells that was similar to the

Figure 4. Interaction of FIH-1, HIF-1α, and VHL in vitro. (A) GST-fusion proteins containing HIF-1α residues 429–608 or 757–826 were expressed in E. coli, purified, and incubated with 35S-labeled in vitro-translated FLAG-tagged VHL or HA-tagged FIH-1, captured on glutathione–Sepharose beads, and analyzed by SDS-PAGE and autoradiography. [B] 35S-labeled in vitro-translated FIH-1 residues 1–349 or 126–349 was incubated with unlabelled FLAG–VHL [top] or GST–HIF-1α(531–826) [middle], which were pulled down on beads containing anti-FLAG antibody or glutathione, respectively, and analyzed by SDS-PAGE along with aliquots of the input FIH-1 polypeptides [bottom]. [C] 35S-labeled in vitro-translated FLAG–VHL truncated at its C terminus as indicated was incubated with unlabelled lysate-treated GST–HIF-1α[429–608] [top] or GST–HA–FIH-1 [middle], which were captured on glutathione–Sepharose beads and analyzed by SDS-PAGE along with aliquots of the input VHL polypeptides [bottom]. [D] GST or the indicated GST–HIF-1α fusion protein was preincubated in reticulocyte lysate (odd-numbered lanes) or buffer (even-numbered lanes), incubated with 35S-labeled FLAG–VHL, captured on glutathione–Sepharose beads, and analyzed as described above.

Figure 5. Interaction of FIH-1 and HIF-1α in human cells. Human 293 cells were co-transfected with expression vectors encoding HA–FIH-1, FLAG–VHL, and HIF-1α, as indicated. The transfected cells were untreated, exposed to MG132, or subjected to hypoxia (1% O2) prior to lysis. Aliquots of whole cell lysate (WCL) and anti-HA immunoprecipitate [IP] were analyzed by immunoblot (IB) assay with antibodies that recognize HIF-1α [top] and HA [bottom].
effect of FIH-1 expression [Fig. 6A]. Immunoblot assays demonstrated that GalA protein levels were unaffected by changes in O2 concentration or by overexpression of FIH-1 or VHL [Fig. 6B], demonstrating that these proteins specifically repressed GalA transactivation domain function. Similar assays were performed to analyze the effect of FIH-1 and VHL on transactivation mediated by GalG, which contains HIF-1α(757–826) and includes the binding site for FIH-1 but not for VHL. FIH-1 repressed GalG-mediated transactivation whereas VHL did not [Fig. 6A]. Next, we tested GalL, which contains HIF-1α(531–575), and thus includes the binding site for VHL but not for FIH-1. Neither FIH-1 nor VHL alone inhibited GalL-mediated transactivation, whereas the combination of FIH-1 and VHL resulted in significant transcriptional repression. These results suggest that VHL-mediated recruitment of FIH-1 to HIF-1α was required for transcriptional repression of GalL. The effect of VHL alone on GalA may reflect the presence of sufficient endogenous FIH-1 for functional activity when the target protein also contains an FIH-1 binding site, whereas the lower affinity (noncooperative) binding of FIH-1 to VHL alone requires higher FIH-1 levels that are attained by transfection of the expression vector. Finally, we tested pGalH encoding HIF-1α(786–826), which does not include the binding site for either VHL or FIH-1. Notably, transcriptional activation mediated by GalH is not O2 regulated [Jiang et al. 1997; Fig. 6A]. Neither FIH-1, VHL, nor the combination of FIH-1 and VHL significantly inhibited GalH-mediated transactivation.

VHL and FIH-1 interact with histone deacetylases in vitro

A general property of corepressors is the recruitment of histone deacetylases (HDACs) to DNA-binding transcription factors [for reviews, see Semenza 1998; Cress and Seto 2000]. In vitro-translated 35S-labeled VHL interacted with GST–HDAC-1, GST–HDAC-2, and GST–HDAC-3 fusion proteins but not with GST [Fig. 7A]. The binding of FIH-1 to HDACs 1–3 was more modest. However, binding of in vitro-translated HDAC-1 or HDAC-3 to GST–HA–FIH-1 but not to GST alone [data not shown] indicates that this interaction is specific. VHL residues 1–213 or 1–155 bound to the GST–HDAC fusion proteins whereas VHL[1–58] and VHL[1–57] did not [Fig. 7B]. HIF-1α was pulled down with GST–HDAC fusion proteins very efficiently in the presence of VHL and very inefficiently in its absence [Fig. 7C]. Taken together with the cotransfection assays, these studies demonstrate that VHL functions as a corepressor by recruiting HDACs to HIF-1α.

Discussion

In this paper, we report the identification of FIH-1 and demonstrate its interaction with residues 757–826 at the C terminus of HIF-1α, which encompass part of the inhibitory domain and TAD-C. FIH-1 is the founder of a family of proteins with members extending from human, mouse, and rat to worm and fly, which is consistent with

Figure 6. Functional interaction of FIH-1 and VHL to repress HIF-1α transactivation domain function. (A) Hep3B cells were cotransfected with reporters pSV-Renilla and pGSE1bLuc, expression vector encoding the GAL4 DNA-binding domain alone (Gal0) or a GAL4–HIF-1α fusion protein, and expression vectors encoding no protein, FIH-1, or VHL. The GAL4-fusion proteins (containing the indicated HIF-1α residues) tested were GalA [531–826], GalG [757–826], GalL [531–575], and GalH [786–826]. The relative luciferase activity represents the ratio of firefly:Renilla luciferase for each construct normalized to the result for Gal0. (B) Immunoblot analysis of lysates from transfected cells using monoclonal antibodies against the GAL4 DNA-binding domain (DBD), FLAG, and HA to detect expression of GalA [top], FLAG–VHL [middle], and HA–FIH-1 [bottom], respectively.
the evolutionary conservation of HIF-1α (and VHL) in both vertebrates and invertebrates (Jaakkola et al. 2001). Forced expression of FIH-1 (or VHL) inhibited the transcriptional activity of a reporter gene that was dependent on either the intact HIF-1 heterodimer (p2.1) or isolated HIF-1α transactivation domains [pG5ElbLuc] under hypoxic or nonhypoxic conditions. The effect of expressing antisense FIH-1 RNA (Fig. 3) suggests that FIH-1, like VHL, functions as an O2-dependent negative regulator of HIF-1α. Under conditions of protein overexpression required to detect coinunoprecipitation of FIH-1 and HIF-1α, regulation of the interaction by cellular O2 concentration could not be demonstrated [Fig. 5]. However, VHL becomes limiting under conditions of HIF-1α overexpression (Tanimoto et al. 2000), and this effect probably occurred in the coinunoprecipitation experiment despite cotransfection of a VHL expression vector because HIF-1α expression was not O2-regulated.

In addition to interacting with HIF-1α, FIH-1 also interacts with VHL, allowing the formation of ternary complexes containing HIF-1α, FIH-1, and VHL (Fig. 8). VHL interacts with HIF-1α and FIH-1 via distinct residues within its β domain and HIF-1α interacts with VHL via TAD-N while interacting with FIH-1 via residues in the inhibitory domain and TAD-C. Because each protein interacts with the other two via independent binding sites, ternary complex formation is likely to involve cooperativity, an hypothesis that is supported by the transactivation studies (Fig. 6A). The failure of VHL to mediate repression of GalL, which contains a binding site for VHL but not for FIH-1, suggests a requirement for functional interaction of VHL and FIH-1 in transcriptional

Figure 7. Interaction of VHL and FIH-1 with histone deacetylases. (A) GST and GST–HDAC fusion proteins were incubated with 35S-labeled FLAG–VHL (top), HA–FIH-1 (middle), or HIF-1α (bottom), captured on glutathione–Sepharose beads, and analyzed by SDS-PAGE and autoradiography. (B) GST–HDAC fusion proteins were incubated with 35S-labeled FLAG–VHL truncated at its C terminus as indicated and analyzed as described above. (C) GST–HDAC fusion proteins were incubated with 35S-labeled FLAG–VHL and/or 35S-labeled HIF-1α.

Figure 8. Negative regulation of HIF-1α protein stability and transcriptional activity under nonhypoxic conditions mediated by VHL and FIH-1. Elongins B and C and cullin 2 are required for E3 ubiquitin–protein ligase activity, whereas HDACs repress transactivation.
repression of HIF-1α. A critical question that remains to be answered is whether O2-dependent interaction of HIF-1α and VHL is required for recruitment of FIH-1. An alternative but not mutually exclusive hypothesis is that FIH-1 and VHL functionally interact by recruiting distinct HDAC complexes to HIF-1α, as described below.

HIF-1-mediated gene transcription is precisely modulated by cellular O2 concentration via regulation of the expression and activity of the HIF-1α subunit (for review, see Semenza 1999). The recent elucidation of the O2-dependent binding of VHL has provided a molecular basis for the regulation of HIF-1α protein stability [Ivan et al. 2001; Jaakkola et al. 2001]. Loss of VHL function in renal clear-cell carcinoma (RCC) lines is associated with constitutive expression of HIF-1α protein and of HIF-1α target genes such as GLUT-1 and VEGF [Maxwell et al. 1999]. From these results it was not clear whether HIF-1α protein overexpression was sufficient to activate target gene transcription in RCC lines, whether other mutations in these cells eliminated the O2-dependent negative regulation of HIF-1α transactivation, or whether VHL also regulated transactivation. Starting with the identification of FIH-1, we have shown that VHL is an important regulator of HIF-1α TAD function, indicating that VHL controls the two major mechanisms for post-translational regulation of HIF-1α [Fig. 8]. These results indicate that loss of VHL function in RCC may be sufficient for constitutive transcription of HIF-1 target genes. Different VHL missense mutations are associated with different disease phenotypes [Clifford et al. 2001; Hoffman et al. 2001]. Whereas the effect of specific mutations on HIF-1α and elongin BC binding has been determined, additional genotype–phenotype correlations may be established by analysis of FIH-1 binding to mutant VHL proteins.

The association of VHL with HDAC-1, HDAC-2, and HDAC-3 provides a molecular basis for the repression of HIF-1α transactivation domain function under nonhypoxic conditions. FIH-1 did not interact as strongly with these HDACs, suggesting that it may interact with HDAC-4, HDAC-5, HDAC-6, or HDAC-7. HDAC1–HDAC3 and HDAC4–HDAC7 represent two structurally and functionally distinct groups of proteins [for review, see Cress and Seto 2000]. Therefore, the existence of corepressors capable of interacting with each group may provide additional safeguards against inappropriate HIF-1α-mediated transactivation. An alternative but not mutually exclusive possibility is that FIH-1 binds to another corepressor, such as mSin3A, N-CoR, or SMRT, which in turn interacts with HDACs [Kao et al. 2000]. Finally, FIH-1 itself may possess HDAC activity. Interestingly, HDAC1 mRNA and protein expression are induced by hypoxia [Kim et al. 2001], suggesting that HDAC1 may represent a HIF-1 target gene and that increased HDAC activity may contribute to the overall decreased rate of transcription in hypoxic cells. The biological role of HDAC1 is complex because of the many transcriptional regulators with which it associates [Cress and Seto 2000] and the net effect of HDAC1 overexpression is to stimulate angiogenesis [Kim et al. 2001]. In contrast, VHL and FIH-1 specifically interact with HIF-1α, and overexpression of either of these proteins is predicted to inhibit angiogenesis as a result of decreased HIF-1-mediated VEGF expression.

The results of protein binding and gene transactivation studies suggest that VHL and FIH-1 function cooperatively to repress HIF-1α-mediated transactivation under nonhypoxic conditions. This relationship implies that loss of either VHL or FIH-1 activity may be sufficient to derepress transactivation. This finding is important because of the recent demonstration that phosphatidylinositols-3-kinase [PI3K]/AKT/FRAP signaling stimulated by receptor tyrosine kinases such as HER2 induces HIF-1α protein expression by increasing its rate of synthesis rather than by decreasing its rate of degradation [Laugner et al. 2001]. However, activation of the PI3K/AKT/FRAP pathway does not derepress transactivation domain function. In contrast, activation of the MAP kinase pathway has been reported to increase HIF-1 transcriptional activity without affecting HIF-1α protein expression [Richard et al. 1999; Sodhi et al. 2000], and it will be interesting to determine whether this effect is mediated via decreased binding of FIH-1.

A common finding in brain tumors is loss-of-function mutations in the gene encoding PTEN, a phosphatase that negatively regulates PI3K signaling [for review, see Di Cristofano and Pandolfi 2000]. Genetic manipulation of PTEN expression has been shown to modulate HIF-1α expression in glioma and prostate cancer cell lines [Zhong et al. 2000; Zündel et al. 2000]. Another common genetic alteration in high-grade gliomas is deletion of chromosome 10q23–q26 [Bigner and Vogelstein 1990], which encompasses the FIH1 locus at 10q24. Thus, FIH-1 loss-of-function may contribute to increased HIF-1α-mediated transactivation of downstream target genes such as VEGF in gliomas and other human cancers [Semenza 2000; Zaggag et al. 2000].

The complex functional relationship between HIF-1α, FIH-1, and VHL documented in this study and the finding that binding of VHL to the intact HIF-1α regulatory domain [residues 531–826] does not require preincubation of HIF-1α with cellular lysate [as a source of putative prolyl hydroxylase activity; Fig. 4D], suggest that negative regulation of HIF-1α by VHL may be controlled by factors in addition to the O2-dependent hydroxylation of Pro 564 [Ivan et al. 2001; Jaakkola et al. 2001], a conclusion that is supported by other recent studies [Yu et al. 2001]. Most importantly, the results reported here establish a unifying mechanism for the modulation of HIF-1α protein stabilization and transcriptional activation in response to changes in cellular O2 concentration that is mediated by VHL in association with FIH-1.

Materials and methods

Yeast two-hybrid system and bait vector construction

A cDNA encoding FIH-1 was isolated by use of the yeast two-hybrid assay [MATCHMAKER Two-hybrid System 2, Clon-
The bait vector pGAL4–HIF-1α[576–826] was constructed by PCR amplification of HIF-1α cDNA sequences [using forward and reverse primers containing NdeI and BamHI restriction sites, respectively], restriction endonuclease digestion, and ligation into the vector pA521–1. The prey vectors [human brain MATCHMAKER cDNA library cloned into plasmid pACT II, Clontech] encoded fusion proteins consisting of the GAL4 transactivation domain followed by amino acids encoded by human brain cDNA sequences.

**Library screening**

The physical interaction of the bait and prey proteins within yeast cells functionally reconstitutes an active GAL4 transcription factor, resulting in the expression of genes that contain upstream GAL4-binding sites and mediate histidine auxotrophy (his+) and α-gal expression. To screen for such yeast cells, Saccharomyces cerevisiae strain Y190 was transformed by use of the LiAc/PEG method. YPD medium [850 mL] was inoculated with 150 mL of overnight yeast culture and grown to OD500 = 0.5. The cells were pelleted by centrifugation [5 min at 1,000g], resuspended in 8 mL of TE/LiAc solution, and exposed to 300 µg of pGAL4–HIF-1α[576–826], 600 µg of pACT II/human brain cDNA, and 20 mg of histidine testes DNA (Clontech). The cells were agitated at 30°C for 30 min, mixed with 7 mL of brain cDNA, and 20 mg of herring testes DNA (Clontech). The transformed cells were inoculated onto agar plates [Winston 1987] for transformation of cells. The transformed cells were inoculated onto agar plates lacking leucine and supplemented with 10 µg/mL cycloheximide. Pelleted cells were lysed by sonication in PBS containing 1% Triton X-100 and Complete protease inhibitor cocktail [Roche]. After centrifugation, supernatants were applied to glutathione–Sepharose 4B [Amersham Pharmacia Biotech]. GST fusion proteins were eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8.0) and stored at −80°C. The concentration and purity of eluates were determined by the Bradford method and by SDS-PAGE.

**Transactivation assay**

HEK293 and Hep3B cells were seeded onto 24-well plates at 4 × 10^4 cells per well. The following day, the cells were transfected with plasmids encoding HIF-1α, HA–HIF-1, or FLAG–VHL sequences. C-terminal deletion mutants of VHL were made as follows: pcDNA3.1-FLAG–VHL [Feldman et al. 1999], kindly provided by J. Frydman, Stanford University, CA] was digested with NotI, SacII, or AccI. Where indicated, bacterially produced GST fusion proteins were preincubated with 10 µL of rabbit reticulocyte lysate for 30 min at 30°C. Five microliters of the indicated lysate [10 µL in the case of HIF-1α] and/or 5 µg of the indicated recombinant proteins was mixed in 150 µL of NETN buffer [150 mM NaCl, 0.5 mM EDTA, 20 mM Tris-HCl at pH 8.0, 0.5% NP-40]. After 90 min at 4°C, 20 µL of glutathione–Sepharose 4B or 20 µL of anti-FLAG M2 monoclonal antibody–agarose affinity gel (Sigma) was added. After 30 min of mixing on a rotator, beads were washed three times with NETN buffer. Proteins were eluted in Laemmli sample buffer and analyzed by SDS-PAGE followed by autoradiography.

**Immunoprecipitation assay**

C6/36 cells were harvested in 200 µL of lysis buffer [Dulbecco’s PBS at pH 7.4, 0.1% Tween-20, 1 mM sodium orthovanadate, and 50 mM Tris-HCl at pH 8.0, 0.5% NP-40] and analyzed by SDS-PAGE.
Complete protease inhibitor and drawn through a 20G needle four times. The lysate was incubated on ice for 1 h followed by centrifugation at 14,000 rpm for 15 min. The cleared lysates were brought to a volume of 1 mL with lysis buffer followed by a 2-h incubation with 20 µL of anti-HA affinity matrix beads (Roche) at 4°C on a rotator. The beads were then washed three times with lysis buffer. Protein was eluted by the addition of Laemmli sample buffer and analyzed by SDS-PAGE and autoradiography.

Acknowledgments

We thank Kelly Chiles and Shannon Berg-Dixon for technical assistance. We are grateful to the laboratory of Edward Benz for generously providing yeast two-hybrid reagents and protocols. We thank Judith Frydberg, Edward Seto, and Wen-Ming Yang for generous gifts of expression plasmids. This work was supported in part by the Yamanouchi Foundation for Research on Metabolic Disorders and by a fellowship from the Uehara Memorial Foundation.

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