Journal of Cellular Biochemistry 95:1264–1275 (2005)

Induction of the von Hippel-Lindau Tumor Suppressor Gene by Late Hypoxia Limits HIF-1 Expression

Jörn Karhausen,[1,2] Tianqing Kong,[1] Sailaja Narravula,[1] and Sean P. Colgan[1]*

[1]Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts
[2]Department of Anesthesiology and Intensive Care Medicine, Tübingen University Hospital, and Tübingen D-72076, Tübingen, Germany

Abstract Hypoxia–inducible factor (HIF) remains the central focus of oxygen sensing during hypoxia. HIF is a heterodimeric transcription factor consisting of an oxygen-regulated alpha- and a constitutively expressed beta subunit. The von Hippel-Lindau tumor suppressor (pVHL) is a component of the E3 ubiquitin ligase complex and targets HIF-α to proteasomal degradation, but also is known to exert a significant control on HIF transactivation activity. However, the understanding of the full interaction between HIF and pVHL has been hindered by a lack in the understanding of pVHL regulation. Here, we report that pVHL itself is induced in prolonged hypoxia in a kinetic that parallels the observed downregulation of HIF-1α protein under such conditions. In addition, we document direct HIF-1α binding to the VHL promoter and identify a functional hypoxia response element (HRE) within the VHL promoter. Such induction of pVHL in hypoxia furthermore has functional implications for the HIF dependent hypoxic response, implicating a physiologically relevant feedback mechanism. These results provide an intriguing model, whereby HIF self-regulates expression through VHL and highlight the role of pVHL as a unifying mechanism of HIF regulation. J. Cell. Biochem. 95: 1264–1275, 2005.

Key words: VHL; HIF; transcription factor; promoter

As a pivotal component in the maintenance of oxygen homeostasis, the hypoxia–inducible factor (HIF) is a heterodimeric transcription factor consisting of an oxygen-regulated alpha subunit (HIF-1α and -2α) and a constituitively expressed beta subunit. HIF regulates the activity of growing number of target genes which convey systemic and cellular hypoxia adaptive responses including erythropoiesis, angiogenesis, and glycolytic metabolism (reviewed by Semenza [1999]). The major pathway for HIF regulation is constituted by post-translational mechanisms which effectively ensure that HIF-1α and -2α protein is virtually undetectable with adequate oxygen supply, but allows a robust and rapid response when oxygen becomes limiting [Jewell et al., 2001]. Oxygen sensing is achieved through prolyl hydroxylation [Ivan et al., 2001; Jaakkola et al., 2001], which is a prerequisite for binding of the α-subunit to an ubiquitin-E3 ligase complex. This complex comprises the von Hippel-Lindau gene product (pVHL), the elongins B and C, cullin-2 and rbx-1 [Kaelin and Maher, 1998; Ivan and Kaelin, 2001], where ensuing ubiquitination and proteasomal degradation depend critically on the functional integrity of pVHL [Ohh et al., 1998; Maxwell et al., 1999; Cockman et al., 2000].

It is well established that pVHL is a central component to limit HIF activation. For example, functional inactivation of pVHL has been shown to lead to dysregulated HIF response observed in familial VHL disease, a disorder characterized by the development of variety of typically hypervascular tumors (reviewed by Kaelin and

Nonstandard abbreviations: hypoxia–inducible factor (HIF); von Hippel-Lindau (*VHL*).

*Correspondence to: Sean P. Colgan, Ph.D., Brigham and Women's Hospital, Center for Experimental Therapeutics and Reperfusion Injury, 20 Shattuck Street, 704 Thorn Building, Boston, MA 02115.
E-mail: colgan@zeus.bwh.harvard.edu

Received 19 January 2005; Accepted 8 March 2005
DOI 10.1002/jcb.20489

Maher [1998]). Moreover, biallelic VHL mutations have been shown to play a role in the oncogenesis of renal carcinomas [Linehan et al., 1995] and haemangioblastomas of the central nervous system [Lee et al., 1998]. These examples, and the fact that the HIF pathway seems to contribute to malignant tumor phenotypes [Paul et al., 2004; Semenza, 2004], underline the necessity for regulatory mechanisms that limit HIF activity. Furthermore, it has been shown that reintroduction of VHL into renal cancer cell lines normalizes efficiently the expression of several hypoxia-inducible genes [Iliopoulos et al., 1996; Mukhopadhyay et al., 1997]. This has lead to speculations whether HIF and VHL form a degradation-transactivation loop similar to p53 and Mdm-2 [Blagosklonny, 2001]. In support of this hypothesis, recent evidence indicates that hypoxia selectively induces pVHL in renal cell lines through mechanisms involving Rho kinase [Turcotte et al., 2004].

It has been previously observed that HIF activation becomes limited following prolonged exposure to hypoxia [Wang and Semenza, 1995; Stroka et al., 2001], yet the mechanism(s) which repress HIF expression in late periods of hypoxia remain incompletely understood. It is generally accepted that HIF-1α activation occurs predominantly through post-translational mechanisms [Wang and Semenza, 1995; Huang et al., 1996; Kallio et al., 1999]. Here, we present work, which establishes pVHL as a unifying regulatory element for HIF activity and indicate that VHL induction in late hypoxia occurs through HIF-dependent transcriptional mechanisms. As a consequence, increased expression of pVHL by hypoxia provides a feedback mechanism to limit expression of HIF.

MATERIALS AND METHODS

Cell Culture and Hypoxia

The intestinal epithelial cell lines T84 and $Caco_2$ were cultured as previously described [Colgan et al., 1996]. Hela cells were used for promoter analysis and immunostaining using culture methods described [Comerford et al., 2002]. Cells were exposed to hypoxia (i.e., to an atmosphere of pO_2 20 torr and pCO_2 35 torr, balance being made up with N_2 and water vapor) as described previously [Colgan et al., 1996]. Normoxic controls were exposed to same protocols under conditions of atmospheric O_2 (pO_2 147 torr; pCO_2 35 torr).

Gene Array Analysis

Gene array analysis was carried out as described [Taylor et al., 2000]. T84 cells were exposed to hypoxia (0, 6, 18 h), total RNA was extracted, mRNA was isolated, and DNase treated. The mRNA profile was assessed by using quantitative gene chip expression arrays (Affymetrix, Santa Clara, CA [Lockhart et al., 1996]).

Transcriptional Analysis

For transcriptional analysis, cells were harvested in TRizol (Invitrogen, Carlsbad, California) and RNA isolated by phenol-chloroform extraction. After DNAse digestion (GenHunter, Nashville, Tennessee), reverse-transcription was performed using the Omniscript RT kit (Qiagen, Hilden, Germany) and oligo (dT) primers (Invitrogen). For amplification on an i-Cycler IQ real-time PCR detection system (BioRad Laboratories, Hercules, California) we used the following gene specific primers: HIF-1: Forward-5′-CTATGTAGTTGTGGAAGTTTATGC, Reverse-5′- ACTAGGCAATTTTGCTAAGAATGC; β-Actin: Forward-5′-ACTGGAACGGTGAAGGTGACAG, Reverse-5′- GGTGGCTTTTAGGATGGCAAG; VHL: Forward-5′-GCCGAGGAGGAGATGGAG, Reverse-5′-GTCGAAGTTGAGCCATACG. Comparison of gene expression in a semi-quantitative manner was performed based on the mathematical model of Pfaffl [2001].

Western Blot Analysis

Nuclear and cytoplasmic fraction of cell culture samples were isolated using reagents provided by Pierce (Rockford, IL). Samples were normalized for protein content and resolved by non-reducing SDS-PAGE as described previously [Taylor et al., 1999]. Antibodies used for Western blotting included monoclonal mouse anti-HIF-1α (123–105, 1 μg/ml, Novus Biologicals, Littleton, CO), rabbit polyclonal anti-VHL (FL-181, 1 μg/ml, Santa Cruz Biotechnology, Santa Cruz, CA), and goat polyclonal anti-actin (C-11, 1 μg/ml, Santa Cruz Biotechnology). Blots were washed and species-matched peroxidase-conjugated secondary antibody added. Labeled bands from washed blots were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ).

Immunofluorescence Microscopy

Hela cells were grown to confluence on acid washed 12 mm glass coverslips. Monolayers

were washed once in phosphate buffered saline and fixed for 10 min at room temperature in 1% paraformaldehyde in cacodylate buffer (0.1M sodium cacodylate; pH 7.4, 0.72% sucrose). Monolayers were permeabilized for 10 min in PBS containing 0.2% Triton X-100 and 3% BSA. After washing twice with PBS, the cells were stained for 1 h with anti-VHL (12.5 μg/ml). After washing, the monolayers were incubated with goat anti-mouse Texas Red (1 μg/ml, Molecular Probes, Eugene, OR).

Plasmids and Antisense Oligonucleotides

The luciferase gene reporter assay was used for functional analysis of the VHL promoter as well as for investigation of transcriptional events regulated by the hypoxia response element (HRE). The VHL promoter constructs were a kind gift from Dr. I. Kuzmin (National Cancer Institute, Frederick, MD). As a control for hypoxia, cells were transfected with a PGL3-based HRE plasmid containing four tandem HIF-1 enhancer sequences from the 3′-region of the erythropoietin gene [Sheta et al., 2001] (kindly provided by Dr. Dan Theodorescu, Univeristy of Virginia, Charlottesville, VA).

Directed overexpression of full-length VHL was achieved using the VHL-pFLAG plasmid (kind gift from Dr. V. Haase, University of Pennsylvania, Philadelphia, PA). HIF-1 depletion in epithelial cells was accomplished by using antisense oligonucleotide loading as described previously [Caniggia et al., 2000] using phosphorothioate derivatives of antisense (5′-GCC GGC GCC CTC CAT-3′) and control sense (5′-CCT GGA GTG ATG CCT AAT AT-3′) oligonucleotides. Transfection of either plasmid or oligonucleotides was performed using PolyFect transfection reagent (Qiagen, Hilden, Germany) and standard protocols. Experiments were initiated after overnight transfection and media change. Overexpression of oxygen-stable HIF-1α was achieved through infection with an adenovirus (a kind gift from K. Vincent, Genzyme Corporation) containing a constitutively stable hybrid form of HIF-1α [Jiang et al., 2002].

Site Directed Mutagenesis

Mutations were performed on a luciferase reporter plasmid bearing the minimal hypoxia responsive fragment of the VHL promoter (F11: −83 to +23 bp in Kuzmin et al. [1995]) using the QuickChange site-directed mutagenesis kit (Stratagene, LaJolla, CA) according to the manufacturers instructions. For the mutation of the SP-1/AP-1 site, the original sequence CTCCGCCCCGC (+2 to +12) was altered into CTAAAACCCGC, a putative HIF-1 site representing the core consensus sequence (5′-RCGTG-3′[Wang and Semenza, 1995]) on the antisense strand was altered from GCACGC (−6 to +1) to AATCGC. These changes resulted in destruction of the consensus sequences of the respective transcription factor without significant change for the binding of other putative transcription factor as determined by computational simulation using Matinspector (http://www.genomatix.de).

Luciferase Reporter Assays

Reporter assays were performed using Hela cells transfected with the respective promoter construct and exposed to normoxia and hypoxia as indicated. Luciferase activity was assessed on a TD20/20 luminometer (Turner Designs, Fresno, CA) using the dual luciferase assay system (Promega, Madison, WI). All activity was normalized with respect to a constituitively expressed renilla reporter.

Chromatin Immunoprecipitation (ChIP) Assay

Chromatin immunoprecipitation was performed as previously described [Ramaswamy et al., 2002] with minor modifications. Hela cells were grown to confluence, fixed with 1% formalin, and chromatin isolated, and sheared. Sheared chromatin was incubated with either anti-HIF-1α antibody or anit-SP-1 antibody, and immune complexes were precipitated with Protein G Sepharose beads. Crosslinking was then reversed and DNA was purified using the PCR purification kit (Qiagen). HIF-binding to VHL promoter DNA was quantified by standard PCR using primers (forward: 5′-GCCTCCGTTACA-ACAGCCTA-3′ and reverse: 5′-TCTTCAGG-GCCGTACTCTTC-3′) designed to amplify a 265 bp region of the VHL promoter extending from −142 and +132 bp with respect to the initiation start site. Chromatin incubated with beads without antibody were used to control for nonspecific binding of DNA.

Data Analysis

Real-time PCR and luciferase reporter data were compared by two-factor analysis of variance (ANOVA) or by Student's *t*-test, where appropriate values are expressed as the mean and S.E.M. from at least three separate experiments.

RESULTS

HIF-1α Expression Pattern in Prolonged Hypoxia

In initial studies, we sought to define potential regulatory mechanisms of HIF protein expression responsible for the observed downregulation of HIF-1α protein with prolonged periods of hypoxia. As shown in Figure 1A, Western blot analysis of nuclear lysates derived from two separate epithelial cell lines (Hela and $Caco_2$) revealed rapid induction of HIF-1α, and a graded loss of expression with prolonged hypoxia (i.e., >24 h), with nearly complete loss of expression by 48 h.

At present, mechanisms of HIF repression during hypoxia are incompletely understood. Therefore, we approached this issue from a global perspective. First, we determined whether HIF repression in prolonged hypoxia was transcriptionally regulated. To do this, the expression pattern of Hela cell HIF-1α protein in late hypoxia (>8 h) was examined in the presence and absence of the general transcription inhibitor actinomycin D (1 μg/ml). As shown in Figure 1B, these studies revealed that at 8 h of hypoxia, HIF-1α expression was enhanced by nearly fourfold in the presence of actinomycin D. Moreover, these studies revealed that the loss of HIF-1α at 24 h was nearly abolished by actinomycin D treatment (Fig. 1B). Consistent with previous reports in Hep3B cells [Iyer et al., 1998], our analysis in both Hela cells and $Caco_2$ cells have indicated that HIF-1α is not significantly controlled on the transcription level by hypoxia (data not shown), strongly suggesting that the loss of HIF-1α in late hypoxia involves transcriptional regulation of genes others than HIF-1α.

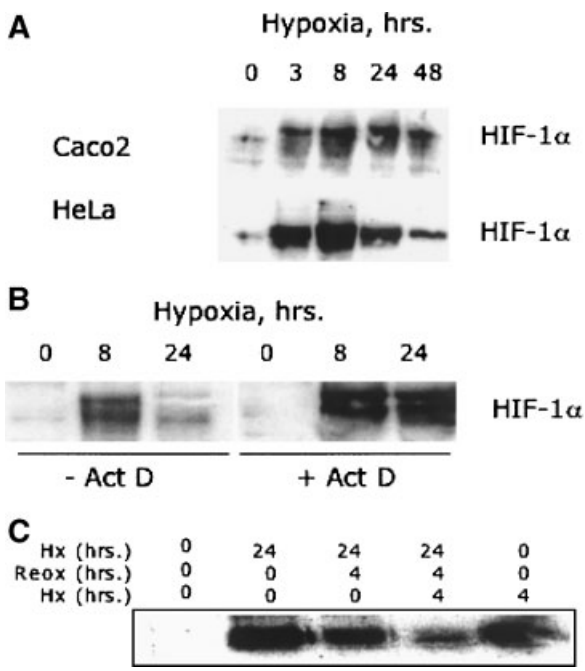

Fig. 1. Kinetics of Hypoxia inducible factor (HIF)-1α protein expression in hypoxia. **A**: Representative Western blot of nuclear lysates from $Caco_2$ and Hela cell lines subjected to indicated periods of hypoxia (pO_2 20 torr). HIF-1α displays a rapid induction in early hypoxia and declines with prolonged exposure to hypoxia. **B**: Influence of general transcription inhibitor actinomycin D (ActD) on the repression of HIF-1α in prolonged hypoxia. Shown here is a representative HIF-1α Western blot of nuclear lysates derived from $Caco_2$ cells exposed to indicated periods of hypoxia in the presence and absence of ActD (1 μg/ml). **C**: Magnitide of HIF-1α expression during hypoxic preconditioning. Represetaive HIF-1α Western blot of nuclear lysates derived from $Caco_2$ cells subjected to a combination of hypoxia (24 h), reoxygenation (4 h), followed by hypoxia (4 h). Note the decrease in HIF-1α levels upon exposure to a second period of hypoxia.

We next determined whether protein(s) produced in late hypoxia are responsible for the kinetic loss of HIF-1α. To do this, we utilized a strategy wherein $Caco_2$ cells were exposed to an initial prolonged period of hypoxia (HIF-1α activation), followed by a period of reoxygenation (to repress HIF-1α expression), followed by an additional period of hypoxia (HIF-1α reactivation). Our logic being that proteins produced during hypoxia should limit reactivation of HIF-1α, with the assumption that these HIF regulatory protein(s) are not rapidly degraded with reoxygenation. As shown in Figure 1C, nuclear HIF-1α protein was evident after the initial period of prolonged hypoxia (24 h), and reoxygenation (4 h) resulted in a significant loss of such expression. Reintroduction of such "preconditioned" cells to hypoxia (4 h) resulted in a significantly attenuated HIF-1α activation response compared to naïve cells exposed to a similar period of hypoxia (4 h). Such results implicate the induction of HIF repressing protein(s) during late hypoxia.

Identification of Hypoxia-Inducible VHL

In order to identify candidate genes for the observed repression of HIF-1α in prolonged hypoxia, a transcriptional profiling approach was utilized. Here, mRNA patterns were compared between epithelial cells exposed either to normoxia or to hypoxia for 6 (standard hypoxia) and 18 h (late hypoxia). With this analysis, we sought to identify genes induced in late hypoxia. Results from this analysis revealed an interest-

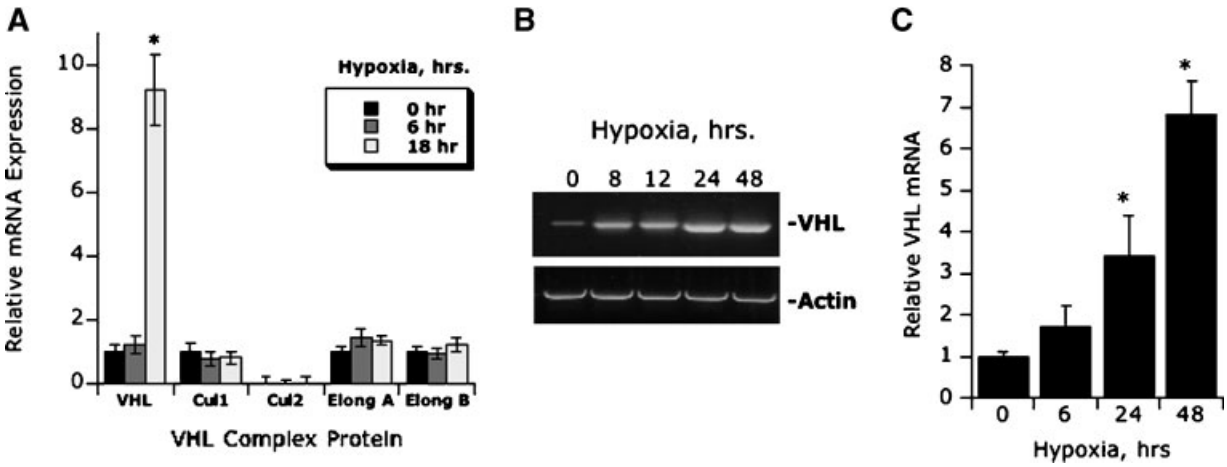

Fig. 2. The von Hippel-Lindau tumor suppressor (VHL) is induced in late hypoxia. **A**: Transcriptional profiling of mRNA derived from T84 cells exposed to normoxia, 6 or 18 h hypoxia (pO_2 20 torr). Microarray analysis ($n = 3$ each) was utilized to profile hypoxia influences on expression of the E3 ubiquitin ligase genes VHL, cullin 2, elongin B and related genes cullin 1 and elongin A. Only VHL was significantly induced by hypoxia, and only at 18 h of hypoxia (where * indicates $P < 0.01$ compared to either normoxia of 6 h hypoxia). Induction of VHL (relative to β-actin) in late hypoxia is further documented by conventional PCR of mRNA generated from T84 cells (**B**) and in real-time PCR of mRNA generated from Hela cells (**C**) exposed to the indicated time in hypoxia (where $*P = 0.05$ compared to normoxia).

ing mRNA expression pattern for members of the E3 ubiquitin ligase complex of proteins involved in the degradation of HIF-1α, namely, VHL, Cullin, and Elongin family members. As shown in Figure 2A, this screening approach identified a 9.2 ± 1.1-fold induction of the VHL gene in late hypoxia, with no significant change with standard hypoxia exposure (1.2 ± 0.3-fold after 6 h of hypoxia). This pattern of VHL induction was relatively specific, since no significant changes were evident in the expression of cullin 1, cullin 2, elongin A, or elongin B (Fig. 2A). These findings make VHL a suitable candidate as a late hypoxia HIF-1α repression factor both by virtue of its known involvement in HIF regulation and because of the parallel dynamics with the observed downregulation of HIF.

We next confirmed these transcriptional profile results using PCR analysis. As shown in Figure 2B, standard PCR of mRNA derived from T84 epithelial cells confirmed VHL hypoxia-inducibility, particularly, at later periods of hypoxia (4.3 ± 0.3 and 3.9 ± 1-fold increase at 24 and 48 h, respectively). Real-time PCR analysis of mRNA derived from Hela cells subjected to a hypoxia time course revealed an expression pattern similar to T84 cells, namely, the significant VHL induction at late periods of hypoxia (24 and 48 h). Of note, these results were more obvious in some cell types (e.g., epithelia, endothelia) compared to others (leukocyte cell lines such as HL-60).

At the protein level, pVHL induction was demonstrated by both immunofluorescent staining (Fig. 3A) and by Western blot (Fig. 3D). Localization of VHL in Hela cells identified a prominent increase in protein in late hypoxia (48 h, Fig. 3B) relative to normoxia (Fig. 3A). In both hypoxia and normoxia, the pVHL staining pattern was diffuse in the cytoplasm, with prominent localization in the nucleus. Western blot analysis revealed this same pattern of induction in late hypoxia (Fig. 3D), with prominent pVHL expression in both the cytoplasmic and nuclear compartments. Taken together, such findings identify hypoxia-inducible pVHL as a relevant member of the E3 ubiquitin ligase complex of proteins.

Molecular Mechanisms of VHL Induction by Hypoxia

Previous work identified hypoxia-inducible pVHL through RhoA-dependent mechanisms [Turcotte et al., 2004]. Given the parallel findings of late HIF-1 repression and transcriptional induction of VHL, we investigated pathways downstream of RhoA, and the possibility that the VHL promoter is directly influenced by hypoxia. To do this, we profiled the influence of hypoxia in transient transfectants expressing various length luciferase reporter constructs (Fig. 4).

These studies revealed substantial baseline activity and hypoxia inducibility in the full

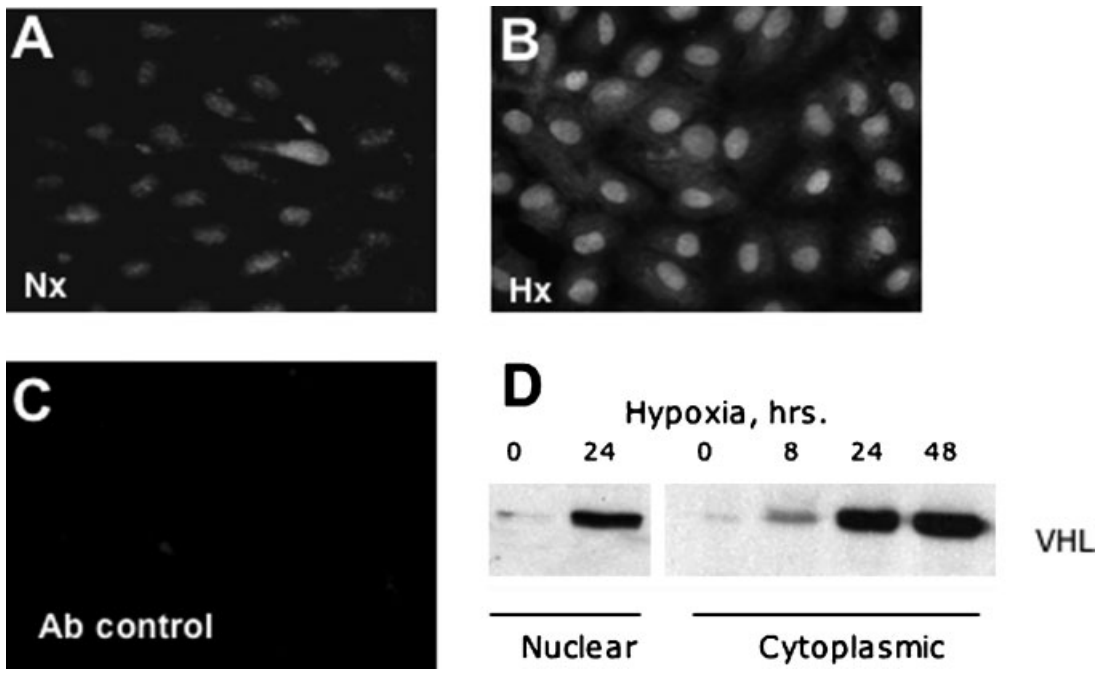

Fig. 3. Induction of VHL protein expression in hypoxia. Hela cells were exposed to normoxia (**A**) or 48 h of hypoxia (**B**) and pVHL was localized using immunofluorescence. Increase of overall signal but lack of distinct changes in expression pattern as both cytoplasmic and nuclear VHL signals are enhanced. **C**: Isotype matched antibody control. **D**: VHL Western blot from Hela cell extracts revealed prominent induction of VHL protein is seen in both nuclear and cytoplasmic fractions.

length promoter (F1) and in those constructs retaining the transcriptional start region [i.e., a 3′ truncated (F2) and a 5′ and 3′ truncated (F11) construct]. While baseline activity was reduced in the both truncated constructs (Fig. 4A), all three constructs displayed hypoxia-inducibility, most prominently in the 106 bp region 5′ and 3′ deletion construct (2 ± 0.1 and 5.6 ± 0.4-fold at 24 and 48 h hypoxia, respectively, $P = 0.05$). This minimal promoter included the transcription start site and the SP-1/AP-1 binding site, previously characterized as a regulatory element for baseline activity [Zatyka et al., 2002]. In close proximity upstream of the transcription start site, we furthermore identified a potential HIF-1 binding site. This HIF-1 site is somewhat atypical as the core consensus sequence (5′-RCGTG-3′) was located on the antisense strand and lacked the typical ancillary HIF binding site [Kimura et al., 2001]. Sequence analysis of this promoter region, including all three transcription factor binding sites (HIF-1, AP-1, and SP-1) showed evolutionary conservation within primates (data not shown).

Site directed mutagenesis was used to address the functional importance of both SP-1/AP-1 and the HIF-1 site in hypoxia-inducibility. For these purposes, the minimal hypoxia responsive promoter construct (F11) was used as a template. As previously determined [Zatyka et al., 2002], mutation of the SP-1/AP-1 site resulted in a marked reduction of baseline activity (to $7 \pm 4.4\%$ of the full length promoter activity, $P = 0.05$). When this construct was exposed to hypoxic conditions, we observed an increase of promoter activity greater than what was seen in the wild type construct (11 ± 1.5-fold after 48 h hypoxia vs. 6 ± 0.8-fold in WT, $P = 0.05$), suggesting that the SP-1/AP-1 site might control the magnitude of hypoxia-inducibility. In contrast, functional mutations within the putative HIF site lead to an almost complete abrogation of the hypoxia response (2 ± 0.6-fold increase after 48 h hypoxia, $P =$ n.s. compared to normoxia), establishing the possibility that the HIF may regulate VHL hypoxia-inducibility. Interestingly, this HIF-1 site mutation also significantly affected baseline expression ($6 \pm 3.2\%$ of full length promoter activity, $P = 0.05$).

We next determined whether this putative HRE in the VHL promoter bound HIF-1α. For these purposes, we utilized chromatin immunoprecipitation (ChIP) to analyze HIF-1α binding in live cells. As shown in Figure 4C, ChIP

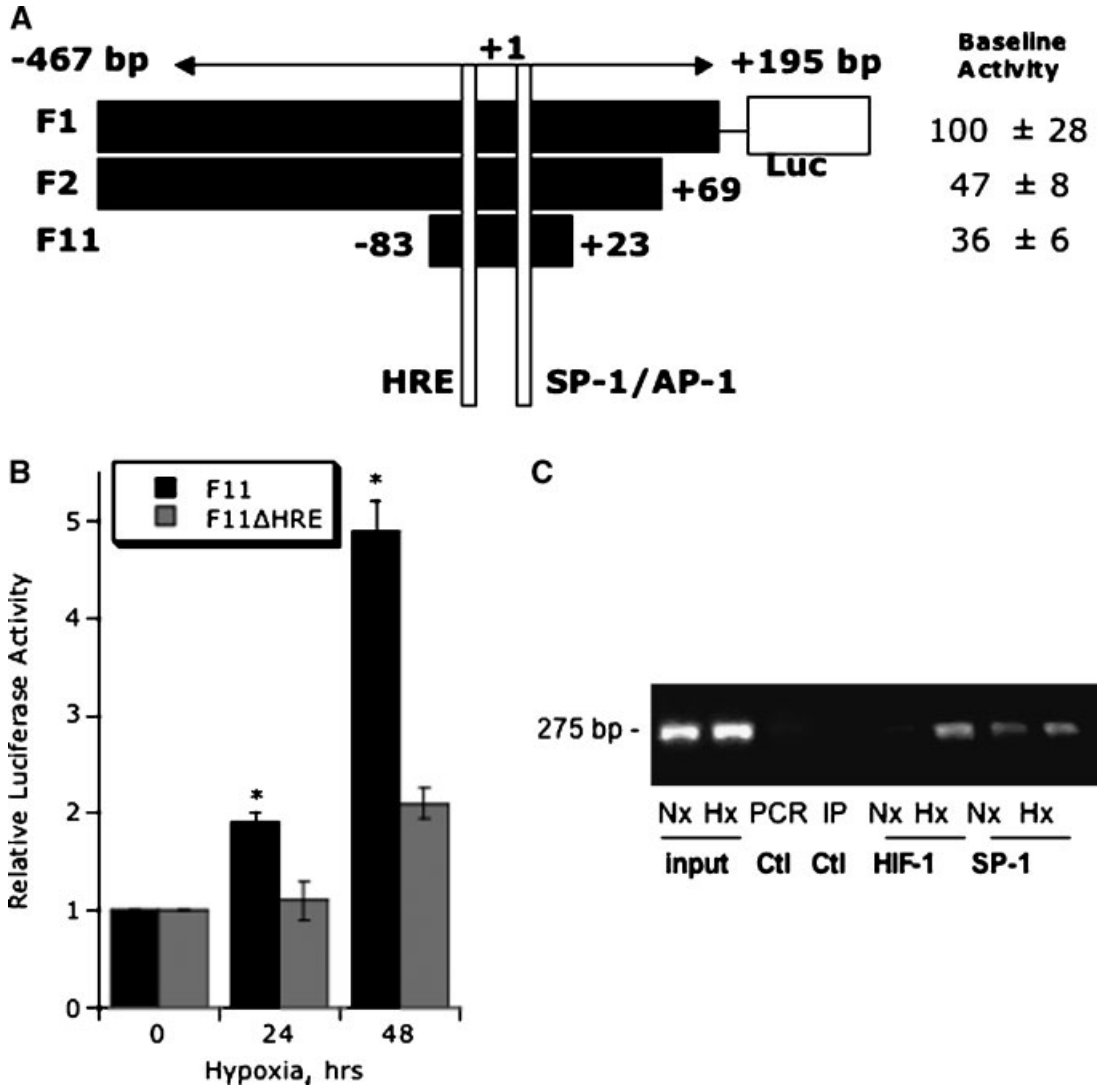

Fig. 4. Analysis of hypoxia inducible VHL promoter; role of HIF-1. **A**: Map of assayed VHL promoter constructs. Hypoxia response was targeted to a region central to the transcriptional start site as included in the full length promoter construct (F1), a 5′ truncated construct (F2), and a 3′, 5′ truncated minimal promoter fragment (F11). Within this, minimal promoter was contained a previously identified SP1/AP1 site and, in close proximity, a previously unappreciated hypoxia responsive element (HRE). Baseline activity of each of these deletion constructs was compared to the full-length promoter. **B**: Hypoxia response of F11 VHL promoter construct (F11) compared to HRE mutated construct (F11ΔHRE). Results are expressed as mean ± S.E.M. luciferase activity (normalized to Renilla control), where *indicates $P < 0.025$ compared to normoxia. **C**: Chromatin immunoprecipitation (ChIP) from Hela cells subjected to either normoxia (Nx) or hypoxia (Hx, 24 h). Untreated chromatin samples show predicted band irrespective of treatment (input), while negative controls for PCR (PCR Co) and immunoprecipitation (IP Co) remain empty. Samples for immunoprecipitation were subdivided and incubated with either anti-SP-1 or anti-HIF-1α antibodies.

analysis of nuclei derived from Hela cells exposed to late hypoxia (24 h) revealed a dominant band of 165 bp in hypoxic, but not normoxic samples. In contrast, ChIP performed with an SP-1 antibody produced bands of almost identical density both in normoxia and hypoxia. No bands were evident in beads only controls, and input samples (pre-immunoprecipitation) revealed the predictable 265 bp band equally under conditions of hypoxia and normoxia. Such results indicate that hypoxia induces HIF-1α binding to the VHL promoter region within −142 to +132 bp relative to the primary site of VHL transcription initiation, while suggesting that SP-1 binding to the VHL promoter is independent of hypoxia.

Role of HIF-1α in Hypoxia Inducible VHL

To verify the significance of HIF-1α for VHL promoter control, we next depleted cells of HIF-1 using antisense oligonucleotides, a condition we have previously shown to decrease HIF-1α protein expression by greater than 60% [Furuta et al., 2001; Synnestvedt et al., 2002]. With this approach, we were able to attenuate the hypoxia response both on the VHL-promoter construct (by $48 \pm 14.0\%$ at 48 h hypoxia, $P < 0.05$ compared to sense control) and in a HRE-reporter construct (by $50 \pm 3.9\%$ after 48 h hypoxia, $P < 0.05$ compared to sense control), which we used as positive control. HIF-1α antisense oligonucleotides did not influence promoter activity in normoxia ($P =$ not significant compared to sense control).

Additional insight was gained by over-expression of oxygen-stable HIF-1α in normoxia. For these purposes, we transduced Hela cells with an adenovirus bearing a constitutively active HIF-1α [Jiang et al., 2002]. As shown in Figure 5B, adenovirus expressing HIF-1α readily activated both the VHL promoter and the HRE construct in normoxia (3 ± 0.6 and 4 ± 0.8-fold increase, respectively, $P < 0.025$ compared to empty virus controls). Such results provide strong evidence that HIF-1α critically controls hypoxia-inducible VHL.

Influence of Increased pVHL in Functional HIF-1α Transactivation

As a final series of experiments, we examined whether increased levels of VHL protein might influence HIF-1α transactivation, and thereby function as a control point for HIF-1α signaling. To do this, we cotransfected Hela cells with a full-length VHL expression vector (VHL-pFLAG) and HRE reporter plasmid, and exposed cells for 48 h of hypoxia (based on our above findings with late hypoxia). As shown in Figure 5C, transfection with increasing plasmid concentrations resulted in dose dependent repression of HIF-1α transactivation, as determined by HRE luciferase activity (maximum reduction of hypoxia response $54.2 \pm 9.8\%$ for 1ug/rx VHL-pFLAG, $P = 0.05$).

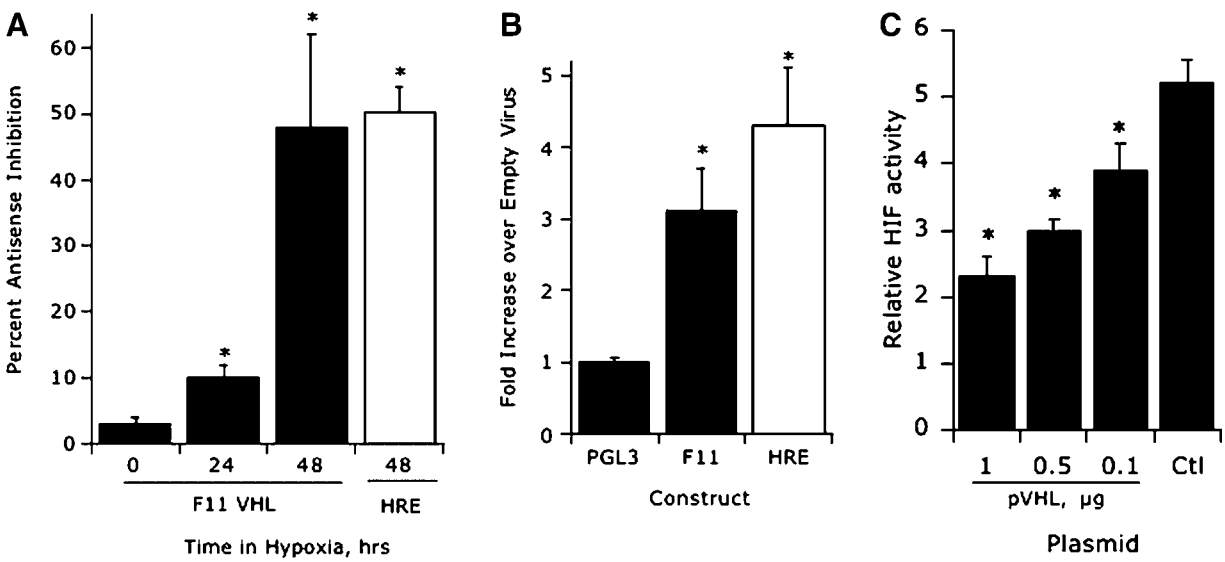

Fig. 5. Role of HIF-1 in VHL induction. **A**: Influence of HIF-1α antisense on VHL promoter activity. Hela cells were loaded with either HIF-1α antisense or HIF-1α sense oligonulceotides, transfected with VHL F11 luciferase constructs, exposed to indicated periods of hypoxia and assayed foe luciferase activity. Results are presented as percent antisense inhibition ± S.E.M. (relative to sense oligonucleotides), where * indicates $P = 0.025$ compared to normoxia. As a control positive for hypoxia, a parallel set of cells were transfected with a luciferase construct containing four tandem HIF binding sites of the EPO promoter (HRE). **B**: Assay of VHL promoter (F11 construct) activity with oxygen-stable HIF-1α. Forced overexpression of oxygen-stable HIF-1α was achieved using an adenovirus expressing constitutively active HIF-1α. Results are presented as fold-increase in activity ± S.E.M. (relative to empty virus), where * indicates $P = 0.05$ compared to PGL3. Empty PGL3 was used as a negative control and the HRE construct was used as a positive control for hypoxia. **C**: Influence of forced VHL expression on HRE activity. Hela cells were co-transfected with HRE (1 μg) and with indicated amounts of plasmid expressing full-length VHL and subjected to hypoxia. Results are presented as HRE activity ± S.E.M. (relative to mock transfected control), where * indicates $P = 0.05$ vs. control treated.

DISCUSSION

Hypoxia is a common physiologic and pathophysiologic occurrence. The rapid induction of the HIF constitutes a critical control point for adaptation to hypoxia at many levels. Conversely, uncontrolled expression of HIF-regulated genes is central to the development of a plethora of pathological states. For this reason, an understanding of mechanisms for HIF repression during ongoing hypoxia are important to understand the dynamics of appropriate hypoxia transcriptional responses. Our findings here contribute to the evolving understanding of HIF biology, and identify hypoxia-induced VHL as a compensatory mechanism to repress HIF activity in late periods of hypoxia.

Since many hypoxia responses consist of persistent, prolonged periods of low oxygen availability, initial studies were done to identify the dynamic range of HIF-1α protein stability in early and late hypoxia. Consistent with previous observations [Wang and Semenza, 1995; Stroka et al., 2001], HIF is rapidly activated, and gradually diminishes during prolonged exposure to hypoxia. Subsequent studies shed some light on potential mechanisms of HIF repression in late periods of hypoxia. First, actinomycin D significantly blocked HIF repression in late hypoxia, implicating the necessity for new transcriptional activity for such HIF repression. Our own analysis revealed no measurable change in HIF-1α mRNA levels under these conditions, suggesting that transcriptional changes in HIF-1α by hypoxia do not account for this finding. Second, a hypoxia/reoxygenation model addressing the expression of potential HIF regulatory protein(s) predicted an oxygen-stable HIF repressor produced late in the hypoxia response, likely requiring the initial period of hypoxia for induction of this repressor activty. Third, microarray analysis examining transcripts regulated in late hypoxia (i.e., >18 h) revealed an interesting profile for members of the E3 ubiquitin ligase complex necessary for the degradation of HIF-1α, namely, VHL, Cullin 2, Elongin B, and related proteins. This screen identified a profound and selective induction of VHL during late periods of hypoxia.

Oxygen availability influences multiple steps in HIF activation, leading to the control of HIF protein levels [Schofield and Ratcliffe, 2004]. However, one of the persistent issues related to the pVHL/HIF interaction remains as to why the loss of VHL function in vivo results in complete and persistent activation of HIF [Iliopoulos et al., 1996], which implicates pVHL as unifying regulator of HIF activity. In support of this hypothesis, we demonstrate that transient transfection of wild-type VHL results in a concentration-dependent loss of functional HIF activity under hypoxic conditions. It is well established that pVHL critically regulates HIF expression through post-translational modification of its alpha subunit [Ohh et al., 1998; Maxwell et al., 1999; Cockman et al., 2000]. Current views maintain that this pVHL/HIF interaction occurs dependent on the availability of hydroxylated HIF-α, though it is not clearly to what extent cellular levels of pVHL and prolyl hydroxylase contriubute to the rate limiting aspects of this reaction. Interestingly though, recent data suggest that the prolyl-hydroxylases are themselves induced in hypoxia through, at least in part, HIF dependent mechanisms [Cioffi et al., 2003; D'Angelo et al., 2003; Marxsen et al., 2004]. Such an induction could counteract the otherwise low binding efficiency these enzymes have for oxygen [Schofield and Ratcliffe, 2004]. In this regard, prolonged hypoxia and the low K_m of mitochondrial cytochrome c oxidase for oxygen (relative to the proly-hydroxylases) should keep the enzyme inactive, and thus HIF dehydroxylated. If, however, respiration decreases in prolonged hypoxia (e.g., through increased production of NO via HIF-1-dependent induction of iNOS), there may, in fact, be an increase in available oxygen due to decreased oxygen consumption. Under such circumstances, NO could facilitate the degradation of HIF-1 in hypoxia. This would likely be due to redistribution of intracellular oxygen by the evolving mechanism of NO-inhibited respiration [Hagen et al., 2003]. In support of this hypothesis, subsets of our own experiments have indicated that NO production (as measured by nitrite intermediates) increases with increasing time in hypoxia (data not shown), particularly, when assessing endothelial cells. In this regard, it is likely that highly vascularlized tissues (i.e., more NO generation) may be relatively more influenced by this response than less vascularlized tissues in vivo. Further studies will be necessary to determine the exact contribution of NO to limitation of HIF activation in prolonged hypoxia.

Conversely, pVHL is shown to be highly involved as a checkpoint of HIF-transcriptional activity. In addition to regulating coactivator recruitment [Lando et al., 2002a,b], the asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH-1), forms a ternary complex containing HIF-1α and VHL, which results to the recruitment of histone deacetylases 1–3 [Mahon et al., 2001] and ensuing transcriptional repression. It has yet to be determined whether oxygen-dependent interaction of HIF-1α and VHL is required for recruitment of FIH-1. Furthermore, other mechanisms of transcriptional repression involving pVHL have also become evident. For example, pVHL recruits a KRAB-A domain protein termed VHLaK in conjunction with the known enhancer of transcriptional repression KAP1/TIF-1β to inhibit HIF-1 transactivation [Li et al., 2003] and interacts directly with SP-1 to inhibit, e.g., VEGF promoter activity [Mukhopadhyay et al., 1997] thereby providing additional levels of regulation. While our data strongly suggest that hypoxia dependent induction of VHL serves as a negative feedback for HIF activation, we did not address the specific mechanisms involved nor whether the effect of VHL induction is restricted on HIF regulation. As such, a number of VHL target genes have been identified [Wykoff et al., 2000], the majority are not regulated by oxygen levels.

This complex involvement of VHL in HIF signaling suggests the existence of regulatory mechanisms that ensure a balance between the necessary HIF activation needed for adaptive responses and HIF repression to prevent autonomous growth. In this regard, it has been previously hypothesized that pVHL and HIF-1 form a degradation-transactivation loop similar to p53 and Mdm-2 [Blagosklonny, 2001], yet, to date, little is known about the regulation of VHL to answer this question conclusively. We show here multiple levels of evidence to demonstrate that VHL is a HIF target gene. These data including increased promoter activity in hypoxia, direct HIF-1α binding to the VHL promoter, a loss of activity with site-directed mutagenesis, and both loss and gain of VHL expression proportional to HIF-1α expression levels. Such findings extend recent work indicating pVHL regulation by hypoxia involving RhoA, and to a lesser extent c-Jun and p38 MAP kinases [Turcotte et al., 2004]. Taken together, these results provide an intriguing model, whereby HIF-1α self-regulates expression through the VHL intermediate, similar to the currently accepted model of I-κB/NF-κB [D'Angio and Finkelstein, 2000].

A lingering question is what controls the late hypoxia expression pattern(s) of VHL? We consistently observed VHL induction by sustained hypoxia, showing a characteristic late induction both at the mRNA and protein level. These observations are similar to previous work indicating the induction of VHL in high density cell culture [Baba et al., 2001], a condition that is known to induce HIF-1 likely due to the development of a hypoxic microenvironment [Sheta et al., 2001]. In contrast, Berra et al. [2001] suggested that decreased HIF-1α stability by hypoxic preconditioning was not likely due to induction of VHL following exposure to hypoxia. These differences are likely explained by their relatively short period of hypoxic stimulation (8 h) compared to our characteristically late hypoxia response (>24 h). Nonetheless, such observations allow for the existence of VHL-independent mechanisms that influence HIF stability (e.g., HIF-1 dependent induction of the prolyl-hydroxylases [Marxsen et al., 2004]), whose time frame overlaps more readily the observation of Berra et al. [2001]. This late induction of VHL is, indeed, somewhat surprising as our data shows that the transcriptional regulator of this induction is HIF-1, a transcription factor that otherwise regulates a rapid response in target genes. In this context, it is of note that the HIF-1 site in close geographical association with a previously established Sp-1 site. The fact that mutation of the Sp-1 site enhanced the hypoxic induction of VHL promoter activity suggests the possibility for functional crosstalk between HIF-1 and Sp-1, where Sp-1 regulates baseline activity of VHL while also limiting the hypoxic response. At this point, the nature of this crosstalk is unclear and may depend either on Sp-1 blocking of the HIF-1 site itself due to the close proximity of both sites or heterotypic interactions between Sp-1 and HIF-1 that are known to take place between Sp-1 and other transcription factors [Suske, 1999]. Alternatively, AP-1 could play an important role in such regulation, particularly, since AP-1 has been shown to cooperate with HIF-1 in the regulation of the VEGF and Cap43 genes in hypoxia [Salnikow et al., 2002].

Taken together, the work presented here supports a negative feedback loop via HIF-dependent VHL induction. The finding that

HIF-1α self-regulation constitutes a novel dynamic aspect of the interplay between adaptive gene expression in hypoxia and prevention of autonomous growth. While distinct mechanisms still need clarification, our work provides a critical link previously postulated by Blagosklonny [Blagosklonny, 2001] essential to the understanding of the relationship between HIF and VHL.

REFERENCES

Baba M, Hirai S, Kawakami S, Kishida T, Sakai N, Kaneko S, Yao M, Shuin T, Kubota Y, Hosaka M, Ohno S. 2001. Tumor suppressor protein VHL is induced at high cell density and mediates contact inhibition of cell growth. Oncogene 20:2727–2736.

Berra E, Richard DE, Gothie E, Pouyssegur J. 2001. HIF-1-dependent transcriptional activity is required for oxygen-mediated HIF-1alpha degradation. FEBS Lett 491: 85–90.

Blagosklonny MV. 2001. Do VHL and HIF-1 mirror p53 and Mdm-2? Degradation-transactivation loops of oncoproteins and tumor suppressors. Oncogene 20:395–398.

Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, Post M. 2000. Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGFbeta(3). J Clin Invest 105:577–587.

Cioffi CL, Liu XQ, Kosinski PA, Garay M, Bowen BR. 2003. Differential regulation of HIF-1 alpha prolyl-4-hydroxylase genes by hypoxia in human cardiovascular cells. Biochem Biophys Res Commun 303:947–953.

Cockman ME, Masson N, Mole DR, Jaakkola P, Chang GW, Clifford SC, Maher ER, Pugh CW, Ratcliffe PJ, Maxwell PH. 2000. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem 275:25733–25741.

Colgan SP, Dzus AL, Parkos CA. 1996. Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. J Exp Med 184:1003–1015.

Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. 2002. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 62:3387–3394.

D'Angelo G, Duplan E, Boyer N, Vigne P, Frelin C. 2003. Hypoxia up-regulates prolyl hydroxylase activity: A feedback mechanism that limits HIF-1 responses during reoxygenation. J Biol Chem 278:38183–38187. Epub 2003 Jul 21.

D'Angio CT, Finkelstein JN. 2000. Oxygen regulation of gene expression: A study in opposites. Mol Genet Metab 71:371–380.

Furuta GT, Turner JR, Taylor CT, Hershberg RM, Comerford KM, Narravula S, Podolsky DK, Colgan SP. 2001. Hypoxia-inducible factor 1-dependent induction of intestinal trefoil factor protects barrier function during hypoxia. J Ex Med 193:1027–1034.

Hagen T, Taylor CT, Lam F, Moncada S. 2003. Redistribution of intracellular oxygen in hypoxia by nitric oxide: Effect on HIF1alpha. Science 302:1975–1978.

Huang LE, Arany Z, Livingston DM, Bunn HF. 1996. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J Biol Chem 271:32253–32259.

Iliopoulos O, Levy AP, Jiang C, Kaelin WG, Jr., Goldberg MA. 1996. Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. Proc Natl Acad Sci USA 93:10595–10599.

Ivan M, Kaelin WG, Jr. 2001. The von Hippel-Lindau tumor suppressor protein. Curr Opin Genet Dev 11:27–34.

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin W, Jr. 2001. HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: Implications for O_2 sensing. Science 292:464–468.

Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, Gassmann M, Gearhart JD, Lawler AM, Yu AY, Semenza GL. 1998. Cellular and developmental control of O_2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 12:149–162.

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ, Maxwell PH, Pugh CW, Ratcliffe PJ. 2001. Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O_2-regulated prolyl hydroxylation. Science 292:468–472.

Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, Gassmann M. 2001. Induction of HIF-1alpha in response to hypoxia is instantaneous. Faseb J 15:1312–1314.

Jiang C, Lu H, Vincent KA, Shankara S, Belanger AJ, Cheng SH, Akita GY, Kelly RA, Goldberg MA, Gregory RJ. 2002. Gene expression profiles in human cardiac cells subjected to hypoxia or expressing a hybrid form of HIF-1 alpha. Physiol Genomics 8:23–32. Epub 2001 Nov 15.

Kaelin WG, Jr., Maher ER. 1998. The VHL tumor-suppressor gene paradigm. Trends Genet 14:423–426.

Kallio PJ, Wilson WJ, O'Brien S, Makino Y, Poellinger L. 1999. Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. J Biol Chem 274:6519–6525.

Kimura H, Weisz A, Ogura T, Hitomi Y, Kurashima Y, Hashimoto K, D'Acquisto F, Makuuchi M, Esumi H. 2001. Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide. J Biol Chem 276:2292–2298.

Kuzmin I, Duh FM, Latif F, Geil L, Zbar B, Lerman MI. 1995. Identification of the promoter of the human von Hippel-Lindau disease tumor suppressor gene. Oncogene 10:2185–2194.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK. 2002a. FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev 16:1466–1471.

Lando D, Peet DJ, Whelan DA, Gorman JJ, Murray LW. 2002b. Asparagine hydroxylation of the HIF transactivation domain: A hypoxic switch. Science 295:858–861.

Lee JY, Dong SM, Park WS, Yoo NJ, Kim CS, Jang JJ, Chi JG, Zbar B, Lubensky IA, Linehan WM, Vortmeyer AO, Zhuang Z. 1998. Loss of heterozygosity and somatic mutations of the VHL tumor suppressor gene in sporadic cerebellar hemangioblastomas. Cancer Res 58:504–508.

Li Z, Wang D, Na X, Schoen SR, Messing EM, Wu G. 2003. The VHL protein recruits a novel KRAB-A domain

protein to repress HIF-1alpha transcriptional activity. Embo J 22:1857–1867.

Linehan WM, Lerman MI, Zbar B. 1995. Identification of the von Hippel-Lindau (VHL) gene. Its role in renal cancer. Jama 273:564–570.

Lockhart DJ, Dong H, Byrne MC, Follettie MT, Gallo MV, Chee MS, Mittmann M, Wang C, Kobayashi M, Horton H, Brown EL. 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. Nat Biotechnol 14:1675–1680.

Mahon PC, Hirota K, Semenza GL. 2001. FIH-1: A novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. Genes Dev 15:2675–2686.

Marxsen JH, Stengel P, Doege K, Heikkinen P, Jokilehto T, Wagner T, Jelkmann W, Jaakkola P, Metzen E. 2004. Hypoxia-inducible factor-1 (HIF-1) promotes its degradation by induction of HIF-alpha-prolyl-4-hydroxylases. Biochem J 381:761–767.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. 1999. The tumor suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature 399:271–275.

Mukhopadhyay D, Knebelmann B, Cohen HT, Ananth S, Sukhatme VP. 1997. The von Hippel-Lindau tumor suppressor gene product interacts with Sp1 to repress vascular endothelial growth factor promoter activity. Mol Cell Biol 17:5629–5639.

Ohh M, Yauch RL, Lonergan KM, Whaley JM, Stemmer-Rachamimov AO, Louis DN, Gavin BJ, Kley N, Kaelin WG, Jr., Iliopoulos O. 1998. The von Hippel-Lindau tumor suppressor protein is required for proper assembly of an extracellular fibronectin matrix. Mol Cell 1:959–968.

Paul SA, Simons JW, Mabjeesh NJ. 2004. HIF at the crossroads between ischemia and carcinogenesis. J Cell Physiol 200:20–30.

Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:E45.

Ramaswamy S, Nakamura N, Sansal I, Bergeron L, Sellers WR. 2002. A novel mechanism of gene regulation and tumor suppression by the transcription factor FKHR. Cancer Cell 2:81–91.

Salnikow K, Kluz T, Costa M, Piquemal D, Demidenko ZN, Xie K, Blagosklonny MV. 2002. The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Mol Cell Biol 22:1734–1741.

Schofield CJ, Ratcliffe PJ. 2004. Oxygen sensing by HIF hydroxylases. Nat Rev Mol Cell Biol 5:343–354.

Semenza GL. 1999. Regulation of mammalian O_2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–578.

Semenza GL. 2004. Intratumoral hypoxia, radiation resistance, and HIF-1. Cancer Cell 5:405–406.

Sheta EA, Trout H, Gildea JJ, Harding MA, Theodorescu D. 2001. Cell density mediated pericellular hypoxia leads to induction of HIF-1alpha via nitric oxide and Ras/MAP kinase mediated signaling pathways. Oncogene 20:7624–7634.

Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D. 2001. HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. Faseb J 15:2445–2453.

Suske G. 1999. The Sp-family of transcription factors. Gene 238:291–300.

Synnestvedt K, Furuta GT, Comerford KM, Louis N, Karhausen J, Eltzschig HK, Hansen KR, Thompson LF, Colgan SP. 2002. Ecto-5′-nucleotidase (CD73) regulation by hypoxia-inducible factor-1 (HIF-1) mediates permeability changes in intestinal epithelia. J Clin Invest 110:993–1002.

Taylor CT, Fueki N, Agah A, Hershberg RM, Colgan SP. 1999. Critical role of cAMP response element binding protein expression in hypoxia-elicited induction of epithelial TNFa. J Biol Chem 274:19447–19450.

Taylor CT, Furuta GT, Synnestvedt K, Colgan SP. 2000. Phosphorylation-dependent targeting of cAMP response element binding protein to the ubiquitin/proteasome pathway in hypoxia. Proc Natl Acad Sci USA 97:12091–12096.

Turcotte S, Desrosiers RR, Beliveau R. 2004. Hypoxia upregulates von Hippel-Lindau tumor-suppressor protein through RhoA-dependent activity in renal cell carcinoma. Am J Physiol Renal Physiol 286:F338–F348. Epub 2003 Oct 28.

Wang GL, Semenza GL. 1995. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem 270:1230–1237.

Wykoff CC, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ. 2000. Identification of novel hypoxia dependent and independent target genes of the von Hippel-Lindau (VHL) tumor suppressor by mRNA differential expression profiling. Oncogene 19:6297–6305.

Zatyka M, Morrissey C, Kuzmin I, Lerman MI, Latif F, Richards FM, Maher ER. 2002. Genetic and functional analysis of the von Hippel-Lindau (VHL) tumor suppressor gene promoter. J Med Genet 39:463–472.