Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein

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Summary

Inactivation of the von Hippel-Lindau tumor suppressor gene is linked to the development of hereditary (VHL Disease-associated) and sporadic clear cell carcinoma of the kidney. The VHL gene product, pVHL, targets the heterodimeric transcription factor HIF for polyubiquitination, and restoration of pVHL function in VHL$^{-/-}$ renal carcinoma cells suppresses their ability to form tumors in nude mice. Here we show that tumor suppression by pVHL can be overridden by a HIF variant that escapes pVHL control. These studies prove that HIF is a critical downstream target of pVHL and establish that activation of HIF target genes can promote tumorigenesis in vivo.

Introduction

von Hippel-Lindau Disease is a hereditary cancer syndrome that displays an autosomal dominant pattern of inheritance (Maher and Kaelin, 1997; McKusick, 1992). The hallmark feature of this disorder is the development of blood vessel tumors (hemangioblastomas) of the central nervous system and retina, often in association with other tumors such as clear cell carcinomas of the kidney and pheochromocytomas. The *VHL* gene resides on chromosome 3p25, and individuals with VHL disease are germline *VHL*$^{+/-}$ heterozygotes. Tumors develop in this setting when the remaining wild-type *VHL* allele is mutated or lost. Thus, at the molecular level, the *VHL* gene is a tumor suppressor gene and conforms to the Knudson two-hit model. In keeping with this notion, biallelic *VHL* inactivation due to somatic mutations is a common feature of nonhereditary clear cell kidney cancers and hemangioblastomas (Brauch et al., 1999; Foster et al., 1994; Gallou et al., 1999; Gnarra et al., 1994; Kanno et al., 1994; Lee et al., 1998; Shuin et al., 1994, 1999). Furthermore, restoration of *VHL* function in *VHL*$^{-/-}$ renal carcinoma cell lines suppresses their ability to form tumors in vivo (Gnarra et al., 1996; Iliopoulos et al., 1995), and somatic inactivation of *VHL* in the mouse causes the development of blood vessel tumors, which loosely resemble hemangioblastomas (Haase et al., 2001).

The *VHL* gene product, pVHL, forms a multimeric complex that contains elongin B, elongin C, Cul2, and Rbx1 (also called ROC1 or Hrt1) (Duan et al., 1995; Kamura et al., 1999; Kibel et al., 1995; Lonergan et al., 1998; Pause et al., 1997, 1999). These complexes ubiquitinate the α subunits of the heterodimeric transcription factor called HIF (hypoxia-inducible factor) in the presence of oxygen (Cockman et al., 2000; Kamura et al., 2000; Ohh et al., 2000; Tanimoto et al., 2000). Consequently, normal cells accumulate HIF only under low oxygen (hypoxic) conditions, whereas cells lacking pVHL contain high levels of HIF irrespective of changes in ambient oxygen (Maxwell et al., 1999). Recent studies have shown that the interaction of pVHL with HIF is governed by oxygen-dependent hydroxylation of conserved proline residues in HIF by members of the Egl9 family (Bruick and McKnight, 2001; Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001).

pVHL contains two subdomains called the α domain and the β domain (Stebbins et al., 1999). The α domain binds to the elongins, which in turn bind to Cul2, while the β domain binds directly to HIF (Ohh et al., 2000). *VHL* mutations associated with the development of hemangioblastoma or renal cell carcinoma typically compromise the function of the α domain, the β domain, or both (Ohh et al., 1999, 2000; Stebbins et al., 1999). Accordingly, these tumors are characterized by high levels of HIF as well as the products of HIF-target genes (Gnarra et al., 1996; Iliopoulos et al., 1996; Krieg et al., 2000; Maxwell et al., 1999; Siemeister et al., 1996; Wiesener et al., 2001; Wizigmann-Voos et al., 1995). Some VHL families display a high risk of pheochromocytoma without the other stigmata of VHL disease. Where tested, such families harbor mutant *VHL* alleles that retain the ability to downregulate HIF (Clifford et al., 2001; Hoffman

SIGNIFICANCE

The *VHL* gene product, pVHL, is frequently mutated in renal carcinoma, and targets HIF for destruction in the presence of oxygen. Tumor-derived pVHL mutants are defective in this regard. Nonetheless, it is unproven that inhibition of HIF is causally linked to tumor suppression by pVHL. Here we report that tumor suppression by pVHL can be overridden by a HIF variant that escapes recognition by pVHL. Thus, inhibition of HIF is necessary for tumor suppression by pVHL. This study shows that activation of HIF-target genes can promote tumor growth and thus establishes that HIF can behave as an oncogene in vivo. This finding has broad implications, as most solid tumors contain hypoxic cells, which accumulate HIF.

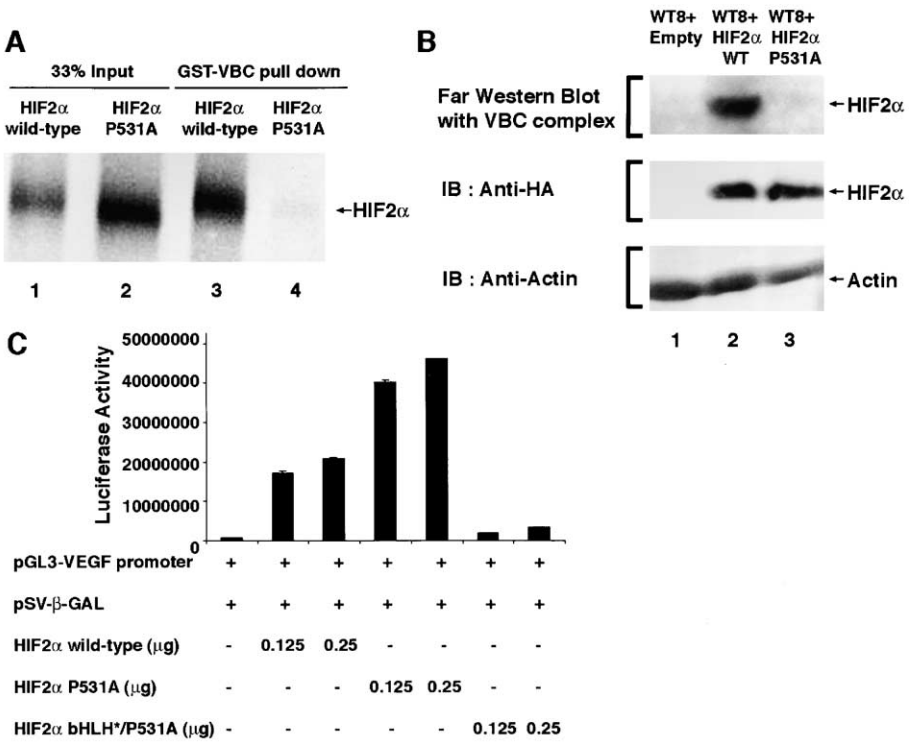

Figure 1. Characterization of HIF2α P531A mutant

A: Binding of ^{35}S-labeled wild-type and P531A HIF2α in vitro translates to immobilized GST-VHL, elongin B, elongin C (VBC) complexes.

B: Far Western blot analysis of WT8 cells stably transfected with a plasmid encoding HA-HIF2α, HA-HIF2α P531A, or the empty vector. Cells were treated with proteasome inhibitor for 8 hr prior to analysis. Blot was probed with VBC complex. Note that endogenous HIF2α is not detected under these assay conditions, presumably due to low abundance.

C: Luciferase activity, corrected for β galactosidase, following transient transfection of U2OS cells with reporter plasmid containing VEGF promoter upstream of luciferase and plasmids encoding the indicated HIF2α species.

et al., 2001). In summary, there is a strong correlation between the risk of developing hemangioblastoma and renal cell carcinoma for a given mutant *VHL* allele and the ability of its protein product to regulate HIF.

HIF induces the expression of angiogenic factors such as VEGF (vascular endothelial growth factor) and PDGF B (platelet-derived growth factor), as well as mitogenic factors such as TGFα (de Paulsen et al., 2001; Kourembanas et al., 1990; Semenza, 1999). Overproduction of angiogenic factors could explain the hypervascular nature of hemangioblastomas and renal cell carcinomas. In support of this, forced expression of VEGF in murine model systems gives rise to immature blood vessels that resemble hemangioblastomas, whereas inhibition of VEGF signaling can inhibit renal cell tumor growth in vivo (Benjamin and Keshet, 1997; Drevs et al., 2000). Overproduction of TGFα and its cognate receptor, EGFR, has been described in both hemangioblastomas and renal cell carcinomas (Atlas et al., 1992; Bohling et al., 1996; de Paulsen et al., 2001; Knebelmann et al., 1998; Lager et al., 1994; Lau et al., 1988; Mydlo et al., 1989; Petrides et al., 1990; Reifenberger et al., 1995; Walke et al., 1991). TGFα acts as a renal epithelial mitogen, and disruption of TGFα/EGFR signaling inhibits renal carcinoma cell proliferation in vitro and in vivo (Atlas et al., 1992; de Paulsen et al., 2001; Humes et al., 1991; Petrides et al., 1990; Ramp et al., 2000).

In summary, both correlative genetic data and biological plausibility suggest that HIF is a critical downstream target of pVHL with respect to the development of hemangioblastomas and renal cell carcinomas. Nonetheless, it has not been formally proven that downregulation of HIF is either necessary or sufficient for tumor suppression by pVHL. For example, it is possible that tumor suppression by pVHL is linked to its ability to bind to another cellular protein that interacts with the β domain in a manner similar to HIF. If true, the ability to bind to HIF might be a surrogate for retention of this function. To address this possibility, we have made a HIF variant that retains the ability to activate HIF-target genes but escapes pVHL control because of a proline to alanine substitution within its pVHL binding motif. Introduction of this HIF variant into renal carcinoma cells previously rendered pVHL-positive restored their ability to form tumors in nude mouse xenograft assays. This establishes that inhibition of HIF is necessary for tumor suppression by pVHL and demonstrates the previously suspected protumorigenic effects of the HIF gene expression program.

Results

786-O renal carcinoma cells lack functional pVHL, and overproduce HIF2α and HIF target genes such as VEGF, PDGF B, and the GLUT1 glucose transporter (Gnarra et al., 1996; Iliopoulos et al., 1996; Maxwell et al., 1999; Siemeister et al., 1996). 786-O cells do not contain detectable amounts of HIF1α (Maxwell et al., 1999). We previously stably transfected 786-O cells with a plasmid encoding wild-type pVHL or, as a control, with the empty vector. Multiple independent subclones producing wild-type pVHL (WT clones) were identified and were unable to form macroscopic tumors in nude mice, in contrast to the empty vector transfectants (RC series) or the parental line (Iliopoulos et al., 1995).

In order to restore activation of HIF target genes in a representative WT clone (WT8), we set out to make a HIF2α variant that would escape recognition by pVHL and yet retain its ability to bind to DNA and activate transcription. Mutation of proline 564 to alanine in the pVHL binding motif of HIF1α leads to loss of pVHL binding and enhanced stability in vitro and in cellulo

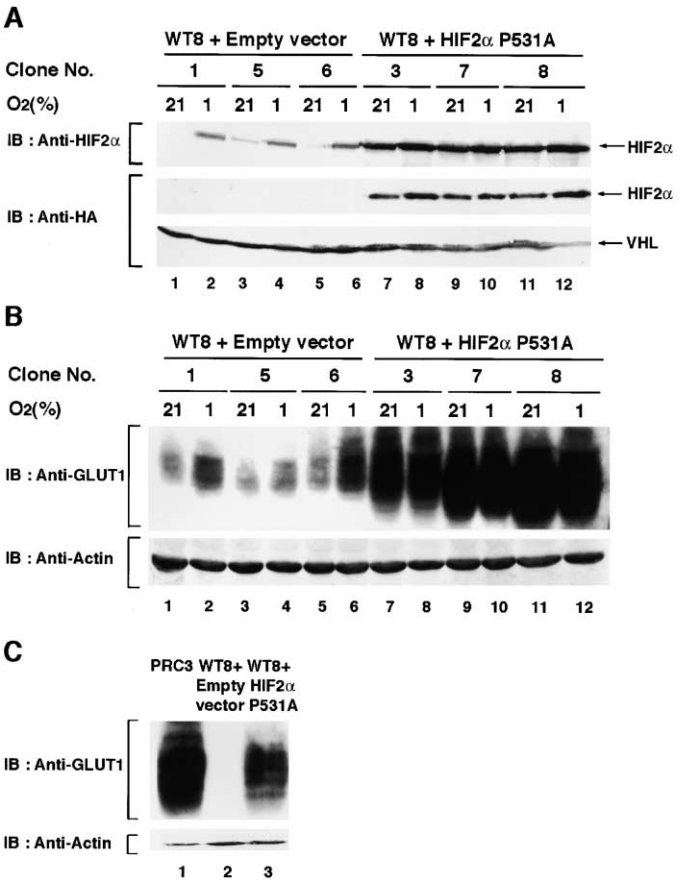

Figure 2. Introduction of HIF2α P531A into WT8 cells by stable transfection

A and B: 786-O *VHL*$^{-/-}$ human renal carcinoma cells engineered to produce hemagglutinin (HA)-tagged wild-type pVHL (WT8) were transfected with a plasmid encoding HA-HIF2α P531A or the empty vector. Following drug selection, individual colonies were expanded, grown in the presence of 1% or 21% oxygen, and immunoblotted (IB) with the indicated antibodies.

C: 786-O cells stably transfected with an empty expression plasmid (pRC3) and representative WT8 clones transfected with a plasmid encoding HA-HIF2α P531A or the empty vector were grown in 21% oxygen and immunoblotted with the indicated antibodies.

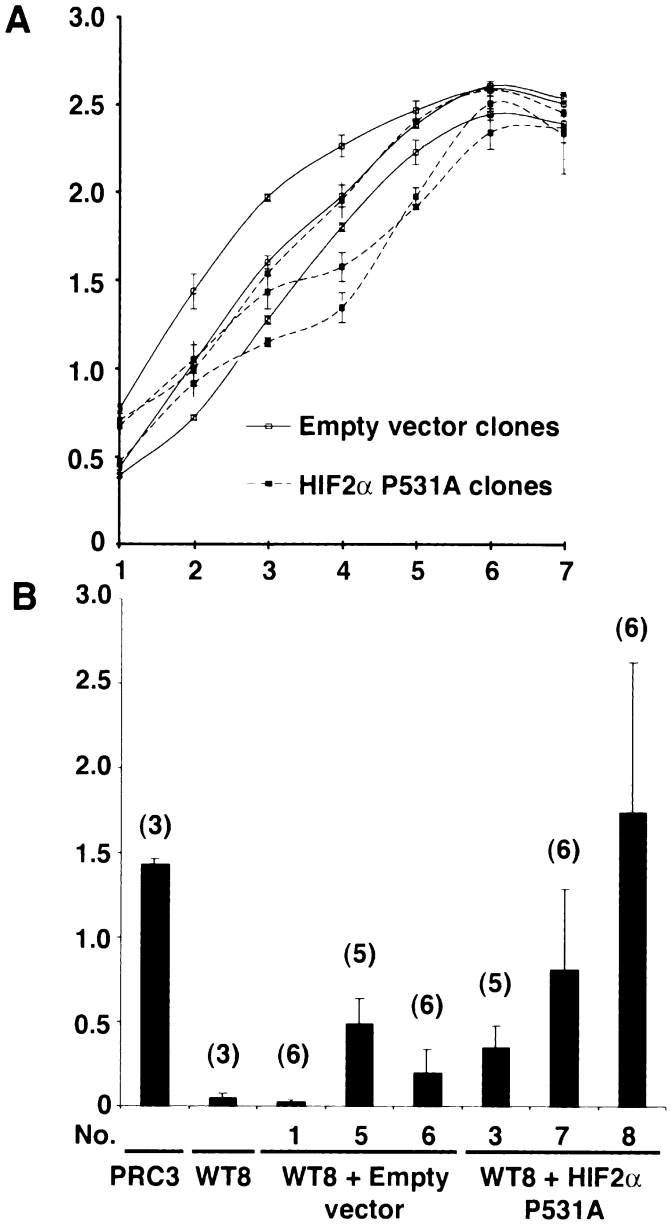

Figure 3. Proliferation of WT8 stable transfectants producing HIF2α P531A

A: Stable WT8 subclones tranfected to produce HIF2α P531A or with the empty vector were grown in 96-well plates in the presence of serum. Viable cell number at the indicated time points after seeding was determined by XTT assay.

B: The indicated WT8 subclones, as well as pVHL-defective pRC3 cells, were injected subcutaneously in the flanks of nude mice. Approximately 10 weeks later, tumors were excised and weighed. The number of tumors analyzed is indicated in parentheses. Error bars = one standard error.

(Ivan et al., 2001). In the first set of experiments, the corresponding HIF2α mutant (P531A) was made as a radioactive protein in a rabbit reticulocyte lysate, which contains HIF prolyl hydroxylase activity, and used in binding studies with immobilized GST-VHL in complex with elongin B and elongin C. As expected, wild-type HIF2α, but not HIF2α P531A, bound to pVHL (Figure 1A). Likewise, pVHL bound to HA-HIF2α, but not HA-HIF2α P531A, in Far Western blot assays of WT8 cells that were engineered to produce either of these two proteins and treated with proteasome inhibitors (Figure 1B). In keeping with this observation, the half-life of HIF2α P531A was prolonged approximately 4-fold relative to wild-type HIF2α when introduced into mammalian cells (Figure 4C and data not shown). Finally, HIF2α P531A retained the ability to activate transcription in cotransfection assays using the HIF-responsive VEGF promoter (Figure 1C). This effect was specific, as it was abrogated by introducing a second mutation within the HIF2α basic HLH domain.

Next, WT8 cells were stably transfected with a plasmid that encodes hemagglutinin (HA)-tagged HIF2α P531A and confers puromycin resistance. In parallel, WT8 cells were transfected with the backbone plasmid. Individual puromycin resistant colonies were expanded and analyzed. HIF2α P531A clones 3, 7, and 8 produced readily detectable levels of HA-HIF2α and were compared to 3 randomly selected empty vector transfectants (clones 1, 5, and 6) (Figure 2A). The presence of an HA tag allowed for the identification of the exogenous HIF2α species as well as comparison with the amount of pVHL in the cell by

anti-HA immunoblot analysis. The total amount of HIF2α present (exogenous and endogenous) was determined by anti-HIF2α immunoblot analysis. The accumulation of HIF2α P531A in the presence of 21% oxygen despite the availability of excess pVHL (Figure 2A, lanes 7, 9, and 11) and the minimal variation observed with changes in oxygen (Figure 2A, compare lanes 7, 9, and 11 with lanes 8, 10, and 12, respectively) further supports that recognition of HIF2α P531A by pVHL is impaired in intact cells. The GLUT1 glucose transporter is the product of a HIF-target gene and is readily monitored by immunoblot analysis. As expected, HIF2α P531A clones 3, 7, and 8 produced high levels of GLUT1 (Figure 2B), which were comparable to the levels observed in 786-O cells lacking pVHL (Figure 2C and data not shown). Another HIF-target, *VEGF*, was also activated in the HIF2α P531A clones as determined by Northern blot analysis (data not shown).

The presence or absence of pVHL does not grossly affect the monolayer growth of 786-O cells on plastic in the presence of serum (Davidowitz et al., 2001; Iliopoulos et al., 1995). Likewise, the presence or absence of HIF2α P531A did not substantially alter the proliferation of WT8 cells (Figure 3A). Moreover, HIF2α did not promote the proliferation of WT8 cells grown in media containing 0.1% serum (data not shown). Following subcutaneous injection into nude mice, however, the WT8 clones producing HIF2α P531A formed larger tumors than the empty vector transfectants, although there was significant clone to clone variation (Figure 3B). HIF2α P531A clones 7 and 8 reproducibly formed macroscopic tumors which, in the case of clone 8, approximated those seen in a representative 786-O clone lacking pVHL (pRC3). The collective difference between the HIF2α P531A clones and empty vector clones was significant ($p < 0.04$) as determined by the Wilcoxon signed rank test.

The variable rescue of tumor forming capability by HIF2α P531A among individual WT8 subclones raised the possibility that genetic heterogeneity arose within the WT8 line before, during, or after selection of the individual clones producing HIF2α P531A. To circumvent the need for clonal selection, and its attendant pitfalls, we next switched to a retroviral-based approach (Figure 4A). WT8 cells were infected with a retrovirus that encoded HIF2α P531A and conferred puromycin resistance. In parallel, cells were infected with an empty retrovirus conferring puromycin resistance alone ("empty vector"). Following infection, puromycin-resistant cells were pooled and analyzed. The accumulation of HIF2α (Figure 4A) and its downstream target GLUT1 (Figure 4B), under normoxic conditions in WT8 cells infected to produce HIF2α P531A, was comparable to that observed in the pVHL-defective PRC3 cells. In keeping with the results obtained with the individual WT8 subclones, retroviral introduction of HIF2α P531A did not significantly alter the proliferation of WT8 cells in vitro (Figure 5A), but fully restored their ability to form tumors in nude mice (Figure 5B). Immunohistochemical analysis of tumors formed by WT8 cells producing HIF2α P531A confirmed that HIF was transcriptionally active in vivo, as shown by diffuse, intense, membranous GLUT1 staining, similar to that observed in PRC3 cells (Figure 6). In contrast, GLUT1 staining in the tumors produced by WT8 cells infected with the empty retrovirus was restricted to membranous staining in focal patches, which were typically associated with areas of necrosis. CD31 staining, which marks endothelial cells and hence reflects angiogenesis, was increased in the RC3 tumors compared to the WT8 cells infected with either

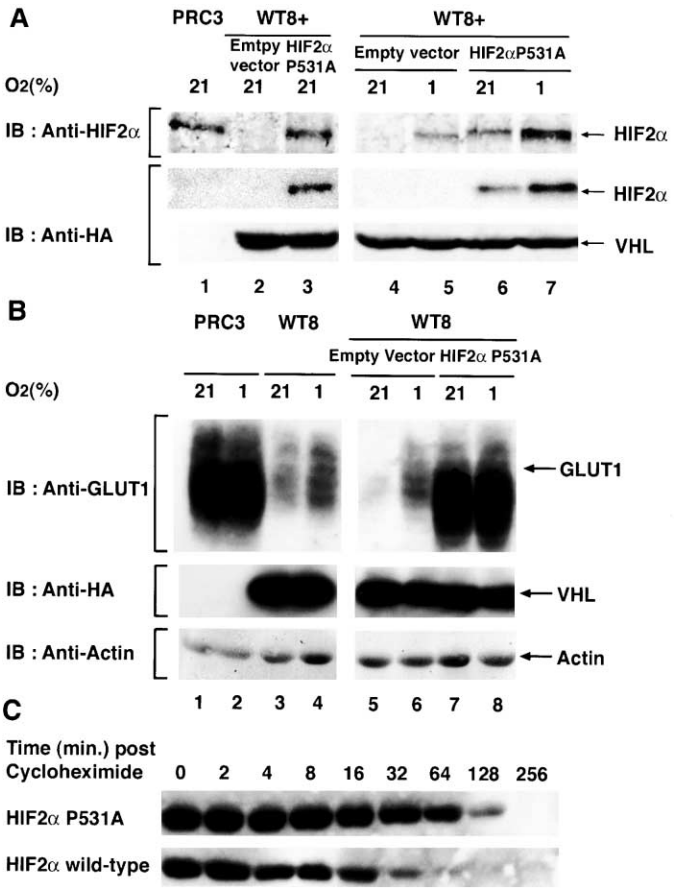

Figure 4. Introduction of HIF2α P531A into WT8 cells by retroviral infection

A and B: pRC3 cells and WT8 cells infected to produce HA-HIF2α P531A or with the empty vector were grown in the presence of 1% or 21% oxygen and immunoblotted (IB) with the indicated antibodies.

C: WT8 cells were retrovirally infected to produce HA-tagged wild-type or P531A HIF2α. At the indicated time points following the addition of cycloheximide, cell extracts were prepared and immunoblotted with anti-HA antibody.

the empty retrovirus or the retrovirus encoding HIF2α P531A (Figure 6). This difference was confirmed by quantification of microvessel density (data not shown). This raises the possibility that angiogenesis following pVHL loss reflects more than dysregulation of HIF alone. The microvessel densities of the tumors formed by the WT8 cells infected with the empty retrovirus or the retrovirus encoding HIF2α P531A did not significantly differ (data not shown), perhaps in keeping with an earlier report that genetic disruption of HIF1α led to a decrease in tumorigenicity without altering microvessel density (Ryan et al., 2000). Of note, WT8 tumor cells expressing HIF2α P531A were highly pleiomorphic and anaplastic, with significant nuclear atypia and frequent mitotic figures. Occasional spindle-shaped tumor cells were observed in addition to bizarre, multinucleated, tumor cells. Histologically, these cells appeared to be more malignant than either the RC3 cells or the WT8 cells.

Discussion

In this study we have shown that activation of HIF target genes is sufficient to override the tumor suppressor activity of pVHL

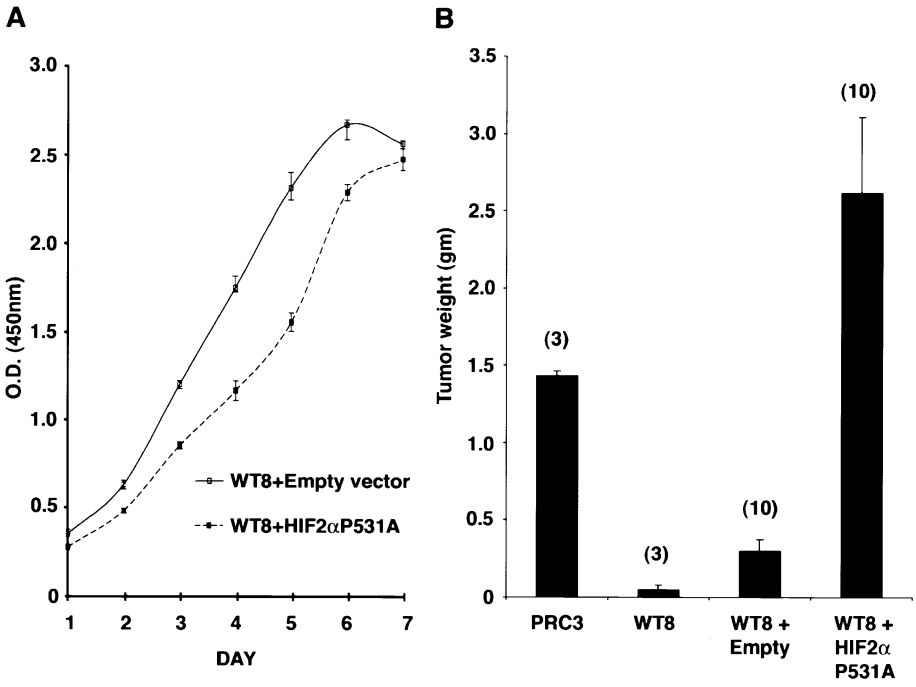

Figure 5. Proliferation of WT8 cells retrovirally infected to produce HIF2α P531A

A: WT8 cells infected to produce HIF2α P531A or with the empty vector were grown in 96-well plates in the presence of serum. Viable cell number at the indicated time points after seeding was determined by XTT assay.

B: pVHL-defective pRC3 cells and WT8 cells infected to produce HIF2α P531A or with empty vector were injected subcutaneously in the flanks of nude mice. Approximately 10 weeks later, tumors were excised and weighed. The number of tumors analyzed is indicated in parentheses. Error bars = one standard error.

against human renal carcinoma cells in vivo. This work has implications with respect to pVHL-defective tumors in particular and solid tumors in general.

All pVHL mutants associated with the development of hemangioblastoma and renal carcinoma examined to date have lost the ability to target HIF for destruction. This correlation, coupled with the knowledge that HIF regulates genes involved in angiogenesis (such as VEGF) as well as mitogenesis (such as TGFα), has led to speculation that downregulation of HIF is linked to pVHL tumor suppressor function. Nonetheless, it remained possible that HIF was a stalking horse for an as yet unidentified pVHL binding protein (or proteins). In this regard, several lines of reasoning suggest that HIFα P531A did not promote tumor growth in our assays merely by binding stoichometrically to pVHL and preventing it from interacting with other targets. First, the binding of HIF2α P531A to pVHL was grossly impaired, as determined by direct binding assays and by half-life determination in cells, although it remains possible that some residual interaction takes place via a recently recognized second HIF hydroxylation site (Masson et al., 2001). Second, the amount of pVHL produced by WT cells exceeds by at least 10-fold the amount present in normal cells (and hence required for tumor suppression) (Iliopoulos et al., 1998) and also exceeds the amount of HIF2α P531A produced following retroviral infection. Therefore, we conclude that downregulation of HIF itself is necessary for pVHL tumor suppressor activity, at least as measured in the nude mouse xenograft assays described here.

Additional studies will be required to determine if downregulation of HIF is also sufficient for tumor suppression by pVHL. In this regard, genetic disruption of HIF1α inhibits tumor growth in model systems as does pharmacological inhibition of some of its targets such as VEGF and TGFα/EGFR (Kung et al., 2000; Maxwell et al., 1997, 1998; Ryan et al., 2000). Establishing that inhibition of HIF is necessary and sufficient for tumor suppression by pVHL would clearly have implications for the rational treatment of pVHL-associated tumors with small molecules.

All cells produce pVHL and at least one HIF family member. Why pVHL inactivation should be intimately linked to the development of human hemangioblastoma and renal cell carcinoma, for example, and not a wide variety of tumors, is unclear. Both hemangioblastomas and renal carcinomas are characterized by an admixture of $VHL^{-/-}$ tumor cells (derived from a poorly characterized CNS "stromal" cell and proximal renal tubular epithelial cell, respectively) and blood vessels that are either $VHL^{+/-}$ (in VHL disease) or $VHL^{+/+}$ (in sporadic tumors) (Chan et al., 1999; Lubensky et al., 1996; Vortmeyer et al., 1997; Zhuang et al., 1995, 1996). Immunohistochemical analysis of these tumors is consistent with factors such as TGFα acting in an autocrine fashion to promote tumor cell proliferation and factors such as VEGF and PDGF B acting in a paracrine fashion to promote endothelial cell and pericyte proliferation, respectively (Bohling et al., 1996; Brown et al., 1993; Flamme et al., 1998; Lager et al., 1994; Lau et al., 1988; Mydlo et al., 1989; Nicol et al., 1997; Reifenberger et al., 1995; Richard et al., 1998; Stratmann et al., 1997; Walke et al., 1991). In this regard, renal epithelial cells appear to be particularly sensitive to the mitogenic effects of TGFα, perhaps accounting in part for the tissue-specific effects of pVHL loss (de Paulsen et al., 2001). Mouse experiments involving either conditional inactivation of VHL or implantation of cells engineered to produce VEGF suggest that inactivation of *VHL*, and consequent overproduction of VEGF, is sufficient to produce blood vessel tumors that have hemangioblastoma-like features (Benjamin and Keshet, 1997; Haase et al., 2001). In the kidney, biallelic *VHL* inactivation is linked to the development of benign renal cysts. Additional genetic alterations are presumably required for conversion to a frank renal carcinoma. Of note, several studies have linked TGFα to renal cyst formation (Lowden et al., 1994; Sweeney et al., 2000).

It is almost certain that pVHL has targets in addition to HIF.

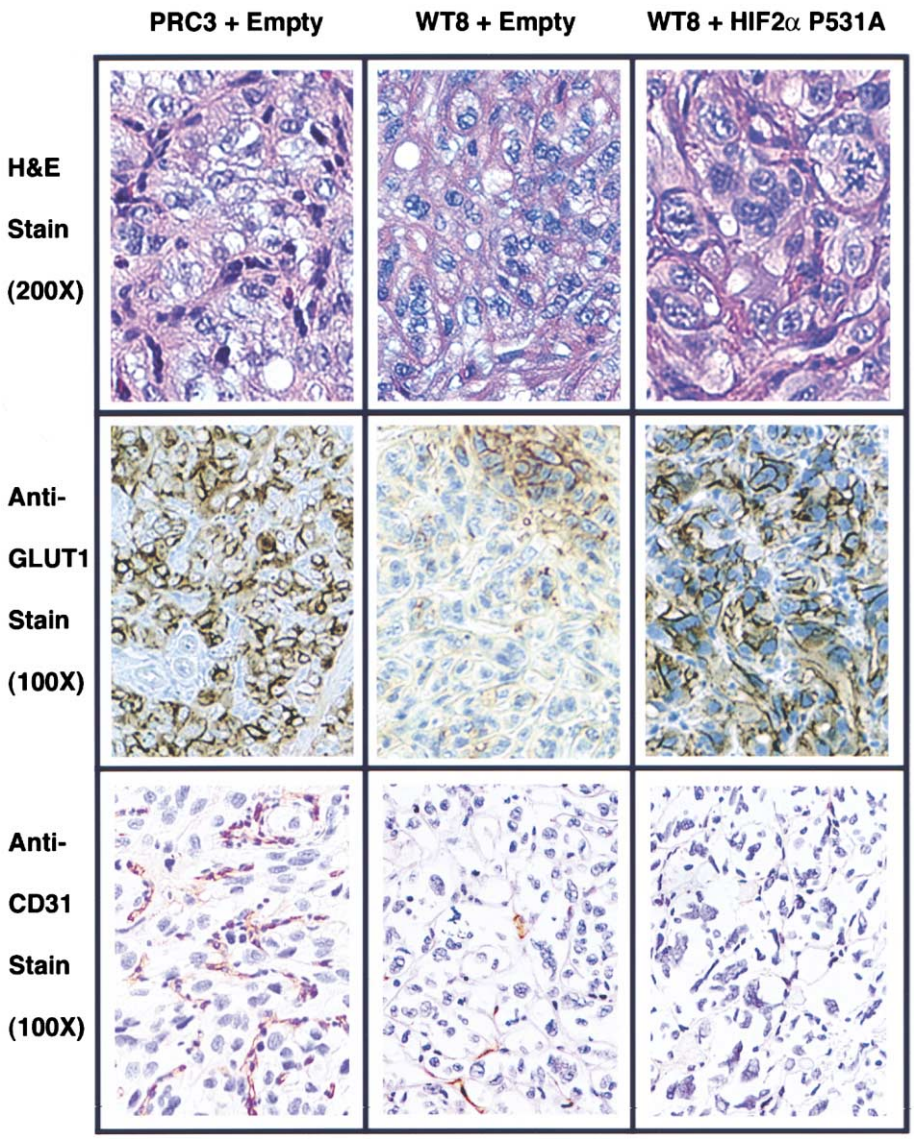

Figure 6. Immunohistochemical analysis of WT8 retrovirally infected to produce HIF2α P531A

Representative immunohistochemical analysis with the indicated antibodies of tumors formed by pVHL-defective pRC3 cells and WT8 cells infected to produce HIF2α P531A or with empty vector.

VHL mutations linked to a "pheochromocytoma-only" variant of VHL disease encode proteins that retain the ability to ubiquitinate HIF (Clifford et al., 2001; Hoffman et al., 2001). Such pVHL mutants may be defective for recognizing one or more proteins that play important roles in regulating the growth and behavior of adrenal medullary cells. Furthermore, it has not been shown that dysregulation of HIF, in any setting, is sufficient to cause tumor growth. In this regard, cutaneous expression of a HIF1α variant lacking the HIF oxygen-dependent degradation/pVHL binding domain led to the appearance of functional blood vessels without the features of hemangioblastoma (Elson et al., 2001). Likewise, tumor development is not a feature of conditions characterized by chronic hypoxia, which would lead to upregulation of HIF. This suggests that the tumor promoting effects of HIF are restricted to certain cell lineages and/or that tumor development in the setting of pVHL loss is due to dysregulation of HIF in addition to loss of other pVHL activities. Finally, $VHL^{-/-}$ renal carcinoma cells harbor additional genetic mutations that might also be required for HIF to promote tumor growth in this setting.

Most solid tumors are characterized by cells that are acutely or chronically hypoxic and hence accumulate HIF (Zhong et al., 1999). Indeed, upregulation of HIF has been suggested to underlie the characteristic changes in tumor cell metabolism referred to as the Warburg Effect (Dang and Semenza, 1999; Seagroves et al., 2001). It has been suspected that HIF promotes tumor growth in vivo, through cell autonomous and nonautonomous changes that would ultimately facilitate growth in a nutrient-poor, oxygen-depleted microenviroment. Our study provides direct evidence that activation of HIF target genes does indeed promote tumor cell growth in vivo and that HIF can, therefore, be viewed as an oncogene.

Experimental procedures

Plasmids

The wild-type HIF2α cDNA in pcDNA3.0-HIF2α-myc (a generous gift of Dr. Steven McKnight) was PCR amplified with Primer A (GCGCGGATCCGC CACCATGACA) and Primer D (GCGCCAATTGTCAGGTGGCCTGGTC), restricted with BamHI and MunI, and ligated into pcDNA3.0-HA and pBABE-

Puro-HA cut with BamHI and EcoRI to make pcDNA3.0-HA-HIF2α and pBABE-HIF2α, respectively. pcDNA3.0-HA-HIF2α P531A and pBabe-puro-HA-HIF2α P531A were generated by two-step PCR. The HIF2α cDNA was first amplified with Primer A and Primer B (CATGGGGATATAAGCTGC CAGTGTCTC) or Primer C (GAGACACTGGCAGCTTATATCCCCATG) and Primer D. Aliquots of these two PCR reactions were then mixed and amplified with Primers A and D. The resulting PCR product was digested with Bam HI/Mun I and ligated with pcDNA3.0-HA vector and pBabe-puro-HA as described above. The HIF2α P531A cDNA in pcDNA3.0-HA-HIF2α P531A was excised by digestion with BamHI and NotI and ligated into IRES-puro-HA cut with these two enzymes to make IRES-puro-HA-HIF2a P531A. To make pcDNA3.0-HA-HIF2α P531A bearing a bHLH mutation (conversion of amino acids residues 24–29, RCRRSK, to ACAASA) the HIF2α P531A cDNA in pcDNA3.0-HA-HIF2α P531A was first amplified with Primer A/Primer E (CTCCGTCTCAGCGCTAGCAGCGCAAGCCGCAGCATC) or Primer F (GAT GCTGCGGCTTGCGCTGCTAGCGCTGAGACGGAG)/Primer D. Aliquots of these two PCR reactions were then mixed and amplified with Primers A and D and ligated with pcDNA3.0-HA vector as described above. All plasmids were authenticated by DNA sequencing. pGL23-VEGF promoter plasmid was a kind gift from Dr. Deb Mukhopadhyay. pSV-β-Gal plasmid was purchased from Promega Corp. (Madison, WI).

Cell culture

786-O renal cell carcinoma subclones stably transfected with either pRc/CMV empty vector (PRC3) or pRc/CMV-HA-VHL (WT8) (Iliopoulos et al., 1995) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Clone I (Hyclone) and 1 mg/ml of G418 in the presence of 10% CO_2 at 37°C. WT8 cells were transfected using FuGENE (Roche) according to the manufacturer's instructions and selected in the presence of puromycin (1.5 μg/ml). Individual drug resistant colonies were isolated using cloning cylinders and expanded.

Phoenix cells (a generous gift of Dr. Gary Nolan, Dept. of Molecular Pharmacology, Stanford University) were maintained in DMEM containing 10% Fetal Clone I in 37°C, 10% CO_2 incubator.

Retroviruses

pBabe-puro-HA-HIF2α (wild-type or P531A), or the backbone vector, were transfected into the Phoenix packaging cell line using FuGene (Roche) according to the manufacturer's instructions. Tissue culture supernatant was harvested 48 hr later, passed though a 0.45 μm filter, and added to WT8 cells in the presence of 4 ug/ml polybrene. Infected cells were selected by growth in the presence of puromycin (1.5 ug/ml).

Immunoblot and far Western blot analysis

Cells were lysed in EBC lysis buffer (50 mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet-P [NP]-40) supplemented with complete protease inhibitor cocktail (Roche Molecular Biochemicals, Mannheim, Germany). Comparable amounts of extract, as determined by the Bradford method, were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Following blocking in TBS with 5% nonfat milk, the membranes were probed with anti-HA rabbit polyclonal antibody (Y-11; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HIF2a rabbit polyclonal antibody (NB100-122; Novus Biologicals, Inc., Littleton, CO) or anti-GLUT1 rabbit polyclonal antibody (Alpha Diagnostic Inc., San Antonio, TX) diluted in TBS with 4% bovine serum albumin. Bound antibody was detected with horseradish peroxidase-conjugated goat anti-Rabbit IgG (PIERCE, Rockford, ILL) and SuperSignal West Pico Chemiluminescent Substrate according to the manufacturer's instructions (PIERCE, Rockford, ILL). Far Western blot analysis was done as described previously (Ohh et al., 2000).

In vitro proliferation assays

Cell proliferation was measured colorimetrically using Cell Proliferation Kit II (Roche Diagnostics) according to the manufacturer's protocol. Briefly, 10^4 cells per well were cultured in 96-well cell culture plates. At the indicated time points, XTT (Sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) was added to the cells. Four hours later the spectrophotometrical absorbance at 450 nm wavelength was measured using a microtiter plate reader.

Cyclohexamide chase assay

WT8 cells that were retrovirally infected to produce wild-type or P531A HIF2α were aliquoted to 35 mm dishes. Once the cells achieved 70%–80% confluence, cycloheximide was added to a final concentration of 100 μg/ml. At the indicated time points thereafter, cell extracts were prepared and immunoblotted with anti-HA antibody.

Nude mouse xenograft assays

Nude mouse xenograft assays were performed as described (Iliopoulos et al., 1995). Briefly, cells were released by trypsination and resuspended in PBS. 10^7 viable cells, as determined by trypan blue staining, were injected subcutaneously into the flanks of nude mice. Both flanks were used for each mouse. One flank was injected with cells producing HIF2α P531A and the other with cells transfected or infected with empty vector. Bidimensional measurements were obtained every week and the animals were sacrificed 10–11 weeks after injection. Autopsy and tumor weight measurements were performed by animal care technicians who were unaware of the HIF status of the tumors. Tumors were cut in half and fixed in either paraffin or frozen in OCT compound for immunohistochemical analysis.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Loda et al., 2000). Briefly, 5 μm tissue sections of formalin-fixed, paraffin-embedded tumors were stained with a polyclonal rat anti-GLUT-1 antibody (Alpha Diagnostic Int., San Antonio, TX) (dilution 1:500, incubation time 1 hr at room temperature) or monoclonal rat anti-mouse CD31 antibody (PECAM-1; BD Pharmingen, San Diego, CA) (dilution 1:100, incubation time 1 hr at room temperature). The reaction was carried out in an automated immunohistochemistry instrument, the Optimax Plus (BioGenex, San Ramon, CA). Antigen-antibody reactions were revealed with standardized development times, using the streptavidin method with diaminobenzidine (DAB) as substrate.

Microvessel quantitation

Microvessel quantitation was performed as previously described (Ogawa et al., 1995; Weidner et al., 1991). Briefly, the area of highest microvascular density was selected by scanning at 40× magnification. Then, the microvessel count (MVC) was determined by examining three fields within this area at 200× magnification (0.785 mm^2 per field). Any CD31-positive endothelial cell or cluster of cells clearly separated from adjacent microvessels, tumor cells, and other elements of connective tissue was considered a single countable microvessel. Branching structures were counted as a single vessel unless there was a break in the continuity of the structure. The presence of either a lumen or erythrocytes in the lumen was not required to classify a structure as a vessel. Undefined endothelial cells, which appeared to be fragments, were not counted as microvessels. For each tumor, the MVC was assessed by two independent pathologists who were unaware of the tumor genotypes and the average value of these two counts was taken as the MVC of the tumor.

Acknowledgments

We thank Dr. Massimo Loda and the DF/HCC Prostate Pathology Core for excellent help with the immunohistochemical studies, Dr. Donna Neuberg for biostatistical help, Julie Kujawa for help with the animal studies, an anonymous reviewer for helpful suggestions, and Dr. S. McKnight and Dr. Deb Mukhopadhyay for reagents. W.G.K. is an Assistant Investigator of the Howard Hughes Medical Institute and is supported by the Murray Foundation and by the National Institutes of Health.

Received: February 8, 2002
Revised: March 25, 2002

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