Endogenous von Hippel-Lindau Tumor Suppressor Protein Regulates Catecholaminergic Phenotype in PC12 Cells

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ABSTRACT

Loss of von Hippel-Lindau (VHL) gene function leads to VHL disease, which is characterized by vascular tumors of the central nervous system, renal clear cell carcinomas, and pheochromocytomas. Pheochromocytomas express high levels of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. PC12 cells that express VHL antisense RNA had 5–10-fold reduced levels of endogenous pVHL and 2–3-fold increased levels of TH protein and mRNA. Nuclear run-on analysis revealed an augmentation of TH gene transcription with enhanced efficiency of transcript elongation in the 3′ region of the gene. Transient coexpression of the VHL antisense RNA with a TH promoter reporter construct increased TH promoter activity by 2–3-fold. A decrease in pVHL accumulation also resulted in an increase in TH mRNA accumulation and transcription of the TH gene during hypoxia. This is the first evidence that endogenous pVHL is a physiological regulator of the catecholaminergic phenotype. Thus, loss of pVHL function may be causative in pheochromocytoma-associated hypercatecholaminemia and arterial hypertension.

INTRODUCTION

VHL disease is an autosomal, dominantly inherited cancer syndrome affecting 1 in 36,000 people with a penetrance of 80% by the age of 65 (1). The disease is associated with a loss of function of the gene encoding the pVHL (2, 3), and patients primarily develop RCCs, hemangioblastomas, and pheochromocytomas (1). pVHL functions as a component of a multiprotein complex that includes elongins B and C, Cullin2, and Rbx-1. This complex has recently been shown to have E3 ubiquitin ligase activity toward α subunits of HIF (4–8). This is the presently accepted mechanism by which loss of pVHL in VHL-associated tumors results in an accumulation of HIFα during normoxia. This accumulation in turn induces expression of various genes containing hypoxia-responsive elements, of which the most relevant for VH disease is VEGF (9–12).

Pheochromocytoma tumors arise from adrenal medulla chromaffin cells, which synthesize and secrete large amounts of catecholamines, leading to sustained or episodic arterial hypertension as well as other symptoms of hypercatecholaminemia (13). Pheochromocytomas associated with VHL disease (14–17) produce primarily tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis. There is a strong correlation between catecholamine levels and concentrations of TH mRNA in pheochromocytoma tumors (19–21). TH mRNA levels are 2–6-fold higher in various pheochromocytoma tumors compared with the levels measured in normal adrenal medullas (19–21). TH belongs to the group of hypoxia-inducible genes (Ref. 22 and references therein) and is stimulated by hypoxia by binding of c-fos and junB to the AP1 site within the TH promoter (23, 24). Although the HIFs have been implicated in hypoxic regulation of TH gene expression, their precise role has not yet been fully demonstrated experimentally. The role of VEGF in the development of pheochromocytoma tumors is less clear than its role in the occurrence of hemangioblastomas and RCCs in VHL disease. However, increased levels of VEGF have been reported in patients with adrenal medulla tumors (25, 26).

Our laboratory has recently reported that overexpression of human wild-type pVHL in rat pheochromocytoma PC12 cells represses TH mRNA and protein levels at the level of transcription (22). To characterize the function of the endogenous pVHL in regulation of TH gene expression, we developed PC12 clonal cell lines that express significantly reduced levels of VHL protein because of expression of VHL antisense mRNA. These cell lines have increased steady-state levels of TH mRNA and protein. This effect is mediated at the level of transcription. Decreased levels of endogenous pVHL were also associated with an increase in steady-state mRNA for VEGF, another hypoxia-inducible gene that is up-regulated in VHL-associated tumors.

MATERIALS AND METHODS

Plasmid Constructs. The rat VHL cDNA was obtained by reverse transcription from RNA isolated from PC12 cells, followed by PCR using the sense primer 5′-atgcgccaagagcttagcctg-3′ and the antisense primer 5′-tacg-ctcttcagggcagctagtcc-3′. The amplified fragment was subcloned into a pcR2.1 TOPO vector (Invitrogen, Carlsbad, CA), and subjected to DNA sequencing analysis. A restriction fragment containing the VHL antisense cDNA was obtained by digesting the plasmid with HindIII and XbaI. The resulting DNA fragment was subcloned into the HindIII and XbaI sites of the pcR CMV vector (Invitrogen). The vector without an insert (pcRC CMV) was used as a control. The TH reporter construct used in these studies included sequences derived from the rat TH promoter, from −773 to +27 bp relative to the transcription start site, fused to the chloramphenicol acetyltransferase gene (kindly provided by Dr. D. M. Chikaraishi, Duke University, Durham, NC). The VEGF reporter plasmid contained the −2273 to +51 bp KpnI-Nhel fragment of the VEGF promoter in the pGL2 luciferase vector (Promega, Madison, WI; kindly provided by Drs. J. Abraham, Scios, Inc., Sunnyvale, CA, and J. Caro, Thomas Jefferson University, Philadelphia, PA). The PRDIIA-CAT construct includes the nuclear factor-κB binding site from the IFN-β enhancer (kindly provided by Dr. M. G. Wathethe, University of Cincinnati, Cincinnati, OH).

Cell Culture. All PC12 clonal cell lines were cultured and exposed to hypoxia (1% O2) exactly as described previously (22). Stable transfections were also carried out according to previously published procedures (22).

Northern Blot Analysis. Northern blot analysis was performed exactly as described previously (Ref. 22 and references therein).

Western Blot Analysis. Cells were lysed in a buffer containing 50 mM HEPES (pH 7.9), 150 mM NaCl, 5 mM MgCl2, 20% glycerol (v/v), 0.5% Triton X-100 (v/v), supplemented with standard protease inhibitors for 15 min at 4°C. Lysates were subjected to SDS-PAGE on gradient gels containing 3–27% or 5–10% polyacrylamide. The proteins were transferred to a nitrocellulose membrane (0.2 μm) using a semidry transfer system (Bio-Rad, Hercules, CA). Membranes were first blocked with 5% milk in PBST (PBS + 0.1% Tween 20)
for 1 h and then incubated with the primary antibody [CA-101bTHrab] and VHL Ig32, 1:500 dilution (PharMingen) in PBST with 5% milk overnight at 4°C. Membranes were then washed three times at room temperature in PBST, incubated with a horseradish peroxidase-coupled secondary antibody in 5% milk in PBST for 1 h, and washed three more times in PBST. Immunoreactivity was visualized by enhanced chemiluminescence and exposure to X-ray film (ECL; Amersham, Chicago, IL).

**Nuclear Run-on Assays.** Nuclear run-on assays were performed essentially as described by Krohl et al. (22). Briefly, 4 × 10⁶ cells were lysed with NP40 lysis buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 3 mM MgCl₂, and 0.5% NP-40] supplemented with a cocktail of protease and phosphatase inhibitors (2 mM sodium orthovanadate, 10 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 mg/ml leupeptin, 2 mg/ml aprotinin, and 1 mM DTT) on ice for 2 min. The lysates were centrifuged for 3 min at 500 × g at 4°C. Nuclear pellets were resuspended in 100 µl of nuclei resuspension buffer (50 mM Tris-HCl, 20% glycerol, 5 mM MgCl₂, 0.1 mM EDTA), and an equal volume (100 µl) of reaction buffer [10 mM Tris-HCl; 5 mM MgCl₂; 300 mM KCl; 0.5 mM each of ATP, CTP, and GTP; and 1 µM [³²P]UTP (800 Ci/mmol, NEN)] was added. The reactions were incubated at 30°C for 10 min. Nuclear RNA was then extracted using the TRI-Reagent protocol (Molecular Research Center).

DNA fragments were immobilized on a nylon membrane (Amersham) and cross-linked to the membrane by UV light. The membranes were prehybridized for 1 h in prehybridization buffer (1% SDS, 0.1 M NaCl) at 42°C and then hybridized in high-efficiency hybridization buffer (Molecular Research Center) with a × 10⁶ cpm/ml for 48 h at 42°C. Membranes were then washed in 1× SSC with 0.1% SDS twice for 20 min at 42°C and once for 20 min at 60°C. Each strip was then incubated with 5 µg/ml RNase A in 2× SSC for 30 min at 25°C. Strips were then washed twice for 15 min at 25°C. Hybridized radioactivity was quantified using a PhosphorImager (Molecular Dynamics). The levels of hybridized radioactivity were normalized to the background radioactivity and to protein concentrations in the original cytoplasmic extracts of each. Thirteen fragments corresponding to the full-length TH gene were amplified using PCR. The sequences of upstream and downstream primers are shown in Table 1.

**RESULTS**

To study the effects of endogenous pVHL on TH gene expression in PC12 cells, we developed clonal cell lines that express a rat VHL antisense RNA. PC12 cells were stably transfected with either a pRC expression vector containing a cDNA that encodes the rat antisense VHL RNA or the empty pRC plasmid as a control. These clones were designated pRCVHL(as) and pRC, respectively. Endogenous pVHL expression was decreased by ~5–10-fold in several pRCVHL(as) clones compared with pRC (Fig. 1A). The VHL mRNA was also similarly reduced (data not shown). Down-regulation of endogenous pVHL correlated with a 2–3-fold increase in TH protein and TH mRNA steady-state levels during normoxia (Fig. 1B) and augmented accumulation of TH mRNA during hypoxic conditions compared with cells expressing physiological concentrations of pVHL (Fig. 1C).

![Figure 1](Fig. 1. Decrease in pVHL levels induces expression of TH and VEGF. A, Western blot analysis of endogenous rat pVHL (pVHL) in total cellular lysates from stably transfected clones (C) of pRC (Lanes 1–3) and pRCVHL(as) (Lanes 4–6) cells. B, TH immunoreactivity levels analyzed by Western blot in the same pRCVHL(as) clones (top panel), and steady-state levels of TH mRNA analyzed by Northern blot (middle panel). C, Northern blot analysis of steady-state mRNAs for TH, VEGF, and GAPDH in cells exposed to normoxia or hypoxia for 16 h. Fold increase represents the relative increase in TH, VEGF, and GAPDH mRNAs compared with the corresponding signal measured in pRC control cells (designated as 1). Ethidium bromide-stained rRNA is shown for comparison of RNA loading in the bottom panels of B and C.]

![Table 1](Table 1. Sequences of primers used in PCR amplification of the fragments of the TH gene)

<table>
<thead>
<tr>
<th>TH fragment</th>
<th>Upstream primer, 5’–3’</th>
<th>Downstream primer, 5’–3’</th>
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<tr>
<td>In1a</td>
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<td>gtagacctgccggaagc</td>
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* Ex, exon; In, intron.
hybridized to 13 DNA fragments, each ~500 bp in size (Fig. 2, A and B), covering the full-length of the TH gene (Fig. 2A). Representative examples of such run-on reactions performed with RNA from pRC cells and from two pRCVHL(as) clones are shown in Fig. 2B. The fold difference in the hybridized signal between pRC and each of the two pRCVHL(as) clones is shown for each fragment at the bottom of Fig. 2B. The average data for several independent repeats of the run-ons for each clone are shown in Fig. 2C. The nuclear run-on blots showed uneven levels of hybridization to several fragments of the TH gene in control cells. For example, the probes from exon 6 toward the 3′ region of the TH gene showed lower levels of hybridization to the radioactive transcripts. Under conditions when the transcripts were of similar length (Fig. 2B) and, on average, contained similar composition of nucleotides, the hybridized radioactivity corresponded to the density of the actively elongating RNA polymerase II complexes within this fragment of the gene. Thus, these results indicate that the 3′ portion of the TH gene contains multiple regions with decreased processivity of transcript elongation.

The decreased concentration of pVHL in pRCVHL(as) cells correlated with an overall increase in transcription of the TH gene. The increases in the hybridization signal in the pRCVHL(as) clones compared with the control clone were in the range of 40–200% in the 5′ region of the TH gene and in the range of 200–400% in the 3′ region (exons 8–13). The general constitutive decrease in TH transcript processivity measured in the pRC clone beyond exon 6 appeared to be attenuated in the pRCVHL(as) cells, resulting in augmented amounts of the full-length transcripts. We also measured some increase in transcription rate at the beginning of the gene, indicating that the decrease in pVHL level leads also to an increase in the initiation of transcription.

Exposing PC12 cells to hypoxia induces transcription of the TH gene (Fig. 3). The absolute amounts of hybridized radioactivity in PC12 cells with normal endogenous levels of pVHL showed that although hypoxia induces TH gene transcription, the general decrease in processivity of transcription from the 5′ to the 3′ region of the gene is maintained (Fig. 3A). In contrast, when cells with reduced amounts of pVHL were subjected to hypoxia, transcription was induced along the full length of the TH gene, and there was an increase in the transcript processivity toward the 3′ region of the gene similar to what is seen during normoxia (Fig. 3B). This augmentation of transcriptional processivity was even more pronounced when the fold induction in response to hypoxia was compared with the values obtained under normoxic conditions in both pRC and pRCVHL(as) cells (Fig. 3C).

The effects of expressing either antisense or wild-type pVHL on the activity of the −773 TH CAT and −2273 VEGF-Luc reporter constructs were assessed in parental PC12 cells during normoxia and hypoxia (Fig. 4A). Hypoxia induced the activities of the
TH and VEGF promoters by 2.5–3.5-fold and 3.5–4.5-fold, respectively. Cotransfection of pRCVHL(as) significantly activated both promoters during normoxia and hypoxia (Fig. 4, A and B). At the same time, the hypoxic inducibility of the promoters’ activities was maintained (Fig. 4, A and B). Cotransfection of the wild-type pVHL construct resulted in only a modest attenuation of the TH and VEGF promoter activities during normoxia. However, transient cotransfection of the wild-type pVHL significantly attenuated the hypoxic inducibility of the TH and VEGF promoters (Fig. 4, A and B). In contrast, neither hypoxia, expression of pVHL, nor expression of VHL antisense affected the activity of the PRDIIA promoter, suggesting that these effects were specific for the hypoxia-responsive TH and VEGF promoters (Fig. 4C).

We also found that the reduction in pVHL levels did not affect TH mRNA stability (data not shown). These data are consistent with a previous report showing that overexpression of wild-type pVHL or pVHL deletion mutants had no effect on TH mRNA stability (22).

Discussion

In this study, we demonstrate that endogenous rat pVHL is a physiological regulator of constitutive and hypoxia-inducible expression of the TH and VEGF genes in PC12 cells. The decrease in pVHL levels produced by the antisense approach is associated with increased expression of the TH and VEGF mRNAs and TH protein. The effects of pVHL appear to be specific for the TH and VEGF genes; decreases in pVHL concentration failed to affect GAPDH gene expression during normoxia or hypoxia. This observation is of interest because GAPDH is also a hypoxia-inducible gene, regulated by HIF (27).

The endogenous pVHL regulates TH gene expression at the level of transcript elongation. Only a few genes are known to be regulated at the level of transcript elongation, and in those that are, the regulatory pause sites are known to be located in the proximal region of the initial exons or introns. In addition, the induction of VEGF gene expression attributable to the loss of pVHL function does not appear to involve regulation at the level of transcription elongation (28). These results extend our previous data, where overexpression of human pVHL in PC12 cells repressed expression of the TH gene at the level of transcript elongation (22). One of the drawbacks of the previous approach was that the levels of the expressed pVHL were higher than those achieved under physiological conditions and could thus possibly yield results that do not occur at physiological levels of.
pVHL. In addition, in the case where the exogenous pVHL is overexpressed in excess of the endogenous pVHL, we might have failed to measure some of the effects of the overexpressed pVHL because of the saturation of the responses by endogenous pVHL. The molecular mechanism underlying regulation of TH transcript elongation by pVHL is presently under investigation. It is possible that the pVHL complex has E3 ubiquitin ligase activity toward some regulators of elongation and thus targets them for degradation, resulting in inhibition of transcriptional elongation.

Accumulation of both TH and VEGF mRNAs is further augmented in response to hypoxia in PC12 cells with decreased levels of pVHL. One possible explanation for this finding is that the antisense approach does not completely abolish accumulation of pVHL. Thus, the remaining functional pVHL contributes to the repression of both genes during normoxia, and this repression is alleviated by hypoxia. The other possibility is that VHL-independent mechanisms contribute to the hypoxic inductibility of both genes.

We found that pVHL can regulate the activity of the TH and VEGF promoters during both normoxia and hypoxia. Cotransfection of wild-type pVHL only minimally affected the activity of either promoter under normoxic conditions. However, the hypoxia-induced activation of the TH and VEGF promoters was substantially reduced by coexpression of wild-type pVHL. In contrast, the decrease in pVHL level resulted in increased activity of both promoters during normoxia and hypoxia. These observations are consistent with our previously published results showing that transcription of the TH promoter into PC12 cells overexpressing pVHL resulted in only a modest (~20%) reduction in its activity (22). A possible interpretation is that endogenous pVHL fully saturates regulation of the TH promoter during normoxia and that a further increase in pVHL concentration has no major effects on the promoter activity (22). Our observation that VEGF promoter activity was not regulated by overexpressed pVHL during normoxia differs from the previously reported finding that pVHL reduces VEGF promoter activity during normoxia by inhibiting Sp1 activity (29). Similarly, these investigators reported that VHL inhibits the activity of other Sp1-regulated promoters, including the CMV promoter (29).

We have determined that in our experimental system, at the doses of pVHL used, the activity of the CMV-reporter construct was unaffected by pVHL (data not shown). It is possible that much higher amounts of pVHL would be needed to measure inhibition of the VEGF promoter activity during normoxia. In that respect, although the TH gene has a single Sp1 element, it is not thought to play a major role in regulation of TH gene expression (30).

The precise molecular mechanisms by which changes in pVHL levels can affect the hypoxia-inducible TH and VEGF promoters remain unknown. The VEGF promoter contains a well-characterized hypoxia-responsive element, which interacts with the HIF transcription factor (31). It has also been proposed that the AP1 site is involved in the regulation of the VEGF promoter by hypoxia (32). Regulation of the TH promoter by hypoxia appears to involve a c-Fos-Jun B dimer interacting with the AP1 site (23, 24). The role of HIFs interacting with a putative hypoxia-responsive element is presently under investigation. Thus, it is possible that a decrease in the pVHL level affects ubiquitination and therefore concentrations of HIFα subunits, which in turn activate HIF-responsive promoters during normoxia. Again, because the activity of pVHL is not completely abolished by the expression of antisense RNA, hypoxia can further stimulate the accumulation of HIFα, which can then activate both promoters. In the case of pVHL overexpression, an analogous interpretation is more difficult to make. Recent results showed that hydroxylation of proline 564 in HIF1α by a proline hydroxylase is necessary for the interaction of HIFα with pVHL and for the subsequent ubiquitination and degradation of HIF1α (7, 8). This hydroxylation does not occur during hypoxia. Thus, in the absence of hydroxylation, an increase in pVHL concentration should not be sufficient to augment ubiquitination of HIF1α or to reduce HIF1α levels during hypoxia. However, at present, very little is known about the nature of this proline hydroxylase, and it is certainly possible that increased concentrations of pVHL may somehow affect the activity of this enzyme or its sensitivity to cofactors. On the other hand, it is also possible that VHL affects the activity of transcription factors other than HIFs that may be involved in regulation of both promoters.

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