von Hippel-Lindau Protein Induces Hypoxia-regulated Arrest of Tyrosine Hydroxylase Transcript Elongation in Pheochromocytoma Cells*

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Rat pheochromocytoma (PC12) cells were stably transfected with either wild type or mutated human von Hippel-Lindau tumor suppressor protein (hpVHL). These proteins have opposing effects on regulating expression of the gene encoding tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis. Whereas wild type hpVHL represses levels of TH mRNA and protein 5-fold, a truncated pVHL mutant, pVHL(1-115), induces accumulation of TH mRNA and protein 3-fold. hpVHL-induced inhibition of TH gene expression does not involve either a decrease in TH mRNA stability or repression of TH promoter activity. However, repression results from inhibition of RNA elongation at a downstream region of the TH gene. This elongation pause is accompanied by hpVHL sequestration in the nuclear extracts of elongins B and C, regulatory components of the transcription elongation hetrotrimer SIII (elongin A/B/C). Hypoxia, a physiological stimulus for TH gene expression, alleviates the elongation block. A truncated pVHL mutant, pVHL(1-115), stimulates TH gene expression by increasing the efficiency of TH transcript elongation. This is the first report showing VHL-dependent regulation of specific transcript elongation in vivo, as well as dominant negative activity of VHL mutants in pheochromocytoma cells.

Pheochromocytoma tumors arise from chromaffin cells of the adrenal medulla. The primary phenotype of these tumors is the ability to synthesize and release large amounts of catecholamines. This activity is directly attributable to augmented activity and increased gene expression for tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine synthesis (1, 2). The increase in catecholamine levels is clinically significant, resulting in elevated blood pressure and leading to life-threatening hypertensive crises (3, 4). Pheochromocytomas are either sporadic or inherited. Familial pheochromocytomas occur most frequently in multiple endocrine neoplasia type II linked to the ret proto-oncogene (5) or in von Hippel-Lindau (VHL) disease (6, 7) linked to the VHL tumor suppressor protein (8, 9). We hypothesized that a gene correlated with pheochromocytoma tumorigenesis might regulate catecholamine synthesis. We chose to examine the effects of pVHL on TH gene expression because pheochromocytomas can be the predominant or only tumor arising in some individuals with VHL disease, thus suggesting a specific role for the VHL tumor suppressor protein in the pathogenesis of this tumor (10).

VHL disease (11) is a hereditary autosomal dominant disorder involving renal clear cell carcinomas, hemangioblastomas, and pheochromocytomas, which are associated with heterogeneous mutations and loss of heterozygosity of the von Hippel-Lindau tumor suppressor gene (8, 9). In cell lines derived from renal clear cell carcinoma tumors that have mutated form of pVHL, wild type pVHL has been shown to down-regulate the expression of a number of genes that may be involved in the pathogenesis of VHL disease-associated tumors, including vascular endothelial growth factor (VEGF) (12–14), glucose transporters (12, 14), and carbonic anhydrases (15). The molecular mechanism of this regulation is poorly understood, and both post-transcriptional (12–14, 16) and transcriptional (17) mechanisms have been reported.

The best characterized molecular function of pVHL is its binding to elongins B + C, which were first described as regulatory subunits of the transcription elongation complex SIII (elongins A/B/C) (14, 18–20). The main component of SIII, elongin A (100 kDa), is part of the RNA polymerase II transcription complex (21, 22). Elongin A activity in isolation is minor, but by binding to the active site of elongin C (14 kDa), it becomes highly activated (23). The elongin A-C complex is relatively unstable by itself but becomes stabilized upon the binding of elongin B (19 kDa) to elongin C (23). The elongin B N terminus is homologous to ubiquitin, suggesting a potential role in the regulation of protein stability and degradation (23). The main activity of SIII is stimulation of processive transcription by suppressing polymerase pausing (21–23). SIII does not affect transcription initiation by the RNA polymerase II preinitiation complex. hpVHL, human pVHL; rpVHL, rat pVHL; VEGF, vascular endothelial growth factor; wt, wild type; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase.

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1 The abbreviations used are: TH, tyrosine hydroxylase; VHL, von Hippel-Lindau; pVHL, von Hippel-Lindau tumor suppressor protein;
Regulation of TH Transcript Elongation by VHL and Hypoxia

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from either Fisher or Sigma, and enzymes were from Promega or Life Technologies, Inc.

Cell Cultures—Rat PC12 cells were grown in Dulbecco's modified Eagle's medium/F12 medium supplemented with 15 mM HEPES buffer, 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin. Stably transfected clones were generated by transfection of plasmid DNAs (pRC, pRCVHL(wt), pRC VHL(1–115)) using LipofectAMINE reagent (Life Technologies, Inc.) followed by selection with 400 μg/ml G418. All experiments were performed on cells that were 95–100% confluent at the time of collection. Exposures of cells to hypoxia (5% O2, 5% CO2, balanced with N2) were performed in an O2-sensitive, O2-microelectrode system (Yellow Springs Instruments) followed by culture in the normal atmosphere as described previously (27, 28).

Northern Blots and Chloramphenicol Acetyltransferase Assays—Total cellular RNA was separated on formaldehyde-agarose gels and transferred and probed with respective cDNA probes labeled by nick translation with [32P]UTP (NEN Life Science Products). TH mRNA stability was measured after inhibition of transcription with 5 μg/ml actinomycin D as described previously (28). The half-lives were calculated for each experiment from linear regression lines fitted to all time points (4, 8, 16, and 24 h after treatment) of each individual experiment (28). The average half-lives are given as the mean ± S.E. for all experiments.

Vectors containing fragments of TH promoter cloned upstream from the chloramphenicol acetyltransferase (CAT) reporter gene and CMV-β-galactosidase constructs were transiently transfected into clones of PC12 cells using LipofectAMINE. CAT and β-galactosidase activities were measured using assay systems from Promega according to the manufacturer's protocols. CAT activity is normalized to β-galactosidase activity and protein concentration.

Nuclear Run-on Assays—2 × 10⁷ cells were washed and lysed with buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40) for 3 min on ice. Lysates were centrifuged (500 × g, p), and the nuclei were resuspended in 2× nuclear resuspension buffer (50 mM Tris, pH 8.3, 5 mM MgCl₂, 0.1 mM EDTA, 40% glycerol) and counted. The number of nuclei corresponded to the number of cells. Run-on reactions were immediately performed for 10 min at 30 °C in reaction buffer (final concentrations: 15 mM Tris, pH 7.4; 2.5 mM MgCl₂; 150 mM NaCl; 0.25 mM ATP, GTP, and CTP each; and 1 μM [α-32P]UTP at 800 Ci/mmol (NEN Life Science Products). Nuclear RNA was extracted using TRI reagent (MRC, Inc., Cincinnati, OH) and hybridized to the PCR fragments of the TH gene that was immobilized on neutralized charged nylon membranes (Biodyne A membrane, Life Technologies, Inc.). A concentration of 1 × 10⁶ cpm/ml was hybridized to each strip in a standard Northern hybridization buffer for 48 h at 42 °C. Blots were washed in 1× SSC and 0.1% SDS at 42 °C and treated with 10 μg/ml RNase A in 2× SSC. Labeled transcripts were also hybridized to the 150-base-long DNA oligonucleotides corresponding to the sense template within the exon's sequence, to evaluate the presence of antisense transcription.

Hybridized radioactivity was measured using a PhosphorImage system (Molecular Dynamics, Sunnyvale, CA). To account for differences in the hybridization efficiency among different fragments of the gene, the results were normalized relative to respective signals that were hybridized to four fragments of the TH gene in the nuclei from pRC cells. Average results were expressed as the mean ± S.E. in the hypoxia experiments, because of concerns about reoxygenation, cells were not counted but approximately equal numbers of cells/nuclei were used, and the data were further normalized based on the micrograms of protein in the cytoplasmic lysates.

Immunoprecipitations and Western Blot Analysis—Nuclear extracts were obtained by lysing nuclei (prepared as described above) in high salt buffer (20 mM HEPES, 1 mM EDTA, 420 mM NaCl, and 20% glycerol with standard proteinase and phosphatase inhibitors) for 20 min on ice. For immunoprecipitations, 0.5 μg of the monoclonal anti-VHL (PharMingen) or monoclonal anti-elongin B antibody was incubated with 1 mg of nuclear proteins in a final volume of 1 ml overnight at 4 °C. Immunoblotting was followed by the addition of anti-mouse antibody coupled to agarose for 2 h and then five washes with 20 mM HEPES, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40. The proteins were separated by SDS-polyacrylamide gel electrophoresis (12.5 or 3–27% gradient gels, Owl Scientific, Inc.) and transferred onto nitrocellulose membranes. Blots were blocked in PBST (phosphate-buffered saline with 0.1% Tween 20) with 5% milk for 1 h and probed overnight with the monoclonal antibodies against the hemagglutinin tag (3F10, Roche Molecular Biochemicals, 1:3000), VHL (PharMingen, 1:500), elongin A (1:300), and elongin B (1:20 000), elongin C (1:1000), Transduction Laboratories), polyclonal antibody against elongin B (1:1000), or polyclonal anti-TH antibody (Protos Biotech, New York, 1:3000). Signal was visualized with chemiluminescence reagents (Amersham Pharmacia Biotech).

RESULTS

Repression of TH Gene Expression by hpVHL—A hemagglutinin (HA)-tagged human wild-type hpVHL (hpVHL), its truncated mutant, pHVLI (1–115), and a pRC cytomegalovirus vector with no insert were stably expressed in PC12 cells. Expression...
levels of the hpVHL and endogenous wild type rat pVHL (rpVHL) in selected clones of transfected PC12 cells are shown in Fig. 1a. Protein levels of the overexpressed hpVHL are on average 5–10 times higher compared with rpVHL (Fig. 1a, top, lanes 3 and 4). Note that as analyzed by Western blot, endogenous rpVHL is expressed as two isoforms, a predominant smaller isoform (lower band) and a minor larger isoform (upper band). Expression of the larger, minor isoform was slightly, but reproducibly, repressed by overexpression of hpVHL. Protein levels of the pVHL(1–115) were much lower than in hpVHL in all analyzed clones and could not be detected by Western blot analysis with anti-pVHL antibodies. However, pVHL(1–115) was detectable by immunoprecipitation with an anti-HA antibody followed by anti-HA blotting (not shown). pVHL(1–115) includes only the first exon of human pVHL; therefore, it does not interact with elongins B/C (17). Overexpression of hpVHL strongly inhibited accumulation of TH protein (Fig. 1a, bottom) and TH mRNA (Fig. 1b) in all clones of the cells transfected with the wild type hpVHL (pRCVHL(wt) cells) as compared with clones transfected with the pRC vector only (pRC cells). In contrast, expression of the mutant pVHL(1–115) increased accumulation of TH protein and TH mRNA in all analyzed clones (pRCVHL(1–115) cells) (Fig. 1, a and b). This effect was specific for TH mRNA, in that there was no effect of hpVHL or pVHL(1–115) expression on mRNA levels of tubulin (Fig. 1b) or glyceraldehyde-3-phosphate dehydrogenase (not shown). These observations suggest a potential gain-of-function or dominant negative activity for pVHL(1–115) in regulating TH gene expression in PC12 cells.

Because pVHL down-regulates the expression of several hypoxia-regulated mRNAs at the level of mRNA stability (12–14) and hypoxia regulates TH gene expression in PC12 cells at the level of both transcription and RNA stability (27, 28), we initially anticipated that pVHL might decrease the stability of TH mRNA. Surprisingly, the TH mRNA half-life was increased by 30% (12.1 ± 0.9 h, n = 7, p < 0.02) in pRCVHL(wt) cells compared with the control pRC cells (9.2 ± 0.5 h, n = 7). Expression of pVHL(1–115) had no measurable effect on TH mRNA half-life (9.9 ± 0.6 h, n = 5). Thus, the regulation of TH gene expression by pVHL appears to occur at the level of RNA synthesis.

hpVHL Inhibits TH Gene Transcription at the Level of Tran-
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FIG. 3. Attenuation of hypoxic inducibility of TH gene by hpVHL. a, Northern blot analysis of induction of TH mRNA by hypoxia (5% O_2) for indicated periods of time. Ethidium bromide-stained gels document loading of RNA. b, steady-state levels of TH mRNA measured at indicated times in pRC, pRCVHL(wt), and pRC VHL(1–115) cells were normalized to the TH mRNA measured in normoxic pRC cells (100%). c, example of nuclear run-on analysis of TH transcripts from pRC and pRCVHL(wt) cells during normoxia (21% O_2) and hypoxia (5% O_2). d, average results of run-on analysis of elongated TH transcripts in pRC, pRC VHL(wt), and pRC VHL(1–115) cells during hypoxia (n = 6). Results are expressed as percent of the respective signal measured in normoxic pRC cells (100%). The line showing 100% is the same as in Fig. 2c.

The effects of hpVHL on TH gene transcription were studied using run-on assays in isolated nuclei of stably transfected PC12 cells. In these assays, transcription is not initiated de novo, but rather, pre-initiated RNA transcripts are elongated. We measured levels of radioactively labeled TH run-on transcripts by hybridizing them to four fragments of DNA encompassing the full length of the TH gene (Fig. 2a). As expected, in pRC cells the hybridization signal gradually decreased along the TH gene, reflecting a decrease in the efficiency of in vitro elongation of full-length TH transcripts (Fig. 2b, lanes 1 and 5). In pRCVHL(wt) cells, we measured a dramatic decrease in the labeled transcripts hybridized to the distal part of the TH gene (exons 8–13, probes c and d) to 5–20% of the value measured in pRC cells (Fig. 2, panel b, lanes 2–4, and panel c). In contrast, there was only a small, insignificant decrease in the levels of labeled transcripts hybridized to the proximal part of the TH gene (exon 1–7, probes a and b). Note that the data shown in Fig. 2c are normalized relative to respective signals that were hybridized to four fragments of the TH gene in the pRC cells to account for differences in the hybridization efficiency among different fragments of the gene. These data indicate that hpVHL represses TH transcription elongation without affecting TH promoter activity. To confirm that hpVHL does not regulate TH promoter activity, we performed transient transfections of chimeric constructs containing from −773 to −109 base pairs of TH promoter (relative to the transcription start site) cloned upstream of the CAT reporter gene (Fig. 2d). We failed to measure repression of promoter activity in pRCVHL(wt) cells (Fig. 2d) even if a longer fragment (~4.8 kb) of the TH promoter was used (data not shown).

In pRCVHL(1–115) cells, levels of early TH transcripts were increased 2-fold over levels measured in pRC cells (Fig. 2, panel b, lanes 6–8, and panel c). We measured an additional significant increase in the levels of full-length transcripts relative to the pRC cells (Fig. 2, panel b, lanes 6–8, and panel c). Analysis of TH promoter activity showed a significant 2.3 ± 0.3 (n = 8, p < 0.05)-fold induction of −773 base pairs of TH promoter (Fig. 2d).

Effects of hpVHL on Hypoxic Regulation of TH Gene Expression—hpVHL was reported to augment hypoxic inducibility of O_2-regulated genes, VEGF, platelet-derived growth factor β chain, and Glut-1 (12–14), primarily by decreasing constitutive expression of their mRNAs in normoxia. We tested the effects of hpVHL on the inducibility of TH mRNA during hypoxia (Fig. 3). When normalized to its own normoxic control, the maximum induction of TH mRNA in pRCVHL(wt) cells was 10-fold. A 2-fold induction was measured in pRCVHL(1–115) cells, compared with a 4-fold induction measured in pRC cells (Fig. 3, a and b). However, the highest steady state levels of TH mRNA achieved in pRCVHL(wt) cells during hypoxia reached only the base-line levels measured in pRC cells during normoxia. Thus, augmentation of hypoxic inducibility by hpVHL becomes apparent only when compared with hpVHL-mediated, base-line repression of TH mRNA accumulation during normoxia. However, hpVHL attenuates the overall hypoxic expression of TH mRNA. In pRCVHL(1–115) cells, TH mRNA was 2-fold higher than in pRC cells at each time point of hypoxia (Fig. 3b); thus, the “de-repressing” effect of pVHL(1–115) on TH mRNA is maintained during hypoxia.

Analysis of TH gene transcription using nuclear run-on assays showed that hypoxia abolished the hpVHL-induced elongation block of TH transcription (Fig. 3, c and d). TH transcripts hybridizing to four DNA probes encompassing the TH gene were induced by exposure to hypoxia for 16 h in pRCVHL(wt) cells to the levels found in pRC cells during normoxia (Fig. 3, c and d). Note that in Figs. 3d and 2c, 100% corresponds to the levels of TH transcripts in pRC cells during normoxia. In pRCVHL(wt) cells, however, hypoxia did not stimulate the synthesis of early transcripts (exons 1–2, probe a) beyond the level measured in control cells during normoxia. In the pRCVHL(1–115) cells, hypoxia stimulated both the early and the full-length transcripts (exons 1–2, probe a) to the same extent, indicating uniformly increased transcription of the TH gene (Fig. 3d).

Effects of Hypoxia on hpVHL-Elongin B/C Interactions—To assess the potential involvement of elongins in the regulation of TH transcript elongation by hpVHL and hypoxia, we evaluated their expression in nuclear extracts from pRC, pRCVHL(wt), and pRCVHL(1–115) cells during normoxia and hypoxia (Fig. 4). We found no consistent changes in the absolute amounts of elongins A, B, or C in the nuclear extracts from pRCVHL(wt) or pRCVHL(1–115) cells as compared with pRC cells during normoxia or hypoxia (Fig. 4c). Note that in nuclear extracts the expression of hpVHL is only 2–5-fold higher than the endogenous rat pVHL, as compared with the 5–10-fold difference in the whole cell lysates (Fig. 1a). In addition, levels...
either normoxia (21% O₂) or hypoxia (5% O₂) for 16 h. Proteins were
tracts from pRC, pRCVHL(wt), and pRCVHL(1–115) clones exposed to
elongin C), rpVHL, and hpVHL, respectively, in nuclear protein ex-
Western blot analysis of elongins (EB, elongin A; EA, elongin C).

FIG. 4. Effects of hypoxia on binding of pVHL to elongins. a, Western blot analysis of elongins (EA, elongin A; ER, elongin B; EC, elongin C), rpVHL, and hpVHL, respectively, in nuclear protein extracts from pRC, pRCVHL(wt), and pRCVHL(1–115) clones exposed to either normoxia (21% O₂) or hypoxia (5% O₂) for 16 h. Proteins were separated on 3–27% gradient gel. Blots were probed with indicated antibodies (blot). b, co-immunoprecipitation of elongins B and C with the antibody against pVHL in nuclear extracts from pRC and pRCVHL(wt) clones exposed to normoxia or hypoxia for 16 h. Immunoprecipitated proteins were separated on 12.5% SDS-polyacryl-

The molecular mechanism by which hypoxia abolishes the elongation block of TH transcript synthesis is currently under investigation. Hypoxia might stimulate activity of the SIII factor (elongins A/B/C), because augmented interaction of elongins A and B occurs during hypoxia. It is also possible that, other than SIII, elongation/transcription factors become in-

of the larger form of endogenous pVHL are decreased by over-
expression of the hpVHL (Fig. 4a, lanes 3 and 4). To evaluate the protein-protein interactions between pVHL and elongins B/C during normoxia and hypoxia, we co-immunoprecipitated elongins C and B with an anti-pVHL antibody in nuclear ex-
tracts (Fig. 4b). Clearly, in nuclear extracts from pRCVHL(wt) cells, the overexpressed hpVHL co-immunoprecipitated substan-
tially more elongin B and C as compared with the endoge-
nous rpVHL (lanes 3 and 4) during both normoxia and hypoxia. Interestingly, hypoxia markedly decreased levels of the lower rpVHL band in anti-pVHL immunoprecipitates. The levels of hypoxia used in these experiments (5% O₂) did not affect interaction of pVHL with elongins B or C in the nuclear ex-
tracts. Next, we performed co-immunoprecipitation of elongins A and C and of pVHL with an anti-elongin B antibody (Fig. 4c). Hypoxia induced a decrease in the levels of hpVHL and endoge-
nous rpVHL co-immunoprecipitated with elongin B. There was also a concomitant increase in the levels of elongin A associated with elongin B in both pRC and pRCVHL(wt) cells during hypoxia (Fig. 4c). Note that anti-elongin B antibody immunoprecipitated only the low molecular weight isoform of rpVHL. We failed to measure any consistent differences in the

amount of elongin C co-immunoprecipitated with elongin B during normoxia and hypoxia, but we found an increase in the association of elongin C with elongin B in the pRCVHL(wt) cells during hypoxia.

DISCUSSION

In this study we show that in PC12 cells, pVHL(wt) represses expression of TH gene at the level of transcript elongation in a gene-specific manner. The elongation-inhibitory activity of pVHL results, most likely, from sequestering of the elongation regulatory factors, elongins C and B. This is the first, and to date the only, physiological evidence that a specific gene is regulated by pVHL and elongins at the level of transcript elongation as predicted from the biochemical studies. It is also the first report showing that a tumor suppressor protein linked to pheochromocytoma tumorigenesis regulates the important biological and clinical feature of pheochromocytoma, i.e., augmented catecholamine synthesis. The novelty of this finding is underscored by the fact that another gene involved in the pathophysiology of the VHL disease, VEGF, has been reported to be regulated by pVHL at the level of mRNA stability (12–14) or promoter activity (17) but not at the level of transcript elongation (13).

The mechanism of gene specificity in the regulation of TH transcript elongation by pVHL is under investigation. The results show that a pause site exists on the TH gene at or just downstream from exon 8. The localization of the pause site may be sequence-specific. In that respect, exon 8 of the TH gene has several short stretches of T residues in the DNA sense strand, which have been shown to lead to transcription pausing or termination in other genes (29–32). We are presently mapping more precisely the block site within the TH gene as well as the 3'-ends of the shorter arrested transcripts. The role of the TH gene proximal region in mediating gene specific regulation of TH transcript elongation by the SIII factor is also being evaluated.

Hypoxia, a physiological stimulus of TH gene transcription in PC12 cells, overcomes the pVHL-induced elongation block and thus promotes efficient elongation of the full-length trans-

cripts. Hypoxia did not stimulate hybridization of TH tran-
scripts at the level of exons 1–2 of the TH gene, indicating failure to activate TH promoter activity in the presence of the overexpressed hpVHL. This demonstrates that hypoxia regulates transcript elongation specifically and independently from promoter activity. Although regulation of gene expression by hypoxia has been studied intensely at the level of transcription initiation and mRNA stability, transcript elongation has not been considered previously as an important regulatory step in this process.

The mechanism of gene specificity in the regulation of TH transcript elongation by pVHL and hypoxia remains to be determined. The molecular mechanism by which hypoxia abolishes the elongation block of TH transcript synthesis is currently under investigation. Hypoxia might stimulate activity of the SII factor (elongins A/B/C), because augmented interaction of elongins A and B occurs during hypoxia. It is also possible that, other than SIII, elongation/transcription factors become involved in regulation of processive TH gene elongation during hypoxia. We have observed that rat pVHL, similar to human pVHL (33–35), is expressed in multiple isoforms. Immunopre-

cipitation of the predominant lower molecular weight VHL isoform by anti-VHL monoclonal antibody is significantly de-

creased during hypoxia, suggesting that the epitope recognized by this antibody may be occluded and/or modified during hypoxia. The role of these isoforms in pVHL activities during normoxia and hypoxia remains to be determined.

Using PC12 cells that express endogenous pVHL, we discovered a dominant negative activity of the pVHL deletion mutant that relieves endogenous pVHL repression of TH transcript

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elation. In this study we focused only on the truncated mutant of pVHL. The activity of other mutants with point mutations at individual amino acids specifically associated with pheochromocytomas remains to be determined. The molecular mechanism of this dominant-negative activity is not understood at present, but it is not likely to depend on changes in the absolute levels of elongins in the nucleus. Dominant-negative activity of other tumor suppressor proteins, such as p53, has been reported previously (36). Based on genetic studies, dominant-negative activity of mutant pVHL may be predicted in pheochromocytomas (37, 38).

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