

Hypoxia upregulates von Hippel-Lindau tumor-suppressor protein through RhoA-dependent activity in renal cell carcinoma

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Turcotte, Sandra, Richard R. Desrosiers, and Richard Béliveau. Hypoxia upregulates von Hippel-Lindau tumor-suppressor protein through RhoA-dependent activity in renal cell carcinoma. *Am J Physiol Renal Physiol* 286: F338–F348, 2004. First published October 28, 2003; 10.1152/ajprenal.00254.2003.—A key task for the multifunctional von Hippel-Lindau protein (pVHL) is regulation of the activity of hypoxia-inducible factor-1 α (HIF-1 α) by targeting it to the proteasome for degradation under normoxia. pVHL binding to HIF-1 α is lost under low O₂ tension, leading to transcription of several genes involved in the hypoxia response. However, regulation of pVHL by hypoxia remains to be investigated. We evaluated the effects of hypoxia on pVHL expression in carcinoma and endothelial cells. We showed that hypoxia stimulates pVHL levels (2.5-fold) in renal Caki-1 cells expressing wild-type VHL (VHL^{+/+}). This upregulation was independent of VHL status, because hypoxia also increased pVHL expression in renal 786-O cells carrying mutated VHL (VHL^{-/-}). Hypoxia did not affect pVHL expression in endothelial cells. Hypoxia-induced pVHL in Caki-1 cells was RhoA dependent, because inhibition by exotoxin C3 prevented pVHL stimulation. Furthermore, inhibition of Rho kinase by Y-27632 blocked pVHL induction by hypoxia. During normoxia, pVHL expression was also induced in cells transfected with dominant-active RhoA. Furthermore, disruption of actin organization by chemical agents or by hypoxia stimulated pVHL expression in kidney cells. On the other hand, inhibition of MAP kinases p38 and JNK, but not MAP kinase kinase (MEK1/2), reduced pVHL upregulation by 30 and 72%, respectively, during hypoxia, supporting a significant role for these signaling pathways. Expression and phosphorylation of c-Jun were stimulated in cells transfected with dominant-active RhoA. Together, these findings demonstrate that hypoxia induces pVHL expression in renal cancer cells, and this induction is mediated by RhoA-dependent pathways.

Rho GTPase; cytoskeleton; hypoxia-inducible factor-1 α

ACTIVATION OF ONCOGENES or inactivation of tumor-suppressor genes disrupts regulation of the cell cycle and increases cellular proliferation. At some point, the increasing tumor size limits O₂ diffusion, leading to a decrease in available O₂ and a reduction in cellular energy levels (1). The adaptation to hypoxia involves regulation of several genes that stimulate erythropoiesis, angiogenesis, and intracellular metabolism (45). These responses are mediated by hypoxia-inducible factor (HIF)-1, a transcription factor stabilized under hypoxic conditions that contains HIF-1 α and HIF-1 β subunits (53). Whereas HIF-1 β is constitutively expressed, expression and activation of HIF-1 α are regulated by O₂ tension. A drop in O₂ level stabilizes HIF-1 α , permitting its nuclear translocation and binding to HIF-1 β . Under normoxic conditions, HIF-1 α is

rapidly degraded by the ubiquitin-proteasome system through a conserved O₂ degradation domain (ODD) (18). von Hippel-Lindau protein (pVHL) is a component of an E3 ubiquitin protein-ligase complex that binds HIF-1 α in the ODD (7, 35, 38). This interaction occurs in the presence of O₂ and iron and requires hydroxylation of two prolyl residues (Pro⁴⁰² and Pro⁵⁶⁴) of HIF-1 α in the cytoplasm by prolyl-hydroxylases (6, 10, 21, 22). Also, hydroxylation of HIF-1 α asparagine residues in the nucleus prevents its interaction with coactivator p300 and reduces the transcriptional activity of HIF-1 (30).

VHL is a tumor-suppressor gene located on chromosome 3p25 (48). Mutations of the VHL gene inactivate the protein, causing a hereditary cancer syndrome and increased risk of developing cerebellar or retinal hemangioblastoma, renal cell carcinoma (RCC), pancreatic carcinoma, or pheochromocytoma (26, 54). This gene produces two proteins of 213 and 160 amino acid residues, with apparent molecular masses of 30 and 19 kDa, respectively, after SDS-PAGE (20, 44). pVHL30 and pVHL19 display similar biochemical properties and are primarily located in the cytoplasm but are also found in the nucleus, mitochondria, and endoplasmic reticulum (13, 20, 44, 46). pVHL is associated with four partners: elongins B and C, cullin-2, and Rbx1 (VBC/Cul-2). Elongin C and cullin-2 are similar to the yeast proteins SKP1 and CDC53, components of ubiquitin ligases referred to as SCF complexes (32, 39). Under normoxic conditions, HIF-1 α is ubiquitinated by pVHL and targeted to the 26S proteasome for degradation. A high rate of VHL mutations is found in sporadic RCC, and the majority of RCC lack functional pVHL. These mutations prevent interaction with HIF-1 α , which accumulates and stimulates expression of several genes encoding for growth factors, such as vascular endothelial growth factor, that are responsible for the high vascularization of RCC (19, 31, 47).

Actin cytoskeletal disruption is a hallmark of the cell response to hypoxia (25, 49). Regulation of actin organization is mediated by members of the Rho GTPase family (14, 50). Each of these proteins has two conformations: an active, GTP-bound state at membranes and an inactive, GDP-bound state in the cytosol when complexed with GDP-dissociation inhibitor (34). The conversion between these two forms is controlled by different classes of regulatory proteins (4, 5). Previous studies demonstrated that constitutively activated Rho proteins protect against disruption of stress fibers caused by chemical depletion of ATP by antimycin A (12, 41). In addition, we (52) and others (17) have shown that hypoxia increases expression and activation of Cdc42, Rac1, and RhoA and that these induced

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Rho proteins are essential to HIF-1 α expression and activation in renal cell lines.

Because pVHL cannot target HIF-1 α to proteosomal degradation during hypoxia (13), we investigated whether pVHL expression was also modulated under low O₂ tension. Surprisingly, our results demonstrated an increase in VHL protein and mRNA expression under hypoxic conditions in RCC carrying functional or mutated pVHL. We therefore investigated the role of Rho GTPases and cytoskeletal organization as molecular mechanisms contributing to the upregulation of pVHL during hypoxia and normoxia. We observed that inhibition of RhoA by C3-ADP-ribosyltransferase and inhibition of effector Rho kinase by Y-27632 prevented hypoxia-induced pVHL. In addition, inhibition of p38 and JNK pathways strongly prevented the induction of pVHL by hypoxia. This latter pathway was also likely RhoA dependent, because c-Jun expression and c-Jun Ser⁷³ phosphorylation were induced in cells transfected with dominant-active RhoA. Furthermore, pVHL expression was induced by overexpression of dominant-active RhoA and by cytoskeletal disruption with chemical agents as during hypoxia in kidney cells. This report clearly shows that increased pVHL expression in RCC during hypoxia is RhoA dependent and likely results from cytoskeletal disruption.

MATERIALS AND METHODS

Materials. Low-glucose Dulbecco's modified Eagle's medium (DMEM), high-glucose DMEM, MEM, and penicillin-streptomycin were obtained from GIBCO-BRL Life Technologies (Burlington, ON, Canada). Fetal bovine serum (FBS) and fetal calf serum (FCS) were obtained from Medicorp (Montreal, PQ, Canada). Vinblastine, cytochalasin D, taxol, and McCoy's medium were purchased from Sigma (St. Louis, MO). PD-98059, SB-203580, and SP-600125 were purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Y-27632 and SB-202474 were provided by Calbiochem (La Jolla, CA). The pVHL antibody was obtained from BD Pharmingen (Mississauga, ON, Canada). RhoA, Rho kinase, LIMK, ezrin, radixin, and moesin (ERM), CD44, vimentin, and α -tubulin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The JNK, c-jun, and phospho-Ser⁷³ c-jun antibodies were purchased from Cell Signalling (Pickering, ON, Canada). Rhodamine-phalloidin, Alexa Fluor goat anti-mouse IgG, Alexa Fluor goat anti-rabbit IgG, and ProLong Antifade kit were purchased from Molecular Probes (Eugene, OR).

Cell culture. Bovine aortic endothelial (BAE) cells were purchased from Clonetics and cultured in low-glucose DMEM containing 10% heat-inactivated FBS. 786-O cells were obtained from American Type Culture Collection and cultured in RPMI 1640 containing 10 mM HEPES and 4.5 g/l glucose, supplemented with 10% FBS. Panc-1, U-87, and Caki-1 cells were purchased from American Type Culture Collection and maintained in high-glucose DMEM, MEM, and McCoy's medium, respectively, each containing 10% FCS. All cells were supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin and cultured at 37°C in a humidified atmosphere of 5% CO₂. All experiments were carried out with confluent cultures.

Hypoxic conditions. An environmental chamber was used for hypoxia treatments. O₂ was maintained at 1% by a compact gas O₂ controller (Proox model 110, Reming Bioinstruments, Redfield, NY). Cells were maintained at a controlled temperature of 37°C in 5% CO₂-balance N₂.

Immunofluorescence and image analysis. The subconfluent Caki-1 or BAE cells were plated on glass coverslips and exposed to hypoxia or normoxia for 1 h. Cells were fixed in 3.7% formaldehyde in PBS for 10 min at room temperature and washed in PBS. Then cells were permeabilized in 0.2% Triton X-100 for 5 min at room temperature

and washed in PBS. The coverslips were incubated with primary antibody (α -tubulin or vimentin) diluted 1:50 in PBS containing 0.2% BSA for 45 min at room temperature. Cells were washed three times in PBS-BSA, and the bound primary antibody was detected using a fluorescein-conjugated secondary antibody diluted 1:1,000 in PBS-BSA and incubated for 45 min. Coverslips were washed three times in PBS-BSA and mounted in ProLong Antifade according to the manufacturer's instructions. For actin, coverslips were incubated with 0.1 μ g/ml rhodamine-phalloidin for 45 min at room temperature. Coverslips were washed in PBS and mounted as described above. Cells were examined with a confocal microscope (Nikon eclipse TE-2000-U), and images were analyzed using Northern Eclipse 6.0 software.

Treatment with modulators of cytoskeletal organization. Caki-1 cells were treated with 0–100 nM taxol, acrylamide, cytochalasin D, or vinblastine for 24 h in serum-free medium under normoxic conditions.

Lysate preparation and Western blot analysis. After treatment, cells were washed twice in cold PBS and lysed in ice-cold buffer containing 150 mM NaCl, 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, and a cocktail of protease inhibitors (Calbiochem). Cells were incubated on ice for 30 min. Lysates were centrifuged for 10 min at 1,000 g at 4°C. Proteins in postnuclear supernatants were quantified with the micro-bicinchoninic acid method. Identical amounts of proteins were solubilized in Laemmli buffer (62.5 mM Tris·HCl, pH 6.8, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, and 0.00625% bromophenol blue), boiled for 4 min, separated on 12.5% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (NEN Life Science Products, Boston, MA), and then immunodetected with pVHL, RhoA, Rho kinase, ERM, CD44, LIMK, c-jun, phospho-c-jun, or α -tubulin antibodies. Blots were exposed to Fuji film, and the autoradiograms were scanned with a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Purification of recombinant fusion proteins. The expression vector pGEX-2T, containing cDNAs encoding the fusion protein glutathione S-transferase-toxin C3 transferase from *Clostridium botulinum* (GST-C3; a gift of Dr. A. Hall, University College London, London, UK), was expressed in *Escherichia coli*. Fusion proteins were purified from isopropyl- β -D-thiogalactopyranoside-induced exponential-phase bacterial cultures by standard procedures. The GST moiety of the fusion protein was removed by incubating GST-C3, while bound to glutathione-Sepharose beads, for 4 h at room temperature with thrombin protease (Pharmacia, Uppsala, Sweden). Contaminating thrombin was removed by incubation with *p*-aminobenzamidine linked to agarose beads (Sigma). Protein concentrations were measured using the Bradford assay (Pierce, Rockford, IL). To verify the purity of recombinant C3 toxin, aliquots of each fraction were analyzed by SDS-PAGE and stained with Coomassie blue.

Treatments with exotoxin C3 and kinase inhibitors before hypoxia. Exotoxin C3 does not easily enter cells, and, to permit its entrance, C3 toxin at 50 μ g/ml was added to confluent cells, and the cells were incubated for 24 h. Then the cells were exposed to hypoxia for 8 h. The efficiency of ADP ribosylation caused by C3 toxin on RhoA protein was observed on 12.5% SDS-polyacrylamide gels by shift mobility of the RhoA protein after immunodetection with RhoA antibody. Alternatively, cells in serum-free medium were exposed to normoxia for 1 h with or without 10 μ M PD-98059, SB-202474, SB-208530, SP-600125, or Y-27632 before hypoxic incubation.

Transfection with RhoA mutants. Caki-1 cells (60% confluent in 100-mm plates) were transfected with vector (pcDNA3.1) or pcDNA3.1-RhoAV14-Myc (dominant-active RhoA mutant with a Gly¹⁴Val mutation; a gift from W. Moolenaar, The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cells that had been serum starved for 1 h and then incubated with vectors for 5 h at 37°C in a humidified atmosphere and 5% CO₂ were transfected with lipofectamine (GIBCO-BRL Life Technologies) as a carrier. To allow cell recovery, the medium was replaced by complete McCoy's me-

dium containing 10% FCS, and the cells were incubated overnight. The levels of RhoA mutant in the transfected cells were determined by immunodetection of cell lysates with an anti-Myc epitope monoclonal antibody (Santa Cruz Biotechnology). Also, the efficiency of transfection was 35–40% when measured by incorporation of green fluorescent protein under the same transfection conditions.

Preparation of whole cell lysates for determination of *c-Jun* and phospho-Ser⁷³ *c-Jun* levels. After transfection with vector alone (pcDNA3.1) or with dominant-active RhoA (RhoAV14), cells were washed twice in cold PBS containing 10 mM NaF to avoid dephosphorylation of serine and threonine residues on *c-Jun*. Cells were lysed in ice-cold buffer containing 10 mM HEPES-Tris, 1.5 mM MgCl₂, 10 mM KCl, 10 mM NaF, and a cocktail of protease inhibitors (Calbiochem). Cells were incubated for 10 min on ice and disrupted with a Polytron (3 times for 15 s each). Total lysates were separated by Western blot analysis as described above.

RNA isolation and RT-PCR analysis. After hypoxia, cells were lysed with TRIzol reagent (GIBCO-BRL Life Technologies) according to the manufacturer's directions for total RNA extraction. RNA was quantitated by the ratio of absorbance at 260 nm to absorbance at 280 nm. A SuperScript one-step RT-PCR kit (Invitrogen Life Technologies) was used to carry out the reverse transcription at 55°C for 20 min with 5 µg of RNA, and cDNAs were amplified by PCR in 50-µl reaction mixtures containing 25 µl of 2× reaction mixture, primers at 0.25 µM each, and 1 µl of RT-platinum *Taq* mixture. For VHL, the PCR primers were 5'-AGAGATGCAGGGACACACGAT-3' (sense) and 5'-TCACAACTGTAGAATGTCAATCC-3' (antisense). The PCR conditions for VHL were as follows: 25 cycles at a denaturation temperature of 95°C for 1 min, annealing at 55°C for 1.5 min, and extension at 72°C for 1.5 min. For α -tubulin, the primers were 5'-GCCATTGCCACCATCAAGAC-3' (sense) and 5'-CACACAACCTCCTCATAATCCTTC-3' (antisense). Amplification was carried out for 25 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. A final extension of 7 min at 72°C was carried out for each PCR. Fragments were analyzed on 1.8% agarose gels stained with ethidium bromide.

Statistical analysis. Data obtained from the densitometric analysis are expressed as the ratio of immunodetected protein by Western blot analysis under hypoxic conditions to that under normoxic conditions. RT-PCR products stained with ethidium bromide were also quantified by densitometric analysis. Values are means \pm SE for at least three separate experiments and were analyzed with Student's *t*-test. Only significant differences ($P < 0.05$) are indicated.

RESULTS

***pVHL* expression increases under hypoxic conditions.** To evaluate the effect of hypoxia on pVHL expression, the RCC line Caki-1, which expresses a functional VHL gene (VHL^{+/+}), was used. First, the effect of hypoxia was evaluated in cells at subconfluence and at confluence in the presence or absence of serum (Fig. 1A). Usually, pVHL expression was lower in the absence of serum in normoxic and hypoxic conditions. Importantly, hypoxia increased pVHL expression after 8 h of hypoxia at 50% confluence (1.65-fold, $n = 3$, $P < 0.05$) and at confluence (2.2-fold, $n = 3$, $P < 0.05$) in the absence of serum. This stimulation was also observed at 50% confluence in serum-containing medium (1.54-fold, $n = 3$, $P < 0.05$), suggesting that pVHL may increase *in vivo*. Because the increase in pVHL under hypoxic conditions displayed the greatest stimulation at cell confluence in serum-free medium, our subsequent experiments were carried out under these conditions, where the level of proliferation was low to reduce the contribution of the cell cycle. Also, previous studies indicated that functional VHL induced growth arrest at high cell density

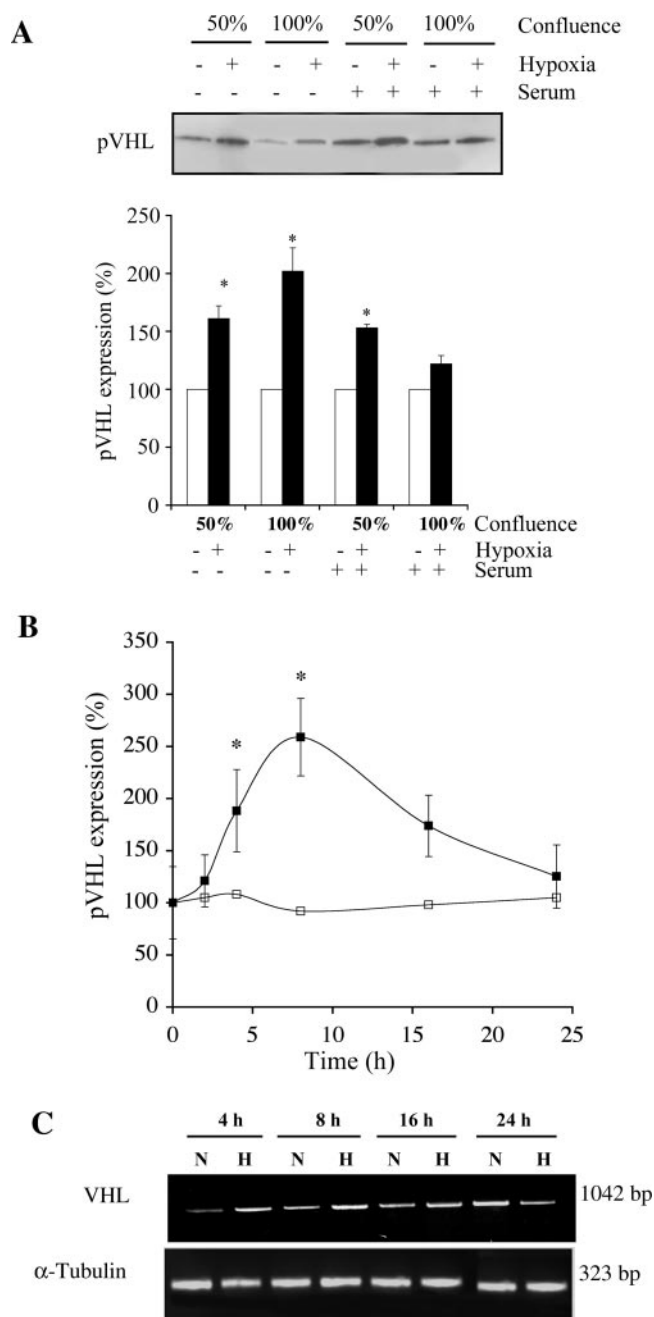


Fig. 1. von Hippel-Lindau protein (pVHL) expression increases under hypoxic conditions in renal cell carcinoma. **A:** Caki-1 cells at subconfluence (50%) or confluence (100%) were exposed to hypoxia (1% O₂, -) or normoxia (21% O₂, +) for 8 h in serum-containing (+) or serum-free (-) medium. Postnuclear supernatant (PNS) samples (20 µg of protein) were separated by SDS-PAGE and subjected to immunodetection using a pVHL antibody after Western blotting. **B:** Caki-1 cells were exposed to hypoxia (■) or kept in normoxia (□) for 0–24 h. PNS samples were separated by SDS-PAGE and immunodetected with pVHL antibody. pVHL expression was quantified by densitometric analysis and expressed as means \pm SE for 3 separate experiments relative to normoxic values. *Significantly different ($P < 0.05$) from normoxia. **C:** total RNA was isolated from Caki-1 cells after normoxic (N) and hypoxic (H) incubations. RT-PCR analysis was performed using primers for VHL and α -tubulin.

(36) and regulated cyclin D1 expression (2, 3). Second, the level of pVHL expression was examined by Western blot analysis for various periods of time in normoxic or hypoxic conditions (Fig. 1B). pVHL expression was increased by hyp-

oxia, whereas its expression remained stable during normoxia. Densitometric analysis showed that pVHL expression displayed the greatest stimulation after 8 h (2.5-fold, $n = 3$, $P < 0.05$) and then gradually returned to control values by 24 h (Fig. 1B). Third, to determine whether changes in pVHL expression were also observed at the mRNA level, RT-PCR analysis was performed on cells exposed for the same amount of time to normoxia or hypoxia. Exposure of cells to hypoxia increased the level of VHL mRNA after 4 and 8 h, and VHL gradually returned to control values after 16 and 24 h (Fig. 1C). Previously, we reported that in Caki-1 cells, HIF-1 α mRNA and protein levels were stimulated by hypoxia, confirming that the cells experienced hypoxic conditions (52). As a negative control, the level of α -tubulin mRNA was examined and was found to be unaffected by the drop in O₂ concentration (Fig. 1C). Thus hypoxia upregulates VHL expression at the mRNA and protein levels.

Effect of O₂ tension on pVHL expression in cancer and endothelial cell lines. To determine whether stimulation of pVHL expression by hypoxia was specific to renal Caki-1 cells or a general response, we subjected other cancer cell lines to hypoxia. To explore whether VHL status influences the cell response to this stress, we examined the effect of hypoxia on 786-O cells, another RCC cell line, where VHL is expressed but is not functional (VHL^{-/-}). The drop in O₂ concentration in these cells increased pVHL expression, as seen in renal Caki-1 cells carrying wild-type pVHL (Fig. 2A). The greatest stimulation was observed after 8 h of hypoxia (1.8-fold, $n = 3$, $P < 0.05$), as found in Caki-1 cells, and then gradually returned to normoxic values after 24 h (Fig. 2B). Stimulation of pVHL expression was also observed in the pancreatic cell line Panc-1 (Fig. 2A). This overexpression was maximal after 8 h of hypoxia as observed in kidney cells (1.6-fold, $n = 3$, $P < 0.05$; Fig. 2B). However, hypoxia did not affect pVHL expression in U-87 glioblastoma cells (Fig. 2A). To evaluate whether the mRNA levels were increased under hypoxic conditions, RT-PCR analysis was performed on these cells exposed to normoxia or hypoxia. In contrast to Caki-1 cells, mRNA levels of 786-O, Panc-1, or U-87 cells were not influenced by the diminution of O₂ concentration (Fig. 2C). Among the responses activated by hypoxia in tumor growth, angiogenesis is a critical process, resulting in new blood vessel formation by endothelial cells. Interestingly, hypoxia did not increase pVHL expression in BAE cells (Fig. 2, A and B). Also, in other endothelial cell lines [human dermal microvascular and umbilical vein endothelial (HMEC-1 and HUVEC-1) cells], pVHL levels were not influenced by the drop in O₂ concentration (data not shown). Thus, among the cell lines tested, hypoxia increased pVHL expression in renal carcinoma cells independent of VHL status and in pancreatic cancer cells, two tissues where tumors are associated with VHL disease.

Hypoxia induces cytoskeletal and microtubular disruption. Among the characterized molecular events known to occur under hypoxic conditions, disruption of actin organization is particularly well understood, in contrast to effects of hypoxia on tubulin distribution. To study the effect of hypoxia on cytoskeletal components, F-actin, α -tubulin, and vimentin were visualized by immunofluorescence in Caki-1 and BAE cells (Fig. 3). Hypoxia resulted in disruption of cortical F-actin structures in Caki-1 cells stained with rhodamine-phalloidin (Fig. 3A). F-actin in cortical regions was attenuated by hypoxia,

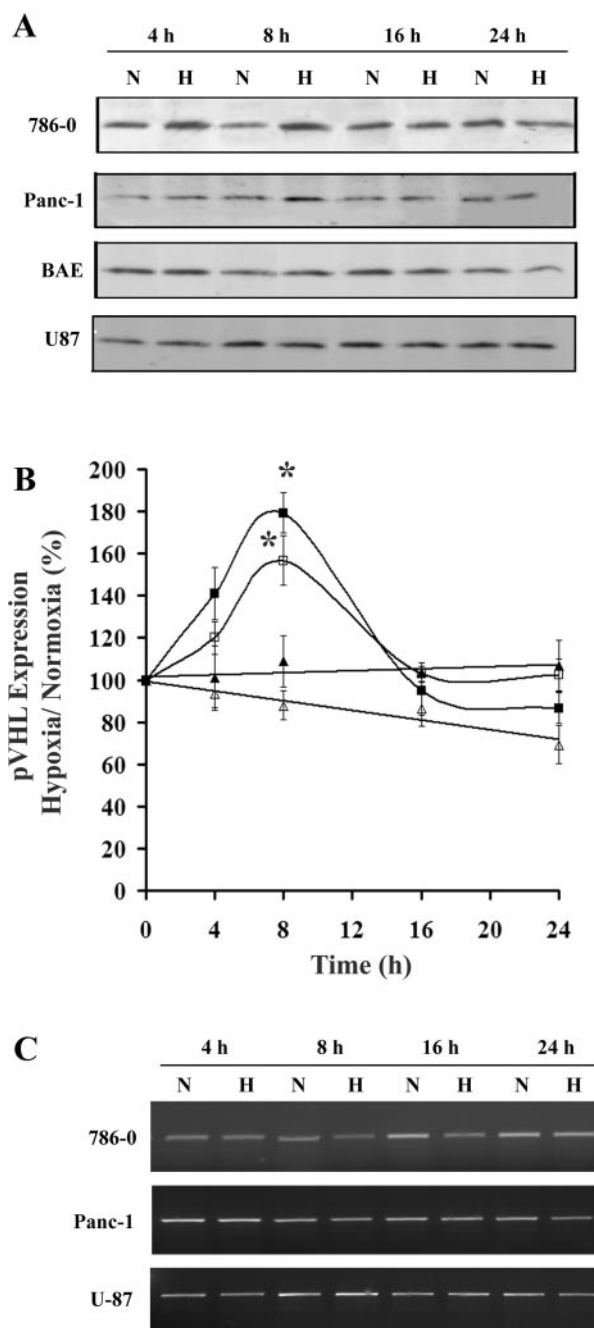


Fig. 2. Effect of O₂ tension on pVHL expression in cancer and endothelial cell lines. A and B: human renal carcinoma 786-O cells (■), pancreatic carcinoma Panc-1 cells (□), glioblastoma U-87 cells (▲), and bovine aortic endothelial (BAE) cells (△) were exposed to hypoxia (H; 1% O₂) or normoxia (N; 21% O₂) for 0, 4, 8, 16, and 24 h. pVHL expression was analyzed by SDS-PAGE and Western blotting in PNS (20 μ g of protein) with pVHL antibody (A). pVHL expression was quantified by densitometric analysis and represented as ratio of hypoxic to normoxic conditions at each time (B). Values are means \pm SE from 3 independent experiments. * $P < 0.05$. C: total RNA of 786-O, Panc-1, and U-87 cells was isolated after hypoxic or normoxic incubations, and RT-PCR analysis was carried out with primers for VHL (1,042 bp).

oxia, as previously shown by other studies (41). Interestingly, hypoxia induced stress fiber assembly, which was expected, because Rho GTPases were activated in these conditions, as we reported previously (52). However, no effect of hypoxia on actin organization was observed in BAE cells (Fig. 3A). Then

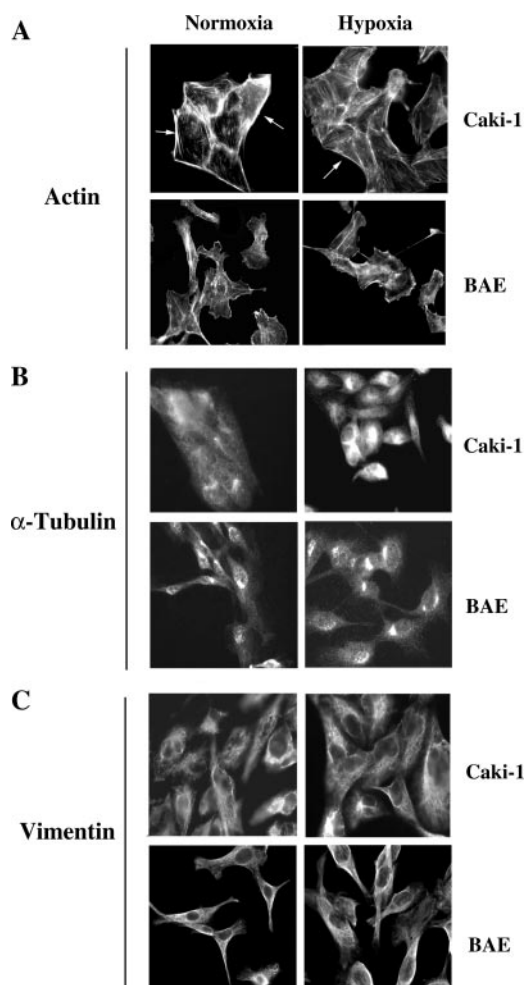


Fig. 3. Hypoxia disrupts actin organization and microtubule networks in Caki-1 cells. Caki-1 and BAE cells plated on coverslips were exposed to hypoxia or normoxia for 1 h. Cells were fixed, permeabilized, and stained for F-actin using rhodamine-phalloidin (A), α -tubulin (B), or vimentin (C). Each image is 100 μ m. Arrows, cortical actin in Caki-1 cells.

α -tubulin was visualized in Caki-1 and BAE cells to examine the effect of hypoxia on microtubule organization (Fig. 3B). The diminution of O_2 concentration induced microtubules to become more bundled in Caki-1 cells. Also, cells developed multiple regions of nucleation. In BAE cells, microtubule bundles were less marked than in Caki-1 cells (Fig. 3B). Finally, the distribution of intermediate filaments was observed in cells under normoxic or hypoxic conditions (Fig. 3C). The organization of vimentin networks was little affected by hypoxia in carcinoma and endothelial cells (Fig. 3C). Together, these results show that hypoxia induces a disruption of cytoskeletal components, particularly F-actin and α -tubulin, which could be early events participating in pVHL stimulation in RCC.

Cytoskeletal disruption induces pVHL expression. A role for pVHL in the organization of the actin cytoskeleton and focal adhesion formation has been postulated (24). To investigate whether cytoskeletal disruption observed in Caki-1 cells by immunofluorescence (Fig. 3) could participate in pVHL expression, cells were pretreated with various concentrations of cytochalasin D, paclitaxel (taxol), vinblastine, or acrylamide,

and the effects of these compounds on pVHL expression were examined. Disruption of the actin cytoskeleton by cytochalasin D and disruption of microtubule polymerization by vinblastine and taxol increased pVHL expression, whereas acrylamide, which disrupts intermediate filaments, had no effect (Fig. 4A). Stimulation of pVHL expression by these agents was maximal at 1 nM but returned to control values at 10 and 100 nM. This return to control values at high drug concentrations could be explained by the activity of P-glycoprotein. Vinblastine is a substrate for P-glycoprotein, and our transport studies in vitro demonstrated that 20–100 nM vinblastine was exported out of cells, whereas a weak concentration remained inside the cells (23). At 1 nM, cytochalasin D and vinblastine significantly stimulated (1.8-fold, $n = 3$, $P < 0.05$) the amount of pVHL (Fig. 4B). Similarly, pVHL expression was significantly increased by taxol treatment (2.3-fold, $n = 3$, $P < 0.05$), which promotes the formation of highly stable microtubules that resist depolymerization (Fig. 4B). Together, these results showed a clear link between the disruption of actin and microtubule networks by chemical agents or hypoxia and pVHL expression.

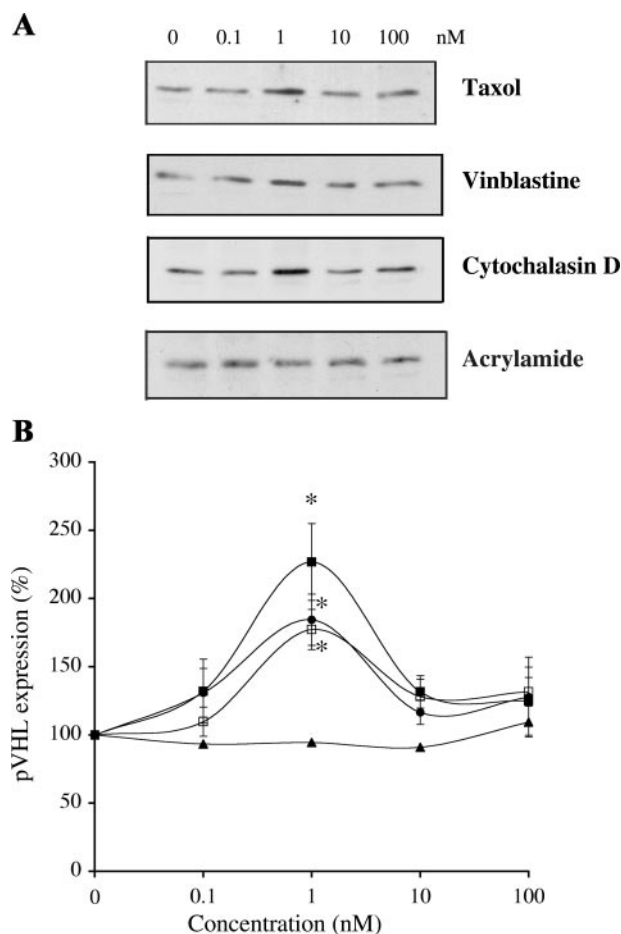


Fig. 4. Disruption of cytoskeleton by chemical agents induces pVHL expression. A: Caki-1 cells were pretreated with various reagents disrupting different components of the cell cytoskeleton for 24 h under normoxic conditions. Then, PNS samples (20 μ g of protein) were separated by SDS-PAGE and subjected to immunodetection using the pVHL antibody. B: expression of pVHL in the presence of taxol (■), vinblastine (□), cytochalasin D (●), and acrylamide (▲) was quantified by densitometric analysis and expressed as means \pm SE for 3 separate experiments relative to control values. *Significantly different ($P < 0.05$) from control.

Because Rho proteins regulate the organization of the actin cytoskeleton, we next investigated a role for these GTPases in pVHL expression.

Dominant-active RhoA stimulates pVHL expression in normoxia. We previously showed that RhoA GTPase activity and expression are upregulated during hypoxia in renal Caki-1 cells, with a maximum at 4 h, and that this upregulation is essential to HIF-1 α activation (52). To determine whether this GTPase could also enhance pVHL expression, we transfected Caki-1 cells with dominant-active RhoA (RhoAV14). This protein was tagged with Myc, and the expression of mutated RhoA was confirmed with a Myc antibody (data not shown). Cells expressing dominant-active RhoA showed significantly enhanced pVHL expression (1.7-fold) in normoxic conditions compared with cells transfected with the vector alone (Fig. 5). The same results were obtained when cells were transfected with wild-type RhoA (data not shown). The vector itself did not significantly affect pVHL level (Fig. 5). These results show that active RhoA stimulates pVHL expression, although to a lesser degree than does hypoxia (Fig. 5). This discrepancy likely resulted from the transfection efficiency of Caki-1 cells, which was estimated to be 35% when a plasmid encoding green fluorescent protein was used (data not shown).

pVHL upregulation during hypoxia is abolished by exotoxin C3. To evaluate the RhoA contribution in pVHL induction during hypoxia, we used exotoxin C3, which selectively inhibits RhoA, RhoB, and RhoC without affecting Cdc42 or Rac1. To determine toxin activity, ADP ribosylation efficiency was examined by Western blot analysis, and a shift in the mobility of RhoA was observed, indicating that all RhoA was inhibited

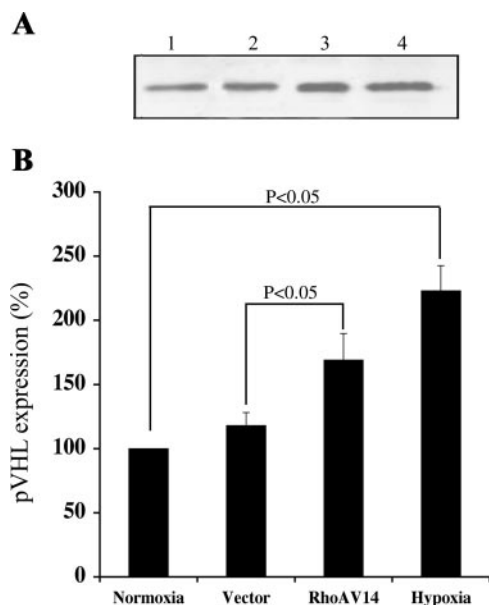


Fig. 5. Dominant-active RhoA stimulates pVHL expression in normoxia. *A:* Caki-1 cells were transfected with pcDNA3.1 vector (lane 2) or with dominant-active RhoA (pcDNA3-RhoAV14-Myc, lane 3) for 24 h using Lipofectamine. Caki-1 cells under normoxia (lane 1) or hypoxia (lane 4) for 8 h were used as negative and positive controls, respectively. PNS samples (20 μ g of protein) were separated by SDS-PAGE and then analyzed by Western blotting. *B:* pVHL immunodetection was quantified by densitometric analysis, and pVHL levels in RhoAV14-transfected cells were compared with values in cells transfected with vector only, while pVHL during hypoxia was compared with normoxia. Values are means \pm SE from 3 experiments.

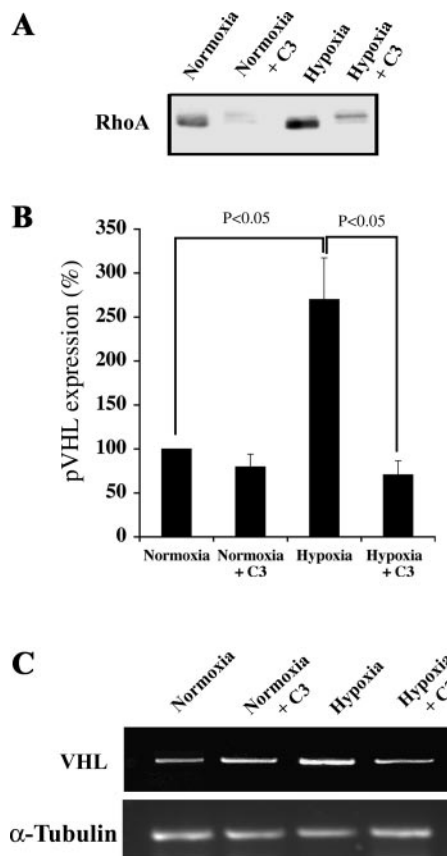


Fig. 6. pVHL expression is abolished by exotoxin C3 (C3). Caki-1 cells were pretreated with 50 μ g/ml of C3 or not pretreated for 24 h in normoxia. Then, cells were placed in hypoxia or kept in normoxia and lysed. *A:* efficiency of ADP ribosylation by C3 was verified by immunodetection of RhoA, inasmuch as ADP ribosylation reduces protein mobility. *B:* Western blot analysis of effects of C3 on pVHL expression. Expression of pVHL was quantified by densitometric analysis and expressed as means \pm SE for 3 separate experiments relative to normoxic values. *C:* total RNA was isolated from cells pretreated with C3 for 24 h or not pretreated and then incubated for 8 h under normoxic or hypoxic conditions, and RT-PCR analysis was performed using VHL and α -tubulin primers.

(Fig. 6A). Pretreatment with exotoxin C3 had no significant effect on pVHL expression under normoxic conditions (Fig. 6B). As expected, pVHL expression was increased (2.8-fold) by hypoxia, but exotoxin C3 completely prevented the induction of pVHL expression by hypoxia (Fig. 6B). RT-PCR analysis indicated that pretreatment with exotoxin C3 also prevented the increase in VHL mRNA observed in hypoxia, whereas α -tubulin remained stable (Fig. 6C). These data are in agreement with data from RhoA transfection experiments, demonstrating that RhoA is a crucial intermediary in the induction of pVHL during hypoxia.

Rho kinase inhibition prevents pVHL induction by hypoxia. To understand how RhoA stimulates pVHL expression during hypoxia, we examined the expression of Rho kinase, an effector of RhoA, previously reported to be regulated by low O₂ tension (51). The levels of RhoA and Rho kinase were higher during hypoxia than during normoxia (Fig. 7A). LIMK, an effector of Rho kinase that induces formation of actin stress fibers, was increased during hypoxia (Fig. 7A). In addition, Rho kinase activates ERM, which binds the actin cytoskeleton to plasma membranes. It has been reported that ERM family

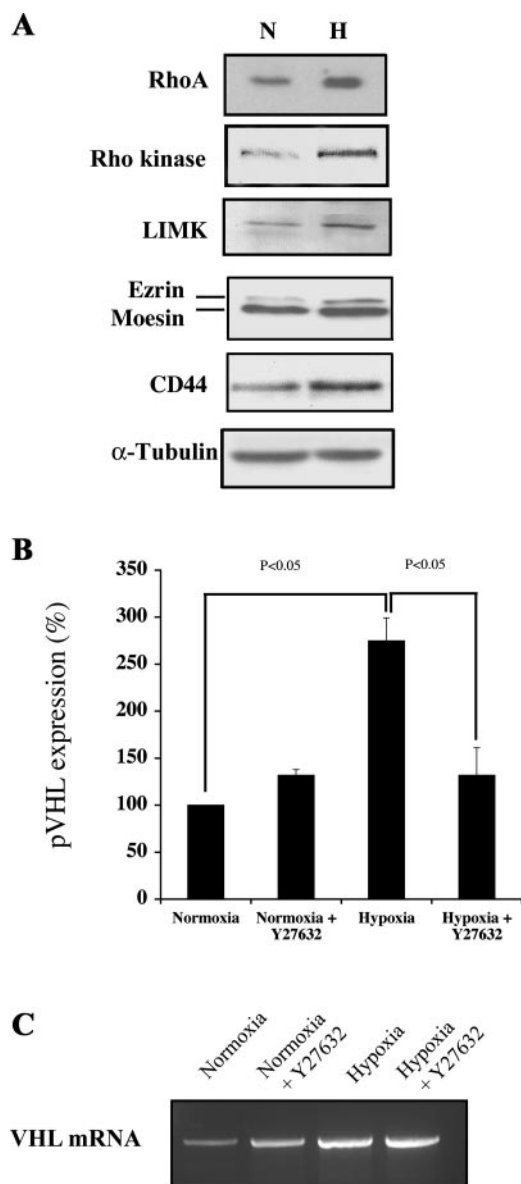


Fig. 7. Rho kinase inhibition prevents pVHL induction by hypoxia. **A**: Caki-1 cells were exposed to normoxia (N) or hypoxia (H), and PNS samples were separated by SDS-PAGE and immunodetected with RhoA, Rho kinase, LIMK, ezrin-radixin-moesin (ERM), CD44, and α -tubulin antibodies to assess the effect of hypoxia on their expression. **B**: cells were pretreated with 10 μ M Y-27632 (a Rho kinase inhibitor) for 1 h in normoxia or not pretreated and then exposed to normoxia or hypoxia for 8 h. PNS samples were separated by SDS-PAGE and immunodetected with pVHL antibody. **C**: Total RNA was isolated from Caki-1 cells in normoxia and hypoxia pretreated with or without Y-27632. RT-PCR analysis from each fraction was carried out with primers for VHL (1,042 bp). Values are means \pm SE from 3 experiments.

members are associated with the cytoplasmic domain of CD44. Interestingly, expression of ERM proteins and CD44 was also increased during hypoxia (Fig. 7A). The level of the negative control, α -tubulin, remained stable under these conditions. We next examined whether Rho kinase was involved in pVHL induction. Pretreatment with the pharmacological Rho kinase inhibitor Y-27632 did not affect pVHL expression under normoxic conditions (Fig. 7B). However, Y-27632 inhibited accumulation of pVHL during hypoxia by 90%, clearly demon-

strating an essential role for Rho kinase in this process (Fig. 7B). However, pretreatment with Rho kinase inhibitor had no effect on VHL mRNA levels during hypoxia (Fig. 7C). These results demonstrate that RhoA is required and essential for VHL protein accumulation in RCC under hypoxic conditions.

p38 and JNK are involved in stimulation of hypoxia-induced pVHL. To gain insight into other signaling pathways induced by Rho proteins in pVHL accumulation by hypoxia, the contribution of the three major mitogen-activated protein kinases (MAPKs) was assessed. Caki-1 cells were pretreated with inhibitors of MAPK kinase (MEK1/2; PD-98059), p38 (SB-203580), and JNK (SP-600125) and subjected to hypoxic conditions for 8 h. SB-202474 was used as negative control for the specificity of SB-203580 inhibition on p38 MAPK. The effects of these MAPK inhibitors on hypoxia-induced pVHL expression were analyzed by Western blotting and densitometry (Fig. 8). The presence of the p38 inhibitor, but not its inactive analog, significantly reduced pVHL induction by 30%, and the JNK inhibitor blocked hypoxia-induced pVHL by 72%, whereas the MEK inhibitor had no effect (Fig. 8). The efficiency of PD-98059 treatment was confirmed by a decrease in phosphorylated extracellular response kinase (ERK1/2; data not shown). During normoxia, none of the MAPK inhibitors significantly affected pVHL expression. Together, these results support the assertion that the hypoxia-induced increase in pVHL was mediated by Rho GTPases, which are known to act through the MAPK p38 and JNK pathways.

c-Jun expression and phosphorylation are enhanced by active RhoA. To determine whether the JNK pathway implicated in the hypoxia-induced pVHL expression occurred through RhoA-dependent activity, Caki-1 cells were transfected with dominant-active RhoA14. Interestingly, the results indicated that activated RhoA increased expression of JNK protein (1.5-fold, $n = 3$, $P < 0.05$; Fig. 9A). We next examined the effect of constitutively active RhoA on c-Jun expression, an effector of JNK. Transfection with RhoA14

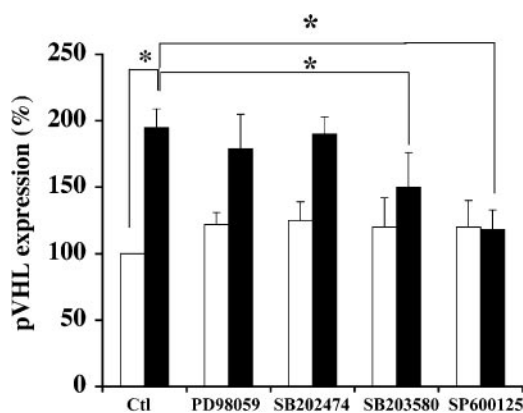


Fig. 8. p38 and JNK are involved in stimulation of hypoxia-induced pVHL. Caki-1 cells were pretreated with 10 μ M PD-98059 (MEK inhibitor), SB-202474 (negative control for p38 inhibitor), SB-203580 (p38 inhibitor), or SP-600125 (JNK inhibitor) in normoxia for 1 h. Cells were then incubated in normoxic or hypoxic conditions for 8 h. PNS (20 μ g of protein) samples were separated by SDS-PAGE, and cells were subjected to immunodetection with the pVHL antibody. Densitometric analysis of pVHL expression is shown for cells in normoxia (open bars) and hypoxia (solid bars). Values are means \pm SE for 3 independent experiments. Data are expressed relative to normoxic values observed in untreated cells (Ctl). *Significantly different from normoxia or from hypoxia.

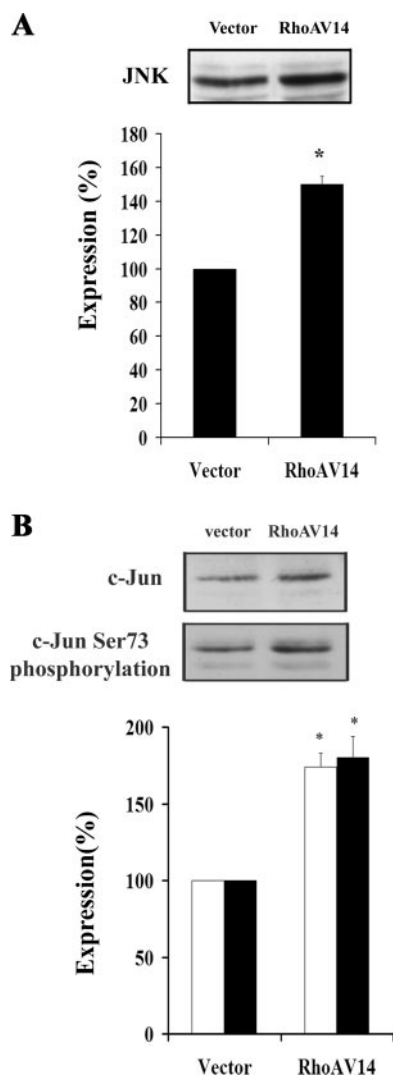


Fig. 9. Dominant-active RhoA (RhoAV14) stimulates expression and phosphorylation of c-Jun. Caki-1 cells were transfected with vector alone (pcDNA3.1) or RhoAV14 for 24 h using lipofectamine. Whole lysates (40 μ g of protein) were separated by SDS-PAGE and then analyzed by Western blotting (*insets*). *A*: densitometric analysis of effects of RhoAV14 on JNK expression. *B*: densitometric analysis of regulation of c-Jun expression (open bars) and c-Jun Ser⁷³ phosphorylation (solid bars) by RhoAV14. Values are means \pm SE from 2–3 independent experiments. *Significantly different ($P < 0.05$) from vector.

increased expression of c-Jun (1.7-fold, $n = 2$, $P < 0.05$) and c-Jun Ser⁷³ phosphorylation (1.8-fold, $n = 2$, $P < 0.05$) compared with cells transfected with the vector only (Fig. 9B). Together, these results show that the c-Jun pathway is involved in hypoxia-induced pVHL expression and mediated by RhoA.

DISCUSSION

A drop in cellular O₂ concentration is frequently observed in pathological conditions, such as during tumor growth. The reduction of O₂ tension activates transcription of several genes that are generally mediated by stabilization of HIF-1. Several studies have demonstrated an essential role for pVHL in regulation of the transcriptional factor HIF-1, particularly regarding angiogenesis during hypoxia (29, 35). Recent studies have demonstrated regulation of the HIF-1 α level by binding

of pVHL to the ODD of HIF-1 α in the presence of O₂ and iron, resulting in prolyl hydroxylation (21, 22). This interaction permits polyubiquitination of HIF-1 α and its degradation by the proteasome. We previously showed an essential role for RhoA in upregulation of HIF-1 α mRNA and protein during hypoxia in RCC (52). In the present study, our understanding of the molecular mechanisms involved in the hypoxia response is extended, and our findings indicate an upregulation of pVHL during hypoxia in RCC that is mediated by Rho-dependent pathways.

Among studies analyzing pVHL-HIF-1 α interactions, none have considered the effect of hypoxia on pVHL expression. In RCC, our data demonstrate that hypoxia upregulates the endogenous level of pVHL in Caki-1 and 786-O cells. Renal Caki-1 cells possess a VHL gene encoding a functional pVHL, whereas kidney 786-O cells contain a VHL sequence that encodes a mutated and inactive pVHL, indicating that hypoxia-induced pVHL is independent of the pVHL activity state. Because VHL mRNA is induced by low O₂ tension in Caki-1 cells, hypoxia could regulate VHL expression at the gene level or by stabilization of its mRNA. These possibilities remain to be examined. We also observed a rise in pVHL during hypoxia in the pancreatic carcinoma cell line Panc-1, which is intriguing, because the pancreas, similar to the kidney, is a tissue where tumors develop in patients with VHL disease (26). In contrast to Caki-1 cells, the level of VHL mRNA remains constant in 786-O and Panc-1 cells. This is consistent with stimulation of the protein, which is lower in these cells than in Caki-1 cells. Our results are supported by another study in which pVHL increased in cytotrophoblasts, placental cells that proliferate under low O₂ tension (11). However, in the glioblastoma cell lines, the pVHL level was unaffected by hypoxia. Because hypoxia stimulates tube formation by endothelial cells, a process that is dependent on Rho kinase, we further studied the effect of low O₂ tension on pVHL expression in these cells. However, pVHL expression was unaffected in the endothelial cells. Because hypoxia prevents interaction between pVHL and HIF-1 α , our finding that pVHL is upregulated suggests a new role for pVHL during hypoxia in renal and pancreatic cell carcinoma.

Because actin disorganization is an early hallmark of the cell response to hypoxia, we investigated the contribution of different cytoskeletal components as potential regulators of pVHL expression. In this study, we demonstrated that 1 h of hypoxia was sufficient to disrupt F-actin patterns, because the labeling

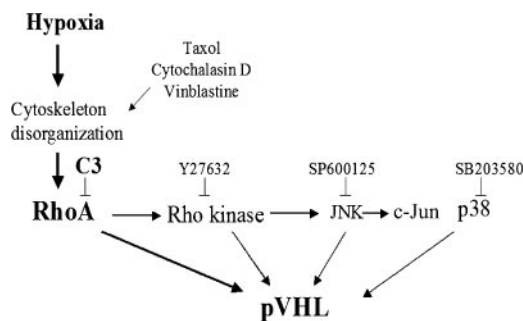


Fig. 10. Model for pVHL induction during hypoxia through RhoA-Rho kinase pathway. Key steps identified using transfection experiments and specific inhibitors to investigate signaling pathways regulated by the GTPase RhoA in induction of pVHL are summarized.

of cortical F-actin was attenuated in this condition. Interestingly, pVHL expression was increased after treatment with an agent, e.g., cytochalasin D, that disrupts the actin cytoskeleton under normoxic conditions in this RCC model. Also, we observed that diminution of O₂ concentration induced microtubules to become more bundled and to develop multiple regions of nucleation in Caki-1 cells. This effect was observed also after treatment of carcinoma cells with taxol, which stabilizes microtubules, and colchicine, which disrupts microtubules, and, in a similar manner, altered tubulin staining in other studies (27, 40). Furthermore, we blocked microtubule polymerization by exposure to vinblastine, which also induced pVHL expression. These data indicate that disruption of actin filaments and microtubules leads to pVHL upregulation. However, intermediate filaments do not appear to regulate pVHL levels. There could be several explanations for the relationship between changes in cytoskeletal organization and pVHL expression. Recently, it has been demonstrated that pVHL binds to tubulin and can protect microtubules from depolymerization (15). Another interesting link is the demonstration that actin filaments are stabilized through formation of focal adhesions by pVHL (24). These observations support the possibility that pVHL induction, when actin and microtubule networks are disrupted by chemical agents or hypoxia, serves to protect or stabilize the cytoskeleton against cell injuries.

In addition to examining the functions of hypoxia-induced pVHL, it is essential to identify the molecular mechanisms involved in pVHL induction. The small G proteins of the Rho family are involved in reorganization of the actin cytoskeleton, in cell migration, and in regulation of gene transcription (14, 50). Whereas RhoA induces stress fiber formation, Rac1 and Cdc42 promote focal complexes with lamellipodia and filopodia, respectively (37). In addition, Rho GTPase activity is stimulated by microtubule depolymerization (28). These data suggest that Rho GTPases could regulate pVHL levels during hypoxia, because this protein is also involved in cytoskeletal organization. As expected, our results clearly demonstrate that RhoA is involved in pVHL induction. First, transfection of Caki-1 cells with dominant-active RhoA stimulates pVHL expression during normoxia. Second, pretreatment with C3 toxin, which inactivates RhoA, RhoB, and RhoC, prevents induction of pVHL in Caki-1 cells during hypoxia. These results indicate that RhoA is likely involved in induction of pVHL by hypoxia in these cells, because RhoA becomes induced (3.5-fold), whereas RhoB remains stable and RhoC is not immunodetected (52). Finally, we observed that Rho kinase expression, as well as expression of Rho kinase substrates such as LIMK and ERM proteins, is increased under hypoxic conditions in Caki-1 cells. Similarly, it has been reported that hypoxia increased Rho kinase expression and activity in endothelial cells (51). Importantly, we have shown that pretreatment with a pharmacological inhibitor for Rho kinase, Y-27632, strongly prevents pVHL induction by hypoxia. VHL induction occurs at the protein and mRNA levels when cells are treated with C3 toxin, whereas the Rho kinase inhibitor blocks VHL induction by hypoxia at the protein level only. These results could be explained by the capacity of RhoA to activate the transcription of genes, whereas Rho kinase acts at the protein level, suggesting that this kinase could modulate stabilization or synthesis of pVHL. These results demonstrate a critical role for the RhoA-Rho kinase pathway in pVHL induction during

hypoxia. It is interesting to speculate that depletion of ATP and GTP during hypoxia (9) provokes a depolymerization of microtubules, a process that is known to stimulate RhoA activity (28). This activated RhoA could then upregulate pVHL, which binds and stabilizes microtubules (15).

In addition to RhoA/Rho kinase activation, we have studied whether other signaling pathways could be implicated in pVHL induction under hypoxic conditions. Using MEK1/2, p38, or JNK inhibitors, we observed that p38, but principally JNK, pathways are involved in pVHL induction by hypoxia. The lack of MEK1/2 inhibition on pVHL induction during hypoxia is supported by our previous observation that Ras, in contrast to Rho GTPase, levels are unaffected on reduction of O₂ tension in Caki-1 cells (52). Furthermore, our results suggest that JNK-dependent pVHL induction during hypoxia is also mediated by RhoA. This is supported by transfection experiments showing that constitutively active RhoA increases JNK expression. When activated, JNK stimulates c-Jun by phosphorylation on serine residues, which can then bind c-Fos to form the transcription factor AP-1 (43). Indeed, we found that c-Jun and phospho-Ser⁷³ c-Jun are increased in the presence of dominant-active RhoA. Moreover, our results demonstrate that hypoxia increases c-Jun protein expression and c-Jun Ser⁷³ phosphorylation (data not shown). In agreement with our data, it has been reported that hypoxia induces AP-1 by an increased level of c-Jun protein (43). Also, it has been demonstrated that RhoA is involved in JNK activation in response to H₂O₂ stimulation (42). Another study found that activated RhoA can stimulate *c-jun* expression and activity of the *c-jun* promoter (33). However, in some studies, Cdc42 and Rac1 stimulate activation of JNK and p38 by protein-activated kinase effector (8, 16). Because p38 and JNK are critical steps in several signaling pathways, it could not be ruled out that other signaling cascades, in addition to RhoA, could regulate pVHL expression.

Figure 10 summarizes the sequence of major findings in our study investigating induction of VHL by hypoxia. Our results demonstrate for the first time that the RhoA-Rho kinase pathway upregulates the VHL protein, whereas RhoA stimulates VHL mRNA levels under hypoxic conditions. Hypoxia also increases RhoA and Rho kinase expression, which is essential to pVHL induction, because their inhibition prevents its induction. Furthermore, the kinase JNK and its effector c-Jun are implicated in this process, because activated RhoA increases their expression and their activation, whereas JNK inhibition blocks pVHL induction during hypoxia. Finally, a contribution of MAPK p38 is also observed in pVHL induction by hypoxia.

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