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The von Hippel-Lindau Tumor Suppressor Gene Product Interacts with Sp1 To Repress Vascular Endothelial Growth Factor Promoter Activity

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The von Hippel-Lindau tumor suppressor gene (*VHL*) has a critical role in the pathogenesis of clear-cell renal cell carcinoma (RCC), as *VHL* mutations have been found in both von Hippel-Lindau disease-associated and sporadic RCCs. Recent studies suggest that vascular endothelial growth factor (VEGF) mRNA is upregulated in RCC- and von Hippel-Lindau disease-associated tumors. We have therefore assessed the effect of the *VHL* gene product on VEGF expression. VEGF promoter-luciferase constructs were transiently cotransfected with a wild-type *VHL* (wt-*VHL*) vector in several cell lines, including 293 embryonic kidney and RCC cell lines. wt-VHL protein inhibited VEGF promoter activity in a dose-dependent manner up to 5- to 10-fold. Deletion analysis defined a 144-bp region of the VEGF promoter necessary for VHL repression. This VHL-responsive element is GC rich and specifically binds the transcription factor Sp1 in crude nuclear extracts. In *Drosophila* cells, cotransfected VHL represses Sp1-mediated activation but not basal activity of the VEGF promoter. We next demonstrated in coimmunoprecipitates that VHL and Sp1 were part of the same complex and, by using a glutathione-*S*-transferase–VHL fusion protein and purified Sp1, that VHL and Sp1 directly interact. Furthermore, endogenous VEGF mRNA levels were suppressed in permanent RCC cell lines expressing wt-VHL, and nuclear run-on studies indicated that VHL regulation of VEGF occurs at least partly at the transcriptional level. These observations support a new mechanism for VHL-mediated transcriptional repression via a direct inhibitory action on Sp1 and suggest that loss of Sp1 inhibition may be important in the pathogenesis of von Hippel-Lindau disease and RCC.

von Hippel-Lindau disease is a neoplastic disorder characterized by central nervous system, kidney, adrenal gland, pancreas, and retina tumors (50). Renal cell carcinoma (RCC) is the most frequent cause of death by von Hippel-Lindau disease. Recently, the von Hippel-Lindau gene (*VHL*) was identified at chromosome 3p25-26 (43). The critical role of *VHL* in clear-cell RCC has been confirmed in studies demonstrating biallelic *VHL* gene defects in von Hippel-Lindau disease-associated RCCs and in the majority (up to 70%) of sporadic RCCs (8, 13, 20, 21). The first clues to VHL function as an inhibitor of transcription elongation came from its interaction with the elongin C component of the elongin (SIII) complex (3, 18, 40). Convincingly, a high percentage of VHL missense mutations in RCC prevent interaction with elongin C (40). Failure of VHL to inhibit elongin-mediated transcription elongation would result in overexpression of genes regulated at this level, but no target genes have been identified.

von Hippel-Lindau disease associated tumors are highly vascularized. Both von Hippel-Lindau disease-associated and sporadic hemangioblastomas (72) and RCCs (6, 67) overexpress the potent angiogenic factor vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF) and its receptors KDR and Flt1, suggesting these genes may be VHL targets. We demonstrate that *VEGF* is indeed a target for the *VHL* tumor suppressor gene product and that transcriptional repression of the *VEGF* promoter depends on a direct interaction between VHL and the ubiquitous transcriptional activator Sp1.

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MATERIALS AND METHODS

Plasmids. The *VHL* cDNA was PCR amplified from a human fetal kidney cDNA library (Stratagene) with primers 5′-TTTTTGAATTCAATGCCCCCGG AGGCGGAGAACTGGG-3′ and 5′-TTTTTTTCTAGAAGTTAGAGGGTAG GCAACTACACGT-3′. The product was subcloned into pCMV2FLAG vector (Kodak) to generate pCMV2FLAGVHL. The plasmid encoding pCMV2FLAG VHL(1-115)[pCMV2FLAGΔVHL] was made as described above, except that the reverse primer in the PCR was 5′-TTTTTTTCTAGATCACACTGGAGCC ATCGACACCTACGCCGCC-3′. *Drosophila* Sp1 expression vector pPacSp1 was a gift from R. Tjian (12). *Drosophila* expression vector pRact-HAdh was a gift from L. Cherbas (66). pRact-VHL1-213 and pRact-VHL1-115 were likewise generated by PCR using forward primer 5′-TTTTTTGGAT CCGAAGTCACC ATGCCCCGGAGGGCGGA-3′ containing a *Drosophila* ATG context (19) and reverse primer 5′-TTTTTTCTGCAGTCAATCTCCCATCCGTTGATG-3′ or 5′-TTTTTTCTGCAGTCAGTGACCTCGGTAGCTGT-3′, respectively. pGST-VHL1-213 was a gift from W. G. Kaelin (40). pGST-VHL1-115 was generated in pGEX-4T3 (Pharmacia), also by PCR (primers 5′-TTTTTTGAATTCCATGCC CCGGAGGGCGGA-3′ and 5′-TTTTTTCTC GAGTCAGTGACCTCGGTAG CTGT-3′), so that no amino acids beyond amino acid 115 of VHL were included in the fusion protein.

The *VEGF* reporter constructs used in transient-transfection assays contain sequences derived from the human *VEGF* promoter driving expression of firefly luciferase. A 2.6-kb (bp −2361 to +298 relative to the transcription start site) *VEGF* promoter fragment was used as described earlier (66). The truncated promoter-reporter plasmids were constructed as follows: for the 1.5-kb construct (bp −1226 to +298), primers 5′-GCGCGCAAGCTTACGCGTATGAGTCTG GGCTTGGGCTGATAG-3′ and 5′-CCTCGTCGACTCGAGATCCACAGTG ATTTGGGGAAGTAG-3′ were used; for the 0.35-kb construct (bp −194 bp to +157), primers 5′-GGTCGAGCTTCCCCTTCATTCCGG-3′ and 5′-AACCCG GATCAATGAATATCA-3′ were used; for the 0.2-kb construct (bp −50 to +157), forward primer 5′-GCGCGGATCCAGCCATGCGCCCCCCCC-3′ and a reverse primer identical to the one used for the 0.35-kb construct were used;

and for the 0.07-kb construct (bp −50 to +18), the same forward primer as for the 0.2-kb construct and reverse primer 5′-CCTCCCAAGCTTTGCCCCAAGC CTCCGCG-3′ were used. The *VEGF* promoter constructs were made by PCR from the 2.6-kb *VEGF* promoter fragment and subcloned into pGL-2 Basic vector (Promega) as described earlier. All PCR-generated constructs were confirmed by sequencing.

Cell culture and transfection. Human fetal kidney 293 cells (ATCC CRL1573), human glioblastoma-astrocytoma cells U-87 MG (ATCC HTB-14), two renal carcinoma cell lines (786-O and TK-10), and Cos-7 cells were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum (HyClone Laboratories). 786-O clonal cell lines stably transfected with either pRC, pRC-HAVHL, or pRC-HAVHL(1-115) (gifts from W. G. Kaelin, [36]) or pCMV2FLAG or pCMV2FLAGVHL (made by us) were grown in complete medium supplemented with G418 (1 mg/ml). *Drosophila* Schneider S2 cells (ATCC CRL1963) were maintained in Schneider medium with 10% heat-inactivated serum (Sigma) at 22°C.

All cells were transfected by calcium phosphate precipitation (23). Mammalian cells were transfected with a total of 5 μg of plasmid DNA per 60-mm-diameter dish, including 1.5 μg of promoter-reporter construct and different amounts (as noted) of VHL expression vector. Empty pCMV2FLAG expression vector served as filler DNA, so that all groups had the same amount of pCMV2FLAG. *Drosophila* cells were transfected with a total of 7.2 μg of plasmid DNA per 60-mm-diameter dish. For the Sp1 dose-response curves (see Fig. 3a), transient transfections included 1.5 μg of reporter plasmid, increasing amounts of Sp1 expression vector, and pBluescriptII SK+ (Stratagene) as filler DNA. For the VHL-Sp1 experiments (see Fig. 3b), 1.5 μg of reporter plasmid with or without 5 ng of pPacSp1 was cotransfected with increasing amounts of VHL expression vectors. Empty pRact-HAdh expression vector served as filler; therefore, no pBluescript was used. Duplicate dishes were transfected for all experiments. Luciferase assays (Promega) were performed per the manufacturer's protocol 42 to 48 h after transfection. Luciferase activity was normalized by the total amount of cellular protein as assayed by the Bradford protein assay (Bio-Rad).

Western analysis. Protein samples were mixed with 2× sample buffer (125 mM Tris-HCl [pH 6.8], 20% glycerol, 10% β-mercaptoethanol, 4% sodium dodecyl sulfate [SDS], and 0.0025% bromophenol blue), boiled, and run on 7.5 to 12% polyacrylamide gels (Ready gels; Bio-Rad) with Tris-glycine-SDS running buffer (Bio-Rad). Agarose beads with bound proteins were handled in the same manner and directly loaded on the gel. Size-separated proteins were transferred (Trans-Blot SD; Bio-Rad) to a nylon membrane (Immobilon-P; Millipore) semidry and stained with Ponceau S (Sigma). For immunodetection, membranes were blocked in washing buffer (1× phosphate-buffered saline [PBS] with 1% milk and 0.1% Tween 20) with 5% milk and incubated in washing buffer without Tween 20 with affinity-purified anti-Sp1 rabbit serum (Santa Cruz Biotechnology) or a VHL monoclonal antibody (gift of W. G. Kaelin (40). The secondary antibodies were donkey anti-rabbit immunoglobulin (Ig) and sheep anti-mouse Ig linked to horseradish peroxidase (Amersham), respectively, which were detected by chemiluminescence (Pierce).

Immunoprecipitations. Cells were washed twice with 10 ml of cold PBS, lysed with ice-cold lysis buffer (50 mM Tris [pH 7.5], 1% Nonidet P-40 [NP-40], 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, leupeptin [10 μg/ml], 0.5% aprotinin, 2 mM pepstatin A), incubated for 10 min on ice, and centrifuged for 10 min at 4°C. Immunoprecipitations were carried out at antibody excess (1.0 μg of IgG) with 0.5 mg of total protein. Antibodies included affinity-purified rabbit polyclonal antibodies directed against Sp1 or Gal4 (Santa Cruz Biotechnology) or mouse monoclonal antibodies against the FLAG epitope (Kodak Co.) or the VHL epitope, as described above. Immunocomplexes were captured with protein A-agarose beads (Bio-Rad). After three washes with cell lysis buffer, bead-bound proteins were subjected to Western analysis, as detailed above.

Nuclear extract preparation, partial protein purification, and electrophoretic mobility shift assays (EMSAs). HeLa S3 crude nuclear extracts were prepared according to a standard protocol (16), with modifications (11, 36). Wheat germ agglutinin (WGA) partial purification of HeLa S3 nuclear extract was performed on a 2-ml WGA-agarose affinity column (Vector Laboratories) (36). 786-O whole-cell extracts were made by washing cells twice in PBS, pelleting, resuspending in Dignam buffer C (20 mM HEPES [pH 7.9], 25% glycerol, 0.42 M KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol [DTT]), freeze-thawing once, rocking for 30 min at 4°C, and pelleting debris from the supernatant of soluble proteins. Whole-cell extracts from transfected *Drosophila* Schneider cells were made by washing cells twice in PBS, pelleting, and resuspending in 1× reporter lysis buffer (25 mM Tris [pH 7.8], 4 mM EDTA, 2 mM DTT, 10% glycerol, and 1% Triton X-100; Promega) containing 0.1 mM $ZnSO_4$. Extract protein was quantitated by the Bradford assay (Bio-Rad).

EMSAs were performed as previously described (11). Briefly, EMSA binding reaction mixtures (25 μl) contained 20 mM HEPES (pH 8.4), 100 mM KCl, 20% glycerol, 0.1 mM EDTA, 0.2 mM $ZnSO_4$, 0.05% NP-40, and 1 μg of bovine serum albumin (BSA). Extract protein and 200 ng of poly(dA-dT) · poly(dA-dT) were added at room temperature 10 min prior to addition of ~0.1 ng of radiolabeled oligonucleotide probe. After 20 min of room temperature incubation, samples were run on 4% acrylamide gels in 1× TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. For EMSA supershift studies, 2 μg of monoclonal (Santa Cruz Biotechnology) or polyclonal Sp1 antibody or, as controls, monoclonal FLAG antibody or maltose binding protein antiserum (New England Biolabs) was added for 40 min at room temperature following the standard binding reaction.

Radiolabeled oligonucleotides used in EMSA studies were two PCR-generated fragments of the VEGF promoter, each containing the four putative Sp1 binding sites, a 106-bp product (bp −112 to −7, relative to the transcription start site) (with 5′-CTGAGGCTCGCCTGTCCC-3′ and 5′-CCGCTACCAGCCGA CTTT-3′ as primers), and a 71-bp product (bp −112 to −41) (with lower-strand primer 5′-GGCGCATGGTCCGCCC-3′ instead). The following unlabeled, double-stranded consensus site oligonucleotides were from Promega and contained the indicated sequences (upper strand only shown): Sp1 (5′-ATTCGATCG GGGCGGGGCGAGC-3′), AP2 (5′-GATCGAACTGACCGCCCGCGGCCC GT-3′), and NF-κB (5′-AGTTGAGGGGACTTTCCCAGGC-3′).

In vitro binding assays. Glutathione-*S*-transferase–VHL protein fusions under the control of a *lac* operator (pGEX plasmids; Pharmacia) were expressed in *Escherichia coli*. pGST-VHL1-213 was a gift from W. G. Kaelin (40). Overnight cultures of BL21 cells (Stratagene) containing pGST-VHL1-213, pGST-VHL1-115, and pGEX-4T3 plasmids were diluted 1:10, grown for 1 h in Luria-Bertani medium, and then induced with isopropyl-β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Cell pellets were briefly sonicated in binding buffer (20 mM Tris-HCl [pH 7.5], 150 mM KCl, 1 mM EDTA, 0.5% NP-40). Cleared cell lysates were mixed with glutathione-agarose beads (Pharmacia) and rocked for 30 min at 4°C. The beads were washed three times with binding buffer containing 0.10% SDS, which substantially reduced binding of *E. coli* GroEL (11a), and then twice with binding buffer alone. Beads were mixed with purified Sp1 protein (50 ng; Promega) and BSA (5 μg) as a carrier in binding buffer, incubated with agitation for 20 min on ice, and washed four times with binding buffer. Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), as described above, and tested for Sp1 immunoreactivity.

Northern blot analysis. RNA, isolated by the single-step acid-phenol extraction method (10), was separated on a formaldehyde-agarose gel, transferred to a GeneScreen (DuPont) membrane by using 10× SSC, and probed with random-primer-labeled cDNAs in a solution containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1% BSA, 1 mM EDTA, and sonicated herring sperm DNA (50 μg/ml) at 68°C. Blots were washed three times with a solution containing 40 mM sodium phosphate (pH 7.2), 0.5% SDS, 0.5% BSA, and 1 mM EDTA at 68°C and autoradiographed.

Nuclear run-on transcription assay. Nucleus isolation and in vitro transcription were performed by a modification of previously described procedures (24, 64). Briefly, cells were scraped and lysed in NP-40 buffer (10 mM Tris [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40) and nuclei were resuspended in 200 ml of glycerol storage buffer (40% glycerol, 10 mM Tris [pH 8.3], 5 mM $MgCl_2$, 0.1 mM EDTA) and stored in liquid nitrogen or used immediately. For transcription reactions, 200 μl of nuclei (1×10^7 to 5×10^7) was mixed with 200 μl of reaction mixture (containing 10 mM Tris [pH 8], 5 mM $MgCl_2$, 300 mM KCl, 5 mM DTT, and 1 mM concentration each of ATP, CTP, and GTP) and 200 μCi of [α-^{32}P]UTP (3,000 Ci/mmol; DuPont NEN), and α-amanitin (2 μg/ml) was or was not added. The reaction mixtures were then incubated at 30°C for 30 min. After the incubation, the RNAs were purified by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method (64). Prior to hybridization, RNA was heated at 80°C for 10 min. Probes were generated by PCR using the appropriate cDNA clones in plasmids as templates, purified, and applied (1 μg of DNA) to a Nytran membrane with a slot-blot apparatus (Minifold II Manifold; Schleicher & Schuell). The size of the probes was 550 bp for *VEGF* (position −8 to 542 relative to the initiation codon), 600 bp for human β-actin probe, 590 bp for Glut1, and 1,200 bp for platelet-derived growth factor B (PDGF-B).

Filters were prehybridized in 10 mM TES [*N*-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid], 0.2% SDS, 10 mM EDTA, 0.3 M NaCl, 1× Denhardt's solution, *Torulla* yeast RNA (250 μg/ml), and salmon sperm DNA (20 mg/ml) at 65°C for 1 h. Filters were hybridized with the run-on products (5×10^6 to 10×10^6 cpm) in 1.5 ml of hybridization solution at 65°C for 2 to 3 days. Standardization was achieved by adding the same amount of radioactivity to all hybridizations in a single experiment. The filters were washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS for 30 min at 65°C and in 0.2× SSC–0.1% SDS for 45 min at 65°C and then were subjected to an RNase A (10-mg/ml; Ambion) wash in 2× SSC for 30 min at 37°C before a final wash in 2× SSC for 1 h at 37°C. Filters were scanned and radioactivity was measured on a Molecular Dynamics PhosphorImager with ImageQuant software. The amount of sample hybridizing to the *VEGF* probe was normalized by dividing the *VEGF* signal by that for β-actin within each experiment.

RESULTS

wt-VHL but not mutant VHL represses *VEGF* promoter activity. The *VEGF* promoter has recently been defined (68). We began our analysis by using a 2.6-kb promoter fragment (bp −2361 to +298, relative to the transcription start site) ligated to a luciferase reporter gene (53, 68). We asked whether the activity of this construct could be repressed in transient assays by the wild-type VHL (wt-VHL) gene product.

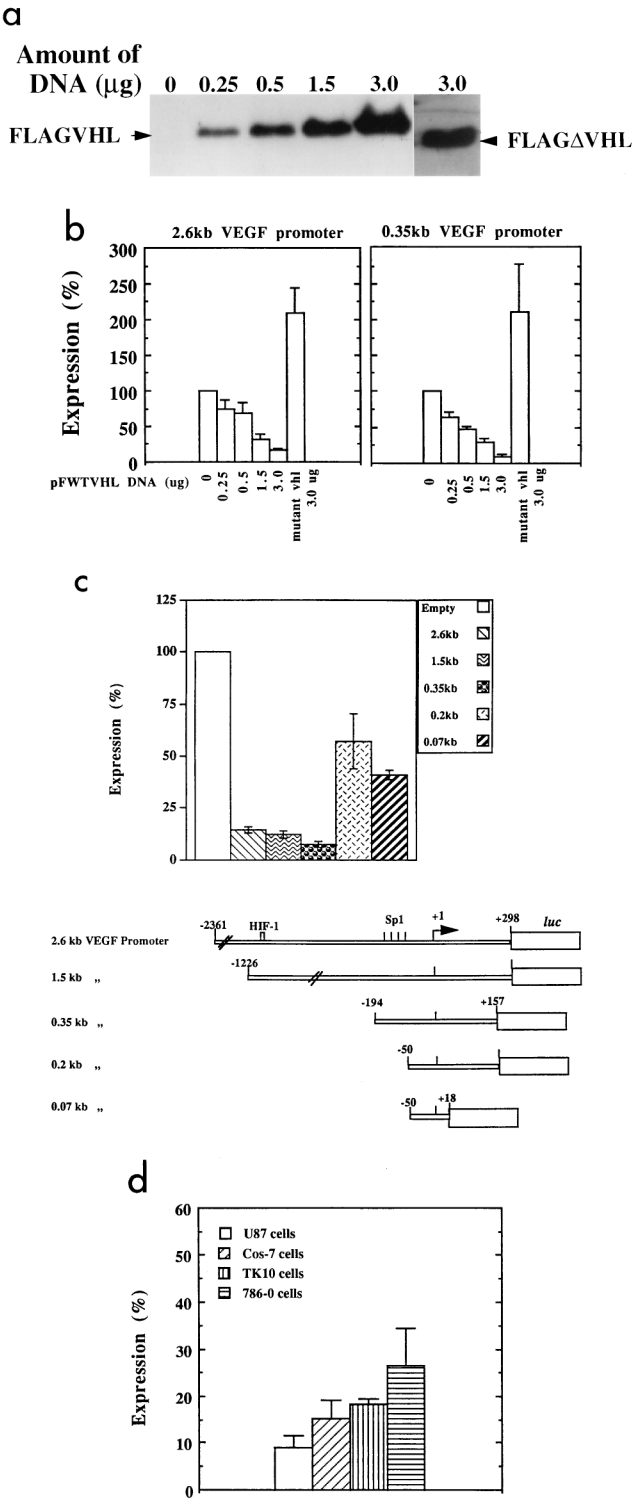

FIG. 1. Inhibition of *VEGF* promoter activity by wt-VHL depends on a promoter *cis* element. (a) Expression of FLAG-tagged VHL proteins was confirmed by Western analysis using an anti-FLAG monoclonal antibody. Each lane contained whole-cell extract (30 μg of protein/lane) from 293 cells transiently transfected with pCMV2FLAGΔVHL or increasing amounts of pCMV2FLAGVHL expression vectors, respectively. (b) VHL inhibits *VEGF* promoter activity in a dose-dependent manner in 293 cells. *VEGF* promoter-luciferase reporter constructs (1.5 μg) were cotransfected with ΔVHL and increasing amounts of VHL expression vectors, as described above. Cells were harvested for luciferase assay 48 h after transfection, and expression in each experiment was normalized to a control empty expression vector (pCMV2 FLAG). (c) Deletion analysis of the *VEGF* promoter revealed a VHL-responsive *cis* element from bp −196 to −50. VHL expression vector (3 μg) and reporter construct (1.5 μg) were contransfected in 293 cells. Percent expression is relative to the activity of the same reporter construct cotransfected with an empty expression vector. Below the bar graph are schematized *VEGF* promoter deletion constructs. The hypoxia-inducible factor 1 (HIF-1) site is centered at bp −965 (49), while the consensus GC boxes reside between bp −94 and −51 (68). (d) VHL inhibits *VEGF* promoter activity in different cell lines. VHL expression vector (3 μg) was cotransfected with the 0.35-kb *VEGF* promoter-luciferase reporter construct (1.5 μg), and data were normalized, as described above. (b to d) Error bars, standard deviation of the means.

For this purpose, 293 cells were transfected with an expression plasmid containing a human full-length *VHL* cDNA epitope tagged in its N terminus with FLAG sequence (pFwtVHL). The methionine utilized for this cDNA corresponds to that designated by Iliopoulos et al. (32) and is conserved in both rat and human VHL (17). First, we examined whether transfection of this construct into 293 cells produces a wt-VHL gene product of the expected size. Figure 1a shows that with increasing amounts of transfected plasmid DNA, expression of the wt-VHL protein increased in a dose-dependent manner. The size of the protein (~31 kDa) is similar to that described previously (32). Similarly, we constructed a mutated VHL expression vector in which we deleted C-terminal amino acids 116 to 213. The selection of this mutated vector was based on the fact that this mutant occurs naturally and prevents binding to elongin C (3, 18, 40) (pFΔVHL). This tagged, truncated VHL protein was expressed at approximately the same level as that of wt-VHL but was smaller, as expected (Fig. 1a). Next we examined the effect of wt-VHL protein and mutated VHL protein on *VEGF* promoter activity. Cotransfected wt-VHL significantly repressed the 2.6-kb *VEGF* promoter-luciferase construct (Fig. 1b, left panel) in a dose-dependent manner. Reporter activity was inhibited nearly 90% with 3.0 μg of wt-VHL expression plasmid DNA (pFwtVHL). In contrast, this amount of ΔVHL expression vector DNA activated VEGF promoter activity about twofold.

VHL repression of the *VEGF* promoter is mediated by a GC-rich *cis* element. To define the region of the *VEGF* promoter responsive to VHL, we constructed a series of 5′ deletions of the base 2.6-kb promoter-reporter vector (shown schematically in Fig. 1c) and cotransfected these deletion mutants with the wt-VHL expression plasmid. VHL repressed reporter activity in the 2.6- and 0.35-kb (bp −194 to +157) *VEGF* promoter constructs roughly 80 and 90%, respectively, while the 0.2-kb (bp −50 to +157) construct still retained 50% activity with VHL (Fig. 1c). These results suggest that sequence from bp −194 to −50 of the *VEGF* promoter confers most of the VHL response. We also tested the effect of increasing amounts of wt-VHL protein on activity of the 0.35-kb construct and, as with the 2.6-kb construct, observed a dose-dependent inhibition (Fig. 1b, right panel).

To determine whether VHL-mediated repression was cell type specific, we tested four other cell lines: human glioblastoma-astrocytoma U87 cells, kidney-derived fibroblast-like Cos-7 cells, and two renal cell carcinoma cell lines, 786-O cells, in which the *VHL* gene is mutated, and TK-10 cells, in which it is not. Similar data were obtained with all cell types. In comparison with an empty expression vector, wt-VHL repressed reporter activity 3- to 10-fold when either the 0.35-kb construct (Fig. 1d) or the 2.6-kb construct (data not shown) was used.

Sp1 binds the VHL-responsive *cis* element. The 144-bp *VEGF* element (bp −194 to −51) that conferred VHL-mediated transcriptional repression is GC rich and contains four closely spaced consensus GC boxes (bp −94 to −51) (68). We

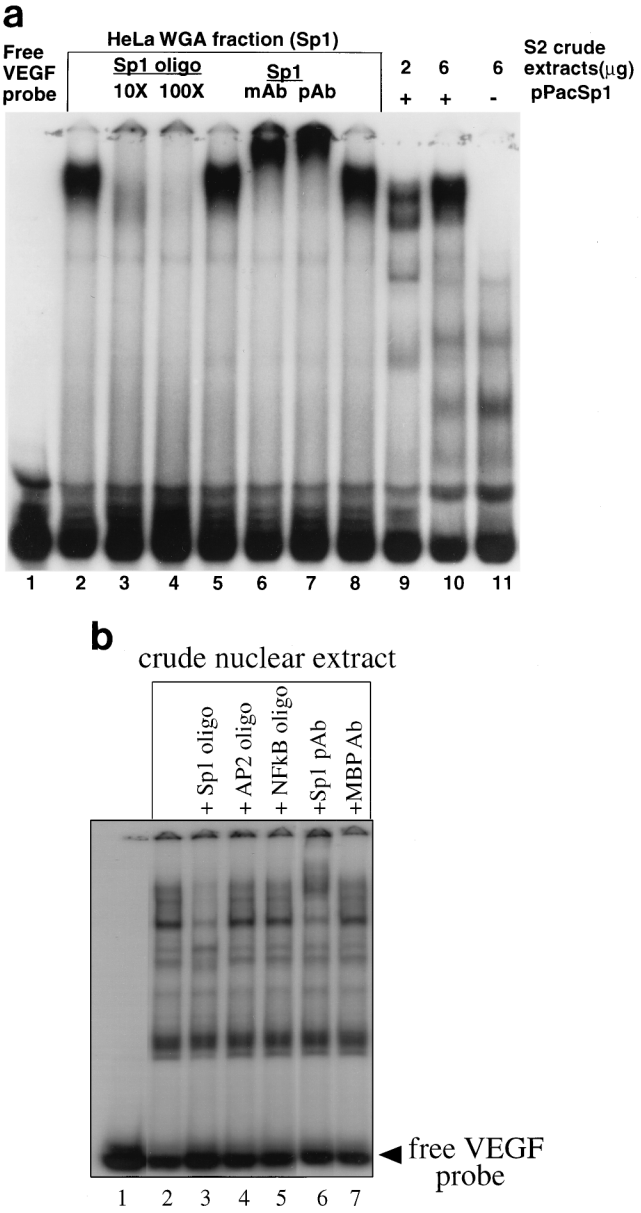

FIG. 2. Sp1 specifically binds the VHL-responsive element of the *VEGF* promoter. (a) By using a 106-bp *VEGF* promoter fragment (bp −112 to −7) as the probe, gel mobility shift assays were performed with HeLa S3 cell partially purified nuclear extract (WGA fraction) (lanes 2 to 8) and whole-cell extract of *Drosophila* S2 cells transfected with (lanes 9 and 10, 2 and 6 μg of protein, respectively) and without (lane 11, 6 μg of protein) Sp1. Unradiolabeled Sp1 consensus oligonucleotide (oligo) (10- or 100-fold molar excess) was added to the binding reaction mixtures run in lanes 3 and 4, respectively. Sp1 monoclonal antibody (mAb) (lane 6) and polyclonal antibody (pAb) (lane 7) were used to supershift the Sp1-DNA complex; a FLAG monoclonal antibody served as the negative control (lane 8). (b) A 72-bp *VEGF* promoter probe (bp −112 to −41) was tested with 293 cell crude nuclear extract (lanes 2 to 7) to demonstrate Sp1 binding. Specific protein-DNA complexes were competed away with 100-fold molar excess Sp1 consensus oligonucleotide (lane 3) but not with 100-fold molar excess AP2 (lane 4) or NF-κB (lane 5) oligonucleotides. The Sp1 antiserum (lane 6), but not a control antiserum against MBP (lane 7), supershifted the Sp1-DNA complexes.

therefore tested whether Sp1 might bind this element in gel shift studies (Fig. 2). We generated by PCR a 106-bp (bp −112 to −7) *VEGF* promoter fragment containing the four GC boxes and performed gel shifts with partially purified Sp1 protein (WGA fraction), whole-cell extract from S2 cells transfected with and without Sp1, and 293 cell crude nuclear extract. The WGA fraction used in these studies was enriched 15-fold for Sp1 protein (11a). Sp1 consensus oligonucleotide competition and antibody supershift studies were performed as well. When the WGA fraction is used, a single prominent DNA-protein complex is formed (Fig. 2a, lane 2) that can be competed away with a 10-fold molar excess of consensus GC box probe (Fig. 2a, lane 3), suggesting that the protein was binding a comparable Sp1-like site in the *VEGF* promoter. We then used two different affinity-purified Sp1 antibodies, a monoclonal antibody directed against Sp1 amino acids 520 to 538, and an antiserum against amino acids 436 to 454, to demonstrate the complex did contain Sp1. Both Sp1 antibodies (Fig. 2a, lanes 6 and 7), but not a control (anti-FLAG) monoclonal antibody (Fig. 2a, lane 8), were able to supershift the complex, strongly supporting the argument that Sp1 bound the *VEGF* promoter. We also demonstrated a similar complex with slightly higher electrophoretic mobility in whole-cell extracts from Sp1-transfected *Drosophila* Schneider S2 cells (Fig. 2a, lanes 9 and 10) (the Sp1 cDNA in the expression vector is not full length) that was completely absent from control-transfected S2 cells without Sp1 (Fig. 2a, lane 11).

Finally, Sp1 in crude nuclear extracts from 293 cells bound this *VEGF* promoter region (Fig. 2b). Because of the complexity of bands formed with crude nuclear extracts on the 106-bp probe, we generated a 72-bp *VEGF* promoter fragment (bp −112 to −41) that also contained the four perfect GC boxes. As shown in the competition experiments (Fig. 2b, lanes 3 to 5), only the Sp1 consensus oligonucleotide (lane 3) substantially interfered with upper-band complex formation. A GC-rich consensus oligonucleotide for AP2 did not compete (lane 4), nor did an NF-κB oligonucleotide (lane 5). In these competition experiments, a 100-fold molar excess of consensus site oligonucleotide was used. The incomplete competition with the Sp1 oligonucleotide (lane 3) may result from two phenomena. First, the bands which are not reduced in intensity are likely to be due to non-Sp1 factors that bind regions other than the GC boxes in the *VEGF* probe. Second, the bands which are reduced are only partially reduced in intensity, perhaps because the molar excess of competitor sites is really only 25× (not 100×). Nevertheless, the most convincing evidence that Sp1 in crude extracts can bind this *VEGF* promoter region is shown in lane 6. The affinity-purified Sp1 antiserum that lacks cross-reactivity with other Sp1 family members can supershift some of the protein-DNA complexes formed on the *VEGF* fragment. A control rabbit antiserum against maltose binding protein does not affect complex formation (lane 7). These results indicate that endogenous Sp1 likely binds this GC-rich region of the *VEGF* promoter.

The VHL-responsive element is a powerful Sp1-driven enhancer. We then demonstrated that Sp1 was capable of transactivating the *VEGF* promoter through this 144-bp GC-rich region. In *Drosophila* Schneider cells, which lack Sp1, we performed dose-titration experiments with increasing amounts of pPacSp1 (provided by R. Tjian) (12) cotransfected with the 2.6, 0.35, and 0.2-kb *VEGF* promoter deletions (Fig. 3a). The 2.6- and 0.35-kb constructs were Sp1 responsive, achieving 55- and 118-fold induction with 100 ng of Sp1 expression vector. The 0.2-kb construct lacking the 144-bp GC-rich region, however, was transactivated only sixfold. This experiment suggests that the *VEGF* promoter region responsible for the Sp1 response was the 144-bp region (bp −194 bp to −51). Moreover, these observations indicate that *VEGF* promoter region responsible for VHL-mediated repression is also an important Sp1-responsive enhancer.

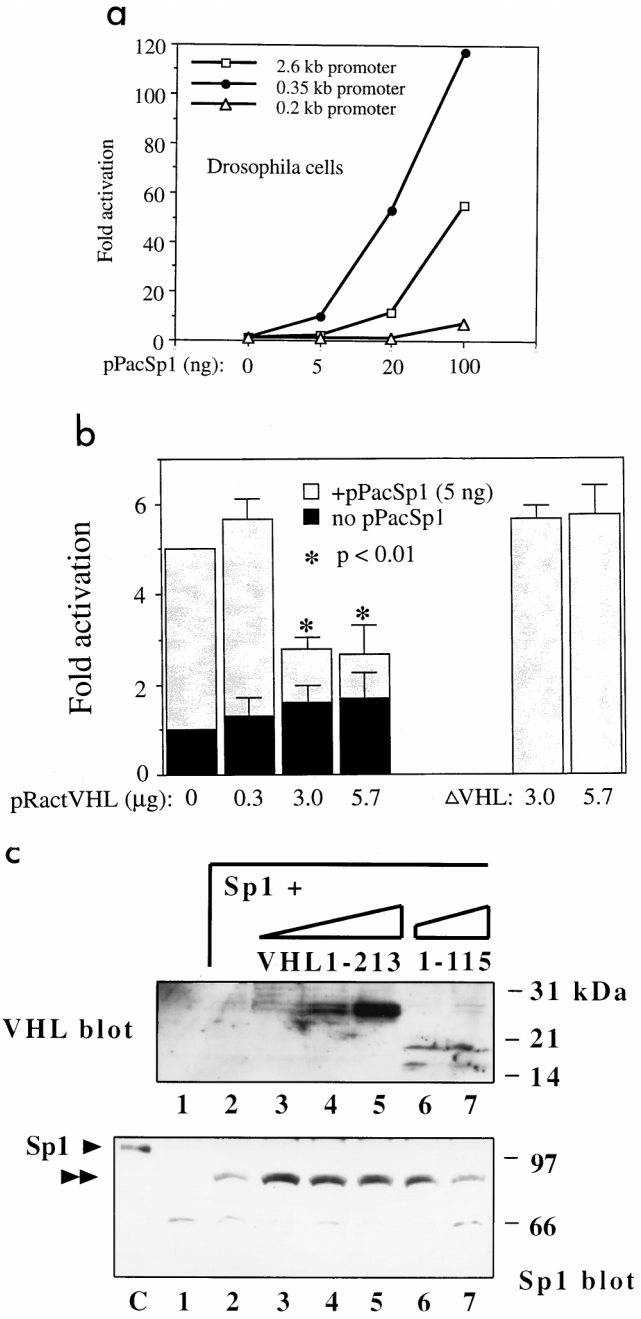

FIG. 3. Transfection experiments in *Drosophila* S2 cells. (a) The VHL-responsive *cis* element, which binds Sp1, is also potently transactivated by Sp1. The deletion series of *VEGF* promoter-reporter constructs (1.5 μg) were cotransfected with increasing amounts of an Sp1 expression vector. Fold activation was relative to the same reporter construct transfected without pPacSp1. Deletion of the *VEGF* promoter region from bp −194 to −50 reduced Sp1-responsiveness of the *VEGF* promoter by ~20-fold, when cotransfected with 100 ng of pPacSp1. (b) In a dose-dependent manner, VHL inhibits Sp1-mediated-, but not non-Sp1-mediated, transcription. The *VEGF* 0.35-kb promoter-luciferase construct (1.5 μg) was cotransfected with increasing amounts of pRact-VHL or -ΔVHL, in the presence (open bars) or absence (black bars) of pPacSp1 (5 ng). Activities shown are relative to the promoter-reporter construct transfected without VHL and with Sp1 (open bars), or without VHL or Sp1 (black bars). *, $P < 0.01$, by analysis of variance with the Scheffe F test, compared with each of the gray bars without asterisks. Error bars, standard deviations. (c) Western blots comparing the relative amounts of VHL or Sp1 proteins expressed in transfected *Drosophila* cells from panel b. Lanes 1 to 7 (40 μg of protein each) in the upper panel correspond to those in the lower panel. Lane 1 cells were transfected without VHL protein or Sp1 and reflect the leftmost black bar from panel b. Lanes 2 to 7 reflect transfections from the gray bars in panel b from left to right, respectively. Lane C contains 0.2 ng of purified Sp1 as a positive control (single arrowhead). Despite large increases in expressed VHL protein with increasing pRact-VHL DNA (upper panel, lanes 3 to 5), Sp1 protein expression is not diminished (lower panel, lanes 3 to 5). Sp1 expressed from pPacSp1 (double arrow) is smaller than the control full-length protein because the pPacSp1 coding sequence is truncated.

VHL inhibits Sp1-mediated, but not non-Sp1-mediated, *VEGF* transcription. We next tested whether VHL was able to counteract the Sp1-mediated transactivation of the *VEGF* promoter observed in *Drosophila* Schneider cells. In this context, in which endogenous Sp1 is lacking, it would also be possible to determine whether VHL repressed Sp1-mediated versus non-Sp1-mediated transcription. We again used the *Drosophila* expression vector pPacSp1 to express Sp1 in these cells. *VHL* and Δ*VHL* genes were inserted into another *Drosophila* expression vector, pRact-HAdh (provided by L. Cherbas) (66). pRact-HAdh was chosen over pPac because, in preliminary experiments, large amounts of cotransfected empty pRact-HAdh expression vector did not inhibit Sp1 activity, whereas large amounts of empty pPac vector did (11a). As shown in Fig. 3b, in a dose-dependent manner, VHL inhibited the Sp1-mediated transcriptional increase about twofold with either 3 or 5.7 μg of pRact-VHL DNA (Fig. 3b, gray bars with asterisks), an effect that approached the level of luciferase activity in the absence of Sp1 (Fig. 3b, black bars). These twofold effects were highly statistically significant in comparison with each of the other four groups transfected with Sp1 (Fig. 3b, gray bars). In contrast, equivalent amounts of transfected pRact-ΔVHL did not inhibit the Sp1 effect on the *VEGF* promoter. Increasing amounts of input VHL expression vectors led to increasing amounts of VHL proteins of expected size (Fig. 3c, upper panel); however, the level of ΔVHL protein expression for a given amount of input DNA appears lower than that of full-length VHL protein. Nevertheless, the VHL protein level with 3 μg of input DNA (lane 4) is comparable to that for ΔVHL protein with 3 and 5.7 μg of input DNA (lanes 6 and 7), levels at which significantly different effects of VHL and ΔVHL protein were noted on Sp1-mediated *VEGF* promoter activity (Fig. 3b). As shown, increasing amounts of transfected pRact-VHL and VHL protein did not affect the amount of Sp1 protein produced from pPacSp1 (Fig. 3c, lower panel), supporting the argument that the VHL-mediated inhibition of Sp1 occurred at the protein-protein level. In these experiments, empty pRact-HAdh expression vector was used as filler DNA so that all groups had equivalent amounts of pRact-HAdh.

Importantly, transfected VHL did not inhibit basal, or non-Sp1-mediated, transcription in the S2 cells (Fig. 3b, black bars). This observation indicates that VHL-mediated transcriptional repression has some specificity for Sp1, which tempted us to determine whether VHL and Sp1 physically interact.

VHL and Sp1 interact in vivo. To determine whether VHL and Sp1 interact in vivo, we transiently transfected the VHL expression vectors into 293 and HeLa cells. We performed immunoprecipitations with an affinity-purified Sp1 antiserum and then Western blot analysis with a VHL monoclonal antibody to assess the presence of VHL in the immunocomplex with Sp1. NaCl (150 mM) was included in the binding and washing buffers (see Materials and Methods). We could readily detect VHL protein in the Sp1 immunocomplex in both cell types (Fig. 4b and e), confirming that VHL and Sp1 can form a complex in solution. The truncated VHL protein with the C terminus deletion only minimally forms a complex with Sp1 (Fig. 4b and e). This VHL deletion mutant lacks a region

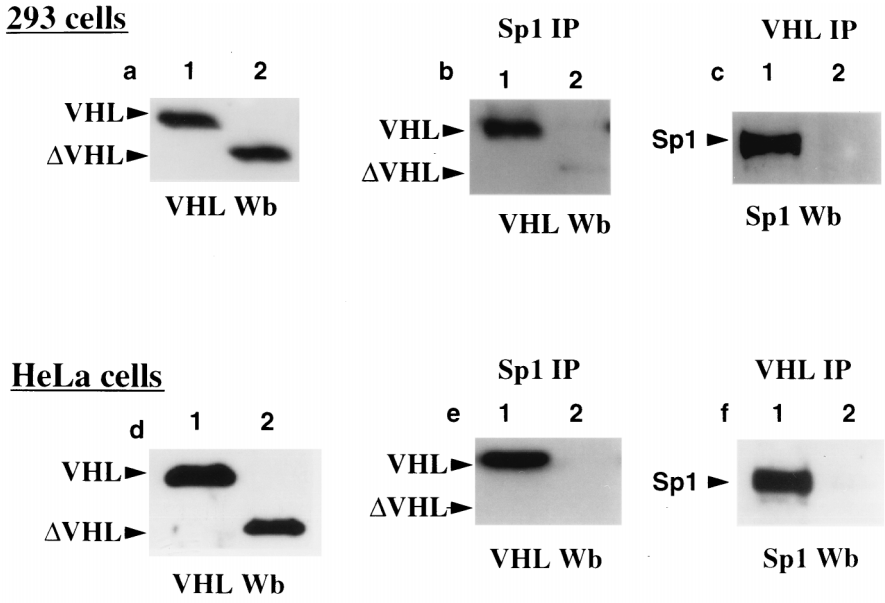

FIG. 4. Coimmunoprecipitation of VHL and Sp1 in vivo. Sp1 and VHL, but not Sp1 and ΔVHL, coimmunoprecipitate in whole-cell extracts from 293 (a to c) and HeLa cells (d to f) transiently transfected with VHL and ΔVHL expression vectors. (a to f) Lane 1 extracts were from VHL-transfected cells, and lane 2 extracts were from ΔVHL-transfected cells. VHL Western blots of lysates from the transfected cells, which indicate comparable expression of the VHL and ΔVHL and comparable affinity of the VHL antibody for both proteins, are shown (a and d). Immunoprecipitation (IP) was performed by incubating extracts with rabbit Sp1 antiserum or mouse VHL monoclonal antibody followed by protein A-Sepharose. The protein A beads were washed and then boiled in SDS-buffer to release bound proteins. After SDS-PAGE, Western blotting (Wb) was performed with VHL or Sp1 antibodies.

frequently mutated (32) and prevents interaction with elongin C. Of note, the amounts of protein expressed with the wt-VHL and ΔVHL constructs were comparable, as shown in a Western blot probed with the VHL monoclonal antibody (Fig. 4a and d). The Western blot samples were aliquots of the same cell extracts used for the coimmunoprecipitations described above. Next, we performed coimmunoprecipitations with a VHL monoclonal antibody and probed a Western blot with Sp1 antiserum. Figures 4c and f show that VHL antibody can immunoprecipitate Sp1 in both cell types. In a control experiment, we used a polyclonal antibody against Gal4 protein in an attempt to immunoprecipitate VHL protein from the transfected cells. No VHL protein was detected (data not shown). These data indicate that Sp1 and VHL interact in vivo but do not indicate whether this interaction is direct.

VHL protein and Sp1 interact in vitro. To test whether Sp1 and VHL protein interact directly, in vitro association assays were performed with GST-VHL fusion proteins and recombinant Sp1. Bacterially expressed GST protein alone or GST fused to full-length VHL (amino acids 1 to 213) or the ΔVHL (amino acids 1 to 115) version was bound to glutathione-agarose beads and mixed with recombinant, purified Sp1 in a buffer that approximates intracellular ionic conditions, 150 mM KCl (see Materials and Methods). After extensive washing with the same buffer, the bound proteins were separated by SDS-PAGE, transferred to a nylon membrane, and subjected to Western analysis for Sp1. To compare the relative amounts of GST fusion proteins bound to the beads, the membranes were stained with Ponceau S (Sigma) (Fig. 5a). While Fig. 5a demonstrates that comparable amounts of each fusion protein were included in the assay, in fact more GST-VHL1-115 protein than GST-VHL1-213 was present, indicating that this experiment was been weighted in favor of ΔVHL. Nevertheless, a strong interaction between Sp1 and GST-VHL1-213 was observed, while no interaction between Sp1 and GST alone was detected and only a minimal interaction with GST-VHL1-115 was detected (Fig. 5b). The amount of Sp1 that binds GST-VHL1-213 in this assay approached 10% of the input Sp1. This assay has been performed multiple times with comparable results. We are now defining the respective interacting domains of VHL and Sp1. These data are consistent with the coimmunoprecipitation results and indicate that VHL and Sp1 interact strongly and directly, while ΔVHL and Sp1 do so only to a minimal extent.

VHL, but not mutant VHL, suppresses endogenous *VEGF* mRNA levels in stably transfected cell lines. To determine whether our studies in transient assays could be extended to the endogenous *VEGF* gene, we used stably transfected RCC

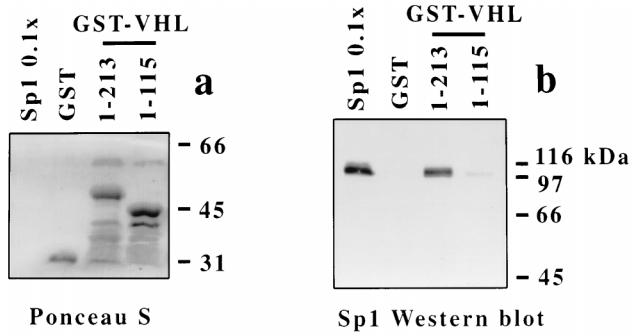

FIG. 5. VHL and Sp1 interact directly in vitro. Bacterially expressed GST, GST-VHL1-213, or GST-VHL1-115 protein bound to glutathione-Sepharose was incubated with recombinant, purified Sp1 protein (50 ng) in binding buffer containing 150 mM KCl for 20 min at 4°C. Beads were washed extensively, and bound proteins were separated by SDS-PAGE and transferred to a nylon membrane. (a) Ponceau S staining of the membrane reveals comparable amounts of GST fusion proteins bound to the beads (heaviest band in each lane). (b) Western analysis of the same membrane with Sp1 antiserum indicates a strong interaction between Sp1 and GST-VHL1-213, but not GST-VHL1-115. One-tenth the amount of Sp1 protein used in the binding reactions (5 ng) was included for comparison (Sp1 0.1x).

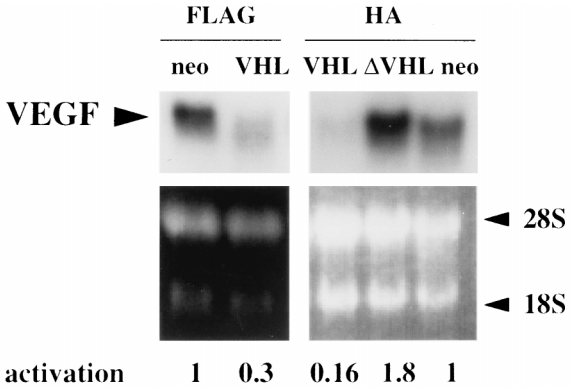

FIG. 6. wt-VHL inhibits endogenous *VEGF* message in stably transfected RCC lines. (a) Total RNA (5 μg) from 786-O cells stably transfected with expression vectors containing wt-VHL (either FLAG tagged or HA tagged), ΔVHL (HA-tagged), or empty vector alone (neo) was size separated, blotted, and probed with ^{32}P-labeled *VEGF* cDNA. The ethidium bromide-stained agarose gels in the lower panels are shown to compare RNA loading; 18S and 28S rRNA bands are indicated. The fold activation was calculated by densitometry, with GAPDH expression as a normalization control (data not shown).

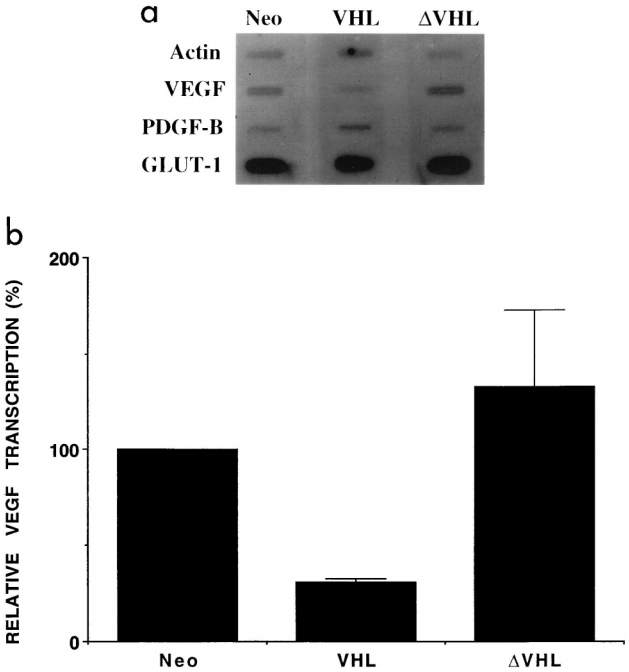

FIG. 7. Transcriptional regulation of *VEGF* mRNA. Nuclear run-on assays using nuclei isolated from 786-O RCC lines stably transfected with expression vectors containing HA-tagged wt-VHL (VHL), ΔVHL, or empty vector alone (Neo) were performed as described in Material and Methods. (a) Representative autoradiogram of one of three experiments, each performed with different preparations of nuclei. (b) Results were quantified with a Molecular Dynamics PhosphorImager. The fold decrease in the *VEGF* transcription rate for each of three independent experiments was calculated by comparing the *VEGF*/actin gene ratios. The mean ± standard error decrease in the transcription rate for VEGF in 786-O cells expressing wt-VHL (compared to 786-O expressing neo alone) for all three experiments was 3.25 ± 0.19. Error bars, standard errors.

cells as an appropriate model. For this work, we used clonal, stably transfected 786-O renal carcinoma cells, which normally lack an endogenous wild-type copy of the *VHL* gene. We introduced the pCMV2FLAG and pCMV2FLAGVHL expression vectors into 786-O cells and generated one clonal line of each. In addition, we obtained 786-O lines expressing cytomegalovirus-hemagglutinin (CMV-HA), CMV-HAVHL, or CMV-HAΔVHL (32). The VHL proteins expressed in these cell lines are tagged with immunoreactive epitopes at their amino termini. The tags were placed there to preserve function, because the VHL protein C-terminal region is thought to interact with elongin C. Importantly, the amounts of HA-tagged VHL and ΔVHL proteins expressed in these lines in Western blots were comparable (data not shown).

Several candidate VHL target genes with relevance to RCC and VHL disease were analyzed at the mRNA level by Northern blot analysis. Strikingly, *VEGF* mRNA levels were repressed three- to sevenfold in the cell lines expressing wt-VHL (Fig. 6). Message levels were not altered in lines generated from either empty expression vector and were even slightly increased in response to overexpression of the C-terminal VHL truncation. Of note, a similar twofold increase in *VEGF* promoter activity was seen in transient assays with the ΔVHL expression vector (Fig. 1b). These changes in *VEGF* message correlate well with the changes in *VEGF* promoter activity observed in response to transfected wt-VHL and mutated VHL. These data indicate that *VEGF* is indeed a VHL target gene and also suggest that *VEGF* regulation by VHL occurs at a transcriptional level.

Evidence for transcriptional control of *VEGF* mRNA abundance by VHL. Because the VHL gene suppresses both the endogenous *VEGF* mRNA levels in 786-O cells and Sp1-dependent *VEGF* promoter activity in different cell lines, we tested whether VHL could indeed decrease endogenous *VEGF* transcription. We therefore performed nuclear run-on assays to study *VEGF* transcription in RCC cells, using the housekeeping β-actin gene as a control for normalization purposes. Figure 7 shows that reintroduction of wt-VHL in 786-O cells decreases *VEGF* transcription about threefold. Importantly, expression of the C-terminal VHL truncation (ΔVHL) did not decrease *VEGF* transcription. Since it has been recently shown that *PDGF-B* and *Glut1* mRNA levels are downregulated by VHL (33), we assessed whether this effect was also partly transcriptional. As illustrated in Fig. 7a, no change in PDGF-B or Glut1 transcription was observed in 786-O cells expressing wt-VHL. Therefore, while VHL regulates *VEGF* mRNA levels at least partly at the transcriptional level, downregulation of *PDGF-B* and *Glut1* seems mainly posttranscriptional, as suggested by Iliopoulos et al. (33).

VHL and Sp1 interact in vivo in RCC cells. Finally, we wished to determine whether the VHL-Sp1 interaction could

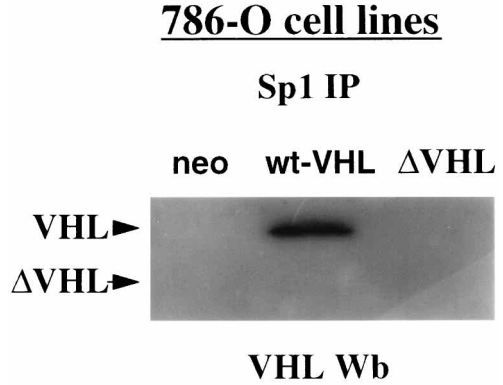

FIG. 8. wt-VHL and Sp1 interact in vivo in stably transfected 786-O RCC cells. Sp1 coimmunoprecipitations were performed as described in the legend to Fig. 4. VHL, but not ΔVHL, interacts with endogenous Sp1.

occur in a cell line affected by VHL mutations, to provide additional biological relevance of the interaction. We therefore performed additional coimmunoprecipitation studies using the stably transfected 786-O cell lines described above. Sp1 antiserum was used to immunoprecipitate Sp1 and associated proteins from 786-O cell lysates. The presence of VHL in the complex was assessed by Western analysis using a VHL monoclonal antibody. Figure 8 demonstrates that in RCC cells, endogenous Sp1 interacts with stably transfected wt-VHL, but not with ΔVHL, as only wt-VHL was found in Sp1 immunocomplexes. This result provides additional proof that VHL can interact with Sp1 in a highly relevant context and that loss of this interaction through *VHL* mutation, as observed with the ΔVHL truncation, may have important consequences.

DISCUSSION

We have identified the *VEGF* promoter as a target for the VHL tumor suppressor gene product and have elucidated a novel mechanism of VHL-mediated transcriptional repression. First, we observed that VHL repression of the *VEGF* promoter depends on a promoter *cis* element that is a powerful Sp1-driven enhancer. Second, VHL inhibited Sp1-mediated, but not non-Sp1-mediated, transcriptional activation in a dose-dependent manner. Third, VHL and Sp1 were found to interact directly in solution. This work therefore identifies VHL as a direct inhibitor of Sp1 activity. Sp1 overactivity from loss of VHL might consequently contribute to renal oncogenesis. Fourth, we further demonstrated that reintroduction of VHL reduced endogenous *VEGF* message about fivefold, supporting the argument that *VEGF* is a bona fide VHL target gene. Finally, downregulation of endogenous VEGF message by VHL occurs, at least in part, at a transcriptional level based on nuclear run-on studies, consistent with VHL's effect being mediated by a promoter *cis* element. In contrast to wt-VHL, a naturally occurring truncated VHL failed to interact with Sp1 or mediate the observed VHL effects.

Many transcription factors that interact with Sp1 have been identified. The largest group of these act synergistically with Sp1 on DNA to increase transcription and include transcription factors of many classes. Only a small subgroup of these have been shown to interact with Sp1 in solution, in the absence of DNA, such as E2F (38, 48), GATA1-3 (51), YY1 (44, 61), RelA(p65) (56), and BPV-E2 (47).

A still smaller group of Sp1-interacting transcription factors that, as described here for VHL, impair Sp1's ability to activate transcription has been found. The cell cycle regulatory protein p107, an Rb family member, binds Sp1 in solution and inhibits Sp1-mediated transcription (14), effects similar to those observed for VHL on Sp1. Like Rb, p107 interacts with many proteins, including viral oncoproteins, cyclins, cyclin-dependent kinases, and E2F. Overexpression of p107 inhibits cell proliferation (75). Infected cell polypeptide 4 (ICP4), a 175-kDa herpes simplex viral product, is a homodimerizing sequence-specific DNA binding protein that can either activate or repress transcription. ICP4 negatively autoregulates its own promoter and thereby inhibits Sp1-mediated transcription (26), but ICP4 is not specific for Sp1 (25). ICP4 inhibits transcriptional activation by many activators, forming a tripartite complex on DNA with TBP and TFIIB that presumably sterically hinders activator-initiation complex interactions (25). Two other candidate negative Sp1 regulators have been identified, but neither protein has yet been cloned. p74 is an evolutionarily conserved nuclear factor that directly interacts with the N-terminal-most portion of the Sp1 transactivation domain and which may be responsible for the apparent repressor activity of this subdomain (54). Sp1-I is a 20-kDa Rb-associated factor that impairs Sp1's DNA binding ability, and the Sp1-I effect can be overcome with excess Rb protein (9). Another larger group of factors competes with Sp1 for DNA binding, and thus these factors interfere with Sp1 action. Not surprisingly, these include several zinc finger proteins, such as GATA1, Sp3 (28), Egr1 (1, 7), T3 receptor (57, 73), and HNF4 (31), as well as Pit-1 (60), NF-I (55), G10BP (65), and sterol binding factors (15). Some of these also act synergistically with Sp1 in different DNA sequence contexts (see above). The VHL gene product then is one of few negative Sp1 regulators that directly bind Sp1.

Although ubiquitous, Sp1 is important in differential gene expression and may even contribute to tumorigenesis. Sp1 levels and function may change with differentiation, transformation, and cell growth, suggesting that these changes have important biologic consequences. For example, Sp1 is expressed at vastly different levels in different tissues (58), and, as shown by immunocytochemistry, Sp1 is spatially and temporally regulated in gastric development (58) and in nephrogenesis (11). Sp1 expression also increases 10-fold with simian virus 40 (SV40) viral transformation (35, 59). Sp1's transactivation potential may be enhanced by expression of oncogenes v-*raf-1* (52) or v-*rel*, -*ras*, or -*src* (63), while Sp1's DNA binding affinity may be altered by growth factors, such as transforming growth factor β1 (34), granulocyte-macrophage colony-stimulating factor (5), and insulin-like growth factor I (IGF-1) (2, 37). Cytokines may affect Sp1 activity as well, as tumor necrosis factor alpha increases Sp1 message several-fold (30) and promotes synergy of Sp1 with p53 (5, 27). These changes in Sp1's transactivation potential or DNA binding are likely due to changes in Sp1 itself, i.e., its phosphorylation state (5, 35, 45, 75) and/or in its interacting partners, such as p53 (4, 27) or perhaps even VHL, which is also ubiquitously expressed. Furthermore, important effects on specific Sp1 target genes are likely to be observed with changes in Sp1. Sp1 can also act synergistically and/or compete with many tissue-restricted transcription factors, as noted above. In this way, tissue-restricted genes may be profoundly affected by changes in Sp1 activity.

An important aspect of VHL's tumor suppressor function may therefore be its modulation of Sp1 activity. For example, a direct relationship between overexpression of Sp1 and epidermal growth factor receptor (EGFR) message has been observed in gastric carcinoma (42). *EGFR* is also overexpressed in RCC (71, 74) and is regulated by transcription elongation (29), which suggests that EGFR may be another Sp1-VHL target. Moreover, the precursor cells of RCC, proximal tubule cells, have very low levels of Sp1, as shown by immunocytochemistry (11), which may make them particularly susceptible to changes in Sp1 activity resulting from loss of VHL.

In contrast to the many described Sp1 protein-protein interactions, only several VHL-interacting proteins have been identified. Coimmunoprecipitation experiments identified 16- and 9-kDa proteins that differentially associated with wt-VHL but not with naturally occurring missense mutants of VHL (17, 41), and protein sequencing confirmed them as elongin B and C components of the elongin complex (18, 41). Elongin C links elongin B to the much larger, 110-kDa elongin A, and the smaller proteins catalyze the transcription elongation function of elongin A (3). VHL has homology with elongin A over a 13-amino-acid stretch that is necessary for the elongin C interaction (40). In this way, VHL is thought to compete for elongin C binding with elongin A, thereby accounting for VHL's in vitro inhibition of elongin-mediated transcription (18, 40). Like Sp1, VHL is a ubiquitous factor with different levels of

expression in tissue (39). *VHL* message is highest in kidney, eye, and lung (39), sites which include von Hippel-Lindau disease targets, suggesting VHL may also contribute to tissue-specific gene expression. Although we have not shown that there is differential association of Sp1 with wt-VHL over VHL with substitution mutations, our observation that Sp1 binds wt-VHL but not ΔVHL suggests that loss of the Sp1-VHL interaction is likely to have clinical relevance, at least for a subset of von Hippel-Lindau disease patients. Importantly, there are many VHL substitution mutations that map outside the elongin binding domain (40), suggesting that failed interactions of VHL with other proteins like Sp1 may be important and perhaps affect the same cellular pathway. We are now mapping the VHL and Sp1 domains responsible for the VHL-Sp1 interaction. In addition to Sp1 and elongins C and B, other proteins likely interact with VHL. VHL binding protein (VBP-1), the function of which is unknown, as well as several other candidate interacting proteins were recently identified through yeast two-hybrid screening (69). In VHL coimmunoprecipitation experiments, Kishida et al. demonstrated on a protein gel many potential VHL-interacting candidates in addition to elongins C and B (41), and bands were present that could represent Sp1.

Because Sp1 is potentially important to the transcription of many genes, we examined the effect of cotransfected VHL on other GC-rich promoters. All promoter-reporter constructs tested, including CMV (major immediate early), Rous sarcoma virus, SV40, the Wilms' tumor 1 gene (*WT1*), and the K-cadherin gene, were inhibited by VHL, but only two- to threefold (data not shown). With none of these constructs, assayed in 293 cells, did repression by VHL approach the level (≥10-fold) observed with the *VEGF* promoter (Fig. 1c). With these constructs too, however, VHL inhibits Sp1 activity. We have identified a critical enhancer in the *WT1* promoter that contains a novel, high-affinity Sp1 site, and mutations in this site completely abolish enhancer activity (11). Interestingly, we have found that the contribution of this Sp1-driven enhancer to the activity of the remaining *WT1* promoter is two- to threefold higher in 786-O cells, which lack wt-VHL, than in any other cell line (11). Furthermore, replacement of VHL in 786-O cells downregulates activity of the *WT1* enhancer to the levels in the other lines. This observation suggests that endogenous wt-VHL might serve as a constitutive inhibitor of Sp1 activity. For this reason, we were unable to find a suitable transfection control plasmid, such as CMV-, Rous sarcoma virus-, or SV40-driven β-galactosidase, as all were inhibited by VHL. One explanation for the particular VHL responsiveness of the *VEGF* promoter may be that it is more highly Sp1 dependent than the other promoters tested. However, the SV40 promoter has six GC boxes and is less VHL responsive than the *VEGF* promoter, perhaps refuting this notion. Alternatively, additional factors may be cooperating with VHL at the *VEGF* promoter's VHL-responsive site that contribute to VHL activity. The lesser effectiveness of VHL on *VEGF* promoter activity in *Drosophila* cells (Fig. 3b), which might lack such a cofactor, is consistent with the latter hypothesis. Such a rationale might also be extended to *WT1*, which is not downregulated by VHL, by Northern analysis (11a), and *PDGF-B* chain, which is not transcriptionally repressed by VHL (Fig. 7a and b), despite the fact that both genes have GC-rich promoters. Inhibition of transcription elongation of *VEGF*, but not of these other genes, by VHL is a possible explanation, but Gnarra et al. (22) found no effect of VHL on *VEGF* elongation in nuclear run-on assays (22) (see below). These observations suggest that the specific effect of VHL on *VEGF* transcription, although involving Sp1, likely includes additional factors.

Our studies and those of others (22, 33, 62 [the latter two having been published while this paper was in revision]) demonstrate that the *VEGF* gene is downregulated by wild-type VHL in RCC cells. Moreover, we provide evidence that transcriptional regulation accounts at least partly for this effect. Based on run-on experiments, Gnarra et al. (22) did not find evidence for transcriptional regulation of *VEGF* by VHL in two different RCC cell lines (UOK 101 and UOK 121). However, they were not able to show a VHL-induced decrease in *VEGF* mRNA stability, whereas Iliopoulos et al. (33) noted such an effect in 786-O cells. These observations suggest that *VEGF* mRNA regulation may be heterogeneous in different RCC cell lines. Comparison of our observed VHL-induced decrease in the steady-state level of *VEGF* mRNA (sevenfold [Fig. 6, compare HA-VHL and neo]) with the decrease in transcriptional activity estimated by our run-on experiments (threefold [Fig. 7b]) suggests that there is an additional level of *VEGF* message control. In UOK 101 and UOK 121 cells, repression of *VEGF* message by VHL does not appear transcriptional (22), whereas in 786-O cells it is likely due to both decreased transcription (our data) and mRNA half-life (33).

Dual levels of *VEGF* mRNA regulation by a single agent are not unusual. Interleukin-1β increased *VEGF* transcription 2.1-fold and *VEGF* mRNA half-life 1.6-fold in rat aortic smooth muscle cells, leading to a 4-fold increase in the mRNA steady-state level (46). More recently, IGF-1 was shown to increase *VEGF* transcription fivefold and *VEGF* mRNA half-life threefold in a colon carcinoma cell line (70). As further evidence of the complexity of *VEGF* control, the IGF-1 upregulation of *VEGF* mRNA levels in this study was also highly variable (2- to 12-fold) among different colon carcinoma cell lines. Given these observations, it is therefore not surprising that the VHL appears to regulate *VEGF* message at multiple levels.

In conclusion, we have shown that *VEGF* is the first identified VHL target gene and that repression of *VEGF* message occurs at least partly at a transcriptional level. The VHL effect on *VEGF* was found to be dependent on an Sp1-responsive *cis* element. Moreover, VHL was found to directly bind Sp1 and inhibit Sp1 activity. These observations further suggest that increased Sp1 activity, from loss of VHL, may be important in the pathogenesis of VHL disease and its associated vascular tumors.

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D. Mukhopadhyay, B. Knebelmann, and H. T. Cohen contributed equally to this work.

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