Biochemical and Biophysical Research Communications **266,** 43–50 (1999)
Article ID bbrc.1999.1767, available online at http://www.idealibrary.com on IDEAL®

An Important von Hippel-Lindau Tumor Suppressor Domain Mediates Sp1-Binding and Self-Association

Herbert T. Cohen,*[,1] Mi Zhou,* Adam M. Welsh,* Sharzad Zarghamee,† Holger Scholz,† Debabrata Mukhopadhyay,† Takeshi Kishida,‡ Berton Zbar,‡ Bertrand Knebelmann,† and Vikas P. Sukhatme†

**Renal and Hematology/Oncology Sections, Boston Medical Center and Boston University School of Medicine, Boston, Massachusetts 02118; †Renal Division, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215; and ‡Laboratory of Immunobiology, NCI-Frederick Cancer Research Center, Frederick, Maryland 21702*

Received October 28, 1999

***VHL* is the causative gene for both von Hippel-Lindau (VHL) disease and sporadic clear-cell renal cancer. We showed earlier that VHL downregulates vascular endothelial growth factor transcription by directly binding and inhibiting the transcriptional activator Sp1. We have now mapped the VHL Sp1-binding domain to amino acids 96-122. The 96-122 domain is disproportionately affected by substitution mutations, which interfere with the VHL–Sp1 interaction. Deletion of the 96-122 domain prevents VHL effects on Sp1 DNA binding and on VHL target gene expression, indicating the domain contributes importantly to VHL tumor suppressor activity. Nevertheless, prevention of the VHL–Sp1 interaction only partially abrogates VHL's transcriptional repressor activity, supporting the existence of VHL transcriptional effectors in addition to Sp1. VHL also directly interacts with the Sp1 zinc fingers and self-associates via the 96-122 domain, which furthermore suggest the domain may bind other metalloproteins and contribute to VHL dominant-negative effects.** **© 1999 Academic Press**

The von Hippel-Lindau tumor suppressor gene *VHL* was identified in 1993 as the causative gene for von Hippel-Lindau (VHL) disease (1; reviewed in 2), an inherited cancer family syndrome comprising several types of well-vascularized neoplasms, including clear-cell renal cancers. The *VHL* gene has also been found to be mutated in nearly all cases of sporadic clear-cell renal cancer (3–6), indicating that *VHL* is the major clear-cell renal cancer gene as well.

Functional analysis of the 213 amino acid VHL protein has been hampered by its lack of major homologies. Most attention has focused on the VHL 157-169 domain encoded by exon 3. This domain is responsible for VHL's interaction with the Elongins C and B, and VHL mutations in this region disrupt the interaction (7–10). The VHL-Elongin complex also supports an interaction with cul-2 (11), which may be part of a ubiquitin-ligase complex that targets cell cycle proteins for degradation (12). Despite the importance of the VHL Elongin-binding domain, many VHL amino acid substitution mutations map outside this region, suggesting other VHL domains may contribute to tumor suppressor activity.

Reintroduction of VHL into renal cancer cell lines downregulates expression of several hypoxia-inducible genes, such as *VEGF* (13–16), *PDGF-B* chain and *glut-1* (15), and *TGF-alpha* (17), which are all overexpressed in renal cancer. *VEGF* may be a particularly important VHL target gene (18–20). Although VHL can also decrease *VEGF* mRNA stability (14, 15), we showed that VHL inhibits *VEGF* transcription and promoter activity, and that the mechanism partly involves VHL directly binding the transcriptional activator Sp1 and inhibiting Sp1 activity (16). We have now carried out additional studies on the interaction of VHL and Sp1.

MATERIALS AND METHODS

In vitro binding assays. GST pull down assays were performed as described earlier (16). In brief, GST fusion proteins were expressed in *E. coli* strain BL21 (DE3) (Stratagene). Cell pellets were sonicated in binding buffer (20 mM Tris–HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40). Fusion proteins were mixed with glutathione-agarose beads (Pharmacia) and washed ×3 with binding buffer containing 0.10% SDS. Beads were mixed with 50 ng purified protein, either Sp1 (Promega) or a maltose binding protein (MBP) fusion protein, and bovine serum albumin (5 μg) in binding buffer, incubated with agitation for 20 min on ice, and washed ×4 with binding buffer. Bound proteins were resolved by SDS–PAGE and Western blotting.

Western analysis. Western blotting was performed as described (16) using affinity-purified anti-Sp1 (Santa Cruz Biotechnology) or

[1] To whom correspondence should be addressed at Renal and Hematology/Oncology Sections, E-428, Boston University Medical Center, 88 E. Newton Street, Boston, MA 02118. Fax: (617) 638-7326. E-mail: htcohen@bu.edu.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

anti-MBP sera (New England Biolabs), or FLAG M5 (Kodak), hemagglutinin (HA) (Boehringer-Mannheim), or human VHL monoclonal antibodies (Pharmingen) (21).

Plasmids. pFLAG-CMV2-VHL1-213 and 1-115, pRC-HA-VHL 1-213 and 1-115, pGEX-4T3-VHL1-213 and 1-115, and −194/+157 bp *VEGF* promoter-reporter constructs were described earlier (10, 16, 21). Point mutations and small internal deletions mutations (in parentheses) were introduced into the VHL coding sequence by site-directed mutagenesis (QuikChange, Stratagene). Details can be obtained by contacting the authors.

Cell culture and transfection. Human fetal kidney 293 cells (ATCC CRL 1573) and 786-O human renal cancer cells were maintained in Dulbecco's modified Eagle medium (D-MEM) with 10% fetal bovine serum (Life Technologies). 786-O clonal cell lines stably transfected with either pRC, pRC-HA-VHL, pRC-HA-VHL(1–115) (kindly provided by Dr. W. G. Kaelin, Dana-Farber Cancer Institute (21)), or pFLAG-CMV2, pFLAG-CMV2-VHL, and pFLAG-CMV2-VHL Δ96-122 were grown in complete media supplemented with G418 (0.4–0.8 mg/ml). Cells were transfected by calcium-phosphate precipitation as previously described (16, 22, 23).

Immunoprecipitations. As described earlier (16), cells were washed twice with 10 ml cold phosphate-buffered saline (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_3V0_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 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 IgG) with 0.5 mg total protein. Immunocomplexes were captured with protein A agarose beads (Bio-Rad), washed with cell lysis buffer, and subjected to Western analysis.

Northern blot analysis. Northern analysis was carried out as described (16).

Yeast 2-hybrid analysis. VHL self-association was tested using the LexA yeast 2-hybrid system and standard protocols (Clontech). VHL cDNA was cloned into both bait and prey yeast expression vectors, pLexA and pB42AD, respectively. Human Elongin C was cloned into pB42AD as a positive control for interaction with VHL. pLexA-Lamin C and pB42AD SV40 T Ag (Clontech) and the empty expression vectors served as negative controls. To test for protein-protein interactions, EGY48(p8opLacZ) was cotransformed with pLexA and pB42AD (empty or with insert) and plated onto synthetic dropout minimal medium (SD) + gal/+ raf/− U/− H/− W/− L X-gal plates to determine reporter expression. Colonies that turned blue within 72 hrs were considered positive. Autoactivation by the pLexA-VHL construct was ruled out by plating singly transformed yeast on SD + gal/+ raf/− H/− U X-gal plates and also on SD + gal/+ raf/− H/− U followed by a colony lifts, as a higher sensitivity lacZ assay.

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA). To limit variability between preparations, nuclear extracts were made on a large scale (5–10 × 10^7 cells), according to a standard protocol (24). Nuclear extract protein concentration was quantitated in duplicate by both Bradford assay and absorbance at 230 and 260 nm, and confirmed by SDS–PAGE and reversible protein staining (Sigma). Binding reactions employed double-stranded consensus Sp1 oligonucleotide (Promega) and 2 μg nuclear extract protein under conditions described previously for Sp1 EMSAs (16, 23).

RESULTS

Amino acids 96-122 comprise the VHL Sp1-binding domain. We have mapped the VHL domain responsible for the VHL-Sp1 interaction in an *in vitro* binding assay with VHL truncations linked to glutathione *S*-transferase (GST), and these data are summarized in Fig. 6. Because the VHL 1-115 truncation did not bind Sp1, we made a series of longer VHL C-terminal truncations linked to GST and tested them for their ability to bind recombinant, purified human Sp1 protein (Promega). As shown, VHL 1-115 again does not bind Sp1, but a slightly larger VHL truncation from amino acids 1-122, containing just an additional 7 amino acids, binds Sp1 as well as larger VHL truncations 1-130, 1-143, (Fig. 1A) and full-length VHL (data not shown). This experiment defines the C-terminal boundary of the VHL Sp1-binding domain and indicates that VHL amino acids 116-122 may be particularly important for the interaction.

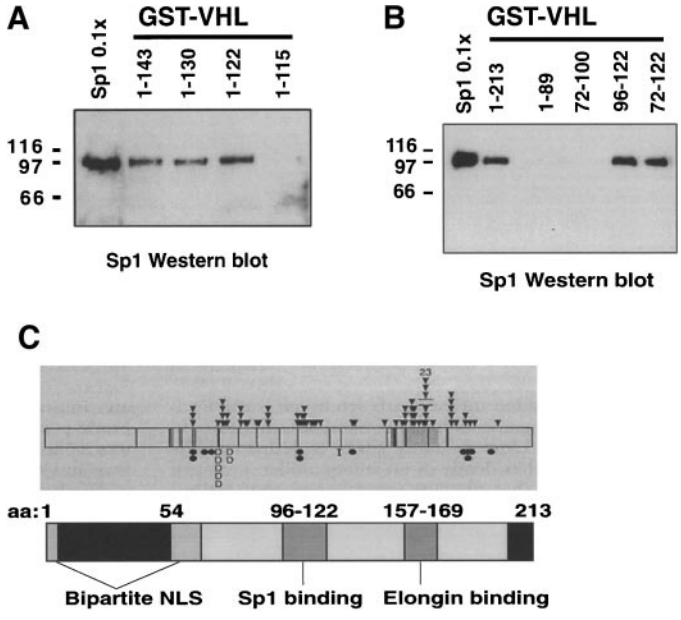

FIG. 1. Mapping the VHL Sp1-binding domain. Bacterially expressed GST-VHL truncations (~1–2 μg) bound to glutathione-agarose beads were tested for an interaction with purified, recombinant Sp1 protein (50 ng). An interaction was detected by Sp1 Western blot. (A) Mapping of the C-terminal boundary of the VHL-Sp1 interaction. (B) Mapping of the N-terminal boundary of the VHL-Sp1 interaction. (C) Schematics of human VHL protein. The upper graphic (from (7)) indicates some mutations found in VHL families. Inverted arrows above represent missense mutations, circles below represent stops, vertical lines indicate frameshifts, and Ds below represent deletions. The lower schematic denotes known VHL regions. VHL's bipartite nuclear localization signal (NLS) (25) is shown. Nonconserved amino acid sequence is shown in black. A cluster of missense mutations affect the 96-122 region.

To narrow the amino terminal boundary of the VHL Sp1-binding domain, we made amino terminal truncations of GST-VHL 1-122. Because VHL 72-122 bound Sp1 well, we split this domain roughly in half, and the interacting half was split again. As shown in Fig. 1B, GST-VHL 72-122 and 96-122 bind Sp1 well, whereas GST-VHL 72-100 does not. In Fig. 2B, GST-VHL 112-122 binds Sp1, but with decreased affinity in comparison with wt VHL, which was a consistent finding. These observations indicate that the VHL 96-122 domain is sufficient for the VHL-Sp1 interaction and that

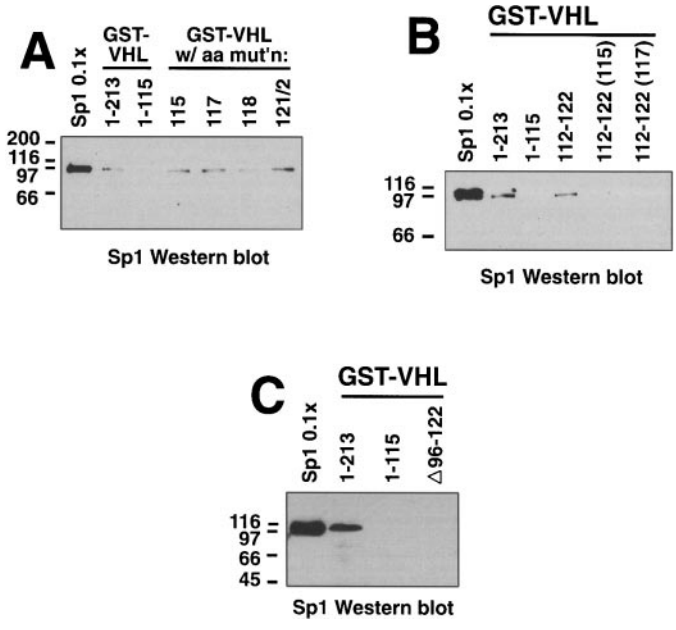

FIG. 2. Naturally-occurring VHL substitution mutations in the 96-122 domain interfere with the VHL-Sp1 interaction. (A) VHL mutation L118P interferes with the VHL-Sp1 interaction. GST-VHL fusions with amino acid (w/aa) substitutions, H115Y, W117C, L118P, ΔD121/A122, were tested for an interaction with recombinant Sp1. Amount of each fusion protein in the binding assay was comparable (data not shown). (B) VHL substitution mutations in the 112-122 context interfere with the VHL-Sp1 interaction. (C) Deletion of the 96-122 region completely prevents the VHL-Sp1 interaction.

amino acids 112-122 may be particularly important for binding. GST-VHL constructs expressing VHL amino acids 1-60, 157-189, and 120-213 did not bind Sp1 (Fig. 1B and data not shown). The VHL Sp1-binding domain is therefore distinct from the VHL Elongin-binding domain. A comparison of the known VHL functional domains and some of the naturally occurring VHL mutations in von Hippel-Lindau disease is shown in Fig. 1C. A cluster of VHL amino acid substitution mutations affects the 96-122 amino acid region.

Naturally-occurring VHL amino acid substitution mutations reduce the VHL-Sp1 interaction. Because of the potential importance of VHL amino acids 112-122, we generated by site-directed mutagenesis several naturally occurring VHL mutations in this region in the full-length GST-VHL construct, H115Y, W117C, L118P, F119L, and ΔD121/A122. We also tested Y98H, S111N, and Y112H and generated the H115Y and W117C mutations in the 112-122 GST-VHL construct. In the GST-VHL 1-213 context, only the L118P mutation substantially reduced Sp1 binding (Fig. 2A, Fig. 6), which was a consistent observation. However, both H115Y and W117C reduced the VHL-Sp1 interaction in VHL 112-122 (Fig. 2B, Fig. 6). We also deleted the 96-122 region from GST-VHL and found that Sp1 does not interact with VHL Δ96-122 (Fig. 2C, Fig. 6). These observations confirm the importance of the VHL 96-122 domain to the VHL-Sp1 interaction and suggest a clinical importance as well.

Reduction of the VHL-Sp1 interaction diminishes the VHL effect on VEGF promoter activity. We generated the same VHL mutations used in the above GST-VHL constructs in a CMV-driven VHL expression vector containing a FLAG tag (pFLAG-CMV2, Kodak). These VHL mutations were tested for their ability to repress the VHL-responsive *VEGF* promoter (−194 to +157 bp, relative to the transcription start site) in 293 cells. Protein expression from these vectors was equivalent (Fig. 3A). wt VHL repressed *VEGF* promoter activity ~8-fold (Fig. 3B, upper panel). Surprisingly, none of the 5 VHL mutations generated significantly interfered with VHL-mediated transcriptional repression, but the L118P mutation did cause a trend toward this. The wild-type and other VHL mutations repressed the *VEGF* promoter to 11–14% of control, whereas the L118P mutation, which interfered the most with VHL-Sp1 binding, only repressed the promoter to 18% of control.

To further examine the importance to transcription of disrupting the VHL–Sp1 interaction, we made pFLAG-CMV2 VHL Δ96-122. This mutated version of VHL was expressed at levels comparable to wt VHL (Fig. 3A). In cotransfections, deletion of the 96-122 domain had a statistically significant effect on VHL-mediated transcriptional repression of the *VEGF* promoter, reducing repression from 5–6-fold to ~3-fold, $P < 0.01$. Together, the L118P and Δ96-122 VHL mutations indicate that partial or complete disruption of the VHL-Sp1 interaction, respectively, cause proportional effects on VHL-mediated transcriptional repression. However, these findings are also consistent with the notion that direct binding and inhibition of Sp1 by VHL do not account for the entire VHL transcriptional repression effect on *VEGF*.

Effects of VHL Δ96-122 on VHL target genes. To help determine the effect of the VHL 96-122 region on VHL target genes and its overall importance to VHL function, we generated 3 clonal stable 786-O cell lines expressing VHL Δ96-122 in pFLAG-CMV2. These lines express VHL Δ96-122 protein at levels comparable to the other VHL-expressing lines tested (data not shown). We compared the VHL Δ96-122 lines with the parent 786-O cells and 786-O lines expressing the empty CMV-driven expression vector, wt VHL, and the 1-115 VHL truncation and by analyzing *VEGF* mRNA and glut-1 protein expression. As shown in Figs. 4A and 4B, unlike wt VHL, VHL Δ96-122 was unable to suppress *VEGF* message or glut-1 protein expression. These data indicate that loss of the 96-122 amino acid region abolishes VHL's ability to inhibit target gene expression, which supports the importance of this domain.

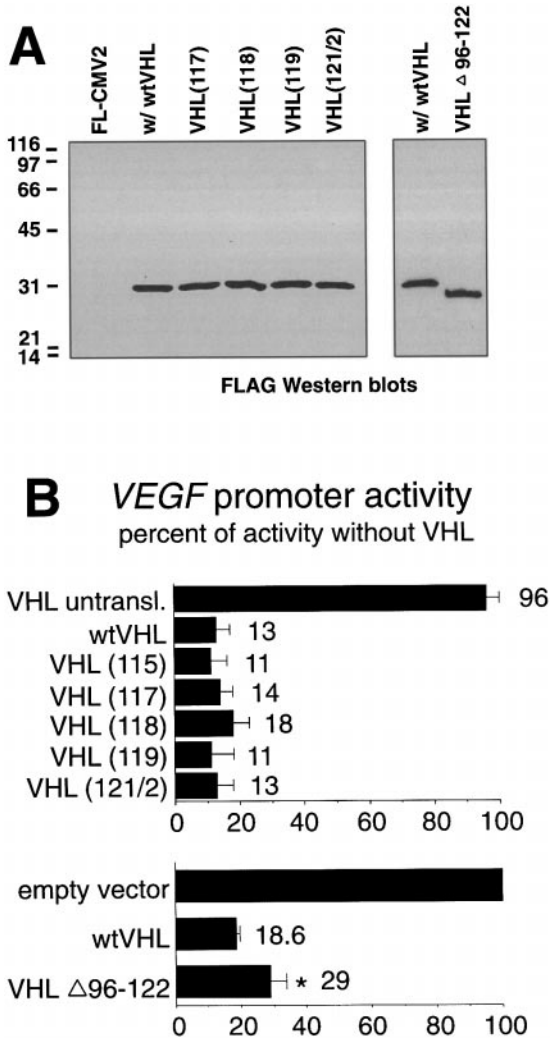

FIG. 3. The VHL 96-122 region is important for VHL-mediated transcriptional repression. VHL mutations H115Y, W117C, L118P, F119L, ΔD121/A122, and Δ96-122 in pFLAG-CMV2-VHL mammalian expression vector were tested for transcriptional repression of the *VEGF* promoter in 293 cells. (A) FLAG Western blots of whole-cell extracts (25 μg protein/lane) from cells transiently transfected with different VHL expression constructs demonstrate comparable protein expression. (B) Cotransfection studies of VHL expression vector (3 μg) with *VEGF* −194/+157 luciferase reporter (1.5 μg) in 293 cells. All versions of VHL (except untranslated) significantly repressed *VEGF* promoter activity versus empty vector and untranslated VHL, $P < 0.001$, by analysis of variance with Scheffe F test. In the lower panel, VHL Δ96-122 exhibited decreased ability to repress *VEGF* promoter activity compared to wild-type VHL, *, $P < 0.01$. n = 4. Error bars, standard deviations.

VHL self-associates through its Sp1-binding domain. We tested whether VHL might also self-associate in multiple contexts. We demonstrated this effect in coimmunoprecipitations *in vivo* (Fig. 5A). 293 cells were transiently cotransfected with 2 versions of wt VHL, one tagged with HA and the other with the FLAG epitope, or FLAG-VHL 1-115. As shown with the FLAG coimmunoprecipitation and HA Western blot detection (Fig. 5A), only the 2 full-length VHL protein interacted (lane 1), but not wt VHL with VHL 1-115 (lane 2). Lanes 5–7 demonstrate comparable amounts of immunoprecipitable HA-VHL protein in the whole cell extracts. Conversely, an HA antibody coimmunoprecipitated FLAG-wt VHL, but not FLAG-VHL 1-115 (data not shown). These observations indicate that full-length VHL molecules are capable of self-associating in intact cells.

We also showed that VHL could self-associate using the yeast 2-hybrid system (Fig. 5B). We cotransformed yeast with plasmids expressing VHL linked to LexA, which contains a DNA binding domain (pLexA-VHL), and with VHL linked to a transcription activation domain (pB42AD-VHL). As shown, cotransforming pB42AD-VHL with pLexA-VHL generates considerable blue color, shown here as dark regions (Fig. 5B, lower 4 growth patches), indicating the presence of a VHL-VHL interaction. As a positive control, cotransformation of pB42AD-Elongin C with pLexA-VHL also resulted in a strong interaction (Fig. 5B, upper 4 patches). pB42AD empty vector with pLexA-VHL is appropriately negative (Fig. 5B, upper middle 4 patches), as is another test insert in pB42AD (Fig. 5B, lower middle 4 patches). These observations indicate that VHL self-association also occurs in this intracellular context.

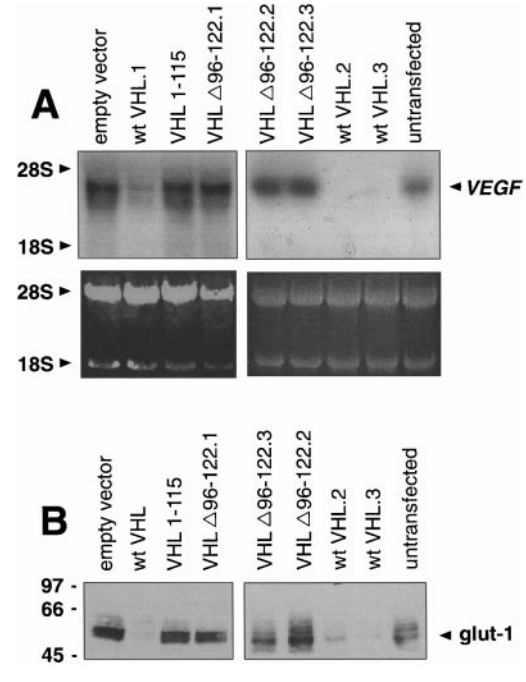

FIG. 4. Deleting the 96-122 domain prevents downregulation of VHL target genes in renal cancer cells. We generated clonal stable 786-O renal cancer cell lines that overexpress VHL Δ96-122 to determine its effect on known VHL target genes. (A) *VEGF* mRNA is substantially downregulated by wild-type VHL, but not by VHL Δ96-122 (upper panel). (B) Glut-1 Western blot of whole-cell extracts from the same stably transfected 786-O cell lines.

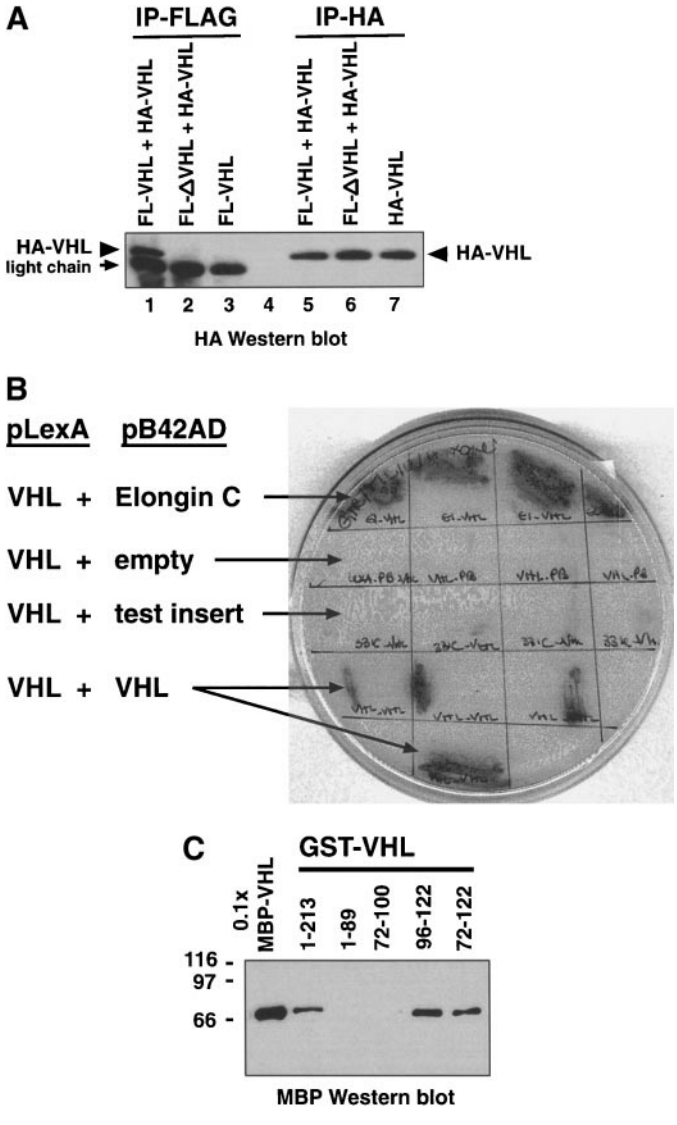

FIG. 5. VHL self-associates in multiple contexts and does so via the Sp1-binding domain. (A) VHL coimmunoprecipitation (IP) *in vivo* using FLAG- (FL) and hemagglutinin- (HA) tagged VHL. 293 cells were transiently transfected with full-length FLAG-VHL and HA-VHL (lanes 1 and 5), FLAG-VHL 1-115 (ΔVHL) and HA-VHL (lanes 2 and 6), or FLAG-VHL alone (lane 3), or HA-VHL alone (lane 7). Lane 4 is empty. An interaction is detected by HA Western analysis. (B) Demonstration of the VHL-VHL interaction using the yeast 2-hybrid system**.** Shown are replicate patches of yeast transformed with pLexA-VHL and pB42AD (Clontech), containing the following inserts: Elongin C (top 4 patches), no insert (pB42AD alone) (upper-middle 4 patches), another test insert (#331) (lower middle 4 patches), and VHL (bottom 4 patches). Blue color from beta-galactosidase activity, indicating a protein-protein interaction, appears dark. (C) VHL also self-associates through the Sp1-binding domain. The GST-VHL truncations noted were tested in *in vitro* binding assays for an interaction with 60 ng purified, bacterially-expressed VHL linked to maltose-binding protein (MBP). An interaction was identified using an anti-MBP antibody.

We demonstrated VHL self-association *in vitro* as well, using GST-VHL and also VHL linked to maltose binding protein (MBP). MBP-VHL was purified on an amylose resin and tested for an interaction with GST-VHL bound to glutathione agarose beads. MBP-VHL that bound the beads was detected by Western analysis with an MBP antibody (Fig. 5C). Surprisingly, mapping studies indicated that the minimum VHL domain sufficient for self association is the VHL Sp1-binding domain, from amino acids 96-122 (Fig. 5C), and the 112-122 region also supported diminished VHL-VHL binding (Fig. 6). With one exception (VHL L118P), there was remarkable concordance between the VHL peptide sequence required for VHL-Sp1 binding and VHL self-association (Fig. 6). In sum, the VHL 96-122 domain is critical for VHL-Sp1 and VHL-VHL protein-protein interactions.

The VHL 96-122 domain binds the Sp1 zinc fingers. We have also mapped the Sp1 domain that binds VHL. We obtained GST-Sp1 constructs from Dr. J. Horowitz (Duke University) (28) that parsed Sp1 as follows: the A domain (amino acids 1-232) and B domain (262-487), which are transcription activation domains, the C domain (500-611), and the C-terminus (604-779), which contains the DNA-binding zinc fingers and the D domain. We also made GST-Sp1 fusions of the Sp1 zinc fingers (amino acids 604-700) and D domain (701-779) separately. *In vitro* binding studies were carried out with these fusions bound to glutathione-agarose beads using purified MBP-VHL protein. The MBP-VHL in-

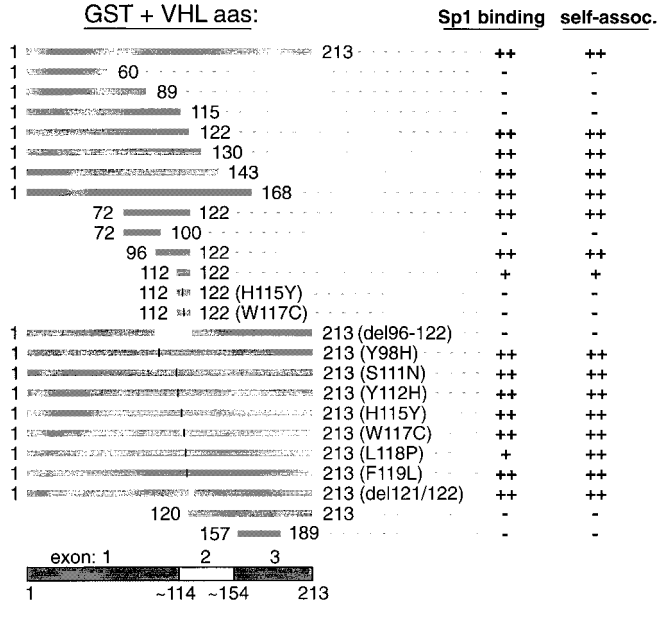

FIG. 6. Summary of GST pull-down assays delineating VHL Sp1-binding and VHL-binding domains. Schematics of VHL amino acids in GST-VHL fusions shown on left, flanked by the included VHL amino acids, with substitutions mutations in parentheses. Amino acid deletions (del) are shown as gaps, substitution mutations as vertical lines. Schematic of VHL exons 1–3 and corresponding amino acids are shown below. On the right is a qualitative assessment of binding, ++ for a strong interaction, + for a moderate interaction, and − for no interaction.

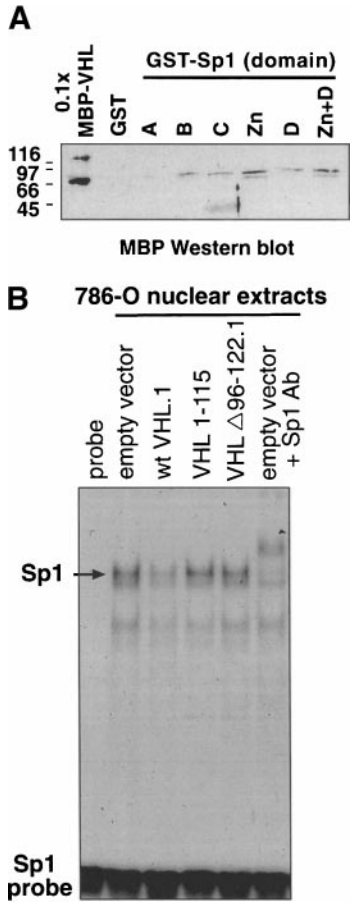

FIG. 7. VHL interacts with the Sp1 zinc fingers, and reintroduction of wild-type, but not mutant, VHL interferes with Sp1 DNA binding. (A) A series of Sp1 truncations fused to GST was tested for binding to MBP-VHL to localize the Sp1 VHL-binding domain. An Sp1-VHL interaction was identified using an MBP antibody. (B) Nuclear extracts from stably-transfected 786-O renal cancer cell lines were tested for Sp1 DNA binding activity on a consensus Sp1 oligonucleotide in EMSAs. Nuclear extract protein concentrations were rigorously normalized (see Methods).

teraction mapped to the Sp1 zinc fingers, although consistent weak affinity was noted in the B, C, and D domains (Fig. 7A). The zinc-finger domain did not bind MBP alone (data not shown). These observations suggests that the VHL 96-122 domain is particularly important for interactions with zinc finger proteins.

Reintroduction of wild-type, but not mutant, VHL into renal cancer cell lines interferes with Sp1 DNA binding. We made nuclear extracts from the 786-O cell lines and tested them for Sp1 DNA binding to a consensus Sp1 site in EMSAs. Nuclear extract proteins were quantitated by 2 methods and confirmed qualitatively by protein electrophoresis and staining (data not shown). Only wt VHL interfered with Sp1 DNA binding, whereas the empty vector, VHL 1-115, and VHL Δ96-122 lines had comparable higher binding (Fig. 7B). The findings shown were reproducible using these and different nuclear extract preparations and in different 786-O cell lines. This observation indicates that the VHL 96-122 region is required for VHL to bind Sp1 and interfere with its activity, and supports the biologic significance of this domain to VHL transcriptional repression.

DISCUSSION

VEGF is overexpressed in renal cell cancer (13–16, 19) and other VHL disease-associated cancers (19) and likely plays a major role in the pathogenesis of these disorders. In further characterizing the VHL-Sp1 interaction and determining its importance, we have made the following new observations. 1. The VHL Sp1-binding domain maps to VHL amino acids 96-122, a region highly-conserved in both rat and mouse VHL (26, 27) and affected by a disproportionate number of VHL mutations. VHL amino acids 112-122 may be most important for the interaction. 2. Several clinically relevant amino acid substitution mutations in this region interfere with the VHL-Sp1 interaction. 3. Deletion of the VHL 96-122 interferes with VHL-mediated repression of transcription, inhibition of Sp1 DNA binding, and downregulation of known target genes in renal cancer cells, supporting the 96-122 domain's key role in VHL function. However, these studies also suggest that factors other than Sp1 are the primary VHL transcriptional effectors. 4. VHL also self-associates via the 96-122 domain, supporting the possibility of VHL dominant-negative effects. 5. The VHL 96-122 domain interacts with the Sp1 zinc fingers, suggesting VHL may bind other metalloproteins.

VHL substitution mutations are found commonly outside the VHL Elongin-binding domain (see Fig. 1C and (29)). Based on cumulated VHL mutation data on 216 families with VHL disease (29), 23 of 139 (16.5%) missense mutations fall within the 96-122 amino acid VHL Sp1-binding region and 17 (12.2%) within the most critical residues, from 111-122. Like the Elongin-binding region, there is a clustering of substitution mutations in the Sp1-binding region (see Fig. 1C). VHL exon 2 includes amino acids 114 to 154, and splice-junction mutations have been described that entirely drop out exon 2 (6) and should therefore interfere with the VHL-Sp1 interaction, VHL self-association, transcriptional repression, or other protein-protein interactions. An alternatively spliced form of VHL has been identified that lacks exon 2 as well. The L118P mutation may be a particularly severe missense mutation, more like a VHL truncation in function and phenotype, as three families with the L118P mutation all have renal cancer without pheochromocytoma (29). Including frameshifts, splice-junction, missense, and nonsense mutations, 28% of mutations in VHL families affect the 96-122 region.

Our studies indicate that the VHL 96-122 domain contributes importantly to VHL function, but we were surprised by the small magnitude of the transcriptional effect of deleting the VHL 96-122 domain. Nevertheless, this statistically-significant ~2-fold effect on transcriptional repression may still be important for VHL function. Indeed, deletion of the 96-122 region prevents VHL downregulation of Sp1 DNA binding in renal cancer cells, which should have a transcriptional consequence. Possible explanations for the residual transcriptional repressor activity of VHL Δ96-122 include a retained ability to inhibit protein kinase C (30) or the HIF transcription factors (31).

During this manuscript's review, VHL was shown crystallographically to be divided into 2 major domains, the Elongin C-binding α domain (residues 157-188) comprising α helical regions and a β domain (residues 63-154 (β strand) and 193-204 (α helix)) (32). The severity of the L118P mutation may be due to strong effects of the proline residue in disrupting β strand number 5 and/or interference with strand 5′s interaction with the more distant 193-204 helical region (32). Intriguingly, the protein-protein interactions described herein are the first identified for the VHL β domain.

In conclusion, we have delineated a second major VHL protein-protein interaction domain. This domain may contribute to VHL function by virtue of its interaction with VHL itself, the transcriptional activator Sp1 or potentially other metalloproteins. Interference with these VHL functions through mutations in this region may therefore contribute to the development of a subset of renal cancers and VHL disease.

ACKNOWLEDGMENTS

We thank J. Horowitz and W. Kaelin for generously providing reagents. This work was supported by NIH Grant DK02280 and a National Kidney Foundation Young Investigator Grant to H.T.C., NIH Grant F32 CA79133 to M.Z., a Fondation pour la Rechereche Medicale grant to B.K., and an NIH/NIDDK grant to V.P.S.

REFERENCES

1. Latif, F., Tory, K., Gnarra, J., Yao, M., Duh, F.-M., Orcutt, M. L., Stackhouse, T., Kuzmin, I., Modi, W., Geil, L., Schmidt, L., Zhou, F., Li, H., Wei, M. H., Chen, F., Glenn, G., Choyke, P., Walther, M. M., Weng, Y., Duan, D.-S. R., Dean, M., Glavac, D., Richards, F. M., Crossey, P. A., Ferguson-Smith, M. A., Paslier, D. L., Chumakov, L., Cohen, D., Chinault, A. C., Maher, E. R., Linehan, W. M., Zbar, B., and Lerman, M. I. (1993) *Science* **260,** 1317–1320.
2. Cohen, H. T. (1999) *Curr. Opin. Nephrol. Hypertens.* **8,** 325–331.
3. Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J. R., Orcutt, M. L., Duh, F. M., Glenn, G., Green, J., Hsia, Y. E., Lamiell, J., Li, H., Wei, M., Schmidt, L., Tory, K., Kuzmin, I., Stackhouse, T., Latif, F., Linehan, W. M., Lerman, M., and Zbar, B. (1995) *Hum. Mutat.* **5,** 66–75.
4. Crossey, P. A., Richards, F. M., Foster, K., Green, J. S., Prowse, A., Latif, F., Lerman, M. I., Zbar, B., Affara, N. A., Ferguson-Smith, M. A., and Maher, E. R. (1994) *Hum Mol. Genet.* **3,** 1303–1308.
5. Foster, K., Prowse, A., van den Berg, A., Fleming, S., Hulsbeek, M. M. F., Crossey, P. A., Richards, F. M., Cairns, P., Affara, N. A., Ferguson-Smith, M. A., Buys, C. H., and Maher, E. R. (1994) *Hum. Mol. Genet.* **3,** 2169–2173.
6. Gnarra, J. R., Tory, K., Weng, Y., Schmidt, L., Wei, M. H., Li, H., Latif, F., Liu, S., Chen, F., Duh, F.-M., Lubensky, I., Duan, D. R., Florence, C., Pozzatti, R., Walther, M. M., Bander, N. H., Grossman, H. B., Brauch, H., Pomer, S., Brooks, J. D., Isaacs, W. B., Lerman, M. I., Zbar, B., and Linehan, W. M. (1994) *Nature Genet.* **7,** 85–90.
7. Kibel, A., Iliopoulos, O., DeCaprio, J. A., and Kaelin, W. G. (1995) *Science* **269,** 1444–1446.
8. Aso, T., Lane, W. S., Conaway, J. W., and Conaway, R. C. (1995) *Science* **269,** 1439–144.
9. Duan, D. R., Pause, A., Burgess, W. H., Aso, T., Chen, D. Y. T., Garrett, K. P., Conaway, R. C., Conaway, J. W., Linehan, W. M., and Klausner, R. D. (1995) *Science* **269,** 1402–1406.
10. Kishida, T., Stackhouse, T. M., Chen, F., Lerman, M. I., and Zbar, B. (1995) *Cancer Res.* **55,** 4544–4548.
11. Pause, A., Lee, S., Worrell, R. A., Chen, D. Y. T., Burgess, W. H., Linehan, W. M., and Klausner, R. D. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 2156–2161.
12. Willems, A. R., Lanker, S., Patton, E. E., Craig, K. L., Nason, T. F., Mathias, N., Kobayashi, R., Wittenberg, C., and Tyers, M. (1996) *Cell* **86,** 453–463.
13. Siemeister, G., Weindel, K., Mohrs, K., Barleon, B., Martiny-Baron, G., and Marme, D. (1996) *Cancer Res.* **56,** 2299–2301.
14. Gnarra, J. R., Zhou, S., Merrill, M. J., Wagner, J. R., Krumm, A., Papavassiliou, E., Oldfield, E. H., Klausner, R. D., and Linehan, W. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10589–10594.
15. Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G., Jr., and Goldberg, M. A. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 10595–10599.
16. Mukhopadhyay, D., Knebelmann, B., Cohen, H. T., Ananth, S., and Sukhatme, V. P. (1997) *Mol. Cell. Biol.* **17,** 5629–5639.
17. Knebelmann, B., Ananth, S., Cohen, H. T., and Sukhatme, V. P. (1998) *Cancer Res.* **58,** 226–231.
18. Takahashi, A., Sasaki, H., Kim, S. J., Tobisu, K.-i., Kakizoe, T., Tsukamoto, T., Kumamoto, Y., Sugimura, T., and Terada, M. (1994) *Cancer Res.* **54,** 4233–4237.
19. Wizigmann-Voos, S., Breier, G., Risau, W., and Plate, K. H. (1995) *Cancer Res.* **55,** 1358–1364.
20. Los, M., Aarsman, C. J., Terpstra, L., Wittebol-Post, D., Lips, C. J., Blijham, G. H., and Voest, E. E. (1997) *Ann. Oncol.* **8,** 1015–1022.
21. Iliopoulos, O., Kibel, A., Gray, S., and Kaelin, W. G. (1995) *Nature Med.* **1,** 822–826.
22. Gorman, C. M., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell Biol.* **2,** 1044–1051
23. Cohen, H. T., Bossone, S. A., Zhu, G., McDonald, G. A., and Sukhatme, V. P. (1997) *J. Biol. Chem.* **272,** 2901–2913.
24. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
25. Lee, S., Chen, D. Y. T., Humphrey, J. S., Gnarra, J. R., Linehan, W. M., and Klausner, R. D. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 1770–1775.
26. Gao, J., Naglich, J. G., Laidlaw, J., Whaley, J. M., Seizinger, B. R., and Kley, N. (1995) *Cancer Res.* **55,** 743–747.

27. Kikuchi, Y., Kobayashi, E., Nishizawa, M., Hamazaki, S., Okada, S., and Hino, O. (1995) *Jpn. J. Cancer Res.* **86,** 905–909.

28. Murata, Y., Kim, H. G., Rogers, K. T., Udvadia, A. J., and Horowitz, J. M. (1994) *J. Biol. Chem*. **269,** 20674–20681.

29. Zbar, B., Kishida, T., Chen, F., Schmidt, L., Maher, E. R., Richards, F. M., Crossey, P. A., Webster, A. R., Affara, N. A., Ferguson-Smith, M. A., Brauch, H., Glavac, D., Neumann, H. P. H., Tisherman, S., Mulvihill, J. J., Gross, D. J., Shuin, T., Whaley, J., Seizinger, B., Kley, N., Olschwang, S., Boisson, C., Richard, S., Lips, C. H. M., Linehan, W. M., and Lerman, M. (1996) *Hum. Mutat.* **8,** 348–357.

30. Pal, S., Claffey, K. P., Dvorak, H. F., and Mukhopadhyay, D. (1997) *J. Biol. Chem*. **272,** 27509–27512.

31. Maxwell, P. H., Wiesener, M. S., Chang, G.-W., Clifford, S. C., Vaux, E. C., Cockman, M. E., Wykoff, C. C., Pugh, C. W., Maher, E. R., and Ratcliffe, P. J. (1999) *Nature* **399,** 271–275.

32. Stebbins, C. E., Kaelin, W. G., and Pavletich, N. P. (1999) *Science* **284,** 455–461.