Inhibition of Transcription Elongation by the VHL Tumor Suppressor Protein

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Germline mutations in the von Hippel–Lindau tumor suppressor gene (VHL) predispose individuals to a variety of tumors, including renal carcinoma, hemangioblastoma of the central nervous system, and pheochromocytoma. Here, a cellular transcription factor, Elongin (SIII), is identified as a functional target of the VHL protein. Elongin (SIII) is a heterotrimer consisting of a transcriptionally active subunit (A) and two regulatory subunits (B and C) that activate transcription elongation by RNA polymerase II. The VHL protein was shown to bind tightly and specifically to the Elongin B and C subunits and to inhibit Elongin (SIII) transcriptional activity in vitro. These findings reveal a potentially important transcriptional regulatory network in which the VHL protein may play a key role.

The identification of tumor suppressor genes whose loss of function results in predisposition to cancer has taken center stage in our attempts to understand human carcinogenesis (1, 2). Germline mutations in these genes are responsible for a number of inherited cancer syndromes, and somatic mutations have been found in most, if not all, sporadic cancers. In addition to their great potential as diagnostic and prognostic tools, tumor suppressor genes can offer valuable insight into the fundamental pathways involved in the control of the cell cycle, genome stability, cell death, and other aspects of carcinogenesis.

The VHL gene is mutated in families with von Hippel–Lindau disease (3–5), a rare disorder (incidence ~1 in 36,000) that predisposes affected individuals to a variety of cancers, including clear-cell renal carcinoma, hemangioblastoma, and pheochromocytoma (4) and references therein. Renal tumors from VHL patients carry germline VHL mutations and show selective loss of the wild-type VHL allele inherited from the unaffected parent (5, 6). As predicted by Knudson’s two-hit hypothesis for tumor suppressor gene inactivation, the majority of patients with sporadic clear-cell renal carcinoma show functional loss of both alleles of the VHL locus in their tumors (7, 8).

The human VHL gene encodes a protein of 213 amino acids with no significant homology to known proteins (3). The rat homolog is 88% identical to human VHL, but lacks an acidic pentapeptide motif that is repeated eight times in the NH₂-terminus of the human protein (9). In previous studies, we found that the human and rat VHL proteins formed oligomeric complexes with several unidentified proteins in cultured mammalian cells (9). One of these complexes, a heterotrimer consisting of VHL and two proteins of 9 kD and 16 kD, did not form when the VHL protein contained certain missense mutations found in human cancers (9). This observation suggested that the 9-kD and 16-kD proteins were potentially involved in the normal tumor suppressor function of VHL (9), and we therefore set out to identify these proteins.

A stably transfected HeLa cell line expressing a rat full-length VHL that had been epitope-tagged with Flag (DYKDDDDK) (10) was used for immunoaffinity purification of VHL and its associated proteins (11). After separation by SDS–polyacrylamide gel electrophoresis (PAGE), the bands corresponding to the associated proteins that we previously denoted p16 and p9 (Fig. 1A) were digested with the protease Lys C. Eluted peptides were purified by reversed-phase high-performance liquid chromatography (HPLC) and sequenced by Edman degradation. The band labeled VHL was also identified by sequence analysis, which confirmed the predicted start codon, amino acid 1 of the rodent VHL open reading frame (9, 12).

As revealed by a database search, a peptide sequence derived from p9 demonstrated a 19 out of 20 amino acid match to a sequence present in Elongin C, a 112-amino acid protein that was originally purified as a subunit of the heterotrimeric transcription elongation factor Elongin (SIII) (13). Elongin (SIII) activates transcription elongation of RNA polymerase II (Pol II), the major polymerase for messenger RNA synthesis. As determined by in vitro assays, Elongin (SIII) suppresses the transient pausing of Pol II that occurs at many sites throughout transcription units (14, 15). Elongin (SIII) also contains a 110-kD subunit (A) and an 18-kD subunit (B) (14, 16, 17). Our previous immunoprecipitation studies had revealed coassociation of VHL with p9 and p16, but not with a 110-kD protein (9). Furthermore, the migration of

Fig. 1. Purification and sequencing of p16 and p9. Extracts from HeLa cells stably transfected with pSx-RBF (rat VHL, tagged with a Flag epitope at the COOH-terminus) were immunoprecipitated with anti-Flag, washed, and eluted with glycine-HCl. (A) The eluate was analyzed by 15% SDS-PAGE and stained with Coomassie brilliant blue. Peptide sequences of VHL-associated p16 and p9 are shown and underlined in the Elongin B (GenBank accession number L42858) and Elongin C (GenBank accession number L29259) sequences (10). (B) The anti-Flag resin eluates from vector-transfected cells (lanes 1, 3, and 5) or from rat VHL-transfected cells (lanes 2, 4, and 6) were analyzed on immunoblots developed with anti-Flag (to detect VHL), anti–Elongin B, or anti–Elongin C.

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the VHL-p9-p16 complexes on sucrose gradients was consistent with a size of ~40 to 50 kD (9). We tested whether p16 was another Elongin subunit by protein sequence analysis, and discovered that a peptide from p16 showed a 20 out of 21 amino acid match to a sequence in Elongin B (17) (Fig. 1A).

We verified the identity of the VHL-associated p9 and p16 as Elongin B and C, respectively, by immunoblot analysis with antibodies directed against Elongin B or C (Fig. 1B). These antibodies recognize their antigen targets on immunoblots but do not directly immunoprecipitate them. The question of whether Elongin A stably binds to VHL was assessed by cotransfection of COS-7 cells with vectors encoding either human VHL tagged with Flag at the NH2-terminus (Flag-VHL) or Elongin A tagged with histidine (His-Elongin A) (18). Protein complexes were purified with antibody to Flag (anti-Flag) or with a nickel–nitrioltri-acetate (nickel-NTA) agarose resin, which binds to the His tag. The resultant complexes were resolved by SDS-PAGE and analyzed on immunoblots developed with antibodies specific for each of the four target proteins (Fig. 2A). The nickel resin purified Elongin A complexed to the B and C subunits but did not purify VHL (lane 2). Conversely, the anti-Flag purified Elongin B and C in a complex with VHL but did not purify Elongin A (lane 6).

To assess the range of interactions between these proteins, we cloned the complementary DNAs (cDNAs) encoding Flag-VHL, His-Elongin A, and Elongin B and C into pGEM3 vectors, and examined the fate of the protein products synthesized in coupled transcription-translation systems (19). In reactions immunoprecipitated with anti-Flag (Fig. 2B), we observed no coprecipitation of Elongin A with VHL, whether the former was synthesized with VHL alone (lane 5) or in combination with the other subunits (lane 9). The small amount of Elongin A in the precipitates (lanes 5 and 9) was no greater than in control lysates containing no Flag-VHL (lane 2). There was no evidence for binding of VHL to Elongin B alone (lane 6), although there was some detectable binding of VHL to Elongin C alone (lane 7). When VHL was translated with both Elongin B and C, the binding of both Elongin B and C to VHL was enhanced (lane 8). The ability of the His–Elongin A to form complexes in this in vitro system was examined by precipitation of the translation products with a nickel-NTA agarose resin. These results precisely mirrored those obtained by anti-Flag immunoprecipitation of VHL (20). There was a small amount of assembly of Elongin A with C but no significant association of Elongin B with A in the absence of C. In contrast, cotranslation of all three subunits resulted in increased binding of Elongin B and C to Elongin A. No VHL was assembled with Elongin A, regardless of the presence of associated subunits (20).

We also assessed the ability of recombinant VHL, Elongin B, and Elongin C to assemble (Fig. 3). The three bacterially expressed proteins were renatured alone or in various combinations, and the resultant

**Fig. 2.** Detection of a complex containing VHL and Elongin B and C. (A) COS-7 cells transfected with pSVL–His-p110 (Elongin A), pSX-Fg7 (human VHL tagged with Flag), or both plasmids were lysed, and proteins were precipitated with nickel-NTA agarose resin, which binds to the His tag (lanes 1 to 4). The supernatants of the nickel-NTA agarose resin precipitates were immunoprecipitated with anti-Flag (lanes 5 and 6). The immunoblot was developed with rabbit polyclonal anti-Elongin A (top), anti-Flag (middle), or a mixture of anti–Elongin B anti–Elongin C (bottom). (B) The cDNAs encoding Flag-VHL, His-Elongin A, and Elongin B and C were expressed in a reticulocyte-coupled transcription-translation system in the presence of 35S-methionine. The translation products were immunoprecipitated with anti-Flag, fractionated by 15% SDS-PAGE, and detected by autoradiography. Lane 10 contains 1/10th of the reaction mixture used for immunoprecipitation in lanes 1 to 9.

**Fig. 3.** HPLC purification of complexes containing VHL and Elongin B and C. Recombinant His-VHL and Elongin B and C were prepared from bacterial inclusion bodies and purified by nickel chromatography (13). VHL was renatured in the presence of various combinations of Elongin B and C (13, 14, 17) in mixtures containing 60 μg of His-VHL, 30 μg of His-Elongin B (17), and 30 μg of His-Elongin C (13). After dialysis, the renatured proteins were applied to a TSK DEAE-NPR column (35 mm by 4.6 mm; Hewlett-Packard) equilibrated in 40 mM tris-HCl (pH 7.9), 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, and 60 mM KCl. The column was eluted at 0.3 ml/min at 8°C with a 3-ml gradient from 0.06 M to 0.6 M KCl in the same buffer. Samples of each column fraction were analyzed by 12% SDS-PAGE, and the proteins were visualized by silver staining. The apparent molecular mass of protein standards are indicated on the left. V, VHL; B, Elongin B; C, Elongin C; M, marker; L, column load; F, column flowthrough.
complexes were analyzed by anion-exchange HPLC on TSK diethyl-amino-ethyl nonporous resin (DEAE-NPR). The individually renatured VHL, Elongin B, and Elongin C proteins exhibited distinct elution DEAE-NPR profiles. Elongin B flowed through the column at 0.06 M KCl, whereas Elongin C and VHL bound to the column and eluted at 0.3 to 0.4 M KCl and 0.44 to 0.5 M KCl, respectively. Upon renaturation and chromatography of mixtures of either VHL plus Elongin C, or VHL plus Elongin B, a substantial fraction of Elongin C coeluted with VHL as a VHL–Elongin C complex. In contrast, very little Elongin B coeluted with VHL. Finally, upon renaturation and chromatography of a mixture of VHL and both Elongin B and C, a portion of the Elongin B and C subunits copurified as an Elongin BC complex that eluted from the column in a discrete peak, and nearly all of the remaining Elongin B and C coeluted with VHL as a VHL–Elongin BC complex. Thus, in three different experimental systems, we consistently observed formation of both the VHL–Elongin C and VHL–Elongin BC complexes, but not of the VHL–Elongin B complex.

The absence of complexes containing both VHL and Elongin A suggested that the VHL–Elongin BC and the Elongin ABC complexes were mutually exclusive and that VHL and Elongin A might compete for binding to Elongin B and C. This hypothesis was tested by analysis of the assembly of recombinant proteins that had been denatured and then renatured (13, 21). Complex formation was detected by immunoprecipitation with anti-Flag (Fig. 4). Equimolar input of each protein resulted in formation of VHL–Elongin BC complexes containing no Elongin A (lane 9). Input of a fivefold molar excess of Elongin A resulted in loss of the VHL–Elongin BC complexes and presumed formation of Elongin ABC complexes (lane 11). VHL has no apparent sequence similarity with Elongin B or C, but does share a stretch of 13 amino acids with Elongin A (16), and it is this region of VHL that has been implicated in the interaction with Elongin B and C (22, 23).

We next investigated the effect of VHL mutations on Elongin interactions. We analyzed several of the VHL mutations that have been identified either in the germ line of patients with VHL disease or in the DNA from sporadic renal cell carcinomas (7, 8, 24). These included three missense mutations (Y98H, R167Q, and R167W) (10) and one frameshift mutation, (157A), in which exon 3 is deleted. COS-7 cells transiently transfected with the appropriate cDNAs expressed wild-type levels of the VHL mutants; these proteins were not grossly misfolded, as judged by their ability to interact with cellular proteins in a manner identical to that of wild-type VHL (9, 22). The 157A mutant showed a greatly reduced ability to bind Elongin B and C. The two R167 mutants (9, 22) showed a slight impairment in binding; however, binding of these mutants was completely lost if the lysates contained the anionic detergent Sarkosyl (0.2%), conditions that had no effect on the binding of wild-type VHL (20). Under either condition, the binding of the Y98H mutant to Elongin B and C was comparable to that of wild-type VHL. The effects of these mutants were also examined in the transcription-translation system (19), and similar results were observed (Fig. 5).

To investigate whether VHL affects Elongin (SII) activity, we included VHL in two different assays of transcription elongation: (i) the adenovirus 2 major late (AdML) runoff transcription assay (17, 24, 25), which measures the ability of Elongin (SII) to stimulate the rate of elongation of RNA transcripts initiated at the AdML promoter in the presence of general initiation factors; and (ii) the oligo(dC)-tailed template assay (25, 26), which permits direct measurement of Elongin (SII) activity in the absence of general initiation factors. In control experiments, we observed that VHL had no detectable inhibitory or stimulatory effect on the basal rate of elongation by Pol II in the absence of Elongin (SII) (Fig. 6A, lanes 1 to 10; Fig. 6B, lanes 1 to 4). In contrast, the presence of highly purified VHL during assembly of the Elongin ABC complex resulted in a substantial inhibition of Elongin (SII) activity, in both the AdML runoff transcription assay (Fig. 6A, lanes 11 to 20) and the oligo(dC)-tailed template assay (Fig. 6B, lanes 5 to 16). The inhibitory activity copurified with VHL protein (Fig. 6C), supporting the idea that the VHL protein is the inhibitor.

If the VHL protein inhibits Elongin (SII) activity by sequestering and thereby negatively regulating the activities of the

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**Fig. 4.** Competition between VHL and Elongin A for binding to Elongin B and C. Recombinant His–Elongin A, His–VHL–Flag, Elongin B, and Elongin C were mixed in different combinations in 6 M guanidine-HCl denaturing buffer. After renaturation (13), the proteins were immunoprecipitated with anti-Flag (lanes 1 to 11) or nickel-NTA agarose resin (lane 12) and analyzed by SDS-PAGE. The immunoblot was probed with anti–Elongin A (top), anti–Flag for detection of VHL (middle), and a mixture of anti–Elongin B and Elongin C (bottom). In lanes 1 to 10, VHL and Elongin A, B, and C were present in equimolar ratios. In lane 11 the molar ratio of Elongin A to VHL was 2:1 and in lane 12 the ratio was 5:1.

**Fig. 5.** Coimmunoprecipitation of Elongin B and C with wild-type (wt) or mutant VHL in vitro. The cDNAs encoding wild-type VHL (Fg7), mutant VHL (Y98H, 157A, R167W) (10), Elongin B, and Elongin C were expressed in a coupled transcription-translation system in the presence of [35S]methionine. The translation products were immunoprecipitated with anti-Flag, fractionated by SDS-PAGE, and detected by autoradiography.

Elongin B and C subunits, then preassembly of the Elongin ABC complex before the addition of VHL, or addition of excess Elongin B and C to assembly reactions containing both VHL and Elongin A, might be expected to reduce the inhibition by VHL. We found that preincubation of Elongin A, B, and C before addition of VHL substantially reduced the ability of VHL to inhibit Elongin activity (27). The addition of excess amounts of Elongin B and C during assembly also reduced VHL inhibition (Fig. 6B, compare lanes 9 to 12 with lanes 13 to 16). These results indicate that VHL can block Elongin (SII) transcriptional activity in vitro and may have a similar function in vivo. It is noteworthy that VHL causes a slight but reproducible inhibition of transcriptional stimulation by Elongin A alone (Fig. 6B, lanes 5 to 8), raising the possibility that it may interact weakly with Elongin A.
In conclusion, we have found that overexpressed VHL can bind tightly and specifically to Elongin B and C and prevent their assembly with Elongin A, the transcriptionally active subunit of the Elongin (SII) complex (Fig. 7). The VHL–Elongin B interaction is also observed with endogenous protein (23), and the correlation of at least a subset of naturally occurring VHL mutants with loss of Elongin BC binding activity indicates that this interaction is likely to be physiologically important in the tumor suppressor function of VHL. The mutations that do not disrupt VHL binding to Elongin BC may alter VHL interaction with other protein targets (9) that may be important to its normal function.

The inhibitory effect of VHL on transcription elongation is consistent with the hypothesis that tumor suppressor genes have negative regulatory effects on critical cellular functions. Our results raise several questions. Does VHL regulate gene expression in vivo and, if so, how? In principle, VHL could regulate gene expression indirectly by controlling other processes coupled to Pol II transcription elongation, such as DNA damage recognition and repair. Alternatively, it could directly regulate the expression of specific genes by controlling the rate of transcription elongation. It is noteworthy that a growing number of eukaryotic genes are now believed to be regulated at the elongation level by mechanisms such as “promoter-proximal” attenuation (28, 29). In addition, the recent discovery that transcriptional activators, including VP-16 and EIA, regulate transcription in part by stimulating the rate of elongation raises the possibility that transcription elongation factors may be targets for gene-specific transcriptional activators in vivo (30, 31). Finally, how is the function of VHL controlled? Identification of
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10. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
11. HelA cells (2 x 10^6) stably transfected with pSG-RFP were lysed in 15 ml of TS buffer (20 mM TRIS-HCl (pH 8.0), 137 mM NaCl, 1% Triton X-100, 0.2% Sarkosyl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (0.5 μg/ml), apro- tinin (1 μg/ml), 1 mM NaF, and 1 mM sodium orthovanadate). The lysate was centrifuged twice at 17,000 g for 30 min at 4°C. The supernatant was mixed with 30 μl of anti-Flag M2-Sepharose conjugate (2.9 mg of antibody/ml (Kodak-I1B) for 3 hours at 4°C, and the immunopreciptates were washed with 1 ml of cold buffer for 3 times. The immunoprecipitate was eluted with glycine-VER (pH 2.5) and analyzed by 15% SDS-PAGE.
12. The proteins were transferred from the gel to nitrocellulose filters (Schleicher & Schuell), and the 32P-labeled bands were excised and prepared for analysis. The proteins were 50% trichloroacetic acid (TCA) in the presence of 50% 1,1,1-trifluoroacetone and 10% acetic acid (TFA). The bands were incubated with Lys C protease (Boehringer Mann-heim) at an estimated ratio of 20:1 (w/w). After 20 hours at 37°C, the samples were treated with 1 ml dithiothreitol (DTT) for 1 hour. The Peptides were eluted with 1 ml of 3 M acetic acid, and the supernatant was resolved on a 20 x 2.1 mm RP-300 reverse phase HPLC using a linear gradient from 0% to 100% B (A = 0.1% TFA in 50% 1,1,1-trifluoroacetone, B = 70% acetonitrile, 0.1% TFA) for 20 min by 0.25 ml/min. The sequences of selected peptides were determined with an Applied Biosystems Model 477A protein se- quencer with an on-line model 120 phenylthiohydantoin analyzer.
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19. COS-7 cells (4 x 10^6) were transfected with pSVL- His-p110 (Elongin A, pSVL-Fig7 (Flag-tagged human VHL), or both plasmids (20 μg each)). Cells were lysed (17), and the proteins were precipitated with Ni-NTA agarose resin (Qigagen, Chatsworth, CA). The supernatants of the nickel-NTA agarose resin precipitates were immunoprecipitated with anti-Flag (2 μg/ml).
20. The translations or cotranslations were done according to the manufacturer's instructions (Promega). The cDNAs for VHL (FGT), His-elongin A, Elongin B, and Elongin C were subcloned into pGEM3 vectors (Promega) and transcribed with SP6 polymerase (final concentration, 2 μg/ml for each DNA) in a coupled transcription-translation reticulocyte lysate. After transcription-translation of the RNA transcripts for 120 minutes and 30°C, the reactions were diluted in 1 ml of TS buffer containing 0.2% Sarkosyl. For immuno-precipitation of Flag-VHL, 2 μg of affinity-purified anti-FLAG and 30 μl of protein A-Sepharose (50% v/v) were added; and reactions were rocked for 120 minutes at 4°C. The immunoprecipitates were washed four times in 1 ml of TS buffer, 0.2% Sarkosyl (anti-Flag precipitates) or 1 ml of TSB, 0.2% Sarkosyl, and 20 m M imidazole (Ni precipitates); analyzed by 15% SDS-PAGE, and processed for autoradiography.
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22. Bacterially expressed His-Elongin A, His-VHL-Flag, Elongin B, and Elongin C were mixed in 6 M guani- dine-HCl denaturing buffer. Denaturing buffer (five volume excess to denaturing buffer) was added to the mixture. After a 90-min incubation on ice, the denatured proteins were dialyzed against 40 mM Hepes (pH 7.3), 100 mM KCl, 50 μM ZnCl2, and 10% glycerol. The dialyzed mixture was diluted 1:8 with Triton X-100 lysis buffer containing 0.2% Sarkosyl and immunoprecipitated with anti-Flag or nickel-NTA agarose. The precipitates were washed with lysis buffer and analyzed on a 9 to 15% SDS-polyacrylamide gel.
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25. Pol II (0.01 unit) and pCP220/S-PX (100 μg) were incubated at 28°C in 20 mM Hepes, 20 mM tris-HCl (pH 7.9), 2% (v/v) polyvinyl alcohol, bovine serum albumin (0.5 mg/ml) 60 mM KCl, 50 mM Na2SO4, 7 mM MgCl2, 0.2 mM DTT, 3% (v/v) glyc- erol, 3 units of recombinant RNAsin (Promega), 50 μM ATP, 50 μM GTP, 2 μM CTP, and 10 μCi of [α-32P]CTP. After 25 min, 100 μM nonradioactive CTP, 2 μM UTP, and VHL or Elongin subunits were added, and the reactions were incubated for a further 7.5 min. Transcripts were analyzed by electrophoresis through 6% polyacrylamide, 7.0 M urea gels.
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