Binding of the von Hippel–Lindau Tumor Suppressor Protein to Elongin B and C

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Germ-line mutations of the von Hippel–Lindau tumor suppressor gene (*VHL*) predispose individuals to a variety of human tumors, and somatic mutations of this gene have been identified in sporadic renal cell carcinomas and cerebellar hemangioblastomas. Two transcriptional elongation factors, Elongin B and C, were shown to bind in vitro and in vivo to a short, colinear region of the VHL protein (pVHL) that is frequently mutated in human tumors. A peptide replica of this region inhibited binding of pVHL to Elongin B and C, whereas a point-mutant derivative, corresponding to a naturally occurring VHL missense mutation, had no effect. These results suggest that the tumor suppression function of pVHL may be linked to its ability to bind to Elongin B and C.

Von Hippel–Lindau disease is a hereditary cancer syndrome characterized by the development of multiple tumors including renal cell carcinomas, retinal angiomas, cerebellar hemangioblastomas, and pheochromocytomas (*1*). The von Hippel–Lindau susceptibility gene (*VHL*) has been identified (*2*), and mutations in this gene have been documented in the majority of VHL kindreds (*3–5*). Tumor formation in the hereditary setting is associated with a loss of heterozygosity at the *VHL* locus with retention of the mutant *VHL* allele, thus depriving the cell of the normal VHL protein (pVHL). Most sporadic renal cell carcinomas and cerebellar hemangioblastomas are similarly deficient in wild-type pVHL, as the result of mutation or hypermethylation of the *VHL* gene (*6–10*). Reintroduction of the wild-type, but not mutant, *VHL* complementary DNA (cDNA) into renal carcinoma cells devoid of endogenous wild-type pVHL impairs the cells' ability to form tumors in nude mice, in keeping with the suspected role of pVHL as a tumor suppressor (*11*). To elucidate the biochemical mechanisms underlying tumor suppression by pVHL, we searched for cellular proteins that bound to wild-type pVHL, but not to tumor-derived pVHL mutants.

We stably transfected 786-O renal carcinoma cells, which lack endogenous wild-type pVHL, with expression plasmids encoding hemagglutinin (HA)-tagged pVHL(wt), HA-pVHL(1–115), or HA-pVHL(167W) (*11*, *12*). pVHL(1–115) lacks residues that are frequently altered by naturally occurring *VHL* mutations (*2–8*, *10*) and, unlike pVHL(wt), does not suppress tumor formation in vivo (*11*). pVHL(167W) is the predicted product of a mutant *VHL* allele that is common in VHL families (*3–5*).

The transfectants were metabolically labeled with ^{35}S-methionine, lysed, and immunoprecipitated with a monoclonal antibody against HA (anti-HA) or a control antibody (Fig. 1, A and B) (*11*, *13*). The immunoprecipitates were washed, fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), transferred to a polyvinylidene difluoride (PVDF) membrane, and detected by autoradiography. Two proteins of ~18 and ~14 kD (p18 and p14) coimmunoprecipitated with wt-pVHL but not with pVHL(1–115), and at diminished levels with pVHL(167W). The recovery of each of the exogenous pVHLs was confirmed by anti-HA immunoblot analysis (Fig. 1B). Neither p14 nor p18 was detected on the anti-HA immunoblot. Similar results were obtained when the cell extracts were immunoprecipitated with a monoclonal antibody against VHL (anti-VHL) (Fig. 1C) (*14*). Together, these immunoprecipitation studies suggested that p14 and p18 bound specifically to the COOH-terminal half of pVHL and that the binding of one or both proteins might be linked to tumor suppression by pVHL.

To map the interaction site on pVHL, we incubated ^{35}S-labeled 786-O cell extracts with glutathione Sepharose that had been preloaded with various glutathione-S-transferase (GST)–pVHL fusion proteins (*13*, *15*). The Sepharose was washed under stringent conditions and then boiled in the presence of SDS to elute the bound proteins. Analysis by SDS-PAGE showed that GST-pVHL(117–213), but not GST alone, bound to two radiolabeled proteins that comigrated with p14 and p18 (Fig. 2A). Partial proteolysis profiles confirmed that these two proteins were identical to p14 and p18 (*16*).

GST-pVHL(157–172) was the smallest GST-pVHL fusion protein to bind efficiently to p14 and p18 (Fig. 2B) (*16*). Furthermore, binding of p14 and p18 to GST-pVHL(117–213) was inhibited by a synthetic peptide (*17*) corresponding to pVHL(157–172), but not by a point-mutant derivative (157–172; 162F) corresponding to a naturally occurring *VHL* mutation (*3*) (Fig. 2C). These results suggest that pVHL(157–172) is sufficient to mediate binding to p14 and p18 in vitro. This region of pVHL is frequently altered by germ-line mutations in pVHL kindreds (*3–5*) (Fig. 3), strengthening the hypothesis that p14 or p18 binding is important in the tumor suppressor function of pVHL. VHL-associated proteins of similar molecular size have been identified indepen-

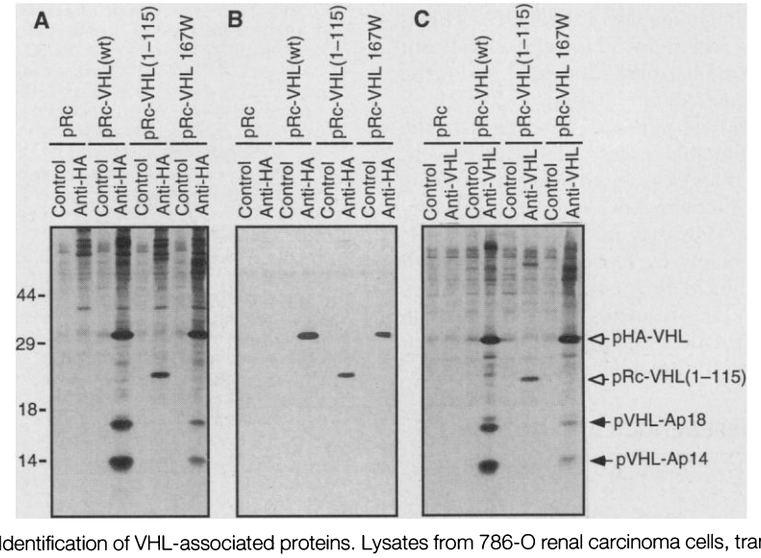

Fig. 1. Identification of VHL-associated proteins. Lysates from 786-O renal carcinoma cells, transfected with the indicated pVHL constructs, were immunoprecipitated with anti-HA (**A** and **B**) or with anti-VHL (**C**). Immunoprecipitates were analyzed by 15% SDS-PAGE and were detected by autoradiography (A and C) or by immunoblotting (B). The positions of the exogenous VHL proteins are indicated by the open arrows (endogenous pVHL is not detectable under these conditions) and the VHL-associated proteins by the closed arrows.

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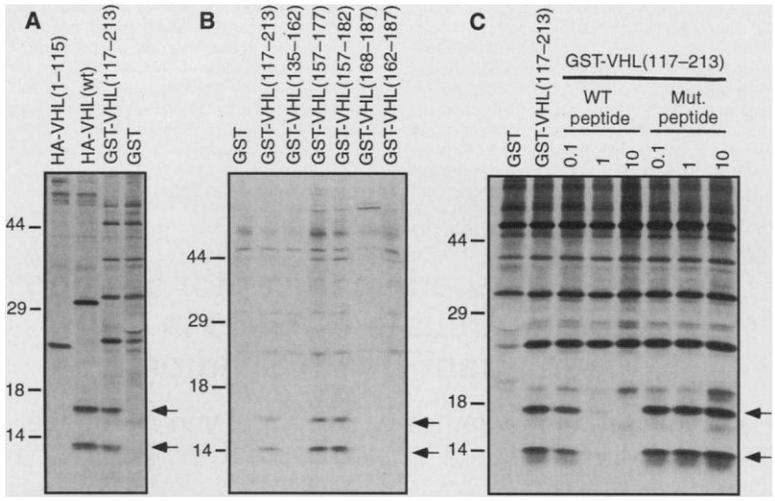
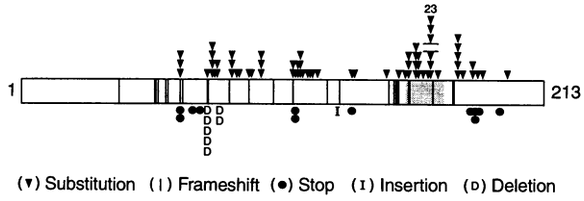


Fig. 2. Mapping the p14 and p18 binding site on pVHL. (**A**) 786-O cells producing HA-VHL(wt) or HA-VHL(1–115) were labeled with ^{35}S-methionine, lysed, and immunoprecipitated with anti-HA. Parental 786-O cells were similarly labeled, lysed, and incubated with glutathione Sepharose preloaded with GST-VHL(117–213) or GST alone. (**B** and **C**) 786-O cells were labeled, lysed, and incubated with glutathione Sepharose preloaded with the indicated GST-VHL fusion proteins. In (C), the indicated peptides (final concentration ~0.1, 1, or 10 μM) were added to the GST-VHL fusion protein before incubation with the radiolabeled extract. The wild-type peptide is TLKERCLQVVRSLVKP (underlined residues are sites of germ-line missense mutations) (*3–5*). The mutant peptide is TLKERFLQVVRSLVKP.

Fig. 3. Distribution of germ-line *VHL* mutations, compiled from (*3–5*). Twenty-three families had codon 167 abnormalities. The shaded region represents the binding site for Elongin B and C.



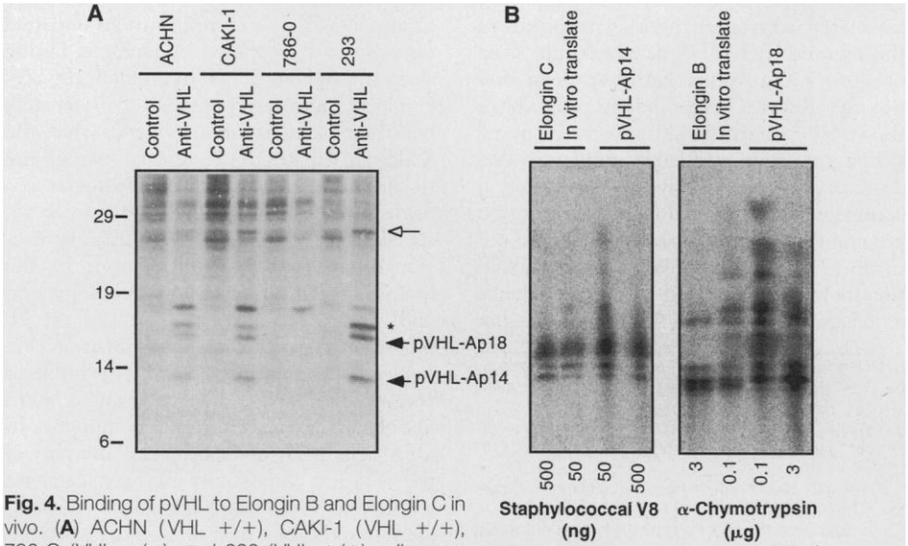


Fig. 4. Binding of pVHL to Elongin B and Elongin C in vivo. (**A**) ACHN (VHL +/+), CAKI-1 (VHL +/+), 786-O (VHL −/−), and 293 (VHL +/+) cells were labeled with ^{35}S-methionine, lysed, and immunoprecipitated with anti-VHL or a control antibody. The immunoprecipitates were washed under high-salt conditions, resolved by 15% SDS-PAGE, and transferred to PVDF. Bound proteins were detected by autoradiography. The identification of pVHL(wt) (open arrow) was confirmed by anti-pVHL immunoblot analysis. The ~19 kD protein immediately above p18 (asterisk) in the ACHN, CAKI-1, and 293 cell anti-VHL immunoprecipitates reacts with a polyclonal antibody to VHL (*16*). (**B**) Comparison of peptides generated by partial proteolysis of Elongin B and C, translated in vitro, with p18 and p14.

dently by other workers (*18*).

To identify the VHL-associated proteins, we pooled the anti-HA immunoprecipitates from 786-O cells producing HA-VHL(wt) and resolved the proteins by preparative SDS-PAGE. Anti-HA immunoprecipitates from 786-O cells producing HA-VHL(1–115) were similarly treated. The proteins were then transferred to a PVDF membrane and p18, identified by its comigration with ^{35}S-radiolabeled p18 and by its absence in the HA-VHL(1–115) immunoprecipitates, was stained with amido black and excised (*19*). We obtained five tryptic peptide sequences from p18, but these sequences showed no homology to proteins in the databases. At this stage, we learned from other investigators performing similar work (*20*) that these sequences were present in Elongin B, a subunit of the Elongin (sIII) transcription elongation complex (*21*). These investigators had also determined that p14 is Elongin C, another subunit of the Elongin (sIII) complex (*22, 23*).

To address whether Elongin B and Elongin C bind to pVHL under physiologic conditions, we prepared cell extracts from ^{35}S-radiolabeled ACHN (VHL +/+), Caki-1 (VHL +/+), and 786-O (VHL −/−) renal carcinoma cells (*11*), and from 293 (VHL +/+) human embryonic kidney cells. Anti-VHL immunoprecipitates from cell extracts containing wild-type pVHL contained two proteins that comigrated with Elongin B and C and that did not react directly with anti-VHL (Fig. 4A) (*16*). In contrast, these two proteins were not detected in immunoprecipitates from 786-O cell extracts, which lack wild-type pVHL, although these extracts did contain Elongin B and C (Fig. 2). The identity of the proteins detected in these native immunoprecipitates as Elongin B and C was confirmed by partial proteolysis (Fig. 4B).

The demonstration that Elongin B and Elongin C bind to pVHL in vivo, and the localization of the interaction site to a region of pVHL that is frequently altered by germ-line *VHL* mutations (*3–5*), strongly suggest that these two proteins contribute to the tumor suppression function of pVHL. It will be of interest to determine whether any genes involved in renal growth and development are regulated at the level of transcription elongation by Elongin (sIII) and pVHL.

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12. The Bam HI–Eco RI *VHL* cDNA fragment from pSP72-VHL(BZ3-4) (*11*) was ligated into pSG5 (Stratagene) to create pSG5-VHL(AB6-2). A C to T change was introduced (*24*) at *VHL* nucleotide position 712, to create pSG5-VHL(167W). A Bst EII–Eco RI *VHL* cDNA fragment from this plasmid was used to shuttle this mutation into pcDNA-HAVHL (*11*) to create pcDNA-HAVHL(167W). The Hind III–Xba I *VHL* cDNA fragment was ligated into pRc/CMV (Invitrogen) to create pRc-HAVHL(167W).
13. Immunoprecipitations and GST-binding experiments were performed as in (*25, 26*), except that the Sepharose was washed four times with NETN(960) [20 mM tris-HCl (pH 8.0), 960 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40] and once with NETN (no NaCl) before addition of sample buffer. For immunoprecipitations, pRC-HAVHL cell lines were labeled with 500 μCi/ml of Expre^{35}S^{35}S protein labeling mix (New England Nuclear) for 4 hours. The 293, ACHN, CAKI-1, and parental 786-O cells were labeled with 1 mCi/ml of this mix for 4 hours. Each immunoprecipitation reaction contained equivalent amounts of cell extract, derived from ~50% of the adherent cells in a nearly confluent 100-mm dish. For GST-binding experiments, parental 786-O cells were labeled with 500 μCi/ml of the labeling mix for 4 hours. Each reaction contained equivalent amounts of cell extract derived from approximately one-eighth of the adherent cells in a nearly confluent 100-mm dish.
14. BNR 5.12 mice were immunized with purified GST-pVHL(1–213) with Freund's complete and incomplete adjuvants, and monoclonal hybridoma cells were prepared. IG32 is an IgG1 that reacts with pVHL in both immunoblot and immunoprecipitation assays. Anti-HA (12CA5) (*27*) and control [anti-RBAP2 (LY11)] (*28*) antibodies have been described previously. Immunoprecipitations were performed with 50 μl of hybridoma supernatant without a secondary antibody. The anti-HA supernatant was diluted 1:50 (v/v) for immunoblot analysis.
15. Segments of the *VHL* cDNA were amplified by the polymerase chain reaction (PCR) with sense and antisense primers containing Bam HI and Eco RI sites, respectively. The products were digested and ligated into pGEX2TK (*28*).
16. A. Kibel, O. Iliopoulos, W. G. Kaelin Jr., unpublished observations.
17. VHL peptides were made with an Applied Biosystems (ABI) synthesizer and dissolved (5 mg/ml) in phosphate-buffered saline with 1 mM dithiothreitol.
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19. After SDS-PAGE, proteins were transferred to a PVDF membrane [Probott (ABI)] in 0.5× Towbin Buffer [12.5 mM tris-HCl (pH 8.0), 96 mM glycine, 10% methanol] and stained with amido black. The filter was destained and p18 was excised and digested with trypsin (*29, 30*). The peptides were separated by narrow-bore high-performance liquid chromatography (HPLC). Selected peptide fractions were subjected to automated Edman degradation on an ABI 477A or 494A (*30*).
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Decreased Muscarinic Receptor Binding in the Arcuate Nucleus in Sudden Infant Death Syndrome

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Muscarinic cholinergic activity in the human arcuate nucleus at the ventral medullary surface is postulated to be involved in cardiopulmonary control. A significant decrease in [^{3}H]quinuclidinyl benzilate binding to muscarinic receptors in the arcuate nucleus is now shown to occur in sudden infant death syndrome (SIDS) infants, compared to infants dying acutely of known causes. In infants with chronic oxygenation abnormalities, binding is low in other nuclei, as well as in the arcuate nucleus. The binding deficit in the arcuate nucleus of SIDS infants might contribute to a failure of responses to cardiopulmonary challenges during sleep.

Sudden infant death syndrome is the leading cause of postneonatal infant death in the United States. SIDS is defined as the sudden death of an infant under 1 year of age that remains unexplained after a thorough case investigation, including performance of a complete autopsy, examination of the death scene, and review of the clinical history (*1*). The syndrome is temporally associated with sleep periods, resulting in the premise that SIDS occurs during sleep or during transitions between sleep and waking. Recent epidemiologic data have shown a positive association between prone sleeping position and SIDS incidence, and decreased SIDS rates have been reported in countries that have initiated intervention programs advocating a supine sleeping position (*2*). The mechanism for the association between decreased prone prevalence and decreased risk for SIDS is unknown, but an interaction possibly exists between prone position and impaired cardioventilatory control, particularly impaired ventilatory and arousal responsiveness (*3*).

We hypothesize that SIDS, or a subset of SIDS, is associated with a deficiency in muscarinic cholinergic receptor (mAChR) binding in the arcuate nucleus of the ventral surface of the medulla (ventral medullary surface, VMS), which results in an impaired response to hypercarbia or asphyxia during sleep and sudden death. In animals, the precise location of the chemosensors is debated, but there is substantial evidence that the VMS participates in the ventilatory response to carbon dioxide (CO_2) and hydrogen ion, cardioventilatory coupling, and pressor responses (*4*). VMS cells are intimately associated with ventrolateral neurons of the brainstem that integrate ventilatory, pressor, and defense responses (*5, 6*). The precise function or functions of the human arcuate nucleus is speculative, as it is not possible to perform direct experiments ablating or stimulating arcuate neurons in living humans. In a previous study comparing the anatomy of the VMS in human infants and cats, we found that the arcuate nucleus is anatomically homologous to a distinct cell population in the cat VMS (*7*). We also reported a subset of SIDS infants with a severe developmental hypoplasia of the arcuate nucleus (*8*). The hypothesis that arcuate neurons are VMS cell populations involved in the response to hypercarbia in humans is supported by functional magnetic resonance imag-

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