Suppression of Growth of Renal Carcinoma Cells by the von Hippel-Lindau Tumor Suppressor Gene

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Abstract

Clear cell renal carcinomas are most frequently characterized by loss of function of both copies of the von Hippel-Lindau (VHL) disease gene, suggesting that the VHL gene product plays an important role in regulating renal cell proliferation. To directly assess the function of the VHL gene product, we transfected the wild-type VHL gene into two renal carcinoma cell lines that lacked normal expression of the gene. Expression of the wild-type VHL gene led to a dramatic suppression of growth in two renal carcinoma cell lines, A498 and UMRC6 in vitro, as measured by colony formation and direct cell counting. Transfection of a naturally occurring mutant VHL gene (nucleotide 713 G to A, Arg to Gin) did not lead to growth suppression of these renal carcinoma cells, nor did transfection of the wild-type VHL gene into two non-renal tumor cell lines that expressed the endogenous wild-type VHL gene. Expression constructs, which included the first ATG at nucleotide 214, were sufficient to produce the strongest growth suppression. These experiments provide direct evidence that the VHL gene product functions to suppress the growth of renal carcinoma cells and also provide a model for mapping the domains of the VHL protein important in suppressing tumor growth.

Introduction

von Hippel-Lindau disease is an inherited disorder characterized by a predisposition to develop tumors in the eyes, brain, spinal cord, kidney, adrenal gland, and pancreas (1). The VHL gene, located at 3p25-26, was recently cloned and shown to be mutated in the germ-line of affected family members, as well as in sporadic RCC (2). VHL-H1 started from nucleotide 214, the first in-frame ATG site within 3p25-26, was recently cloned and shown to be mutated in the germ-line of affected family members, as well as in sporadic RCC (2, 4, 5). The central issue surrounding the first ATG codon extending to the stop codon (without the VHL mutation/deletion was identified in either of them (5)).

Materials and Methods

VHL Gene Expression Constructs. Various VHL gene sequences were cloned into the mammalian expression vector pCR3 (Invitrogen, San Diego, CA) under the control of the constitutive cytomegalovirus promoter and containing the neomycin resistance gene as a selection marker. The construct VHL-gp7 started from nucleotide 190 with an introduced ATG codon and extended to nucleotide 852, the end of the ORF of the VHL cDNA sequence (2). VHL-H1 started from nucleotide 214, the first in-frame ATG site within the VHL cDNA sequence, and extended to nucleotide 852. VHL-H2 started from nucleotide 373, the second in-frame ATG site, and extended to nucleotide 852. A mutant derived from VHL-H1 was generated by making a G to A substitution at nucleotide 713, resulting in an amino acid change from Arg to Gin, and was designated as VHL-H1-713 mut. This 713 mutation is a naturally occurring hot spot mutation identified in many VHL families who were affected by RCC, pheochromocytoma, and other tumors (3, 5, 6). A control construct containing the VHL-H1 sequence in reverse orientation was designated as VHL-H1-antisense. All constructs were confirmed by sequencing.

Cell Lines. Human RCC lines A498 and UMRC6 were grown in medium RPMI 1640 with 10% FCS (GIBCO-BRL, Bethesda, MD) as described previously (15, 16). A498 has a 4-base deletion at nucleotides 639--642 of the VHL gene (5), and UMRC6 has a 10-base deletion at nucleotides 717--726 (5). In addition, chromosome 3p loss was reported in both cases (5)). A human ovarian cancer cell line SKOV3 and a human lung cancer cell line NCI-H23 were also used for this study. Both cell lines expressed the VHL gene, and no VHL mutation/deletion was identified in either of them.

Transfections. Transfections were performed using the cationic lipid, Lipofectamine (GIBCO-BRL), with cells at 60--70% confluence, as described by the manufacturer. Preliminary tests were performed to determine the amount of lipofectamine and DNA required to produce the highest transfection efficiency. For colony counting, A498 line in T-25 cm flasks, 20 /µg/3.5 µg (Lipofectamine/DNA), was used (Table 1). For A498 and UMRC6 lines in 3.5-cm plates, 6.0 /µg/1.0 µg (Lipofectamine/DNA) was used. For SKOV3 and NCI-H23 lines in 3.5-cm plates, 1.2 µg/0.2 µg (Lipofectamine/DNA) was used. Geneticin (G418; GIBCO-BRL) selection started 48 h after transfection. G418 concentrations for each cell line were: A498, 500 µg/ml; UMRC6, 250 µg/ml; SKOV3, 1000 µg/ml; and NCI-H23, 1000 µg/ml. G418-resistant colonies were counted after 18 days of G418 selection, whereas individual G418-resistant cells were counted after 8 days of G418 selection.

RNA Isolation and RT-PCR. Total RNA was isolated from cultured G418-resistant cells using RNAzol (Tel-Test, Friendswood, TX). Endogenous and exogenous VHL mRNA were amplified by differential RT-PCR. The first cDNA strand was synthesized using AMV reverse transcriptase (Promega, Madison, WI) for endogenous VHL, with the antisense primer 5'-TTG TAC GCC ACT GTA TAC TCT GAA AGA GCC CCA GTG CTG TAT C-3', and then amplified by PCR with the sense primer pCR351 (nucleotides 666--695, 5'-CAC ACT GCC AGT GTA TAC TCT GAA AGA GCC -3') and a nested antisense primer, 6b (nucleotides 902--930, 5'-TAC CAT CAA AAG CTA GGA TAC AAT-3'.

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2 The abbreviations used are: RCC, renal cell carcinoma; ORF, open reading frame.

3 M. H. Wei, unpublished data.
Table 1 Colony formation of A498 cells

<table>
<thead>
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<th>Transfected constructs</th>
<th>Exp.1</th>
<th>Exp.2</th>
<th>Exp.3</th>
<th>Exp.4</th>
<th>Exp.5</th>
<th>Exp.6</th>
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<tr>
<td>VHL-H1-antisense</td>
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<td>7,6</td>
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</tr>
<tr>
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<td>42</td>
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<tr>
<td>VHL-H1-713 mut</td>
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<td>45,41</td>
<td>145,102</td>
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</tr>
</tbody>
</table>

² Transfection procedures were described in "Materials and Methods." After 18 days of G418 selection, cells were stained by méthylène blue and counted under a microscope. A group of cells that numbered more than 20 was counted as a colon. The number (if colonies observed.) The VHL-gp7 group and VHL-H 1 group are statistically significantly different from the VHL-H1-antisense groups (P < 0.001, t test); the VHL-H2 group is also significantly different from VHL-H1-antisense group (P < 0.01). There is no difference between the VHL-gp7 and VHL-H1 groups.

For exogenous VHL, the first cDNA strand was synthesized with the antisense primer VHLBGHAS2 located in the vector’s BGH-3’ untranslated region (nucleotides 868-897 of pCR3, 5’-CAG TGG GAG TGG CAC CTT CCA GGG TCA AGO -3’) and then amplified by PCR with the sense primer pCR3Sl and a nested antisense primer VHLBGHAS1 from the vector (nucleotides 802-831 of pCR3, 5’-AAC TAG AAG OCA CAO T CG AGO CTG TAT ACT-3’). The use of these primers enabled us to specifically amplify and identify either the endogenous (274 bp) or the exogenous (306 bp) VHL transcripts.

Results

The cloned VHL cDNA contains two in-frame ATG codons with an ORF extending upstream from the first ATG, making the actual translation initiation site uncertain (2). To examine growth suppression activity, we prepared three wild-type VHL constructs with different NH2-termini, an antisense control, and a mutant VHL construct beginning at the first ATG codon and containing the 713 (A to G, Arg to Gin) hot spot mutation (Refs. 3 and 5; see "Materials and Methods"). To determine whether these constructs could drive synthesis of the VHL proteins, each of the expression vectors was transiently transfected into COS-7 cells and assayed by probing a Western blot with polyclonal antibodies against the VHL protein. Specific expression products of appropriate molecular weights were detected for each of the transfected constructs 48 h after transfection. For VHL-gp7, M3, 33,000, 31,000 and 17,000 expression products were seen. For VHL-H1 and VHL-H1-713 mut, M3, 31,000 and 17,000 proteins were detected, and for VHL-H2, a single band of M3, 17,000 protein was detected. Untransfected COS-7 cells and the transfected antisense construct did not produce detectable VHL proteins (data not shown).

We introduced the VHL expression constructs into two RCC lines lacking normal VHL expression to examine their effects on cell proliferation. Six experiments were performed in which the VHL expression constructs were transfected into the A498 line (Table 1). Following 18 days of G418 selection, cells were stained with methylene blue (Fisher Scientific, Pittsburgh, PA), and colonies containing more than 20 cells were counted (Table 1). Transfection of the VHL-gp7 and VHL-H1 constructs into A498 cells resulted in 5-20-fold fewer colonies than cells transfected with either the antisense control or VHL-H1-713 mut constructs. Transfection of the VHL-H2 construct into A498 cells resulted in only a 2-fold reduction of G418-resistant colonies.

Similar results were also observed in another RCC line, UMRC6. These cells however, grow in a diffuse manner, making it difficult to quantitate growth suppression by colony formation, and instead, after 8 days of G418 selection, cells were stained and photographed. Fig. 1 shows a magnified photograph of UMRC6 cells following transfection with each of the expression constructs and G418 selection. Again, both VHL-gp7 and VHL-H1 induced strong suppressive effects, while the VHL-H2 constructs induced a milder effect, when compared to the antisense control or VHL-H1-713 mut constructs. Transfection of the VHL-H2 construct into A498 cells resulted in only a 2-fold reduction of G418-resistant colonies.

Similar results were also observed in another RCC line, UMRC6. These cells however, grow in a diffuse manner, making it difficult to quantitate growth suppression by colony formation, and instead, after 8 days of G418 selection, cells were stained and photographed. Fig. 1 shows a magnified photograph of UMRC6 cells following transfection with each of the expression constructs and G418 selection. Again, both VHL-gp7 and VHL-H1 induced strong suppressive effects, while the VHL-H2 constructs induced a milder effect, when compared to the antisense control or the VHL-H1-713 mut. In contrast to the growth suppression observed with these RCC lines, the human ovarian carcinoma cell line SKOV3 did not respond to any of the transfected VHL constructs (Fig. 1).

To further quantitate the growth suppression induced by the VHL gene product, individual cells were counted following transfection and 8 days of G418 selection and compared with the VHL antisense control. As seen in Fig. 2, the growth of the A498 line was suppressed...
by VHL-gp7 and VHL-H1 by 75–79%, and the growth of UMRC6 line was suppressed by VHL-gp7 and VHL-H1 by 87–89%. The VHL-H2 construct suppressed the growth of UMRC6 by 69% but had little significant effect on A498. No growth suppression was observed with the VHL-H1-713 mut. In addition, neither the SKOV3 lines nor the NCI-H23 lines showed any significant growth response to the expression constructs.

Although the number of cells surviving after transfection with the wild-type VHL gene and selection with G418 was reduced compared to controls, we wished to determine whether these surviving cells expressed the exogenous, wild-type VHL gene. We measured the expression of exogenous and endogenous VHL genes by RT-PCR with primers designed to amplify either the exogenous or endogenous VHL transcripts. No exogenous VHL transcript was detected in 13 G418-resistant colonies isolated from the VHL-H1-transfected A498 line; in contrast, endogenous VHL transcript was detected in 12 of 13 G418-resistant colonies (data not shown). This experiment was repeated with a larger number of samples. No exogenous VHL transcripts were detected in a pool of 31 G418-resistant, VHL-H1-transfected A498 cells or a pool of 23 G418-resistant, VHL-gp7-transfected A498 cells. Endogenous VHL gene transcripts were detected in both pools. These results indicate that the A498 cells that survived after transfection with VHL-H1 or VHL-gp7 and G418 selection do not express the exogenous VHL gene. In contrast, the A498 cells transfected with VHL-H2 or VHL-H1-713 mut that survived after G418 selection expressed both exogenous and endogenous VHL transcripts (Fig. 3).

Discussion

Recent studies have shown that the VHL gene is mutated in approximately 60% of both inherited and sporadic clear cell renal carcinomas and cell lines (4, 5, 7), suggesting a primary role for this gene in the onset of kidney cancers. We have shown here that reintroducing the wild-type VHL gene into two RCC lines lacking this gene caused strong growth suppression. Introduction of a naturally occurring mutant VHL gene had no detectable effect on cell growth, nor did introduction of the wild-type gene into two non-renal carcinoma lines expressing wild-type VHL genes. Although in these experiments the cytomegalovirus promoter was used to drive VHL expression, the tissue-specific nature of the VHL-directed growth suppression observed suggests that these results are not due to VHL overexpression. Rather, they imply that the VHL gene product plays a critical role in a pathway regulating growth of kidney cells.

The published human VHL gene sequence suggested that an ORF beginning from nucleotide 1 and extending to nucleotide 852 may encode a protein of 284 amino acids. Moreover, it seemed possible that the cloning sequence may extend even further upstream in the 5' direction (2). However, the cloned cDNA contained two in-frame ATG codons at nucleotides 214 and 373, either of which (or both) could conceivably function as a translation initiation site. Recent studies on the VHL gene are providing evidence supporting the latter possibility: (a) all of the germline and somatic mutations identified are located downstream of nucleotide 214 (3–7); (b) the complete cDNA sequence of the mouse and rat VHL homologues show conservation with the human sequence at the first ATG site but not in the region 5' to this site (17, 18); and (c) the human VHL promoter has recently been identified and characterized and found to be located about 60 bases upstream from the first ATG codon (19). Our growth suppression studies provide clear evidence that translation from the first ATG at nucleotide 214 (VHL-H1) is sufficient to produce strong growth suppression in RCC. The inclusion of 24 nucleotides upstream to this site, found in the cDNA (VHL-gp7), did not enhance the growth suppression effects already produced by VHL-H1. Both
constructs induced similar growth suppression in RCC lines. We believe that the results of this study, combined with those mentioned above, strongly suggest that the full-length functional VHL gene product begins at the first ATG at nucleotide 214 and extends to the TAA stop codon at nucleotide 852. The biological significance of the translation product beginning with the second ATG is unclear. This construct (VHL-H2) induced a partial reduction in the growth of UMRC6 and A498 (Fig. 2). The transcripts of this gene construct were detectable in rapidly growing cells (Fig. 3).

The differential RT-PCR experiments analyzed the cells that survived after transfection with different recombiant constructs and G418 selection. The A498 cells transfected with VHL-gp7 and VHL-H1 showed only the endogenous VHL transcripts. The A498 cells transfected with the VHL-H2 or VHL-H1-713 mut constructs showed both the endogenous and exogenous VHL messages. These data suggest that wild-type VHL expression in these lines is incompatible with rapid cell growth.

A single nucleotide change (G to A) at nucleotide 713, resulting in a single amino acid change (Arg to Gin), eliminated the growth suppressive effect of the VHL gene. This naturally occurring mutation has been identified as a hot spot mutation, present in many unrelated VHL families, as well as sporadic RCC (3, 5, 6). Patients carrying this mutation in their germline are affected by pheochromocytoma and RCC, a VHL type 2 disease by previous classification (3). This mutation has been shown recently to inhibit the binding of the VHL protein to two low molecular weight cellular proteins (18). These two in vitro assays for VHL protein function will be valuable in studying the biological and biochemical aspects of the VHL gene function.

The ability to suppress the growth of tumor cells is the most important aspect in understanding the biological function of the VHL gene. Although the exact intracellular mechanism of this suppression still remains unclear, the loss of VHL-directed growth suppression, either by mutation or by transcriptional inactivation (20), seems to be a crucial event to the development of RCC. The demonstration of the growth suppressive effect of the VHL gene in cultured RCC cells provides direct evidence that the VHL gene plays an essential role in the tumorigenesis of RCC.

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References


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