von Hippel-Lindau Protein Promotes the Assembly of Actin and Vinculin and Inhibits Cell Motility

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ABSTRACT

Mutation of the von Hippel-Lindau (VHL) gene is responsible for familial and sporadic renal cell carcinomas as well as for cancers in many other organs. According to recent studies, the VHL protein (pVHL) is a multifunctional tumor suppressor protein associated with the inhibition of angiogenesis, cell cycle exit, fibronectin matrix assembly, and proteolysis. To examine whether pVHL affects other important cellular events such as morphogenesis, adhesion, cytoskeletal organization, or motility, we introduced the VHL gene into human kidney and lung cancer cells and compared its effects with those in parental cells. Compared with non-pVHL-expressing cells, the morphogenesis of pVHL-expressing cells was remarkably changed, with cells having many focal adhesions and stress fibers and a spreading morphology. The attachment ability of non-pVHL-expressing cells was significantly increased by expression of pVHL. Additional studies showed that vinculin was translocalized from the cytoplasm to the cell membrane by the pVHL expression, indicating induction of focal adhesion formation by pVHL. Furthermore, motility of the pVHL-expressing cells was significantly reduced compared with that of non-pVHL-expressing cells (P < 0.05). These results indicate that pVHL stabilized actin organization and inhibited cell motility through focal adhesion formation. Thus, pVHL plays a crucial role in cytoskeletal organization and motility and functions as a unique suppressor protein in malignant cells.

INTRODUCTION

VHL disease is a hereditary cancer syndrome characterized by the development of RCC, vascular tumors of the retina, and tumors of the central nervous system (1, 2). Mutation of the VHL tumor suppressor gene is observed in sporadic clear-cell renal carcinoma (3, 4), hemangioblastoma (5), and lung carcinoma (6, 7), and the VHL gene is therefore implicated in the development of these carcinomas. Several mechanisms underlying tumorigenesis in VHL-associated human neoplasms have been described. pVHL controls the gene expression of transforming growth factor-α (8), the GLUT-1 glucose transporter (9), and VEGF (10–14). It has been shown that VHL-associated tumors are highly vascularized and overproduce VEGF. Recently, it was demonstrated that hypoxia inducible factor, which is required for the transcription for VEGF, interacts with the β-domain of pVHL to cause its degradation (15, 16). Roles for pVHL in fibronectin matrix assembly and reactivity to hepatocyte growth factor have been reported (17). Fibronectin is an extracellular matrix component and functions as a tumor suppressor (18, 19). These studies have shown a specific relationship between pVHL and cytoskeleton-associated proteins. Thus, it is likely that pVHL regulates cytoskeletal organization. However, there is as yet no direct evidence for this.
morphology was assigned to one of three types (round, partially spreading, or fully spreading; 22). These experiments were performed in triplicate and the results presented as means ± SD. Statistical analysis was performed using Student’s t test or the Mann-Whitney U test.

The extent of cell spreading was examined by a cell area assay. The area of each cell was measured using NIH Image 1.62 (NIH, Bethesda, MD). One hundred cells were measured and the results presented as means ± SD. The significance of the differences was determined by Student’s t test.

Quantification of Focal Contact and Stress Fibers. Focal adhesion formation was measured by a modification of the method of Woods and Couchman (23). After cells were cultured for 48 h, they were harvested by trypsinization and inoculated onto chamber slides. Cells were allowed to adhere to a normal chamber glass for 3 h, processed for immunofluorescence, and then stained for vinculin or F-actin. Preparations were examined using a fluorescence microscope (Laser Scan Microscope LSM410 Invert; Carl Zeiss, Jena, Germany). In each microscope field at a magnification of ×200, the number of cells that had a typical pattern of vinculin-rich focal adhesions or stress fibers was counted and expressed as a proportion of the total number of cells in the field. Ten fields were examined, and data are presented as means ± SD (in triplicate assay from three independent experiments). The significance of the differences was analyzed by Student’s t test.

Membrane Fractionation. Cells were washed in ice-cold PBS and then scraped off with a rubber policeman into a buffer containing protease inhibitors [10 mM Tris-HCl (pH 7.4), 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM Na2VO4, 10 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, and 1 mM PMSF]. The cell suspensions were lysed on ice with 100 strokes of a homogenizer, and centrifuged at 1,200 g at 4°C for 30 min. The supernatants were centrifuged at 12,000 × g at 4°C for 30 min. The supernatants were then transferred onto polyvinylidene difluoride membranes. pVHL was detected using an anti-pVHL antibody. Cell lysates were subjected to SDS-PAGE. Proteins were then transferred onto polyvinylidene difluoride membranes. pVHL was detected using an anti-pVHL antibody.

Polyvinylidene difluoride membranes. PONASTERONE A (ecdysone)-inducible clones of 99VHL cells expressed levels of pVHL after treatment with ponasterone A. The maximum level of pVHL was examined by Western blotting analysis. Western Blotting Analysis. Cell lysates were prepared by standard methods (24). Briefly, the blot was incubated with primary antibody overnight at 4°C, and then incubated with horseradish peroxidase-conjugated antirabbit IgG for 1 h at room temperature. The primary antibodies used were mouse monoclonal antihuman VHL antibody (BD PharMingen, San Diego, CA) and mouse monoclonal anti-vinculin antibody (Sigma Chemical Co., St. Louis, MO). The experiments were performed in triplicate and the results presented as means ± SD from triplicate assays.

RESULTS
To determine the morphological alterations induced in cultured cancer cells by pVHL expression, pVHL-expressing cells (99VHL(+)) and UMRC-6-pVHL cells) were compared with their non-pVHL-expressing parental lines (99VHL(−) and UMRC-6 cells). As a control, cellular morphogenesis was also examined in H1299 cells with [H1299v(+) and without [H1299(−)] treatment with ponasterone A, which was used to induce the pVHL expression in the 99VHL cells.

pVHL Expression in H1299, 99VHL, and UMRC-6 Cells. The level of expression of pVHL was examined by Western blotting analysis. pVHL was not detected in H1299 and UMRC-6 cells. Ponasterone A (ecdysone)-inducible clones of 99VHL cells expressed pVHL after treatment with ponasterone A. The maximum level of...
expression of pVHL in 99VHL cells was obtained at 48 h after induction. This indicated that we needed to replenish the ponasterone A every 2 days to maintain adequate pVHL-expression. pVHL was also detected in UMRC-6-pVHL cells (Fig. 1).

**Cell Spreading Was Induced by pVHL Expression.** To investigate whether pVHL stimulates cell spreading, cultured cells on non-coated chamber glass were examined by phase-contrast microscopy. Spreading cells were frequently detected among pVHL-expressing cells (Fig. 2, a' and b'). In contrast, many rounded or only partially spreading cells were found among non-pVHL-expressing cells (Fig. 2, a and b). Change in cell shape in H1299 cells was not induced by treatment with ponasterone A (Fig. 2, c and c').

The morphological changes in the cultured cells were examined with immunofluorescence at various times (10 min to 3 h) after seeding (Fig. 3A). All of the incubated cells were stained with rhodamine-phalloidin. Morphological changes were unremarkable for 10–30 min after seeding (Fig. 3A; a, a', d, e, and f). The shape of all cells was round after 10 min in culture. At 1–3 h, the shape of both types of pVHL-expressing cells (99VHL(+) and UMRC-6-pVHL cells; Fig. 3A; b', c', e', and f') was significantly different from that of non-pVHL-expressing cells (99VHL(−) and UMRC-6 cells; Fig. 3A, b, c, e, and f). and the size of the pVHL-expressing cells was dramatically increased at 1 and 3 h compared with non-pVHL-expressing cells. After 3 h, a large number of pVHL-expressing cells had actin stress fibers (Fig. 3A, c' and f'). However, stress fibers were detected in only a few non-pVHL-expressing cells (Fig. 3A, c and f).

The extent of cell spreading was measured by a cell area assay at 3 h after plating (Table 1). The mean areas of 99VHL(−), 99VHL(+), UMRC-6, and UMRC-6-pVHL cells were 0.28 ± 0.075, 1.03 ± 0.74, 0.23 ± 0.12, and 0.36 ± 0.15 units (means ± SD), respectively. Areas of pVHL-expressing cells were significantly increased compared with those of non-pVHL-expressing cells (P < 0.001). In particular, the mean area of 99VHL(+) cells was three times larger than that of 99VHL(−) cells. No increase in cell area was induced by treatment with ponasterone A.

The number of spreading cells was measured by morphological assay. We categorized cells into three groups: round, partially spread, and fully spread. The proportion of fully spreading cells among 100 pVHL-expressing cells was not significantly different from that for non-pVHL-expressing cells at either 10 or 20 min after seeding. However, a significant increase in the proportion of partially spreading cells and decrease in that of rounding cells among pVHL-expressing cells was observed at 3 h compared with non-pVHL-expressing cells (Fig. 3B; c and f; P < 0.05). The proportion of fully spreading cells among pVHL-expressing cells was significantly increased compared with that for non-pVHL-expressing cells at 1 and 3 h (Fig. 3B; b, c, e, and f; P < 0.05). Cell spreading in H1299 cells was not affected by treatment with ponasterone A.

Because morphological change in cells usually depends on alterations of focal adhesion and actin stress fiber formation, the degree of cell attachment was estimated by a cell attachment assay. pVHL-expressing cells attached to glass more rapidly than non-pVHL-expressing cells, and the number of attached pVHL-expressing cells was significantly larger than the number of attached non-pVHL-expressing cells at all incubation times (10 min to 3 h; Fig. 3C, a and b; P < 0.05). Similar results were observed on the fibronectin- and quantification of cell spreading of cultured cells. Cells were incubated for 10 min (a and d), 1 h (b and e), and 3 h (c and f) after seeding. The results (in triplicate) are presented as the number of cells with a spread morphology in 100 total cells. *P < 0.05 (Student’s t test). **P < 0.05 (Mann-Whitney U test). C. Time course of attachment of cultured cells (10, 20, and 30 min and 1 and 3 h). The number of cells adhering to the chamber glass was measured in 10 randomly selected microscopic fields at a magnification of ×200. *, P < 0.05 (Student’s t test).

![Image](cancerres.aacrjournals.org)
type IV collagen-coated slide glasses: the number of attached cells was significantly increased in the pVHL-expressing lines, and their morphology was spread on such substrates (data not shown). The number of attached H1299 and H1299v cells was not increased by treatment with ponasterone A.

pVHL Promoted Vinculin Assembly on the Cell Membrane. To analyze the focal adhesion formation associated with cell spreading or attachment, vinculin expression was examined by Western blot analysis. All cell lines were found to express vinculin at high levels from 20 min to 3 h after seeding (Fig. 4, a). The data from 30 min and 1 h are not shown; however, the amount of vinculin did not change during this time course. To determine the amount of vinculin contained in focal adhesions, membrane fraction lysates were obtained from both pVHL-expressing and nonexpressing cells. Vinculin in the membrane fraction was remarkably higher in the 99VHL(+) cells than in 99VHL(−) cells, suggesting that pVHL contributed to the assembly of vinculin in the membrane rather than to the induction of its expression (Fig. 4A, b). Similarly, vinculin was detected more in the membrane fraction of UMRC-6-pVHL cells than of UMRC-6 cells (Fig. 4A, c). For quantification of the amount of vinculin, densitometric analyses were performed, and relative amounts of vinculin were determined. The amount of vinculin in the membrane fractions increased with time in pVHL-expressing cells, and the differences in vinculin content between pVHL-expressing cells and non-pVHL-expressing cells were particularly marked: at 3 h incubation, a 3-fold difference was observed between 99VHL(+) and 99VHL(−) cells (Fig. 4B, a; P < 0.05), and a 2-fold increase was seen in UMRC-6-pVHL cells compared with UMRC-6 cells (Fig. 4B, b; P < 0.05).

pVHL Increases the Formation of Focal Adhesions and Intracellular Stress Fibers. Vinculin in focal adhesions was examined by immunofluorescence staining in cells cultured for 3 h. The number of cells that had a typical pattern of vinculin-rich focal adhesions was counted (Fig. 5a). The proportion of cells displaying the typical vinculin-rich pattern was significantly increased in pVHL-expressing cells: 99VHL(+) and UMRC-6-pVHL cells with typical vinculin patterns were encountered five and two times more frequently than those in 99VHL(−) and UMRC-6 cells, respectively (Fig. 5a; P < 0.05). Cells with a typical pattern of actin stress fiber formation were investigated by staining for F-actin (Fig. 5b). pVHL-expressing cells with a rich stress fiber pattern were seen in significantly higher numbers than non-pVHL-expressing cells with the typical pattern (Fig. 5b; P < 0.05).

pVHL Inhibited Cell Motility. The effect of pVHL on cell motility was measured by Boyden chamber assay. The motility activity of the cells was significantly reduced by pVHL expression (Fig. 6; P < 0.05). In particular, the motility of UMRC-6-pVHL cells was one-fourth that of UMRC-6 cells. However, the number of attached cells on type IV-collagen was increased by pVHL expression as mentioned above. These results suggest that pVHL caused inhibition of cell motility through the stabilization of actin stress fibers, which in turn occurred by way of vinculin localized in the cell membrane.

### DISCUSSION

In this study, we have demonstrated that pVHL promotes the well-organized distribution of actin bundles, which results in changes in cell shape and motility. We quantified the degree of morphological change during short incubations using the parameters of cell shape, cell area, and cell attachment. A rounding or partially spreading cell morphology indicates high levels of motility, whereas a fully spreading cell morphology suggests an increase of cell attachment ability. The number of fully spreading cells was significantly increased by pVHL-expression, as was cell area. In contrast, non-pVHL-expressing cells hardly attached or spread on glass, type IV collagen, or fibronectin. pVHL-expressing cells had therefore acquired an increased ability to undergo attachment and to spread, both of which depend on focal adhesion formation. pVHL significantly increased the translocation of vinculin, a component of focal adhesions, to the cell membrane, and promoted stress fiber formation, while reducing cell motility. These results strongly suggest that expression of pVHL results in a stabilization of cytoskeletal organization that inhibits cell motility.

Table 1. Cell areas

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Mean ± SD units</th>
</tr>
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<tbody>
<tr>
<td>99VHL(+)</td>
<td>0.28 ± 0.15*</td>
</tr>
<tr>
<td>99VHL(−)</td>
<td>1.03 ± 0.74</td>
</tr>
<tr>
<td>UMRC-6</td>
<td>0.23 ± 0.12*</td>
</tr>
<tr>
<td>UMRC-6-pVHL</td>
<td>0.36 ± 0.15</td>
</tr>
<tr>
<td>H1299(−)</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>H1299(+)</td>
<td>0.28 ± 0.09</td>
</tr>
</tbody>
</table>

* P < 0.001 (Student’s t test).

[Fig. 4.](#) Western blot analysis of vinculin in cultured cells. pVHL was detected at 20 min and 3 h after seeding in whole cell homogenates (a) and membrane fractions (b) of 99VHL cells, and in UMRC-6 and UMRC-6-pVHL cells (c). The whole-cell fraction of H1299 cells was used as a loading control for vinculin in b and c. Preparations were separated by SDS-PAGE, and vinculin was detected using an anti-vinculin antibody. B, densitometric analysis. *, P < 0.05 (Student’s t test).
Cell subsequent to the initial attachment of cells to substrates involves a rearrangement of cytoskeletal and membrane components that can result in the formation of focal adhesions and the reorganization of stress fibers (27, 28). The well-characterized cytoplasmic proteins vinculin, talin, and α-actinin accumulate at focal contacts and form part of adhesion plaques (29). We showed that pVHL expression stimulated the translocation of vinculin from the cytoplasm to focal adhesions on the cell membrane. Ben-Ze’ev et al. (30) similarly reported that induction of vinculin in 3T3 fibroblasts stimulated by serum and several growth factors caused the formation of a dotted distribution of adhesion plaques. Because loss of focal adhesion is thought to be able to facilitate cell migration, it was suggested that pVHL inhibits cell migration through vinculin translocation to focal adhesions.

Focal adhesion formation is believed to be regulated by many proteins linked to the small GTP-binding proteins Rho, Rac, and cdc42 (31–33). Although stress fiber formation is observed along with focal adhesion, it is now believed that stress fiber and focal adhesion formation are controlled by independent cascades. Rho mediates actin stress fiber formation, whereas Rac and cdc42 induce focal adhesion with lamellipodia and filopodia, respectively. Because the morphological changes induced by pVHL expression are characterized by full spreading with an abundance of focal adhesions and stress fibers, pVHL may be involved in the Rho activation cascades.

We have already reported the binding of pVHL to PKC isoforms (34). Vinculin phosphorylation by PKC was found to be a crucial step in the assembly of epithelial junctional complexes (35). PKC is a link to the PTK-mediated signaling pathways and regulates focal adhesion formation (20). pVHL also binds to fibronectin and promotes extracellular fibronectin matrix assembly (17, 18). These studies indicate that pVHL plays an important role in both cell adhesion to a substrate and cell-cell adhesion. Our data suggest that pVHL regulates not only the assembly of a fibronectin matrix, but also the adhesion to the extracellular matrix through focal adhesion formation. Recently, the PTK family associated with focal adhesion has been observed to react to growth factors and to be involved in motility (20). FAK have been
shown to be an important mediator between growth factor receptors and integrin signaling pathways (36).

Cell motility activity is regulated by a collaboration between Rho and Rab family members which control the assembly and disassembly of actin and focal adhesion (37). Actin polymerization and depolymerization is regulated by many cytoskeleton-associated proteins. Our results suggest that a shift to actin polymerization occurs as a result of pVHL expression. Thus, pVHL causes inhibition of cell motility through a stabilization of actin stress fibers mediated by vinculin.

In summary, we have demonstrated significant effects of pVHL on the stabilization of actin assembly, focal adhesion formation, and cell motility activity. Although recent research on pVHL has focused on hypoxia inducible factor -mediated VEGF up-regulation, our observations suggest that the processes of growth factor-mediated Rho-PTK signaling and actin fiber reorganization may also be modulated by pVHL. pVHL is an important suppressor factor in the multistep process of invasion or metastasis of cancer. Our results suggest that it acts by limiting the ability of cells to move, through the formation of focal adhesions, and by encouraging the adhesion of a tissue to its substrate. From these findings, we conclude that pVHL is a multifunctional and unique tumor suppressor protein.

REFERENCES

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