Inhibition of Insulin-like Growth Factor-I-mediated Cell Signaling by the von Hippel-Lindau Gene Product in Renal Cancer*

Insulin-like growth factor-I (IGF-I)-mediated signaling is thought to be involved in the regulation of multiple cellular functions in different tumors including renal cell carcinoma (RCC). Blocking IGF-I signaling by any of the several strategies abolishes or delays the progression of a variety of tumors in animal models. Herein, we demonstrate that in RCC cell lines, IGF-I-mediated signaling is found to be inhibited in the presence of wild type von Hippel-Lindau (VHL) tumor suppressor gene. Moreover, molecular modeling and biochemical approaches have revealed that β-domain of the VHL gene product by interacting directly with protein kinase Cδ inhibits its association with IGF-IR for downstream signaling. We also demonstrated that RCC has IGF-I-mediated invasive activity where protein kinase Cδ is an important downstream molecule, and this invasiveness can be blocked by wild type VHL. These experiments thus elucidate a novel tumor suppressor function of VHL with its unique kinase inhibitory domain.

Recent studies on renal cell carcinoma (RCC) have mainly focused on identifying molecular prognostic factors, including growth factors (1, 2), oncogenes (3, 4), cell adhesion molecules (5, 6), and proteases (7, 8), that may provide insights into the mechanism of the cancer and its subsequent treatment. Insulin-like growth factors (IGFs) are candidate proliferation markers in renal cell carcinoma because of their overall importance in embryonic and somatic growth, differentiation, and tumorigenesis (9, 10) and because of their particular importance in renal growth and development (11, 12). Although IGFs are mainly secreted in the liver, its autocrine and paracrine activity is observed in most tissues (13, 14).

Most of the biological activities of IGFs are mediated through insulin-like growth factor-I receptor (IGF-IR). The ligand IGF-I triggers an intrinsic tyrosine kinase activity in the receptor, resulting in its autophosphorylation and the presentation of its substrate binding sites (15). Substrates containing either the Src homology domain or phosphotyrosine-binding domain can interact with IGF-IR for various downstream signal transduction cascades that ultimately lead to cell proliferation, differentiation, antiapoptosis, and, in pathological conditions, tumor development (16, 17). Embryonic fibroblasts established from IGF-IR−/− mice resistant to transformation induced by different oncogenes, growth factor receptors, and viral proteins that can be reversed by reconstitution with wild type IGF-IR (17). Also blocking of IGF-IR signaling by any of the several strategies (antisense, dominant negative, or neutralizing antibody against IGF-IR) abolishes or delays the progression of a variety of tumors in animal models (18–20). In addition to its tumorigenic activity, IGF-I also has a role in tumor angiogenesis. Recently, it has been demonstrated that IGF-I promotes the expression of the potent angiogenic factor, vascular permeability factor/vascular endothelial growth factor in colon cancer (21). Thus any factor that can block IGF-I-mediated downstream signaling can potentially inhibit both tumorigenesis and angiogenesis. Recent studies have shown that protein kinase Cδ (PKCδ) plays an important role in IGF-IR-mediated cell proliferation and transformation (22). It associates with IGF-IR and gets tyrosine phosphorylated, resulting in increased activity. It has also been demonstrated that the ATP-binding mutant of PKCδ can inhibit the transforming ability of IGF-I.

The von Hippel-Lindau (VHL) gene on chromosome 3p25–26 encodes a tumor suppressor protein of 30 kDa that has multiple functions, such as down-regulating hypoxia-inducible genes (e.g. angiogenic factor-like vascular endothelial growth factor and hypoxia-inducible factor), regulating cellular ubiquitination machinery and p27 proteins (23–26). Germ line mutations in human VHL gene lead to various VHL-associated diseases that predispose to different kinds of tumors such as renal cell carcinomas, pheochromocytoma, hemangioblastoma, and pancreatic cancer (27–29). The critical role of VHL in clear cell RCC has been confirmed by demonstrating that more than 70% of the sporadic RCCs have biallelic VHL mutations (30). Present studies on VHL indicated that wt-VHL binds with hCUL2-elongin B/C complex (CBC complex), forming CBC-VHL, which may function as E3-type ubiquitin ligase because of their structural similarity with its yeast homologue SCF (31–34). Thus VHL helps ubiquitination of cellular proteins that are ultimately degraded by proteasome 26 S complex. Our previous studies have shown that the VHL gene product can directly interact with PKCδ (35), but here we did not find any decrease in PKCδ level in RCC cells that overexpress wt-VHL. Because PKCδ is an important downstream intermediary of IGF-I signaling cascade (22), we therefore hypothesized that apart from

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‡ The abbreviations used are: RCC, renal cell carcinoma; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; PKC, protein kinase C; VHL, von Hippel-Lindau; wt, wild type; GST, glutathione S-transferase; PKA, cAMP-dependent protein kinase.

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MATERIALS AND METHODS

Cell Culture—Human renal carcinoma cell line (786-O) was maintained in Dulbecco's modified Eagle's medium with 10% foetal bovine serum (hyClone Laboratories). 786-O clonal cell lines stably transfected with either pRC (Neo cells; cells contain empty vector with neo cassette), pRCHAVHL (VHL cells; cells expressing wt-VHL) pRC-HAVHL- (1–115), pCMVFLAGVHL, pCMVFLAGVHL (1–145) were grown in complete medium supplemented with G418 (1 mg/ml) (25). 786-O neo, 786-O HAVHL, and 786-O HAVHL- (1–115) were gifts from W. G. Kaelin.

Immunoprecipitations and Western Blot Analysis—Cells were washed twice with 10 ml of cold phosphate-buffered saline, lysed with ice-cold lysis buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM Na3VO4, 2 mM EDTA, 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 in antibody excess, using 0.5 mg of total protein either with affinity-purified rabbit polyclonal antibody (1 μg of IgG) against PKCδ (Santa Cruz Biotechnology, Inc.) or with mouse monoclonal antibody (1 μg) directed against IGF-IRα subunit (Santa Cruz Biotechnology, Inc.). Immunocomplexes were captured with protein A-agarose beads (Bio-Rad). After three washes with cell lysis buffer, bead-bound proteins were separated by SDS-polyacrylamide gel electrophoresis.

Size-separated proteins were transferred (Trans-Blot SD; Bio-Rad) to a nylon membrane (Immobilon-P; Millipore). For immunodetection, membranes were blocked in washing buffer (phosphate-buffered saline and 0.1% Tween 20) for 1 h at room temperature. Membranes were then incubated with the primary antibody, incubated for 1 h at room temperature with the secondary antibodies (1:1000 dilution; stock concentration, 0.5 mg/ml) and washed again with washing buffer. Beads were then mixed with purified recombinant human PKCδ protein (Panvans) (50 ng) and bovine serum albumin (5 μg/ml) as a carrier in binding buffer. Bound proteins were then resolved in SDS-polyacrylamide gel electrophoresis and subjected to Western blotting.

LIGHT WAVEFLY FLYER

In Vivo Binding Assay—Glutathione S-transferase-VHL protein fusion proteins under the control of a lac operator (pGEX plasmids; Amersham Pharmacia Biotech) were expressed in Escherichia coli. Cells pellets were briefly sonicated in binding buffer (20 mM Tris-HCl, pH 7.5, 150 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40). Cleared cell lysates were mixed with glutathione-Sepharose beads (Amersham Pharmacia Bio tech) and rocked for 1 h at 4 °C. The beads were washed three times with binding buffer. Beads were then mixed with purified recombinant human PKCδ protein (Panvans) (50 ng) and bovine serum albumin (5 μg/ml) as a carrier in binding buffer. Bound proteins were then resolved in SDS-polyacrylamide gel electrophoresis and subjected to Western blotting against anti-PKCδ antibody.

RESULTS AND DISCUSSION

By co-immunoprecipitation experiments using antibodies against PKCδ, we found that IGF-IR and PKCδ are in the same immunocomplex in two different 786-O clonal cell lines (Neo cells; cells contain empty vector with neo cassette, and without any wt-VHL; and ΔVHL cells; cells express VHL-(1–115)) (25). Interestingly, in a 786-O clonal cell line stably transfected with pRC-HAVHL (wt-VHL cells; cells express wild type VHL), this immunocomplex was undetectable (Fig. 1A). These results suggest that in wt-VHL-containing cells, much less IGF-IR is associated with PKCδ as compared with renal cancer cells where wt-VHL is missing. Through immunoblot analysis it appears that the IGF-IR expression level is lower in VHL containing cell as compared with that of other cell lines. But densitometric scanning of the IGF-IR protein level detected in immunoblot suggests that the ratio of IGF-IR expressed in VHL containing cell line to other cell lines (which is 0.7) is significantly higher than that of IGF-IR associated with PKCδ (the ratio is 0.067) in these two cell lines. These data also might imply that VHL can regulate the protein expression level of IGF-IR to some extent.

We did not find any difference in the protein level of PKCδ in any of these cell lines, which also indicate that the direct interaction of PKCδ and VHL does not lead to ubiquitination and subsequent degradation of PKCδ (Fig. 1A). The immunoblot with anti-VHL antibody (Fig. 1A) shows the relative expression level of the wild type and ΔVHL in their respective cell lines.

To investigate whether the inhibition of IGF-IR signaling can block renal cell proliferation, we utilized IGF-IR antibodies that specifically inhibit IGF-IR-mediated cell proliferation. Fig. 1B shows that the blocking antibody indeed inhibited IGF-I dependent cell proliferation in a dose-dependent manner in an
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RCC cell line. The [3H]thymidine incorporation in control (in cpm) was 4707 ± 388; whereas in the presence of 2 μg/ml and 10 μg/ml of anti-IGF-IR antibody, the values were 2726 ± 368 and 448 ± 85, respectively. Therefore, the renal cancer cells have a dependence on IGF-I for their growth, whereas the presence of wt-VHL might be playing an inhibitory role on IGF-IR-mediated cell proliferation. Of importance, it has been described previously that wt-VHL has growth suppressive ability of renal carcinoma cell lines in addition to its antiangiogenic effect (36, 37). Therefore, our results suggest that wt-VHL prevents the association of IGF-IR with PKCδ and thereby inhibits the IGF-IR-mediated signaling pathways.

To determine the region of VHL that binds to PKCδ, we used different deletion mutants of GST-fused VHL. It was found that both the N-terminal mutant of VHL, i.e. VHL-(1–115) (ΔVHL) and VHL-(1–122) can bind PKCδ along with wild type (wt-VHL) (Fig. 2A), whereas no interaction was detected with VHL-(1–89), VHL-(120–213) or VHL-(157–189), the latter known to be the elongin-binding domain from the crystal structure (38). So it can be concluded from this experiment that an N-terminal domain of VHL indeed has an important role in the inhibition of PKCδ binding and inhibition of PKCδ. On the other hand, it is difficult to understand why the VHL-(1–115) mutant shows binding affinity and kinase inhibitory activity to PKCδ, whereas 786-O cells containing this mutant did not show any reduction in complex formation of IGF-IR with PKCδ.

Molecular modeling studies were carried out to understand the mechanism of inhibition of PKCδ by VHL at the molecular level. Recently, the x-ray crystal structure of VHL in association with Elongin B and C was determined (38). The structure of VHL reveals an N-terminal domain at residues 63–154 with a predominantly β-structure and an α-helical domain to residue 204. The interactions of VHL in the complex are through the α-domain, which packs against the helices of Elongin C. The β-domain, on the other hand, makes very little contact with the Elongins. We propose that the segment of VHL that interacts with PKC-δ (residues 105–122) lies in this domain, and we further present a model for this interaction.

Although the three-dimensional structure of PKCδ is not known, a molecular model for the catalytic domain can be built by homology modeling with PKA. A sequence alignment of the catalytic domains of PKA and four representative members of the PKC family (α, γ, δ, and ζ) (Table II) shows their high homology particularly within the secondary structural regions. We used the program LOOK from Molecular Applications Group on a Silicon Graphics Octane workstation to obtain a preliminary model for PKCδ based on the structure of PAK obtained from the Protein Data Bank (code 1atp). Because of high sequence homology between catalytic domains of PKCδ and PKA (residue identity of 30%), the backbone of the modeled structure follows that of PKA very closely with a low RMS deviation of the Ca atoms (0.34).

The x-ray crystal structure of VHL was obtained from the Protein Data Bank (code 1vcb). A scan of the VHL sequence against the PROSITE data base (41) identified a 9-residue segment (105TGRRIHSYR113) closely resembling the consensus names are known to be involved in the phosphorylation of Ser or Thr residues of a large number of proteins. In PKA the catalytic domain consists of two lobes that are involved in binding to MgATP and the peptide substrate, respectively (39). A comparison of the phosphorylation sites has shown the importance of basic residues, in particular Arg, flanking the phosphorylated residue. The catalytic activity of protein kinases can be inhibited by protein kinase inhibitors as well as by the regulatory subunit of the kinases themselves. Two different classes of regulatory subunits exist: the R1-subunit class, which is not phosphorylated, and the RII-subunit class, which is autophosphorylated. A comparison of the sequence of the R-subunit of the kinases shows the presence of a segment resembling the substrate but differing in a few residues that are crucial for inhibition (40).

We were unable to detect any phosphorylation of wt-VHL or its mutants by PKCδ (data not shown), suggesting that VHL is not a substrate for PKCδ. The high degree of amino acid sequence similarity of the region (106–112) of VHL with the naturally occurring protein kinase inhibitors, however, suggests that this region has a good potential for being a kinase inhibitor of PKCδ (Table I). To investigate the inhibitory role of VHL on PKCδ, we performed kinase assays for PKCδ using histone as a substrate in the presence of wt-VHL or its different deletion mutants. The inhibitory activity closely followed the results obtained with the PKCδ binding assay; wt-VHL, VHL-(1–115) and VHL-(1–122) inhibited the kinase activity of PKCδ, whereas VHL-(1–89), VHL-(120–213) and the elongin binding domain of VHL, VHL-(157–189), did not show any inhibitory activity (Fig. 2B). These results again suggest that the N-terminal domain of VHL indeed has an important role in the binding and inhibition of PKCδ. The other hand, it is difficult to understand why the VHL-(1–115) mutant shows binding affinity and kinase inhibitory activity to PKCδ, whereas 786-O cells containing this mutant did not show any reduction in complex formation of IGF-IR with PKCδ.

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Fig. 2. Biochemical and molecular modeling analysis of PKCδ and VHL interaction. A, immunoblot (IB) analysis with anti-PKCδ antibody after incubating different GST-fused VHL mutants with recombinant human PKCδ (50 ng). One-twentieth the amount of PKCδ used in the binding buffer was included for comparison (X0.2). The lower panel indicates the Ponceau staining of the blot showing the expression level of different GST-fused VHL mutant proteins. Fold activation of each experiment was determined by comparison with \([γ^{32}P]ATP \) incorporation in the presence of histone alone. Results are averages of three independent experiments. All experiments are done at least thrice. C, molecular model of the interaction of VHL with PKC. Space-filling model of the complex with Elongin B and Elongin C using WebLab Viewer Lite, v3.20. (Molecular Simulations, Inc., San Diego, CA). Blue, PKCδ; white, VHL; green, Elongin B; orange, Elongin C. D, environment of cofactor MgATP. E, ionic interactions that stabilize the complex. C and D were drawn using Molscript (43).
sequence of the phosphorylation site. As shown in Table I, the tetrapeptide TGRR is also contained in the inhibitor segment that was co-crystallized with PKA. The conformation of the tetrapeptide is very similar in the two structures: a β-turn followed by an extended strand. Our strategy for initial positioning of VHL within the active site of PKC is to align the tetrapeptide in VHL with the corresponding segment in PKC substrate.

The docking of VHL with PKC was carried out using QUANTA (Molecular Simulations, Inc., San Diego, CA) on a Silicon Graphics Indigo2 workstation. Energy minimization of each molecule was carried out independently using CHARMM. The VHL molecule was manually moved, and the atoms of the tetrapeptide were superposed on the corresponding atoms of the PKC substrate. Iterative cycles of energy minimization and manual rotation of the side chain torsion angles were then performed to optimize the interactions between side chains of the two molecules.

The final model of VHL (Fig. 2, C and D) shows the N-terminal β-sandwich of VHL fitting snugly into the cleft between the two lobes of PKC. The major interactions that stabilize the complex involve the two Arg side chains of the consensus phosphorylation site sequence, with Arg107 making ionic contact with the carboxyl side chains of Asp384, Asp427, and Asp434, whereas Arg108 is in close proximity to Asp423, Asp427, Asp463, and Glu490 (Fig. 2E). In the crystal structure of PKA, the Ala residue at the phosphorylation (P) position of the inhibitor peptide is close enough to the MgATP molecule so that an addition of a hydroxyl group (as in Ser or Thr) would enable it to interact with the γ-phosphate of ATP. In VHL the P position is occupied by His110, whose polar side chain can interact favorably with the phosphate, adding stability to the complex (Fig. 2D). Ser111, on the other hand, points away from the complex and inhibits its catalytic activity. This tight binding is necessary for its inhibitory activity in vitro. This observation can be explained by the molecular modeling data, which shows that the region 107–111 of VHL is the potential binding site to the catalytic domain of PKC. But we also found that VHL-(1–115) cannot block the association of PKC and IGF-IR in the 786-O cell line. To answer this anomaly we have synthesized two peptides derived from VHL amino acid sequence: (VHL-(100–118)) CTLPPGTGRRIHSYGHLWL and (VHL-(105–123)) CTGRRRIHSYRGHLWLFRDG (additional Cys in the peptides are for coupling purpose with iodoacetyl matrix) and tested their kinase inhibitory function. Both the peptides were found to inhibit the kinase activity of PKC (Fig. 3A). Interestingly, VHL-(105–123) showed much greater kinase inhibitory activity compared with VHL-(100–118), although both contain the segment that is essential for inhibition (106–111), suggesting that the region 115–123 has a distinct role in binding and down-regulating the catalytic activity of PKC. As a control experiment, an unrelated 30-amino acid peptide with no sequence similarity (CGKPPAPKPSKPKNIKTRSAQKRV) to the two VHL peptides showed very little inhibition at higher concentrations (Fig. 3A).

To further confirm the role of the segment 115–123 in substantially increasing the affinity of VHL for PKC, we performed in gel competitive binding assays. PKC was complexed with each of the VHL fragments 100–118 and 105–123, immobilized to a iodoacetyl bead (Fig. 3B), and eluted out using reciprocal soluble peptides. Fig. 3C shows that PKC can be eluted out only from the complex with VHL-(100–118) immo- bilized to iodoacetyl bead by free VHL-(105–123) peptide. Our studies therefore show the importance of the segment (115–123), and the molecular modeling results suggest that it binds to a region of PKC different from the catalytic domain. Interestingly, the VHL 96–122 region is also sufficient for the interaction of VHL with the Sp1 zinc finger domain, and, similarly, amino acids 112–122 are the most critical for binding (47). Because the regulatory domain of PKC contains cysteine-rich zinc-binding regions, it is possible that VHL binds to this region through the segment 115–123. Because this cysteine-rich domain of PKC is only few amino acids apart from the autoregulatory domain, conformationally this domain of PKC should be very close to the catalytic subunit of the enzyme (42). Therefore, we hypothesize that if VHL can bind to these two regions of PKC simultaneously, forming a very stable complex and inhibiting its catalytic activity, this tight binding is necessary for its inhibitory activity in vivo because it can compete only with the high affinity substrates for PKC. Thus, VHL-(1–115) can bind PKC in vitro, but it is competed out with other molecules in the cell because of its lower affinity, as in this case by IGF-IR. To confirm our hypothesis, we generated
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FIG. 3. Comparison of two different VHL-derived peptides to the abilities of kinase inhibitory activity and making stable complexes with PKCδ. A, kinase inhibition assays were performed of PKCδ using histone as a substrate in the presence of two different peptides derived from the VHL sequence, VHL(100–118; CTLPPGTTGRRHIHSYRGHLWL) and VHL(105–123; CTGRRHIHSYRGH LWL FRDAG). A random 30-amino acid unrelated peptide (CGKPPAPKPASPPKNIKTRSAQRTPNPKRV) was used as control. Fold activation of each experiment was determined by comparison with [γ-32P]ATP incorporation in presence of histone alone. Results are averages of three independent experiments. B, binding affinity of recombinant PKCδ (50 ng) with the VHL peptides (in A) immobilized on iodoacetyl beads. The immunoblot analysis shows the amount of PKCδ present on the bead alone, bead covalently linked to VHL-(100–118), and bead linked to VHL-(105–123). Total input shows recombinant human PKCδ used as positive control. C, the lanes show the immunoblot of the eluants of PKCδ bound to each of the immobilized peptides, by the other or the same soluble peptides.

Stable RCC cell lines containing VHL-(1–143) (without Elongin-binding sites but consisting of complete PKCδ-binding domain). Immunoprecipitation analysis reveals that VHL-(1–143)-containing cell lines completely inhibit PKCδ and IGF-IR interaction, and thus VHL-(1–143) functions as that of wt-VHL addition to its inhibitory effect on vascular endothelial growth factor up-regulation and cell proliferation, can also inhibit the IGF-mediated invasiveness of RCC. Again when we transiently transfected dominant negative of PKCδ (PKCδ KR) to 786-O cells, a marked decrease in invasive activity was observed (Fig. 5C). Taken together these results suggest that the invasive activity of VHL is mediated through IGF-IR signaling where PKCδ is an important downstream molecule in this pathway and can be efficiently blocked by VHL.

Various cellular proteins are found to be complexed with VHL protein that include Elongin B/C, Cullin-2(CUL-2), Rbx1, VHL-binding protein-1, hypoxia-inducible factor-1a, etc. (31–34). All these interactions either help to form the ubiquitina-
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Inhibition machinery or lead to ubiquitination of cellular proteins that are ultimately degraded by proteasome 26 S complex. In this communication, we described a novel domain of VHL, is different from previous findings, that can be directly correlated to its tumor suppresser function by inhibiting IGF-I signaling pathways in renal cancer. On the other hand, our studies suggest an unique mechanism of inhibitory action of wt-VHL as a tumor suppressor and define a novel approach for designing anti-tumor drugs.

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