Communication

The von Hippel-Lindau Gene Product Inhibits Vascular Permeability Factor/Vascular Endothelial Growth Factor Expression in Renal Cell Carcinoma by Blocking Protein Kinase C Pathways*

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Soumitro Pal, Kevin P. Claffey, Harold F. Dvorak, and Debabrata Mukhopadhyay‡
From the Departments of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215

Mutation or loss of function of the von Hippel-Lindau (VHL) tumor suppressor gene is regularly found in sporadic renal cell carcinomas (RCC), well vascularized malignant tumors that characteristically overexpress vascular permeability factor/vascular endothelial growth factor (VPF/VEGF). The wild-type VHL (wt-VHL) gene product acts to suppress VPF/VEGF expression, which is overexpressed when wt-VHL is inactive. The present study investigated the pathways by which VHL regulates VPF/VEGF expression. We found that inhibition of protein kinase C (PKC) represses VPF/VEGF expression in RCC cells that regularly overexpress VPF/VEGF. The wt-VHL expressed by stably transfected RCC cells forms cytoplasmic complexes with two specific PKC isoforms, ζ and δ, and prevents their translocation to the cell membrane where they otherwise would engage in signaling steps that lead to VPF/VEGF overexpression. Other experiments implicated mitogen-activated protein kinase (MAPK) phosphorylation as a downstream step in PKC regulation of VPF/VEGF expression. Taken together, these data demonstrate that wt-VHL, by neutralizing PKC isoforms ζ and δ and thereby inhibiting MAPK activation, plays an important role in preventing aberrant VPF/VEGF overexpression and the angiogenesis that results from such overexpression.

Germ line mutations or loss of the von Hippel-Lindau (VHL)1 gene predispose to a rare hereditary cancer syndrome characterized by the development of renal cell carcinoma (RCC) as well as other tumors including central nervous system hemangioblastomas, pheochromocytomas, and retinal angiomas (1–8). The VHL gene, which maps to chromosome 3p25-p26, is also commonly mutated or silenced in sporadic RCC and, like p53 and Rb, fulfills Knudsen’s criteria for a tumor suppressor gene (5, 6, 9, 10). Consistent with this interpretation, restoration of a normal chromosome 3p to an RCC cell line suppresses its tumorigenicity (11).

Sporadic RCC as well as the RCC and other tumors that arise in patients with the VHL syndrome are characteristically well vascularized, a property that has been attributed to their consistent overexpression of the potent angiogenic factor, vascular permeability factor, also known as vascular endothelial growth factor (VPF/VEGF) (12–16). Several different mechanisms have been shown to up-regulate VPF/VEGF expression in different tumors and also in non-tumorigenic cells; these include hypoxia, cytokines such as transforming growth factor-α and platelet-derived growth factor, hormones, and more recently, certain oncogenes including activated forms of Src and Ras (17–22).

The mechanisms responsible for up-regulating VPF/VEGF expression in RCC are not yet fully understood. Recent reports have shown that the VHL tumor suppressor gene down-regulates VPF/VEGF gene expression at both the transcriptional and post-transcriptional levels (23–25). In addition, the wild-type VHL (wt-VHL) gene product represses VPF/VEGF expression by inhibiting Sp1/transcriptional (23). Several reports suggest that steps in the Ras pathway may also play an important role in up-regulating VPF/VEGF expression in RCC (26, 27).

The present experiments were undertaken to elucidate more fully the role of VHL in regulating VPF/VEGF expression in RCC. We hypothesized that the wt-VHL gene product acts to inhibit steps in a signaling cascade that involves several protein kinases, including PKC. Here we present evidence in favor of this hypothesis, demonstrating that 1) PKC inhibitors repress the overexpression of VPF/VEGF characteristic of RCC cells, 2) wt-VHL protein complexes selectively with PKC ζ and δ, preventing the translocation of these PKC isoforms to the cell membrane and thereby interrupting a signaling cascade that involves MAP kinase, and 3) PKC δ, but not PKC ζ, complexes directly with wt-VHL protein.

EXPERIMENTAL PROCEDURES

Cell Culture—A human renal carcinoma cell line (786-O) was maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (HyClone Laboratories). 786-O clonal cell lines stably transfected with either pcDNA3 (Neo cells; cells contain empty vector with neo cassette), pRCE-HAVHL (VHL cells; cells expressing wt-VHL), or pRCE-HAVHL-(1–115) (VHL-(1–115) or ΔVHL cells; cells expressing deletion mutation of VHL) were gifts from W. G. Kaelin and were grown in complete media supplemented with G418 (1 mg/ml) (28).

RNA Analysis by Northern Blot Hybridization—Total RNA was isolated and Northern blots were performed as described previously (23). Briefly, immunoprecipitations were carried out in antibody excess, using 0.5 mg of total protein with either affinity-purified rabbit polyclonal

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antibody (1 μg of IgG) against PKC isoforms (Chemicon International Inc., CA) or with a mouse monoclonal antibody (1 μg) directed against VHL (gift from O. Iliopoulos and W. G. Kaelin). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Western blot analysis was carried out as described earlier (23).

PKC Assay—Cell samples were prepared and insoluble and soluble components were isolated as manufacturer’s protocol (Pierce). Afterward, extracts were immunoprecipitated with specific antibodies to PKC ζ and δ, and kinase assays were performed using the Pierce colorimetric PKC assay kit according to the manufacturer’s protocol. We used two different synthetic peptides (glycogen synthase, product 29510, and neurogranin peptide, product 29580) as PKC substrates. A standard curve was generated for each experiment using an appropriate PKC standard with known activity (supplied by Pierce), and PKC activity of each sample was measured using that standard curve. Each experiment was performed in triplicate.

In Vitro Binding Experiments—In vitro binding assays were performed as described previously (23). Briefly, the glutathione–Sepharose beads bound with GST-fused VHL proteins were incubated with purified PKC isoforms (50 ng, PanVera Corp., WI). The beads were then incubated for 1 h at 4 °C, washed four times with cold binding buffer, and boiled in SDS sample buffer. Bound proteins were resolved on SDS-PAGE, and blots were performed with antibodies to specific PKC isoforms.

RESULTS

Inhibition of VPF/VEGF Expression by Different Protein Kinase Inhibitors—Earlier work had shown that the wt-VHL gene suppresses VPF/VEGF expression in RCC and that genistein, a potent inhibitor of protein tyrosine kinases, inhibits hypoxia-induced VPF/VEGF overexpression (19). We had also shown that genistein at a level of 100 μM suppressed completely the high base-line VPF/VEGF expression characteristic of RCC cells (data not shown). Taken together, these findings suggested that wt-VHL regulated VPF/VEGF expression by interacting with a signaling pathway that involved protein tyrosine kinases. To test this hypothesis further, we studied the effects of several specific kinase inhibitors on the expression of VPF/VEGF mRNA in a renal carcinoma cell line, 786-O, which constitutively overexpresses VPF/VEGF. Staurosporine, a potent and specific PKC inhibitor, suppressed basal VPF/VEGF expression by 90% at a concentration of 1 μM (Fig. 1). However, VPF/VEGF mRNA levels were not significantly altered by H89, a PKA inhibitor, or by G06976, a selective inhibitor of the α and β PKC isoforms (Fig. 1). These data indicate that activation of VPF/VEGF mRNA in RCC is mediated through PKC but not through its α or β isoforms or by PKA.

Interaction of VHL with Specific PKC Isoforms—To investigate further the interaction between VHL and PKC in regulating VPF/VEGF expression, we looked for a possible physical interaction between the VHL gene product and various PKC isoforms. To that end, we prepared lysates of wt-VHL cells (VHL) and immunoprecipitated with affinity-purified antibodies specific for various PKC isoforms, followed by Western blotting with a monoclonal antibody directed against wt-VHL protein. In the case of immunoprecipitates prepared with antibodies to PKC ζ and PKC δ, Western blots revealed intense bands (ζ > δ). Very weak VHL-positive bands were also detected when immunoprecipitates were prepared with antibodies against the α or ε isoforms of PKC (Fig. 2, a–d). However, no detectable band was found against the β, γ, and μ PKC isoforms (data not shown).

In contrast, anti-VHL reactive protein bands were not detected in Western blots of immunoprecipitates prepared with antibodies to PKC isoforms on cell extracts of either RCC cells transfected with an empty neo cassette (neo-786-O cells, Neo) or stably transfected with a deletion mutation of VHL (VHL-(1–115), ΔVHL) (Fig. 2, a–d). In the case of VHL-(1–115) cells, these findings could not be attributed to a failure of protein expression because VHL-(1–115) cells contained mutant VHL protein in amounts comparable with those of wt-VHL protein present in VHL cells (Fig. 2e). Also, making use of split samples, we determined that the levels of PKC isoforms were similar in all three cell lines (Fig. 2, a–d).

We also performed reciprocal experiments. Extracts were prepared from the same three cell lines and were immunoprecipitated with a monoclonal antibody directed against VHL, followed by Western blotting with antibodies specific for PKC isoforms α, β, δ, ε, γ, ζ, and μ. Only extracts of wt-VHL-expressing cell lines formed complexes that reacted with anti-PKC ζ (Fig. 2f). Surprisingly, we were unable to detect a band that reacted with anti-PKC δ, in wt-VHL extracts, perhaps because of the low blotting sensitivity of the anti-PKC δ antibody. As expected, no bands were detected with antibodies to PKC isoforms α, β, ε, γ, and μ.

Subcellular Localization of VHL and Its Complexes with PKC Isoforms—We next determined the subcellular localization of wt-VHL protein and of its complexes with PKC isoforms ζ and δ. Cytoplasmic and membrane fractions were prepared from extracts of Neo and VHL cells. Western blots with antibodies to VHL revealed that the great bulk of VHL was found in the cytoplasmic fraction of VHL cells; as expected, VHL protein was not detected in cytoplasmic or membrane fractions of the Neo cells (Fig. 3a). We next immunoprecipitated these cytoplasmic extracts with antisera against PKC ζ and δ. We found VHL-reactive bands in cytoplasmic fractions (Fig. 3, b and c) but not in membrane fractions (data not shown) of VHL cells. Thus, PKC ζ and δ complexes were localized primarily to the cytoplasm.

Regulation of PKC Activity by wt-VHL—We immunoprecipitated cytoplasmic and membrane fractions of the RCC cell lines with antibodies to PKC ζ and blotted these with the same antibody. Fig. 3d shows that the amount of PKC ζ present in the membrane fraction of the wt-VHL-expressing cell line is significantly lower than that present in its cytoplasmic fraction. In contrast, relatively more PKC ζ was found in the membrane fractions (as compared with the cytoplasmic fractions) of Neo and VHL-(1–115) (∆VHL) cells. We measured PKC activity in membrane and cytoplasmic fractions of these same immunocomplexes using a colorimetric assay. Levels of PKC ζ activity in the cytoplasmic fraction of wt-VHL-expressing cells were higher than those in cytoplasmic fractions of either Neo or VHL-(1–115) cells but did not quite achieve statistical significance (analysis of variance, p = 0.06, Fig. 3e). However, PKC ζ activity was significantly reduced in membrane preparations of VHL cells compared with membrane preparations of Neo or VHL-(1–115) cells (p < 0.005). Also, the amount of PKC activity found in VHL cell cytoplasmic fractions was significantly greater than that found in membrane prepa-
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**Fig. 2. Association of wt-VHL product with PKC isoforms in RCC cells.** Extracts were prepared from Neo, VHL, and VHL-(1–115) cells. In a–d, extracts were immunoprecipitated with polyclonal antibodies specific for various PKC isoforms as indicated. Immunoprecipitates (IP) were then captured by protein A-Sepharose and washed, and the Sepharose beads were boiled in SDS-buffer and separated by SDS-PAGE. Western blotting (Blot) was performed using antibodies to wt-VHL and PKC antibodies. In e and f, cell extracts were immunoprecipitated with antibodies against wt-VHL and subjected to SDS-PAGE and Western blotting with antibodies against wt-VHL and PKC, respectively.

**Fig. 3. Subcellular localization of wt-VHL complexes with PKC isoforms in RCC and estimation of PKC activity.** Cytoplasmic and membrane fractions were prepared from extracts of RCC cell lines. a, blots of Neo and VHL cell extracts with anti-PKC antibody. Also similar results were obtained when we used neurogranin peptide as PKC substrate (data not shown). Together these data are consistent with the hypothesis that wt-VHL binds PKC ζ and δ in the cytoplasm, preventing their translocation to the cell membrane.

**Interaction of VHL with PKC ζ and δ in Vitro—**The experiments just described did not determine whether the cytoplasmic complexes of wt-VHL with PKC ζ or δ resulted from a direct interaction between these proteins or required the presence of additional components. To distinguish between these possibilities, we performed in vitro association experiments using GST-VHL fusion proteins and recombinant PKC ζ and δ isoforms. Bacterially expressed GST protein alone or GST protein fused to full-length wt-VHL or VHL-(1–115) was bound to glutathione-Sepharose beads, and these were mixed with purified recombinant PKC ζ or δ in a buffer designed to approximate intracellular ionic concentrations (see "Experimental Procedures" for details). After suitable incubation and extensive washing with the same buffer, the bound proteins were separated by SDS-PAGE and subjected to Western blotting with antibodies to PKC ζ and δ. We observed a strong association of PKC δ, but not PKC ζ, with immobilized wt-VHL (Fig. 3f). These results indicate that PKC δ interacted directly with wt-VHL protein; in contrast, wt-VHL did not interact directly with PKC ζ, and therefore the wt-VHL-PKC ζ complex that forms in living cells must involve additional as yet unidentified component(s).

**wt-VHL Inhibits Phosphorylation of MAP Kinase—**MAPK is a downstream target of both PKC ζ and PKC δ (30–32). To determine whether wt-VHL interferes with downstream signaling of these PKC isoforms, we examined the status of the activated form of MAPK (phospho-MAPK) in VHL cells as well as in the Neo and VHL-(1–115) cells. Total levels of MAP kinase were similar in all three cell lines. However, we found significantly lower levels of phospho-MAPK in VHL cells than in the two, non-wt-VHL-expressing cell lines (Fig. 4a). These results provide further evidence that formation of wt-VHL-PKC complexes in VHL cells prevents PKC translocation to the cell membrane and thus subsequent activation of MAPK.

**Inhibition of MAPK Phosphorylation Down-regulates VPF/VEGF Expression—**To determine whether phosphorylation of MAPK has an effect on VPF/VEGF expression, we treated RCC cells with a specific inhibitor of MAPK kinase (MEK), PD98059, and examined VPF/VEGF mRNA levels by Northern blot analysis (33). Fig. 4b shows that PD98059 reduced VPF/VEGF mRNA levels by 80% as compared with the untreated control. These data suggest that MEK activation and MAPK phosphorylation have a role in the overexpression of VPF/VEGF found in RCC.

**Discussion**

The data presented here investigated the mechanisms by which VHL regulates VPF/VEGF expression in RCC. In accord with earlier work, we found that a PKC inhibitor, staurosporine, repressed VPF/VEGF expression in RCC cells whereas inhibitors of PKC α or β or of PKA did not. Also, the wt-VHL tumor suppressor gene product formed selective complexes with two specific PKC isoforms (ζ > δ) in wt-VHL-expressing cells. The interaction of wt-VHL protein with recombinant PKC δ was replicated in vitro; however, wt-VHL did not complex directly with PKC ζ, suggesting that additional, as yet unidentified, components are required for wt-VHL-PKC ζ complex formation. The complexes that formed in cells between wt-VHL and PKC ζ and δ were localized primarily in cytoplasm. Also, more PKC ζ and δ activity was found in the cytoplasm of wt-VHL cells than in either Neo or VHL-(1–115) cells that lacked wt-VHL; conversely, more PKC ζ and δ activity was found in cell membrane fractions of Neo and VHL-(1–115) cells than in wt-VHL-expressing RCC. In addition, although total amounts of MAPK were roughly equivalent in cells that lacked or expressed wt-VHL, significantly less activated (phosphorylated) MAPK was found in cells expressing wt-VHL than in cells...
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mechanisms whereby VHL controls VPF/VEGF expression. Current experiments seek to determine whether these mechanisms affect transcription or act to decrease the stability of VPF/VEGF mRNA.

REFERENCES

1. Chen, F., Kishida, T., Duh, F. M., Renbaum, P., Orcutt, M. L., Schmidt, L. & Zbar, B. (1995) Cancer Res. 55, 4804–4807
2. Chen, F., Kishida, T., Yao, M., Hustad, T., Glavac, D., Dean, M., Gnarra, J. R., Orcutt, M. L., Duh, F. M., Glenn, G., Green, J., Haia, Y. E., Larrniet, J., Li, H., Wei, M. H., Schmidt, L., Tory, K., Kuzmin, I., Stackhouse, T., Latif, F., Linehan, W. M. & Zbar, B. (1995) Hum. Mutat. 5, 66–75
3. Crosse, F. A., Maher, E. R., Foster, K., Richards, F. M., Latif, F., Tory, K., Jones, M. H., Bentley, E., Lerman, M. I., Zbar, B., Affara, N. A. & Ferguson-Smith, M. A. (1993) Hum. Genet. 93, 53–58
4. Crosse, F. A., Richards, F. M., Foster, K., Green, J. S., Prowse, A., Latif, F., Lerman, M. I., Zbar, B., Affara, N. A., Ferguson-Smith, M. A. & Maher, E. R. (1994) Hum. Mol. Genet. 3, 1303–1308
5. Gnarra, J. R., Glenn, G. M., Latif, F., Anglard, P., Lerman, M. I., Zbar, B. & Linehan, W. M. (1993) Urol. Clin. North Am. 20, 207–216
6. Richards, F. M., Phipps, M. E., Latif, F. Yao, M., Crosse, F. A., Foster, K., Linehan, W. M., Affara, N. A., Lerman, M. I., Zbar, B., Ferguson-Smith, M. A. & Maher, E. R. (1993) Hum. Mol. Genet. 2, 879–892
7. Richards, F. M., Maher, E. R., Latif, F., Phipps, M. E., Tory, K., Lush, M., Crosse, P. A., Oostra, B., Gustavson, K. H., Green, J., Turner, G., Yates, J. R. W., Linehan, M., Affara, N. A., Lerman, M. I., Zbar, B. & Ferguson-Smith, M. A. (1993) J. Med. Genet. 30, 104–107
8. Richards, F. M., Payne, S. J., Zbar, B., Affara, N. A., Ferguson-Smith, M. A. & Maher, E. R. (1995) Hum. Mol. Genet. 4, 2139–2143
9. Passe, A. Lee, S., Worrall, R. A., Ch., D. Y., Burgess, W. H., Linehan, W. M. & Klaauin, R. D. (1997) Prog. Natl. Acad. Sci. U. S. A. 94, 2156–2161
10. Linehan, W. M., Lerman, M. I., and Zbar, B. (1995) J. Am. Med. Assoc. 273, 564–570
11. Foster, K., Prowse, A., van den Berg, A., Fleming, S., Hulsbeek, M. M., Crosse, P. A., Richards, F. M., Cairns, P., Affara, N. A., Ferguson-Smith, M. A., Bays, C. H. C. M. & Maher, E. R. (1994) Hum. Mol. Genet. 3, 2169–2173
12. Leung, D. W., Cachianes, G., Kuang, W. J., Goeddel, D. V. & Ferrara, N. (1989) Science 246, 1306–1309
13. Stengger, D. R., Galli, S. L., Drvorak, A. M., Peruzzi, C. A., Harvey, V. S. & Drvorak, H. F. (1989) Science 219, 983–985
14. Wizigmann-Voos, S., Breier, G., Risau, W. & Plate, K. H. (1995) Cancer Res. 55, 1358–1364
15. Takahashi, A., Sasaki, H., Kim, J. S., Tobisu, K., Kakizoe, T., Tsukamoto, T., Kumamoto, Y., Sugimura, T. & Terada, M. (1994) Cancer Res. 54, 4233–4237
16. Brown, L. F., Berse, B., Jackman, R. W., Tognazzi, K., Manseau, E. J., Dvorak, A. M. & Senger, D. R. (1993) Am. J. Pathol. 143, 1255–1262
17. Shweiki, D., Itin, A., Soffer, D. & Keshet, E. (1992) Nature 359, 843–845
18. Plate, K. H., Breier, G., Weiss, H. T. & Risau, W. (1992) Nature 359, 845–848
19. Shweiki, D., Peter, D., Zhou, X. M., Foster, D., Brugge, J. S. & Sukhatme, V. P. (1995) Nature 377, 575–581
20. Mukhopadhyay, D., Tsioskas, L. & Sukhatma, V. P. (1995) Cancer Res. 55, 3161–3165
21. Ralk, J., Mitsuhashi, Y., Bayko, L., Filmus, J., Sasazuki, T. & Kerbel, R. S. (1995) Cancer Res. 55, 4575–4580
22. Debm, M., Brown, L. F., Chaffey, K. P., Yen, K. T., Kocher, O., Jackman, R. W., Berse, B. & Drvorak, H. F. (1994) J. Exp. Med. 180, 1141–1146
23. Mukhopadhyay, D., Knebelmann, B., Cohen, H. T., Ananth, S. & Sukhatme, V. P. (1997) Mol. Cell. Biol. 17, 5629–5639
24. Levy, A. P., Levy, N. S., Iliopoulos, O., Hangan, C., Kaplin, W. G. Jr. & Goldberg, M. A. (1997) Science 275, 575–578
25. Iliopoulos, O., Levy, A. P., Jiang, C., Kaelin, W. G. Jr. & Goldberg, M. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10395–10399
26. Uchida, T., Wada, C., Wang, C., Egawa, S., Ohntai, H. & Koshiba, K. (1994) Cancer Res. 54, 3682–3685
27. Fujita, J., Kraus, M. H., Onoue, H., Srivastava, S. K., Ebi, Y., Kitamura, Y. & Dvorak, H. F. (1983) Science 219, 23512–23519
28. Mukhopadhyay, D., Knebelmann, B., Cohen, H. T., Ananth, S. & Sukhatme, V. P. (1997) Mol. Cell. Biol. 17, 5629–5639
29. Chomcynski, P. & Sacchi, N. (1987) Anal. Biochem. 166, 156–159
30. Berra, E., Diaz-Meco, M. T., Lezano, J., Frutos, S., Municio, M. M., Sanchez, P., Sanz, L. & Moscat, J. (1995) EMBO J. 14, 6115–6157
31. Liao, D. F., Monia, B., Dean, N. & Berk, B. C. (1997) J. Biol. Chem. 272, 6146–6150
32. Ueda, Y., Hirai, S., Osada, S., Suzuki, A., Mizuno, K. & Ohno, S. (1996) J. Biol. Chem. 271, 23512–23519
33. Watts, S. W. (1996) J. Pharmacol. Exp. Ther. 279, 1541–1550
34. Arezona-Sciedf, F., Israel, N., Bachelerie, F., Hazan, U., AACami, J., Dautry, F. & Vireilazer, J.-L. (1989) Oncogene 2, 1359–1362
35. Devary, Y., Rossete, C., DiBiono, J. A. & Karin, M. (1995) Science 261, 1442–1445
36. Dominguez, I., Sanz, L., Arezona-Sciedf, F., Diaz-Meco, M.T., Vireilazer, J.-L. & Moscat, J. (1993) Mol. Cell. Biol. 13, 1290–1295
37. Fincio, T. S. & Baldwin, A. S. (1993) J. Biol. Chem. 268, 1766–1769
38. Oka, H., Chatani, Y., Hoshino, R., Ogawa, O., Kakehi, Y., Terachi, T., Okada, Y., Kawauchi, M., Kohno, M. & Yoshida, O. (1995) Cancer Res. 55, 4182–4187