Activation of Sp1-mediated Vascular Permeability Factor/Vascular Endothelial Growth Factor Transcription Requires Specific Interaction with Protein Kinase C ζ*†

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The transcription factor Sp1 is ubiquitously expressed and plays a significant role in the constitutive and induced expression of a variety of mammalian genes and may even contribute to tumorigenesis. Here, we describe a novel pathway whereby Sp1 promotes the transcription of vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), a potent angiogenic factor, by interacting directly and specifically with protein kinase C ζ (PKC ζ) isoform in renal cell carcinoma. PKC ζ binds and phosphorylates the zinc finger region of Sp1. Moreover, in the presence of the wild type von Hippel-Lindau gene product, the interaction of Sp1 with PKC ζ is inhibited, and in this manner steady state levels of Sp1 phosphorylation are decreased significantly. Co-transfection of renal cell carcinoma cells and human fibrosarcoma cells with a plasmid over-expressing PKC ζ and VPF/VEGF promoter luciferase constructs results in activation of Sp1-mediated transcription, whereas expression of a dominant-negative mutant of PKC ζ repressed this activation. Taken together, our results suggest a new pathway of cell signaling through PKC ζ and provide an insight into PKC ζ and Sp1-dependent transcriptional regulation of VPF/VEGF expression and thus tumor angiogenesis.

Angiogenesis, the formation of new blood vessels from the existing vascular bed, plays a central role in neoplasia, in many non-neoplastic disorders and also in normal adult physiology (1, 2). VPF/VEGF is a multifunctional cytokine that exerts a number of direct effects on vascular endothelial cells, with important roles in vasculogenesis and both pathological and physiological angiogenesis (3, 4). Although constitutively expressed by many tumor cells, transformed cell lines, and some normal cells, VPF/VEGF expression is substantially up-regulated by hypoxia, cytokines, hormones, and certain oncogenes including activated forms of Src and Ras (5–10).

Germ-line mutations or loss of the von Hippel-Lindau (VHL) gene predisposes to a hereditary cancer syndrome characterized by the development of vascular tumors (11–13). The VHL gene, which maps to chromosome 3p25-p26, is commonly inactivated by mutations in sporadic RCC (11–13). Restoration of a normal chromosome 3p to an RCC cell line suppresses its tumorigenicity, suggesting the VHL gene as a tumor suppressor (14, 15). Both VHL-associated and sporadic hemangioblastomas and RCCs overexpress the potent angiogenic factor VPF/VEGF and its receptors KDR and Flk-1, suggesting that these genes may be VHL targets (16, 17). We have demonstrated that VPF/VEGF is indeed a target for the VHL gene product, and the transcriptional repression of the VPF/VEGF promoter depends on a direct interaction between VHL and the ubiquitous transcriptional activator Sp1 (18). Recently, we also found that wt-VHL protein complexes selectively with PKC isoforms, directly with PKC ζ and indirectly with PKC ζ thus inhibiting VPF/VEGF expression (19). To understand the mechanism by which loss of wt-VHL function leads to VPF/VEGF overexpression, we postulated that the PKC ζ isoform might have an important role in activating Sp1-mediated transcription. Here we demonstrated that PKC ζ indeed interacts directly with Sp1 and activates transcription in RCC as well as in human fibrosarcoma cells where basal level of VPF/VEGF expression is very high.

EXPERIMENTAL PROCEDURES

Cell Culture—Human fibrosarcoma (HT1080) and human renal carcinoma (786-0) cell lines were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (HyClone Laboratories).

Plasmids—The VEGF reporter constructs used in transient transfection assays contain sequences derived from the human VEGF promoter driving expression of firefly luciferase. The 0.35- and 0.07-kb deletion mutant constructs were made by polymerase chain reaction from the 2.6-kb VEGF promoter fragment and subcloned into pGL-2 Basic vector (Promega) as described earlier (7). The VHL cDNA was polymerase chain reaction amplified from a human fetal kidney cDNA library and subcloned into pCMV2FLAG vector (18). The overexpressed PKC ζ and a kinase inactive PKC ζ cDNA (PKC ζ K; Lys-275 to tryptophan substitution), both subcloned into pCMV2FLAG vector were generous gifts from Alex Toker. The kinase inactive PKC ζ plasmid (KR; Lys-376 to arginine substitution) was a generous gift from R. Dutta.

Immunoprecipitation and Western Blot Analyses—Cell lysis and immunoprecipitations were performed as described previously (20, 21). Briefly, immunoprecipitations were carried out in antibody excess, using 0.5 mg of total protein with affinity-purified rabbit polyclonal antibody (1 μg of IgG) directed either against PKC isoforms (Chemicon International Inc.) or Sp1 (Santa Cruz Biotechnology, Inc.). Proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE). Western blot analysis was carried out as described earlier (18, 19).

In Vitro Binding Experiments—In vitro binding assays were performed as described previously (18, 19). Briefly, the glutathione-S-phosphate beads bound with GST-fused Sp1, Sp1 deletion mutants (A, B, C, D only and zinc finger), or VHL protein were incubated with purified PKC ζ isoform (50 ng; Panvera Corp., WI) for 1 h at 4°C. The beads were then washed with cold binding buffer. Bound proteins were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. Proteins were stained with Coomassie Blue and then detected by ECL.

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‡ The abbreviations used are: VPF, vascular permeability factor; VEGF, vascular endothelial growth factor; VHL, von Hippel-Lindau; wt, wild type; RCC, renal cell carcinoma; PKC, protein kinase C; kb, kilobase pairs; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; PBS, phosphate-buffered saline.
Activation of Sp1-mediated Transcription by PKC ζ

SDS-PAGE, and blots were performed with antibodies to PKC ζ isoform. **Protein Kinase C Assay**—PKC was assayed in presence of phosphatidylinerine by measuring the incorporation of [32P] into GST-Sp1 or its deletion mutants, using histone as positive control. Aliquots of 50 ng of PKC ζ (Panvera Co.) were incubated in a 50-μl reaction mixture consisting of 30 μl Tris HCl (pH 7.5), 0.01% Triton X-100, 10 μM 2-mercaptoethanol, 0.2 μM phenylmethylsulfonfyl fluoride, 5 μg/ml leupeptin, 0.4 μM EGTA, 10 μM MgCl₂, 20 μg/ml phosphatidylinerine, 20 μg/ml GST-Sp1 or its mutants or 150 μg/ml histone type III-S (Sigma), and 50 μM [γ-32P]ATP for 30 min at 30 °C. The reaction was stopped by addition of ice-cold 25% trichloroacetic acid. Precipitates were collected on phosphocellulose filter paper. The filters were washed with 12% trichloroacetic acid and counted for 32P incorporation using liquid scintillation spectroscopy.

**Transfection Assays**—Cells were plated at 2–3 × 10⁵ cells/60-mm dish 1 day before transfection with VPF/VEGF promoter-luciferase constructs and expression plasmids using calcium-phosphate precipitation. The expression was normalized with a control empty expression vector. Cells were harvested for luciferase assay 40 h after transfection. In all co-transfection experiments, transfection efficiency was normalized by assaying β-galactosidase activity using a β-galactosidase gene under control of the cytomegalovirus immediate early promoter as internal control.

**Immunofluorescent Analysis**—786-0 cells were grown at low density (≤20% surface area) on multi-well Lab Tek chambers. Cells were fixed in methanol:acetone (1:1) at −20 °C for 10 min. The slides were then blocked in bovine serum albumin (1% in PBS) for 2 h and incubated with primary antibodies to PKC ζ (Signal Transduction Laboratories) or Sp1 (Santa Cruz Biotechnology, Inc.) overnight at 4 °C. Slides were washed three times in PBS at room temperature for 5 min each time. Secondary anti-mouse fluorescein isothiocyanate antibody and anti-rabbit tetramethyl rhodamine-5-isothiocyanate antibodies were incubated in bovine serum albumin (1%) PBS for 1 h at room temperature to detect PKC ζ and Sp1, respectively. The slides were then washed three times extensively with PBS for 10 min each time and mounted with aqueous mounting media. Slides were analyzed by confocal microscopy to simultaneously detect fluorescein isothiocyanate and tetramethyl rhodamine-5-isothiocyanate signals. Individual and merged images from low density and high density fields were obtained at magnification ×650.

**RESULTS AND DISCUSSION**

In RCC wt-VHL was found to be in a complex with PKC ζ, but purified forms of these proteins did not form a complex when they were mixed together in vitro (19). We therefore considered the possibility that Sp1 served as an intermediary in the association of VHL and PKC ζ, forming a bridge that joined these molecules into a complex. We tested this hypothesis in cultured cell lysates and also by using recombinant proteins in direct mixing experiments. For analysis in cells, lysates of 786-0 renal carcinoma cells (RCC) were immunoprecipitated with affinity-purified antibodies to specific PKC isoforms, followed by Western blotting with antibody directed against the Sp1 protein. We found a strong band corresponding to Sp1 only in the case of immunoprecipitates prepared with antibodies to PKC ζ (Fig. 1a). Very weak Sp1 positive bands were also observed when immunoprecipitates were prepared with antibodies against β and ε isoforms of PKC, but no detectable band was found with antibodies against the δ and α PKC isoforms (Fig. 1a). The reciprocal experiment, in which RCC lysates were immunoprecipitated with an antibody specific for Sp1, followed by immunoblotting with antibody against PKC ζ, also demonstrated that PKC ζ and Sp1 were present in the same protein complex (Fig. 1a).

These proteins might complex directly with each other, we mixed recombinant forms of different PKC isoforms (human PKCs produced from recombinant baculovirus in insect cells; Panvera Co.) with recombinant human Sp1 (from a recombinant vaccinia virus; Promega Co.). Mixtures were then immunoprecipitated with antibodies specific for each of the PKC isoforms tested and subjected to Western blotting with an antibody specific for Sp1. Fig. 1b reveals that a strong band was detected only when immunoprecipitation was performed with the antibody for the PKC ζ isoform. Very weak bands were found when immunoprecipitates were prepared with other PKC isoforms. The reciprocal experiment was also performed in which the Sp1-PKC ζ isoform mixture was immunoprecipitated with antibody specific for Sp1, followed by immunoblotting with antibody against PKC ζ. The result revealed a strong protein band corresponding to PKC ζ (Fig. 1b). These results suggest that Sp1 interacts directly and specifically with PKC ζ. To confirm these results we performed in vitro association experiments using recombinant PKC ζ and bacterially expressed GST fused to full-length Sp1 or wt-VHL. GST and GST-Sp1 were then mixed with purified recombinant PKC ζ. After appropriate incubation and extensive washing, the glutathione-Sepharose bound proteins were separated by SDS-PAGE and subjected to Western blotting with antibodies to PKC ζ. A strong association of PKC ζ with immobilized Sp1 was observed when GST-full-length Sp1 was mixed with purified recombinant PKC ζ (Fig. 2a). In contrast, when GST-Sp1 was preincubated with GST-VHL and then mixed with recombinant PKC ζ, the association of Sp1 and PKC ζ was significantly reduced (Fig. 2b). These results indicate that the interaction of Sp1 with PKC ζ can be competed with wt-VHL, which also binds to Sp1.

To determine which protein domain of Sp1 binds to PKC ζ, we utilized GST-Sp1 fusion proteins representing four distinct domains, A, B, C, and D, as well as the zinc finger region; all of these domains are important for transcriptional activation (20, 21). Fig. 2b shows that PKC ζ interacts selectively with the zinc finger domain of Sp1. The B and C regions interacted very weakly, and no interaction was detected with either the A or D regions of Sp1.

These results suggest that PKC ζ interacts with a specific region of Sp1 but did not establish whether Sp1 was a substrate for PKC ζ phosphorylation. To test this possibility, we performed PKC ζ kinase assays using full-length GST-Sp1 fusion protein as a substrate. We found that recombinant PKC ζ strongly phosphorylates the full-length Sp1 about 3–4-fold higher than that observed for the GST only (Fig. 2c). These data indicate that Sp1 is a potential substrate for PKC ζ. Interestingly, when GST-VHL was included in the reaction mixture, PKC ζ phosphorylation of Sp1 was significantly reduced to basal levels (Fig. 2c). These results suggest that in the presence of VHL the association between PKC ζ and Sp1 is inhibited, and thus Sp1 phosphorylation is reduced. Moreover, when all the domains of Sp1 were allowed to interact with recombinant PKC ζ separately, PKC ζ strongly phosphorylated only the zinc finger region of Sp1; in contrast, the A, B, C, and
respectively, reveal comparable amounts of GST fusion protein bound to the finger region.

phosphorylates Sp1 through a direct interaction with its zinc fingers of Sp1 and its deletion mutants by PKC z. Together these findings suggest that PKC z binds to and phosphorylates Sp1 through a direct interaction with its zinc finger region.

We next set out to determine whether PKC z had a role in Sp1-mediated VEGF transcription. To this end, 786-O RCC and human fibrosarcoma cells (HT1080) were cotransfected with a 2.6-kb VPF/VEGF promoter-luciferase construct and plasmid containing PKC z cDNA tagged with a FLAG sequence under the control of a cytomegalovirus immediate early promoter (7). VPF/VEGF reporter activity was increased at least 2–4-fold in comparison with cells transfected with PKC expression vector alone (Fig. 3a). To define the region of the VPF/VEGF promoter that is responsive to PKC z, we utilized two different 5' deletions of the 2.6-kb promoter-reporter vector and cotransfected these deletions with a plasmid that overexpresses PKC z. PKC z increased the reporter activity by 3–4-fold in comparison with cells transfected with the VEGF 0.35-kb promoter luciferase construct (1 µg) and increasing amounts (0.6–2.5 µg) of PKC z (KW) cDNA-tagged with FLAG sequence. e, HT1080 and 786-0 cells were cotransfected with the VEGF 0.35-kb promoter luciferase construct (1 µg) and a dominant-negative mutant (KR) of PKC z expression vector (2.5 µg). f, 786-0 cell lysates, transiently transfected with wt- and KW-PKC z overexpression vectors, were immunoprecipitated with Sp1-specific antibody. The immunoprecipitate was subjected to SDS-PAGE and blotted with anti-FLAG M2 monoclonal antibody. Results for a, c, d, e, and f were representative of two independent experiments. b and g were from three independent experiments.

It was previously shown that the VPF/VEGF promoter region responsible for the Sp1 response was a 144-base pair region and that Sp1-mediated transcription is inhibited in the presence of wt-VHL (18). Because VHL inhibits the association between Sp1 and PKC z and thus phosphorylation of Sp1, we tested whether VHL can abrogate the PKC z-mediated Sp1 transactivation of the VPF/VEGF promoter (0.35 kb). Indeed, Fig. 3c shows that overexpression of wt-VHL completely inhibited PKC z-mediated Sp1 transactivation in 786-0 cells (in which endogenous VHL is mutated). Together these data are consistent with the hypothesis that PKC z is a key activator of Sp1-mediated transcription and that Sp1 transcription is modulated by wt-VHL.

To explore whether Sp1 phosphorylation by PKC z is obligatory for VPF/VEGF promoter transactivation, we employed a kinase inactive PKC z (PKC z KW). PKC z KW is a dominant-negative mutant (22). We observed dose-dependent inhibition of Sp1-mediated VPF/VEGF promoter transactivation when PKC z KW was overexpressed in 786-0 cells (Fig. 3d). Similar results were observed in HT1080 cells (data not shown). Interestingly, when we transfected the same cells with a kinase inactive PKC z plasmid (KR) (23), no significant decrease was observed in Sp1-mediated VPF/VEGF transcription (Fig. 3e). Together these results indicate that PKC z is essential for promotion of Sp1-mediated VPF/VEGF transcription.

We next sought to determine whether Sp1 is able to associate with PKC z. Utilizing the cellular extracts of cotransfected
Low Density | High Density
---|---
Sp1 | |
PKC ζ | |
Sp1 + PKC ζ |

786-O cells, we detected PKC ζ KW in the same immunocomplex with Sp1 (Fig. 3f). The kinase inactive mutant of PKC ζ also interacted with Sp1 but could not phosphorylate it, and therefore VPF/VEGF promoter activity was significantly repressed. Taken together, these results suggest that phosphorylation of Sp1 by PKC ζ is an important step of Sp1-mediated transactivation.

To assess whether PKC ζ and Sp1 might be colocaled in cells, immunofluorescence and confocal microscopy were performed making use of specific antibodies to PKC ζ and Sp1 in 786-O cells. Cells were tested as either pre-confluent mitotic and compared with post-confluent monolayers. As expected, Sp1 was predominantly localized to cell nuclei in both pre-confluent and post-confluent cells (Fig. 4, a and b). PKC ζ, however, demonstrated a distinctly nuclear/perinuclear staining in the pre-confluent cells but diffuse cell membrane staining in the confluent cells (Fig. 4, c and d). Merged images of Sp1/PKC ζ as nuclear and perinuclear yellow staining was found mainly in the pre-confluent cells and less intense in the confluent cells (Fig. 4, e and f). Thus, the cells that are actively dividing translocate PKC ζ to nuclear/perinuclear compartments where an interaction with Sp1 is likely to occur. This finding also correlates nicely with the observation that VHL is found predominantly in the nuclear compartment in pre-confluent cultured cells but is cytoplasmic in confluent cells (24), which furthermore supports the notion that VHL and PKC ζ may translate together as part of the same protein complex.

The Sp1 transcription factor is broadly expressed in mammalian cells and performs a major role in the constitutive and induced expression of a variety of genes. Sp1 levels and function may change with cell differentiation, transformation, and growth, and Sp1 may contribute to tumorigenesis (25–27). But, very little is known about the mechanism of activation of Sp1. The results of the present study strongly support a novel role for PKC ζ in the regulation of Sp1-mediated VPF/VEGF transcriptional activation. Interestingly, Sp1 does not appear to be part of the VHL-PKC ζ complex as observed previously (19) but is a direct substrate for PKC ζ activity, which can be blocked by VHL. The mechanism whereby PKC ζ is activated in renal cancer deserves further investigation, although it has been shown earlier that PKC ζ is a critical step downstream of p21^ras^ and also pp60^c-src^ (22, 28, 29). We have presented evidence that a dominant-negative mutant of PKC ζ significantly inhibits the Sp1-mediated transcriptional activation of the VPF/VEGF promoter. Our data suggest that an inhibitor specific for PKC ζ would have potential in inhibiting VPF/VEGF expression and therefore might be useful in anti-angiogenic therapy.

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**Fig. 4.** Intracellular localization of PKC ζ and Sp1 in RCC. Immunofluorescent analysis was performed for intracellular localization of PKC ζ and Sp1, which is dependent upon density and growth status of 786-O cells. 786-0 cells were seeded at low density or high density. Sp1 is predominantly nuclear, indicated with red fluorescence (a and b), and PKC ζ, indicated with green fluorescence, was to be both nuclear and peri-nuclear localized in low density cells (c) and diffuse cell membrane localized in high density cell (d). Colocalization of Sp1 and PKC ζ is indicated in the merged images as yellow staining and is predominantly nuclear and adjacent peri-nuclear localized in the low density cells (e) with less colocalization with Sp1 at high density cells (f).