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Protein Kinase C ζ Transactivates Hypoxia-Inducible Factor α by Promoting Its Association with p300 in Renal Cancer

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Abstract

Hydroxylation at an asparagine residue at the COOH-terminal activation domain of hypoxia-inducible factor (HIF)-1/2 αs is essential for its inactivation under normoxic condition. To date, the mechanism by which HIF-α avoids the inhibitory effect of asparagine hydroxylase in renal cell carcinoma (RCC) in normoxia is undefined. We have shown herein that protein kinase C (PKC) ζ has an important role in HIF-α activation in RCC. By using dominant negative mutant and small interference RNA approaches, we have demonstrated that the association between HIF-α and p300 is modulated by PKCζ. Moreover, a novel signaling pathway involving phosphatidylinositol 3′-kinase and PKCζ has been shown to be responsible for the activation of HIF-α by inhibiting the mRNA expression of FIH-1 (factor inhibiting HIF-1) in RCC and thereby promoting the transcription of hypoxia-inducible genes such as vascular permeability factor/vascular endothelial growth factor.

Introduction

Tumor angiogenesis is an important event for the survival, growth, and subsequent metastasis of tumors (1–4). Although the detailed molecular mechanism involved in neovascularization in and around the tumor is not fully understood, the angiogenic growth factor vascular permeability factor (VPF)/vascular endothelial growth factor (VEGF)-A appears to play an important role (4). This is also the case for renal cell carcinoma [RCC (5, 6)]. RCC is a heterogeneous disease from both phenotypic and genetic points of view. It cannot usually be cured by conventional chemotherapy or by radiation therapy (7). Radical nephrectomy is the therapy of choice only for the initial stage; there is not yet any established therapeutic approach for the advanced metastatic stage (8). Because angiogenesis has been found to be essential for renal cancer progression (5, 6), antiangiogenic therapy, especially in combination with any potential chemotherapeutic agent, will have a promising future in designing an effective therapeutic approach for RCC.

Among the various subtypes of RCC, clear cell carcinoma is a common one (7). Most of the clear cell RCC is sporadic in nature and often develops due to the functional inactivation of the tumor suppressor gene von Hippel-Lindau (VHL) gene product (7). The loss of VHL protein (pVHL) leads to increased transcription of various angiogenic growth factors such as VPF/VEGF (9, 10), transforming growth factor β1 (11), matrix metalloproteinase-2 (5), and so forth. It is also responsible for enhanced VPF/VEGF mRNA stability (12). Previous work from our laboratory showed that pVHL inhibits the transcription factor Sp1 and thereby can suppress VPF/VEGF transcription (10). It has also been demonstrated that pVHL contributes to the ubiquitination and degradation of hypoxia-inducible factor (HIF)-1α under normoxic conditions (9, 13). The transcriptional activator HIF is a key regulator for coordinate expression of extensive arrays of genes required to maintain oxygen homeostasis in every living system during severe oxygen stress conditions. Due to its involvement in various physiological processes during hypoxia, a major goal for the investigators in this field is to understand the molecular mechanism that leads to increased HIF-1 transcriptional activity and the modifications that occur in this signaling event during pathological conditions.

The protein level of the HIF-1α subunit (14) or its isoforms in mammalian origin, namely, HIF-2α, HIF-3α, and so forth (15, 16), in the cell depends on cellular oxygen concentration (14). In normoxia, HIF-α subunits are rapidly degraded by the ubiquitin-proteasome pathway, such that the steady-state levels are low, and the transcriptional complex cannot form. Mechanistically, in high oxygen concentration, HIF-1α is hydroxylated at the proline residues of its oxygen-dependent degradation domain. This posttranslational modification of HIF-1α is necessary for its recognition by the tumor suppressor protein VHL gene product (pVHL) and subsequent degradation by proteasome complex (17, 18). However, stabilization of HIF-1α protein levels *per se* was not sufficient for its transcriptional activation. Recent evidence suggests that, in oxygenated cells, HIF-1α is also hydroxylated at an asparagine residue in the COOH-terminal transactivation domain. As a result, the HIF-1 complex cannot bind to the adaptor protein p300 to execute its transcriptional activity under normoxia (19, 20). Therefore, the present information suggests at least two regulatory signals for HIF activity that depend on the cellular oxygen levels.

It is known that in clear cell renal carcinoma cell lines such as 786-O cells, the VPF/VEGF level is high even under normoxic conditions (21, 22). HIF-1α and its isoform HIF-2α, the predominant one (23), here play an important role for the up-regulation of VPF/VEGF expression. In RCC, HIF-α subunits are not degraded under normoxic conditions due to the functional inactivation of pVHL (9, 24). However, until now, it has not been clear how HIF-1 and HIF-2α subunits escape the inactivation mechanism by asparagine hydroxylase under normoxic conditions in RCC. In this study, we have shown the signaling event and the detailed molecular mechanism by which HIF-1/2α is activated in RCC under normoxic conditions for the transcription of VPF/VEGF. Our studies have established protein kinase C (PKC) ζ as an important intermediary signaling molecule that regulates the association between HIF-1/2α and p300 in RCC. Phosphatidylinositol 3′-kinase (PI3K) is an upstream molecule in this pathway. More importantly, we have shown that this signaling event actually represses the total mRNA level of the asparagine hydroxylase (FIH-1) that regulates the expression of VPF/VEGF in RCC.

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Note: K. Datta and J. Li contributed equally to this work.

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Materials and Methods

Cell Culture. Human renal carcinoma cell line 786-O (American Type Culture Collection) was maintained in DMEM with 10% fetal bovine serum (HyClone Laboratories). 786-O is a human primary clear cell carcinoma cell line with epithelial morphology (American Type Culture Collection CRL-1932).

Nuclear Extract Preparation. 786-O cell suspension was incubated in a hypotonic buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 10 μg/ml aprotinin, 3 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride] for 15 min in ice. Nonionic detergent IGE-PAL (10%; Sigma) was then added to the cell suspension and mixed vigorously. The whole mixture was then centrifuged at 14,000 rpm in an Epppendorf centrifuge for 5 min. The pellets were again suspended in a hypertonic buffer solution [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 10 μg/ml aprotinin, 3 mM DTT, and 0.2 mM phenylmethylsulfonyl fluoride] and mixed on a rotating rack for 25 min at 4°C. The sample was then centrifuged at 14,000 rpm for 10 min, and the supernatant was collected as nuclear extract.

Whole Cell Extract Preparation. 786-O cells were washed twice with 10 ml of cold PBS, lysed with ice-cold lysis buffer [50 mM Tris (pH 7.5), 1% NP40, 150 mM NaCl, 1 mM Na_3VO_4, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 0.5% aprotinin, and 2 mM pepstatin A], incubated on ice for 10 min, and centrifuged at 4°C for 10 min.

Immunoprecipitation and Western Blot Analysis. Immunoprecipitation was performed in 0.2–0.5 mg of cellular protein from nuclear or whole cell extracts with antibodies (1 μg) directed against HIF-2α, HIF-1α, and p300 and pulled down by protein A-agarose beads (Pharmacia). For immunodetection antibodies against PKCζ, HIF-2α, HIF-1α, and p300 (all from Santa Cruz Biotechnology) were used.

Transfection and Luciferase Assay. 786-O cells (1×10^4 to 5×10^5) were seeded in either a 60-mm or 24-well plate 1 day before transfection. Transfection was carried out with Effectene Transfection Kit (Qiagen, Valencia, CA). Briefly, 0.4 μg of the 2.6-kb-long full-length VPF/VEGF promoter-luciferase construct was resuspended in a DNA suspension buffer (60 μl), and 3.2 μl of enhancer were added and incubated at room temperature for 5 min. Effectene (10 μl) was then added, and the whole mixture was incubated for another 10 min. DMEM with 10% fetal bovine serum (350 μl) was added to the DNA mixtures. This promoter contains all of the transcription factor binding sites for the VPF/VEGF gene. Plasmids expressing the kinase inactive form of PKCζ or pSiPKCζ [for RNA interference (RNAi) purpose] at different doses were also transfected to the 786-O cells in a similar way. The antisense oligonucleotide of PKCζ (5′-GACGCACGCGGCCTCACACC-3′) was also transfected as described earlier. Twenty-four h after transfection, cells were washed three times with PBS; nuclear or whole cell extracts were prepared or lysed with reporter lysis buffer (Promega) at room temperature for 15 min followed by luciferase assay. Luciferase activity was measured using the luciferase assay kit (Promega). Data are expressed as the mean ± SD of triplicate values.

The FIH-1 gene is located in chromosome 10q24. The locus ID in the National Center for Biotechnology Information database of FIH-1 is 55662, which contains the full-length FIH-1 gene sequence. The gene has eight exons. We have used the genomatix web site[3] to search for the transcription factor binding sites of the region upstream of the exon I and identified a 6633-bp long sequence to be the potential promoter for FIH-1 gene. Because there are binding sites for transcription factors after the ATG start codon, we considered the entire 6633-bp region along with a 317-bp region after the ATG as the approximate full-length promoter region of FIH-1. The whole 6950-bp region contains the binding sites for many transcription factors. Among those, the potentially important transcription factors are Smad4, Pax-3, cut-like homeodomain protein, c-Myb, AP2, AP1, C/EBP, Runt-related transcription factor 2, PPAR/RXR heterodimers, and so forth. The promoter region of FIH-1 was amplified by PCR from the genomic DNA prepared from HEK293 cells by using DNeasy tissue kit (Qiagen). The primers used for the PCRs are as follows: forward primer, 5′-CGACGCGTACACGCATATTATACTTC-3′; and reverse primer, 5′-CCGCTCGAGCATCTCTTTCACCATTC-3′. The PCR fragment was sequenced to confirm the proper sequence with the database and subcloned into pGL3-Basic vector in the *Mlu*I and *Xho*I sites. The promoter activity of FIH-1 was measured by measuring the luciferase activity as described above.

RNA Preparation and Real-Time PCR. After washing twice with ice-cold PBS, 786-O cells were lysed using lysis buffer from the RNeasy Mini Kit (Qiagen). The total RNA was extracted according to the RNeasy Mini Kit protocol. We used the TaqMan real-time PCR method. The sequences for forward, reverse, and TaqMan middle primers for human FIH-1 and human 36B4 (housekeeping gene) were taken from the PubMed gene bank and synthesized (Integrated DNA Technology): FIH-1 forward primer, 5′-GCCAGCACCCACAAGTTCTT-3′; FIH-1 reverse primer, 5′-CCTGTTGGACCTCGGCTTAA-3′; FIH-1 middle primer, 5′-GTTCTGGAAATTGGCCATCTTCTTCTCATCA-3′; 36B4 forward primer, 5′-ATGCAGCAGATCCGCATGT-3′; 36B4 reverse primer, 5′-TCATGGTGTTCTTGCCCATCA-3′; and 36B4 middle primer, 5′-CACCACAGCCTTCCCGCGAA-3′. Each real-time PCR reaction was performed using 0.5 μg of total RNA, 25 μl of reverse transcription-PCR Master Mix (Applied Biosystems), 1.25 μl of Rnase inhibitor (Applied Biosystems), 50 nM forward primer, 50 nM reverse primer, and 100 nM middle primer. For reverse transcription, a 30-min period at 48°C was run before inactivating the reverse transcriptase at 95°C for 10 min. Forty cycles of 95°C for 15 s and 60°C for 1 min were performed with an ABI Prism 7700 Sequence Detector (Applied Biosystems). All experiments were carried out three times, and from each of the three experiments, triplicate readings were taken, and the average was calculated. The relative RNA amount was calculated as follows: Δ = CT(FIH-1 sample) − CT(36B4 sample). $\Delta\Delta = \Delta$(transfected sample) − Δ(empty vector sample). Relative RNA amount in comparison with the control = $2^{-\Delta\Delta}$. Average and SD from three experiments were calculated.

Reverse Transcriptase and PCR. The cDNA was prepared from the total RNA (5 μg) of the 786-O cells transfected with the different concentrations of PKCζDN plasmid or control plasmid. We used the Superscript First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) for cDNA preparation. Of 30 μl of the total cDNA, 6 μl were used for PCR amplification of FIH-1, and 1 μl was used for the constitutively expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using high-fidelity platinum Taq DNA polymerase (Invitrogen). The total number of thermal cycles for this PCR reaction was 40. One thermal cycle consisted of 15 s at 95°C, 40 s at 56°C, and 40 s at 72°C. The PCR products of FIH-1 and the GAPDH were then resolved into 2% agarose gel (FIH-1 forward primer, 5′-CAGTTGCGCAGTTATAGCTTC-3′; FIH-1 reverse primer, 5′-GCCATCTTCTTCTCATCATAG-3′; GAPDH forward primer, 5′-CCACCCATGGCAAATTCC-3′; GAPDH reverse primer, 5′-TGGGATTTCCATTGACAAG-3′).

RNAi for PKCζ. Endogenous silencing of PKCζ in renal cancer cell line 786-O was achieved by using small interference RNA (siRNA) strategy. The pRetro-SUPER siRNA expression vector (Oligoengine) was used to generate retrovirus that expresses siRNAs of ~21 nucleotides in length with 2–3 nucleotide overhangs complementary to the human PKCζ gene. The oligonucleotide complementary to the human PKCζ gene was cloned into the pRetro-SUPER vector in the *Hind*III and *Bgl*II sites. The sequence is as follows: 5′-GTGAGAGACATGTGTCGTCTTCAAGAGAAGACGACACATGTCTCTCAC-3′. The 786-O cells were infected with siRNA-PKCζ retrovirus and incubated for 72 h. The protein level of PKCζ was detected in the cell lysate by Western blot.

Retrovirus Infection. Retrovirus containing the dominant negative mutant of p85 was prepared by transfecting 293T cells (3×10^5 cells/100-mm tissue culture dish) with pMMP-p85DN plasmid. pMMP-LacZ was also used as a control. Two μg of targeted gene, 1.5 μg of pMD.MLV gag.pol, and 0.5 μg of pMD.G (the last two plasmids are required for virus packaging and were kindly provided by Dr. Richard Mulligan) were transfected with the Effectene reagent. After 48 h of transfection, the culture media were collected, filtered, and used as retrovirus-containing media. Retrovirus-containing media (0.5 and 1.0 ml) was added to 786-O cells (in 60-mm tissue culture dishes). After 16 h of incubation with the virus, fresh medium was added to the cells. After another 24 h of incubation, either mRNA or nuclear extract was isolated from the cells for additional studies.

Results and Discussion

Kinase Inactive Dominant Negative Mutant of PKCζ Inhibits the HIF-α-Mediated Promoter Activity of VPF/VEGF. We have shown previously that PKCζ was an important intermediary for VPF/

[3] www.genomatix.de.

VEGF transcription in RCC where pVHL was nonfuntional (25). Inhibition of PKCζ kinase activity or its expression led to complete down-regulation of VPF/VEGF expression, as observed by the VPF/VEGF promoter luciferase activity as well as Northern blot analysis (26). These results helped us to investigate whether PKCζ had any role in HIF-induced transcriptional up-regulation of VPF/VEGF. Hence, we assayed the transcriptional activity of VPF/VEGF using 2.6-kb VPF/VEGF promoter construct fused with the luciferase reporter gene in the presence of HIF-2 and HIF-1α subunits and with or without the overexpressed kinase inactive mutant of PKCζ. Fig. 1, *A* and *B,* illustrated a substantial increase in luciferase activities when HIF-α subunit expression plasmids were transiently cotransfected with the VPF/VEGF promoter luciferase construct in 786-O cells under ambient (21%) oxygen concentration (normoxia). On the other hand, the VPF/VEGF promoter activation due to HIF-α subunit was inhibited by cotransfecting the kinase inactive mutant of PKCζ (PKCζKW, Lys281Trp), suggesting its importance in the transcriptional activity by both of the HIFs.

The Kinase Inactive Mutant of PKCζ Inhibited the Association of p300 and HIF-α Subunit. We also examined the molecular mechanism that leads to the transcriptional activity of HIF-α subunit by PKCζ. It was known that adaptor protein p300 directly interacts with both of these HIFs to facilitate its activity for increased expression of target genes (19). It thus appeared to be logical to examine the effect of PKCζ on the interaction between p300 and both HIF proteins in RCC. Nuclear extracts were isolated from 786-O cells in normoxia, which were transfected with the dominant negative mutant of PKCζ or an empty vector. Immunoprecipitation reactions were performed with anti-p300 antibody followed by Western blot with anti-HIF-2α or HIF-1α antibody. Fig. 2, *A* and *B,* show clear HIF-2α and HIF-1α protein bands, suggesting the presence of the association of these two molecules with p300 in 786-O cells under normoxia. Interestingly, the association was inhibited in the presence of the overexpressed form of the kinase inactive mutant of PKCζ. These results indicated a positive role of PKCζ for the association of p300 and HIFs that promotes transactivation. Fig. 2, *A* and *B,* shows the individual protein level of p300, HIF-1α, and HIF-2α in RCC, which was unchanged in the presence of overexpressed PKCζ dominant negative mutant. Similar inhibition of association was observed when the immunoprecipitation of HIF-2α was performed followed by Western blot with p300 of the cell lysates of the 786-O cells that were transfected with the PKCζ dominant negative plasmid (Fig. 2*C*). To identify whether PKCζ was essential for the HIF-α/p300 association, we used the siRNA approach against PKCζ. The inhibition of HIF/p300 association in RCC was again observed after depleting the endogenous PKCζ level by siRNA, thus providing a validation of our PKCζ dominant negative approach (Fig. 2, *D* and *E*). The same observation was made using an antisense approach against PKCζ (data not shown). Taken together, our findings suggested an integral role of PKCζ in HIF-mediated transactivation in RCC.

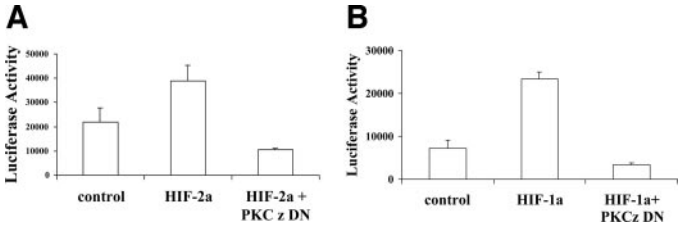

Fig. 1. Protein kinase C ζ can modulate hypoxia-inducible factor (HIF)-1α- and HIF-2α-mediated vascular permeability factor/vascular endothelial growth factor promoter activity in renal cell carcinoma. Plasmid DNA containing the full-length vascular permeability factor/vascular endothelial growth factor promoter (2.6 kb) fused with luciferase reporter gene was transiently cotransfected with (*A*) HIF-2α- or (*B*) HIF-1α-expressing plasmids alone or in combination with protein kinase C ζ kinase inactive mutant-expressing plasmids. Total luciferase activities in the cells in normoxia were measured by a chemiluminescent reader. The data represented here are the average of three independent results.

Recently, it was shown that asparagine hydroxylase inhibited the association between HIF-2/1α and p300 under normoxia (20). The above observations had a 2-fold significance: first, they established that in 786-O cells, even under normoxia, HIF-2/1α can associate with p300 to form a functional transcription initiation complex, suggesting the presence of at least one mechanism in RCC that can circumvent the asparagine hydroxylase activity; and secondly, they established PKCζ as a signaling molecule that is a part of this mechanism. Therefore, it would be interesting to determine whether the activation mechanism of HIF-α by PKCζ involves asparagine hydroxylase, an intermediary molecule, or whether PKCζ acts independently in this situation. Current studies show that a previously discovered protein, FIH-1, was actually the asparagine hydroxylase specific for HIF-1 and HIF-2α (20, 27). We thus wanted to test whether FIH-1 expression or activity can be controlled by PKCζ in RCC.

We first tested whether FIH-1 can function in the same way in RCC as reported by other researchers in other cancer types. When 786-O cells were transiently cotransfected with a full-length (2.6-kb) VPF/VEGF promoter luciferase construct with increasing concentration of FIH-1 expression plasmid, the dose-dependent inhibition of VPF/VEGF promoter activity with increasing concentration of FIH-1 expression was observed (data not shown). We also confirmed that in RCC, FIH-1 inhibits the association of HIF-2α and p300. Nuclear extracts of 786-O cells had been collected with or without the presence of overexpressed FIH-1. Immunoprecipitation of anti-p300 antibody followed by Western blot with anti-HIF-2α antibody showed that FIH-1 indeed inhibits the association in 786-O cells under normoxic conditions (data not shown).

Total mRNA Level of FIH-1 Was Increased in Renal Cancer Cells in the Presence of the Kinase Inactive Form of PKCζ. To examine whether PKCζ had any effect on the expression of FIH-1, the total mRNA level of FIH-1 was assayed after inhibiting PKCζ activity in the 786-O cell line in normoxia. We performed the real-time PCR experiments with the mRNA isolated from 786-O cells after transfecting them with different concentrations of expression plasmid of the kinase inactive form of PKCζ. A dose-dependent increase in the FIH-1 mRNA level was observed in the presence of dominant negative PKCζ (Fig. 3*A*). To validate our real-time PCR result, we performed the reverse transcription-PCR experiment of the mRNA isolated from 786-O cells that were transfected with increasing doses of PKCζ dominant negative plasmid or the control plasmid. The DNA samples were then run into an agarose gel to detect the FIH-1 level (Fig. 3*B*). A clear increase in FIH-1 level was observed in 786-O cells with increasing doses of dominant negative PKCζ. These findings suggested that PKCζ can suppress the total mRNA level of FIH-1 and provided at least one possible explanation why, in the presence of the activated form of PKCζ, p300 can associate with HIF-2 and HIF-1α even under normoxia in renal cancer cells.

PI3K Is Upstream of PKCζ for the Signaling Event That Regulates the Association of p300 and HIF-2α and Also Decreases the FIH-1 mRNA Level. We also examined the upstream signaling molecules of PKCζ, which might be responsible for down-regulation of the association of p300 and HIF-2/HIF-1α. Our previous experiments suggested that PI3K was an upstream molecule in this pathway (26). We used a retrovirus construct expressing a dominant negative PI3K (a kinase inactive mutant of p85, the catalytic domain of PI3K) and infected the 786-O cells in different virus doses. The immunoprecipitation experiment was performed with nuclear extracts isolated

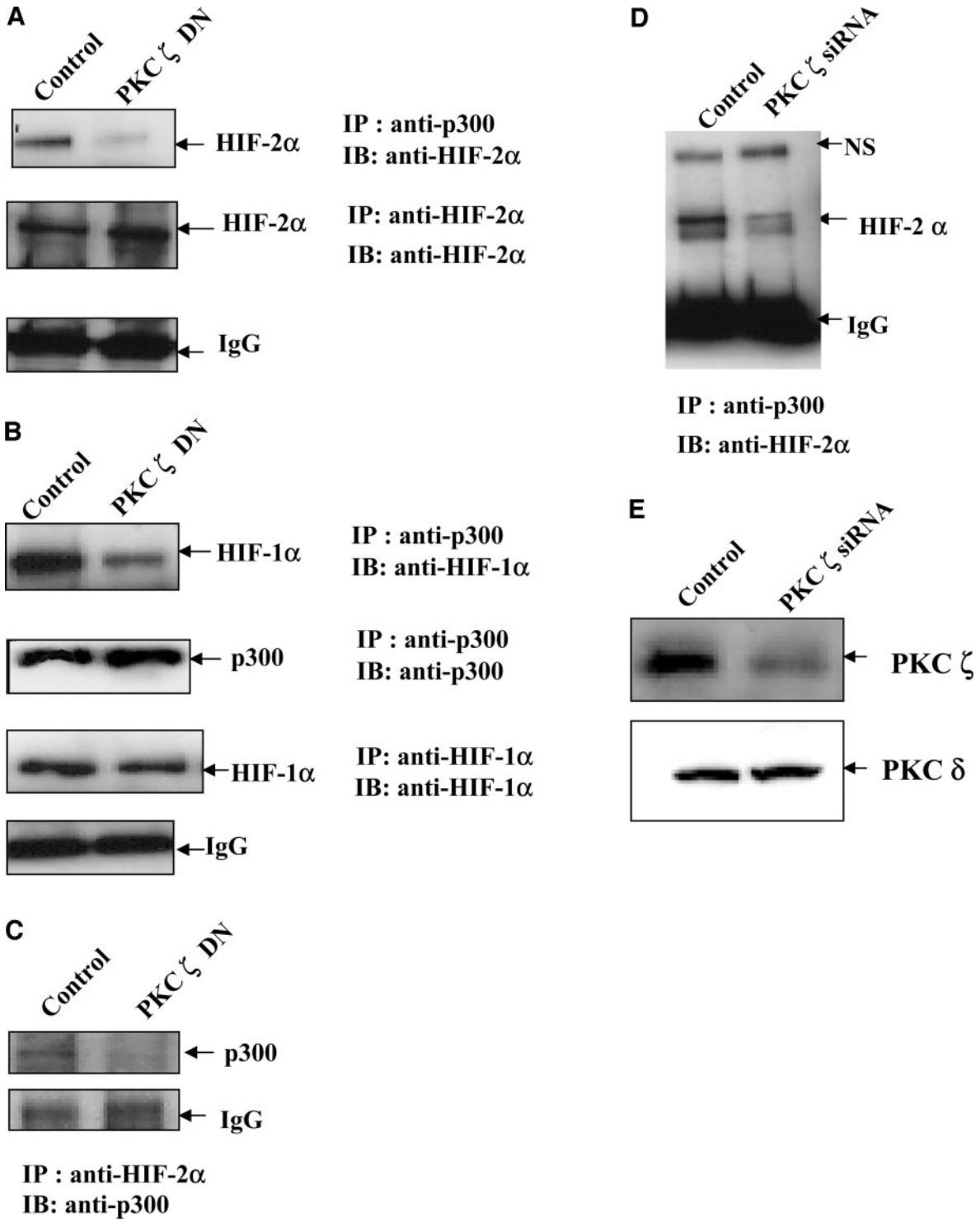

Fig. 2. Protein kinase C (PKC) *ζ* is important for the association of hypoxia-inducible factor (HIF)-2*α* and HIF-1*α* with p300 in renal cell carcinoma (RCC). Nuclear extracts were prepared from RCC 786-O cells either transiently transfected with the kinase inactive mutant of PKC*ζ*-expressing plasmid or the control plasmid in normoxia. *A*, immunoprecipitation (*IP*) of the nuclear extracts with anti-p300 antibody followed by Western blot (*IB*) with anti-HIF-2*α* antibody. *B*, immunoprecipitation (*IP*) experiment with anti-p300 antibody followed by Western blot (*IB*) with anti-HIF-1*α* antibody. Individual protein levels of p300, HIF-1, and HIF-2*α* in the presence of the dominant negative mutant of PKC*ζ* in RCC are shown in the *bottom panels*. *C*, immunoprecipitation (*IP*) of the nuclear extracts with anti-HIF-2*α* antibody followed by Western blot (*IB*) with anti-p300 antibody. *D* and *E*, RNAi approach. Nuclear extracts were prepared from RCC 786-O cells and infected with retrovirus containing siRNA-PKC*ζ* or the control retroviral vector. *D*, immunoprecipitation (*IP*) of the nuclear extracts with anti-p300 antibody followed by Western blot (*IB*) with anti-HIF-2*α* antibody. *E*, the *top panel* shows the PKC*ζ* protein level in cell lysates of 786-O cells with siRNA-PKC*ζ*, and the *bottom panel* shows the PKC*δ* level providing evidence in favor of the specificity of the RNAi approach.

from the infected 786-O cells with anti-p300 antibody followed by Western blot with anti-HIF-2*α* antibody. The association between p300 and HIF-2*α* in 786-O cells was significantly decreased after virus infection (Fig. 3*C*). We also collected total RNA from the infected cells and performed real-time PCR experiments to detect the mRNA level of FIH-1 (Fig. 3*D*). A significant increase in the FIH-1 total mRNA level had been observed in renal cancer cells due to the inhibition of PI3K activity. All of these results suggested a signaling

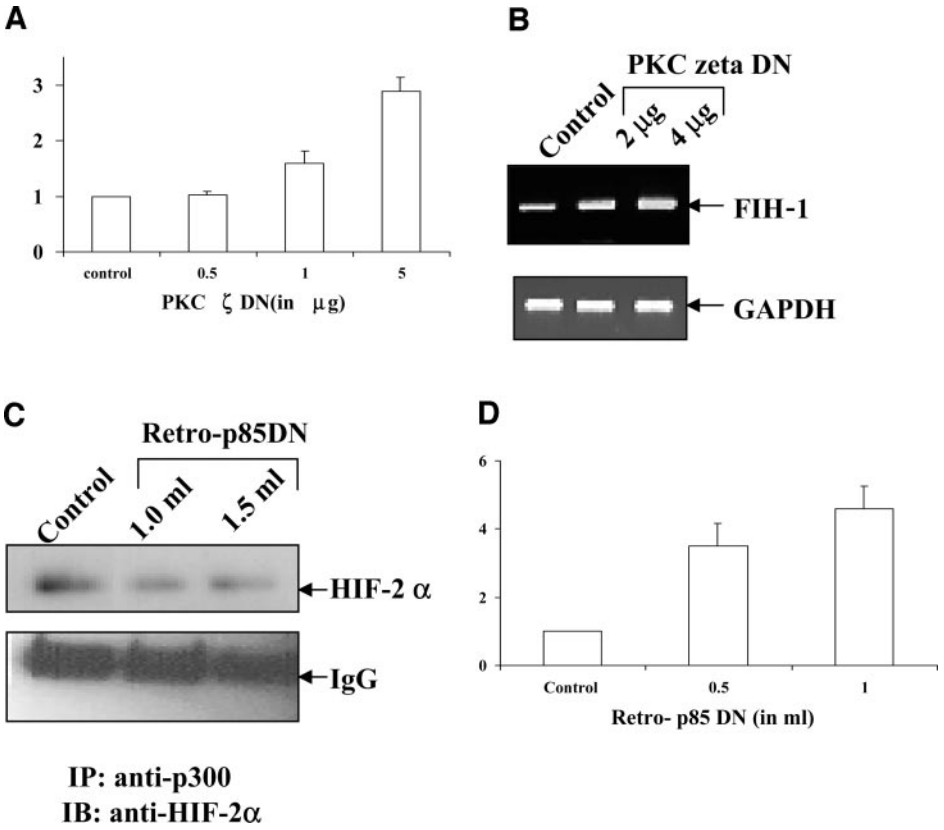

Fig. 3. Protein kinase C (PKC) ζ dominant negative (DN) can increase the total mRNA level of FIH-1. *A*, 786-O cells in normoxia were transfected with different concentrations of PKCζ-DN plasmids (0.5, 1.0, and 5.0 μg, respectively) in 60-mm tissue culture dishes. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. The data represented here are the average of three independent results. *B*, 786-O cells in normoxia were transfected with different concentrations of PKCζ-DN plasmids (2.0 and 4.0 μg, respectively) in 60-mm tissue culture dishes, and mRNA was collected. After reverse transcription of the mRNA, PCR was performed using primers specific to FIH-1 (*top panel*). The DNA products were then resolved into an agarose gel. The *bottom panel* represents the amount of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) DNA as the control. *C*, phosphatidylinositol 3′-kinase is essential for the association of hypoxia-inducible factor 2α with p300 in renal cell carcinoma. Nuclear extracts prepared from RCC 786-O cells were infected with retrovirus carrying p85DN or lacZ (control) in normoxia. Immunoprecipitation (*IP*) of the nuclear extracts with anti-p300 antibody was performed, followed by Western blot (*IB*) with anti-hypoxia-inducible factor 2α antibody. *D*, infection with p85DN-containing retrovirus can increase the total mRNA level of FIH-1. 786-O cells were infected with retrovirus carrying p85DN or lacZ (control) at a dose of 0.5 and 1.0 ml in 60-mm tissue culture dishes. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. The data represented here are the average of three independent results.

pathway that involves PI3K as an upstream molecule of PKCζ for inhibiting the FIH-1 mRNA level.

The involvement of PI3K and PKCζ for FIH-1 transcription was also tested in other cell lines in normoxia. In the presence of the dominant negative form of PI3K and antisense PKCζ, the total FIH-1 mRNA level was increased in A498 cells, a VHL-mutated renal carcinoma cell line (Fig. 4, *A* and *B*). The antisense PKCζ that we used was isoform specific and was reported by several groups, including ours (26, 28–34). A similar result was observed when dominant negative PKCζ was used in A498 cells (data not shown). The FIH-1 mRNA level was also increased in the pancreatic adenocarcinoma cell line ASPC-1 in the presence of increasing doses of PI3K and dominant negative PKCζ (Fig. 4, *C* and *D*). We detected a similar increase in FIH-1 total mRNA in the human fibrosarcoma cell line HT1080 after treating the cells with retro-p85DN (the kinase inactive mutant of the catalytic domain of PI3K) or antisense PKCζ (data not shown). Interestingly, we did not find any change of FIH-1 mRNA level in breast cancer cell line MDA-MB-435 under our experimental conditions. The overall conclusion of all these findings was that the PI3K/PKCζ pathway that controls the total mRNA level of FIH-1 is active in a number of tumor types and not specific for 786-O cells; however, it is also true that different types of signaling events for FIH-1 regulation are also present in some other cancer cell lines.

PKCζ Regulates the Transcription Activity of FIH-1. The promoter region of FIH-1 was cloned into pGL3-Basic vector and transfected with plasmids that express either the dominant negative form of PKCζ or the empty vector. A dose-dependent increase in promoter activity was observed in 786-O cells when cells were transfected with dominant negative PKCζ plasmid (Fig. 4*E*). Importantly, a dose-dependent decrease in FIH-1 promoter activity was observed in 786-O cells when cells were transfected with wild-type PKCζ plasmid (Fig. 4*F*). These results conclusively proved the involvement of PKCζ in regulating FIH-1 promoter activity. This increase in promoter activity in the presence of dominant negative PKCζ was observed in all of the cell lines (*e.g.*, 786-O, A498, ASPC-1, and so forth) that were previously found to have an increase in FIH-1 mRNA by real-time PCR experiments (Fig. 4*G*). These results, taken together, suggested that the increase in mRNA level of FIH-1 due to inhibition of PI3K/PKCζ signaling pathway was at least partially due to the increase in transcription activity of FIH-1 promoter. They also corroborated our previously described real-time PCR results.

The up-regulation of VPF/VEGF is one of the important events for the onset of tumor angiogenesis. It is therefore necessary to understand the mechanism and cellular signaling event for the expression of VPF/VEGF under pathological situations. It is also important to comprehend the differences between the signaling events that up-regulate VPF/VEGF expression under physiological and pathological conditions. This information, in the end, will help to develop an effective antiangiogenic therapy. In this study, we attempted to delineate the molecular mechanism of VPF/VEGF expression in renal cells that can mimic hypoxic regulation of VPF/VEGF in normoxia due to the presence of a specific signaling event. FIH-1, presently identified as the asparagine hydroxylase, specifically hydroxylates HIF-1/HIF-2α under normal physiological conditions in normoxia and inhibits their association with the adaptor protein p300 that leads to shut down the HIF-mediated gene expression (19). Because oxygen is one of the substrates for FIH-1, the enzymatic activity of FIH-1 depends on the cellular oxygen concentration. In the hypoxic condition, FIH-1 is not active due to the low availability of cellular oxygen, resulting in the withdrawal of its inhibitory effect on HIF-1/HIF-2α. Therefore, FIH-1 acts like a cellular oxygen sensing system that prevents the HIF-mediated expression of certain hypoxia-induced genes. On the other hand, tumor cells require the expression of many of those same HIF-induced genes for their survival, rapid growth, and metastasis. Importantly, this expression is often independent of cellular oxygen concentration. Hence, studies on renal cancer cells have

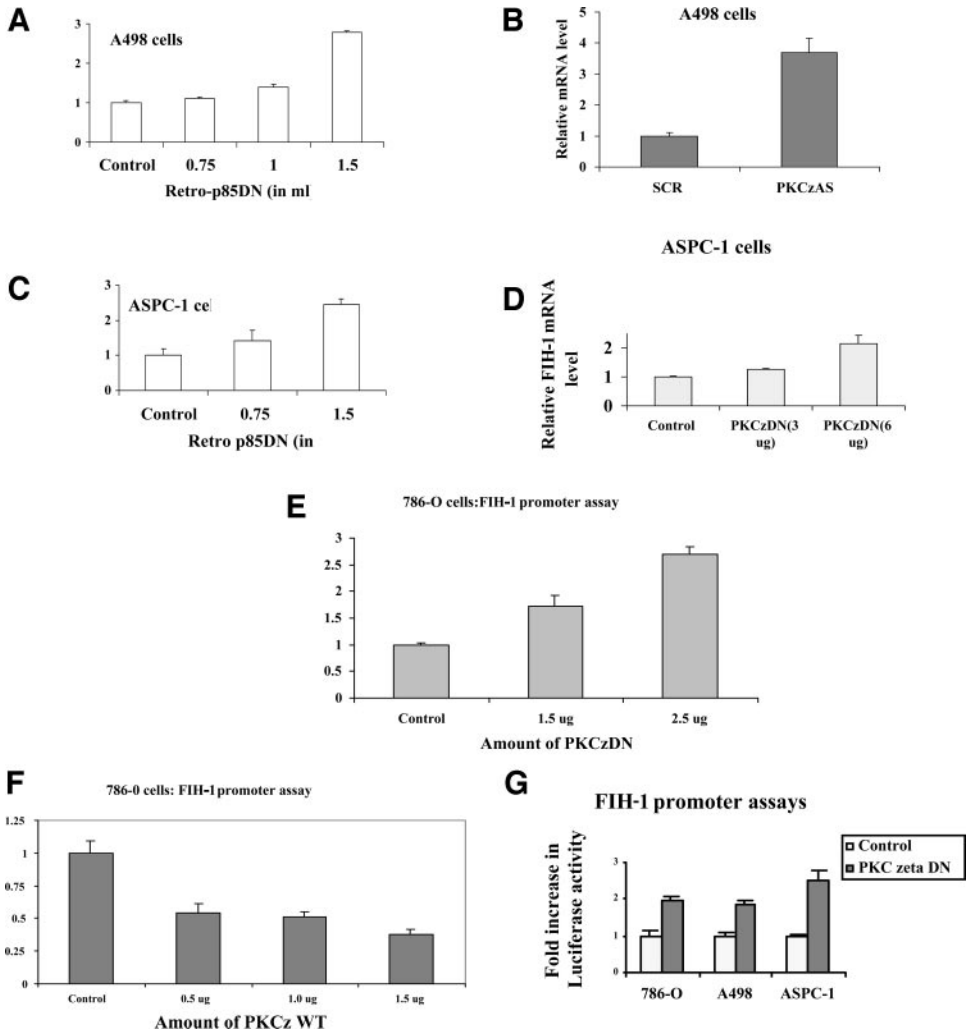

Fig. 4. *A*–*G*, inhibition of the phosphatidylinositol 3′-kinase pathway increases the total mRNA and transcriptional level of FIH-1 in different cancer cells. *A*, A498 cells were infected with increasing doses of retrovirus carrying p85DN or lacZ in 60-mm tissue culture dishes. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. *B*, A498 cells were transfected with antisense oligonucleotide of protein kinase C (PKC) ζ (*PKCzAS*) or scramble (*SCR*) oligonucleotide. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. *C*, ASPC-1 cells were infected with increasing doses of retrovirus carrying p85DN or lacZ in 60-mm tissue culture dishes. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. *D*, ASPC-1 cells were transfected with the dominant negative form of PKCζ or a control plasmid. Real-time PCR was performed using primers specific to FIH-1 with the total RNA isolated from the cells. *E*, FIH-1 promoter luciferase activity assay in 786-O cells after cotransfecting the cells with the FIH-1 promoter plasmid and different doses of dominant negative PKCζ-expressing plasmid or control plasmid in normoxia. The data represented in all of the experiments are the average of three independent results. *F*, FIH-1 promoter luciferase activity assay in 786-O cells after cotransfecting the cells with the FIH-1 promoter plasmid and different doses of wild-type PKCζ-expressing plasmid or control plasmid in normoxia. The data represented in all of the experiments are the average of three independent results. *G*, FIH-1 promoter luciferase activity assay in 786-O, A498, and ASPC-1 cells after cotransfecting the cells with the FIH-1 promoter plasmid and the dominant negative PKCζ-expressing plasmid or control plasmid in normoxia. The data represented in all of the experiments are the average of three independent results.

shown the expression of high levels of HIF-induced genes such as VPF/VEGF and transforming growth factor β (9, 11) under normoxic conditions. It has also been known that HIF-2/HIF-1α is stabilized in renal cancer cells even in normoxia due to the functional inactivation of the VHL gene (9). In this study, we provided evidence in support of a definite mechanism by which HIF-2/HIF-1α is activated in RCC in normoxia after being stabilized.

In renal cancer cells, several signaling molecules such as Ras, PI3K, PKCζ, and so forth are always in the activated form. This triggers signaling events unique to the tumor cells and is necessary for its survival and growth. Our study has shown that a specific signaling event involving PKCζ and PI3K down-regulates the total mRNA level of FIH-1 and thereby helps HIF-2/HIF-1α to be activated. This leads to its association with p300 and subsequently enhances the expression of the target genes under the hypoxic condition. We have also proved, by promoter luciferase assays, that at least part of the total increase in FIH-1 mRNA is due to the increase in the transcription activity of the FIH-1 promoter. The significance of this finding is severalfold. First, it placed PKCζ in a central position for transcription of VPF/VEGF in renal cancer cells, which controls both the transcription factors (HIF-2/HIF-1α and Sp1) for VPF/VEGF transcription. Of importance, previous studies from our laboratory (25) also pointed out the role of PKCζ in the activation of Sp1 for VPF/VEGF transcription. It also pointed out one cellular signaling event by which HIF-α can be activated under normoxic condition. The observation of a similar signaling event in A498, another VHL-deficient RCC cell line, ASPC-1, a human adenocarcinoma of pancreas, and HT1080, a human fibrosarcoma cell line, proves that it is not specific for the 786-O cell line. Our failure to detect the same signaling event in MDA-MB-435 cells may indicate that the mechanism of FIH-1 regulation can vary in cancer types. Because Ras is mutated and oncogenically active in 786-O, A498, ASPC-1, and HT1080 cells, but not in MDA-MB-435 cells, one possible explanation is that the PI3K/PKCζ pathway that regulates FIH-1 expression is active only in the background of active Ras. Additional studies will be needed to prove this hypothesis. Finally, this is the first evidence that suggests the presence of a tumor cell-specific mechanism for controlling the mRNA level of FIH-1.

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