Protein Kinase C-Mediated Modulation of FIH-1 Expression by the Homeomain Protein CDP/Cut/Cux

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Under normoxia, FIH-1 (factor inhibiting HIF-1) inhibits the transcriptional activity of hypoxia-inducible factor (HIF); however, under such conditions, we observed a significant level of HIF activity in renal cell carcinoma (RCC). This phenomenon could be attributed to a decrease in the level of functional FIH that has been identified in our previous work. Nonetheless, the molecular mechanism of FIH regulation in cancer, in particular RCC, was unclear until now. In this communication, we have demonstrated that in RCC, the Cut-like homeodomain protein (CDP/Cut) is involved in FIH transcriptional regulation and is controlled by a specific signaling event involving protein kinase C (PKC) ζ. Furthermore, we have defined a unique CDP/Cut binding site on the FIH promoter, With chromatin immunoprecipitation assays, we show that CDP binds to the FIH-1 promoter in vivo and that this binding is PKC ζ dependent. Moreover, we have also defined a potential phosphorylation site in CDP (serine 987) that modulates FIH expression. CDP/Cut is a transcriptional repressor that decreases FIH-1 expression and subsequently leads to a decrease in the repressor activity of FIH-1. Without this repression, HIF activity increases, allowing for the increased transcription of the genes it regulates, such as the vascular endothelial growth factor and GLUT-1 genes. Both CDP and HIF levels are increased in several cancers and are responsible for the metastatic progression of the tumors. Taken together, our results suggest for the first time a potential connection between CDP and FIH that could lead to the development of future therapeutic interventions.

Hypoxia-inducible factor (HIF) is a heterodimeric transcription factor that regulates a broad range of hypoxic responses in the cells and tissues of an organism (40, 41). These adaptive responses include the up-regulation of target genes involved in angiogenesis, erythropoiesis, matrix metabolism, glycolysis, vasomotor tone control, cell survival, and cell death (9, 27). To date, about 110 hypoxia-inducible genes have been found to be directly regulated by HIF-1 (14, 43).

HIF-1 is composed of two subunits that are both basic helix-loop-helix PAS proteins (40). The α subunit is rarely detectable in normoxia, but strikingly, it is induced under hypoxia in all cell types examined so far (12, 34–36). On the other hand, the β subunit, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), is not influenced by oxygen concentrations (15). It has been found that the hydroxilation of HIF-α subunits is important for their function as a transcription factor. In normoxia, HIF-αes are hydroxylated by prolyl hydroxylases (PHDs) and the asparagine hydroxylase FIH-1 (factor inhibiting HIF), members of the 2-oxoglutarate (2OG) superfamily, and Fe(II)-dependent dioxygenases (1, 11, 12, 14, 19, 21, 28–30, 31, 37). The PHDs, also known as EGLNs, hydroxylate at a specific proline residue (14). This hydroxilation recruits pVHL (von Hippel-Lindau gene product), where pVHL subsequently ubiquitinates HIF-α, designating it for degradation (13, 16, 18, 25, 26, 34, 44), while FIH-1 hydroxylates a specific asparagine residue within the HIF-α activation domain (21, 24). In normoxia, the FIH-1-mediated hydroxylation of the conserved asparagine residue at the COOH-terminal end of the HIF-α activation domain leads to the inhibition of p300/CBP recruitment (11, 17, 21, 22, 28). p300/CBP is a coactivator of FIH-1, and blocking its recruitment represses the transcriptional activity of HIF-1-targeted genes (2, 8, 15). One such target gene is the vascular endothelial growth factor gene, which has been shown to be inhibited by FIH-1 in cancer cells. Again, this modification is inhibited under hypoxia and leads to the transactivation of HIF-1.

Crystallographic studies suggest a unique active-site pocket and interactive sites for HIF-1 on FIH-1 (5, 22). Furthermore, it is very important that the length of the HIF substrate and the sequence surrounding the hydroxilation site play a crucial role in determining the catalytic efficiency of FIH-1 (7, 17, 23). The formation of an FIH-1 homodimer is required for substrate recognition and hydroxylase activity, and a single-point mutation (L340R) was found to be sufficient to disrupt the dimerization and ablate catalysis (20).

The FIH-1 gene is located on chromosome 10q24 (locus ID is 55662). FIH-1 contains the JmjC domain, which may be involved in chromatin structure and dynamics and signal transduction mechanisms (7). Metzen et al. have shown that the intracellular localization of FIH-1 was mainly in the cytoplasm and was not influenced by hypoxia (29). FIH-1 protein is widely expressed in human tissues and is thus potentially available for the regulation of HIF activity across a broad range of cells and culture conditions (38). Recent reports also indicate that FIH-1 may contribute to the regulation of PHD-2 and -3 ex-
pression in hypoxia, thus promoting HIF stability. It is also suggested that FIH-1 has important nonredundant effects in vivo on the expression of a range of HIF transcriptional targets in normoxia and even in severe hypoxia (38).

In summary, FIH-1 is a corepressor that interacts with HIF-α and the von Hippel-Lindau tumor suppressor protein to mediate the repression of HIF-1 transcriptional activity (24). Understanding FIH-1 regulation is essential to elucidate the HIF-1-mediated hypoxic response due to physiological stresses. Recent genetic studies did not detect any mutation in the FIH-1 gene that can be correlated with disease progression (31). This finding suggests a possible epigenetic control of FIH-1 expression responsible for FIH-1-associated pathogenesis. Our previous paper showed an increase in FIH-1 mRNA in renal cancer cells in the presence of the kinase-inactive form of protein kinase C (PKC) ζ. These data suggest that PKC ζ may play an important role in FIH-1 transcriptional regulation. In accordance with our previous results (6), we found that the FIH-1 protein level significantly increased when PKC ζ was blocked in renal cell carcinoma (RCC) cells using PKC ζ interfering RNA (siRNA) or by overexpressing dominant-negative PKC ζ. To further study the mechanism of this event, we have cloned the FIH-1 promoter region and identified a cis element important for PKC ζ-mediated inhibition of FIH-1 transcription. Furthermore, we detected, via in vitro and in vivo assays, PKC ζ-mediated binding of the homeobox protein-CDP (CCAAAT displacement protein), an important regulator of FIH-1 transcription, to the FIH-1 promoter.

**Materials and Methods**

**Cell culture and reagents.** The human renal cancer cell line 786-O (ATCC no. CRL-1932) and the human embryonic kidney cell line HEK293 were maintained in Dulbecco modified Eagle medium with 10% fetal bovine serum (HyClone Laboratories, Logan, UT). 786-O is a human primary clear-cell carcinoma line with an epithelial morphology. Expression wild-type vectors as well as mutant plasmids of CDP/Cut were kind gifts from Alain Nepveu, McGill University, Quebec, Canada. The pCMV/Cut/Mut CDP 6-mutant plasmid contains Ser402, Thr415, Ser789, Thr804, Ser972, and Ser987 and also Ser987 alone (pCMV/Cut CR#-HD/S987A), residues which were replaced with codons for Ala (4). Note that the Ser987 mutant should not be recognized by anti-CDP antibody (Santa Cruz Biotechnology, Inc.) as this is a much smaller version of the wild-type CDP as described earlier (4).

FIH-1 reporter constructs and deletion of the FIH-1 promoter. A 6,950-bp FIH-1 promoter construct was cloned into pGL3-basic vector to create an approximately full-length FIH-1 promoter. Details were described in our previous paper (6). Based on this construct, a series of deletion mutants (see Fig. 1) of the FIH-1 promoter were generated by direct PCR amplification from genomic DNA prepared from HEK293 cells by using the DNacay kit (QIAGEN Inc., Valencia, CA). The length was counted with the transcription start site as 1, and upstream of the ATG codon was a minus count. PCR primers were designed containing an MluI site in the 5′ forward primers and an XhoI site in the 3′ reverse primers. The 5′ forward primer used for the −2.7 kb construct was 5′-CG AGC CGT CTA CAT GGT GTG TTA TAT TGA TC-3′, and the reverse primer is 5′-CCG CTC GAG CAT CTT CTC TAT CAC TC-3′. The 480-bp forward primer is 5′-CCG CTC GAG CAT TCT TTT CAC CAT TC-3′, and its reverse primer is identical to the 2.7-kb reverse primer. The −220-bp forward primer is identical to that of the 480-bp construct, and its reverse primer is 5′-CCG CTC GAG CAT CTT CTC TAT CAC TC-3′.

**Western blot analysis.** The whole-cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the immunodeTECTION antibodies used were FIH-1 (catalog no. BC-100-428A; Novus Biologicals, Littleton, CO), CDP (catalog no. sc-13024; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-mitogen-activated protein (phospho-MAP) kinase (catalog no. 07-467; Millipore, Billerica, MA), phosphosine (catalog no. AB1603; Chemicon, Temecula, CA), and PKC ζ (Santa Cruz Biotechnology). Primary incubations were followed with an appropriate secondary antibody and detected with the ECL enhancer reagent from Amersham Biosciences Corp. (Piscataway, NJ). For immunoprecipitation assays with the phosphosine antibody, anti-PKC ζ antibody was used for Western blotting.

Luciferase reporter assays. 786-O cells and 293 cells (5 × 10⁴) were seeded in a 12-well plate 1 day before transfection. All transfections were carried out using 0.5 μg of FIH-1 promoter constructs using the Effectene transfection kit (described above) and incubated for an additional 48 h. The cells were then lysed with 1× lysis buffer for the luciferase assay.
the Effectene reagent as described above. A total of 1 μg of FIH-1 promoter construct and 0.1 μg of pRL-TK Renilla luciferase vector (Promega, Pittsburgh, PA) were used for each transfection. The pRL-TK Renilla luciferase activity was used as the control for transfection efficiency. In some of the experiments, we equilibrated the luciferase activities by protein concentrations of the cell extracts with respect to the control vectors. Each transfection experiment was performed in triplicate and repeated for a minimum of three times. For cotransfection experiments, cells received 0.3 μg of different deletion constructs of pGL3-FIH-1, 0.1 μg of pRL-TK Renilla luciferase vector, 1 μg of the indicated expression plasmids (PKCζ DN) or siRNA of CDP/Cut, and carrier DNA. Firefly luciferase and Renilla luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). Approximately 48 h after transfection, cells were washed twice with 1× phosphate-buffered saline and harvested with 200 μl of passive lysis buffer. Cell lysates were cleared by centrifugation, and 5 μl of lysate was added to 100 μl of firefly luciferase substrate. Light units were measured in a LB960 microplate luminometer. Renilla luciferase activities were measured in the same well after addition of 100 μl of the Stop and Glo reagent.

The average was calculated among three wells; the ratio between the firefly luciferase and Renilla luciferase was calculated, and the relative change (n-fold) was obtained after comparing the results to the control sample. Data were expressed as the means ± standard deviations of triplicate values.

EMSAs. Double-stranded oligonucleotides generated from the single-strand list in Table I were used as electrophoretic mobility shift assay (EMSA) probes. Sequence W is the wild-type FIH-1 promoter region between 114 bp and 92 bp; sequence M is the mutant sequence. The upper-strand (sense) oligonucleotide (30 ng) was 5′-end labeled using polynucleotide kinase with [γ-32P]ATP (Amersham Pharmacia Biotech). After the labeling reaction, a twofold excess of the lower-strand (antisense) oligonucleotide was annealed to the upper strand. Double-stranded DNA probes were purified from the reaction mixture using a Bio-Gel P-100 column (Bio-Rad). Whole-cell extracts were isolated from 786-O cells and 293T cells. DNA-protein binding was performed in 0.5× Dignam buffer D (20 mM HEPES, pH 7.9, 100 mM KCl, 1% Tween 20, 0.2 mM EDTA) supplemented with 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 10 mM MgCl2, and 100 μg/ml poly(dI-dC). Binding reactions were initiated with a 30,000-cpm addition of DNA probe in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) to 5 to 10 μg of whole-cell extracts. Electrophoresis was performed in 4% acrylamide gels, and the gels were vacuum dried and exposed to film for 16 to 48 h. Competition experiments were carried out in the same way as described above except that increasing amounts of double-stranded wild-type oligonucleotide (200-fold) were mixed with 30,000 cpm of M1 probe (see Table 1) and then added to the binding reaction mixture.

ChIP assay. The chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit from Upstate Biotechnology, Inc. (Lake Placid, NY). Briefly, 106 to 107 cells were used for each ChIP assay. Protein-DNA cross-linking was carried out by the addition of 1% formaldehyde directly to the cell cultures, followed by incubation at 37°C for 10 min. After the cells were thoroughly washed with ice-cold phosphate-buffered saline, the cells were scraped off and harvested. Cells were lysed with 200 μl of SDS lysis buffer (1% SDS, 10 mM Tris EDTA, 50 mM Tris, pH 8.1) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A). Sonication was then performed on ice using a sonicator (Lab-Line Ultra Tip; Lab-Line Instrument, Inc.) preset for 10-s pulses with 10-s intervals. Ten repeated sonication cycles (as previously standardized by us) were applied to achieve chromatin fragmentation in the 200- to 1,000-bp range. Fragmented chromatin was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 2 mM EDTA, 16.7 mM Tris, pH 8.1, and 150 mM NaCl). Diluted chromatin fragments were precleared by incubation with protein A agarose beads under constant rotation for 2 h at 4°C. For immunoprecipitations, an antihuman antibody for CDP-1 or the respective immunoglobulin G (IgG) control was used for an overnight incubation with constant rotation at 4°C. The protein-DNA-antibody complex was pulled down by protein A agarose-salmon sperm DNA beads. After thorough and sequential washings with low-salt, high-salt, and LiCl-containing buffers, the resulting immune complex was eluted with 1% SDS and 0.1 M NaHCO3. Formamide cross-links were reversed by adding 5 M NaCl and heating at 65°C for 4 h. DNA fragments were then recovered by ethanol precipitation following proteinase K digestion and phenol-chloroform extraction.

PCR was performed with FIH promoter-specific primers amplifying the CDK binding domain (forward, 5′-GTG TTAT GTCAT TAC GAG GAG CCA-3′; reverse, 5′-CGGATA CACC ACCCA CGG AA AC-3′). The 200-bp amplicons were analyzed with 2% agarose gel electrophoresis.

RESULTS

FIH-1 protein was increased in the presence of overexpressing dominant-negative PKCζ in 786-O cells. In our previous paper, we have shown that the siRNA or kinase-inactive form of PKCζ increased the total mRNA level of FIH-1 in renal cancer cells and other cell lines, including 293T cells. Because there was no FIH-1-specific antibody commercially available at that time, we were unable to determine whether the level of FIH-1 protein also increased similarly to its mRNA. Here, we confirmed the increase in FIH-1 protein by Western blotting in 786-O cells after they were transiently transfected with the kinase-inactive form of PKCζ (Fig. 1A). As a positive control to illustrate the inhibitory effect of PKCζ, we observed a 50% decrease of phospho-MAP kinase in this condition (Fig. 1B). This result not only confirms our previous findings of PKCζ as an important transcriptional mediator of FIH-1 expression in cancer cells but also paves the way towards understanding how PKCζ exerts its control. Previously, we observed a dose-dependent increase in FIH-1 full-length promoter activity with a dominant-negative form of PKCζ, suggesting PKCζ-mediated transcriptional control of the FIH-1 gene. As we did not observe any change in FIH-1 mRNA stability in the presence of the dominant-negative PKCζ under our experimental setup (data not shown), we have focused our subsequent studies on PKCζ-mediated transcriptional regulation of FIH-1, beginning with the identification of the FIH-1 transcriptional start site.

Determining the transcriptional start site(s) of the FIH-1 gene. The NCBI website indicated a potential FIH-1 transcriptional start site located 20 bp upstream of the first FIH-1 translational start codon (ATG) (gene ID, 55662). In order to experimentally confirm the transcriptional start site, we examined the heterogeneous FIH-1 transcriptional start sites by
using RNA ligase-mediated oligonucleotide-capping rapid amplification of cDNA ends (RML-RACE from Invitrogen Life Technologies). Our results indicated a potential FIH-1 transcription start site located 35 bp, not 20 bp, upstream of the ATG start codon (data not shown). According to our results, this is the first report of an FIH-1 transcription start site.

Potential transcriptional factor(s) regulated by PKCζ for FIH-1 transcription. Previously, we showed that the PKCζ isoform inhibits the activity of the full-length FIH-1 promoter (6.9 kb in pGL3-basic vector). In this study, we tried to elucidate the molecular mechanism of PKCζ-mediated FIH-1 transcriptional regulation. Different deletion fragments of the FIH-1 proximal promoter region were cloned into the pGL3-basic vector containing the luciferase reporter gene (e.g., 2.7 kb, 480 bp, 220 bp, 180 bp, 114 bp, and 92 bp) (Fig. 2A). The basal activities of these deletion constructs are shown in Fig. 2B.

When cotransfected with the 2.7-kb or 480-bp construct of FIH-1, an increased FIH-1 transcriptional activity was observed when the PKCζ dominant-negative plasmid (kinase-inactive PKCζ) was overexpressed, compared to the empty control vector (Fig. 2C).

We further generated a 220-bp fragment of the FIH-1 promoter. The 5’ end of the 220-bp fragment was identical to the 480-bp fragment; however, the 3’ end stops immediately after the transcription start site. Interestingly, the 220-bp fragment showed a similar increase in promoter activity in the presence of dominant-negative PKCζ. These results suggest that a cis element responsible for the PKCζ-mediated transcriptional regulation of FIH-1 exists within the 220-bp region, upstream of the transcription start site (Fig. 2D).

We engineered further deletions within the 220-bp region of the FIH-1, yielding 180-bp, 114-bp, and 92-bp fragments. FIH-1 promoter activities with both the 180- and 114-bp fragments were increased when cells were cotransfected with the PKCζ dominant-negative plasmid. Importantly, we did not observe any PKCζ regulation with the 92-bp FIH-1 promoter construct (Fig. 2D). Therefore, we concluded that the region between 114 bp and 92 bp may be important for regulation of FIH-1 transcription by PKCζ. According to the Genomatix website (http://www.genomatix.de), this region contains a consensus binding site for Cut-like homeodomain proteins (CDP/Cut). Our next series of experiments were to determine the involvement of Cut in PKCζ-mediated FIH-1 transcription.

CDP/Cut is involved in the transcriptional regulation of FIH-1. CDP (CCAAT-displacement protein) is the human homologue of the Drosophila Cut protein (32). CDP/Cut proteins are an evolutionarily conserved family of proteins relative to Renilla luciferase activity. The average firefly/Renilla luciferase activities (in light units per microgram of protein) with empty vector versus PKCζ DN expression vectors were as follows: 2.7 kb, 1.900/1.982 and 5.673/3.543, respectively; 480 bp, 6.197/1.500 and 1.766/1.793, respectively; 220 bp, 12.577/1.320 and 5.103/1.817, respectively; 180 bp, 51.600/3.053 and 21.987/3.064, respectively; 114 bp, 11.093/2.738 and 7.398/3.458, respectively; 92 bp, 38.323/125.083 and 21.470/698.230, respectively. The data are shown as relative activation of luciferase activities compared to that of the control vector and are the averages of three independent experiments. *, P value less than 0.05.
containing several DNA binding domains. CDP was found to be the DNA binding protein for several genes including histone nuclear factor D and CXCL1 (10, 33, 39). These proteins generally act as transcriptional repressors, although some data suggest their involvement in transcriptional activation.

The human CUTL1 gene was mapped to 7q22, a chromosomal region that is frequently rearranged in various cancers. The function of CDP/Cut is regulated by several posttranslational modification events including phosphorylation, dephosphorylation, and acetylation (reviewed by Nepveu [32]). Previous studies showed that the transcriptional activity of Cut proteins was modulated by PKC (4).

Further indications that CDP/Cut plays an inhibitory role in FIH-1 transcription came from the comparison study of the basal FIH-1 promoter activity of 114-bp and 92-bp deletion constructs in 293T and 786-O cells. The luciferase activity (units/µg protein) of FIH-1 promoter was measured by the luciferase assay with the 114-bp promoter plasmid and 92-bp deletion plasmid in both 293T cells and 786-O cells. The averages were obtained from three time experiments and shown as relative activation. * P value less than 0.05.

(B) Inhibition of CDP/Cut increases the FIH-1 promoter activity in the 114-bp deletion construct but not the 92-bp construct. A CDP/Cut siRNA pool or a nontargeting control siRNA pool was transfected into 786-O cells for 48 h and followed by a second transfection with the FIH-1 promoter constructs (114 bp and 92 bp) for 30 h. FIH-1 promoter activity was measured with luciferase assays. We equilibrated the luciferase activities by protein concentrations of the cell extracts with respect to the control vectors shown as relative activation. The absolute values of luciferase activity with control treatment versus Cut siRNA treatment were as follows: 114-bp construct, 5,772/17,880, and 92-bp construct, 39,111/51,120 light units/µg protein, respectively. The data here are the means from three independent results. * P value less than 0.05.

(C) FIH-1 protein was increased with the depletion of CDP/Cut. Human CDP/Cut protein was blocked by its respective siRNA in 786-O cells. Whole-cell lysates were obtained from cells transfected with the siRNA pool (final concentration was 100 nM) or the nontargeting control siRNA pool after 72 h. Western blot assays against anti-FIH-1 antibody, anti-CDP antibody, and anti-β-actin were performed. The top panel shows the FIH-1 protein level in cell lysates. The middle panel shows the CDP/Cut protein level with siRNA-CDP, providing evidence in favor of the efficiency of the RNA interference approach.

Inhibition of Cut leads to the increase in FIH-1 promoter activity. We used Cut siRNA to deplete endogenous Cut protein from both 293T and 786-O cells. For the 114-bp FIH-1 promoter construct, an increase in promoter activity was observed in both 293T and 786-O cells when cotransfected with Cut siRNA compared to the nontargeted control. Interestingly, no significant difference in promoter activity was observed when the 92-bp FIH-1 promoter construct was transfected (Fig. 3B). This result suggests that the Cut protein could repress FIH-1 transcription.

The FIH-1 protein level is increased by inhibition of CDP/Cut. An increase in mRNA does not always lead to an increase in protein levels. In order to determine whether FIH-1 protein also increased due to the inhibition of CDP/Cut, we transfected 786-O and 293T cells with CDP/Cut siRNA. For the control experiments, cells were transfected with nontarget
siRNA oligonucleotide. FIH-1 protein levels were determined by Western blot assays from the cell lysates (Fig. 3C). An increase in FIH-1 protein was observed when the CDP/Cut expression was inhibited by siRNA. These data led to the question of whether CDP/Cut was a transcriptional regulator of the FIH-1 promoter.

**CDP/Cut regulates FIH-1 transcription.** In order to verify the binding of CDP/Cut to the FIH-1 promoter, EMSAs were performed. We constructed a probe of the CDP binding region within the FIH-1 promoter (-114 to -92 bp, wild-type probe) as well as another probe of the same length and similar sequence except with six out of the nine core nucleotides from the CDP binding sequence within the FIH-1 promoter being mutated (mutated probe) (Table 1). A specific DNA-protein complex was detected with 786-O total cell extracts using the wild-type probe (Fig. 4, lane 2) but not with the mutant probe (Fig. 4, lane 9). The protein-DNA complex identified with the radiolabeled probe was specifically competed out with increasing concentrations of excess unlabeled wild-type probe (Fig. 4, lanes 3 to 5) but not with excess unlabeled mutant probe (Fig. 4, lanes 6 to 8), suggesting the specificity of CDP binding to the FIH-1 promoter region.

**PKC ζ regulates the binding of CDP/Cut to FIH-1 promoter DNA.** To address any role of PKC ζ in the binding of CDP to the FIH-1 promoter, we initially examined whether CDP phosphorylation is PKC ζ dependent in RCC cells and later we also evaluated the consequences of this phosphorylation. We immunoprecipitated the cell extracts that overexpressed PKC ζ DN with anti-CDP antibodies and immunoblotted with antiserine antibodies. Figure 5A showed reduced phosphorylation of CDP in the presence of PKC ζ DN without changing any endogenous CDP expression. Then, we performed EMSAs with the wild-type probe and whole-cell extracts obtained from 293T cells transfected with PKC ζ siRNA. A decrease in DNA-protein binding was observed when PKC ζ was depleted with siRNA compared to the nontargeted control siRNA (Fig. 5B). The efficiency of the siRNA was shown in Fig. 5C. These data suggest that PKC ζ increases CDP phosphorylation and binding to the FIH-1 promoter and most likely leads to inhibition of FIH-1 promoter activity.

**ChIP assays to detect the binding of CDP/Cut to the FIH promoter.** To detect the in vivo binding of CDP to the FIH promoter region in 786-O cells, the ChIP assay was performed. Protein-cross-linked chromatin fragments were isolated and the CDP binding region on the FIH promoter was amplified by PCR with specific FIH promoter primers as described in detail in Materials and Methods. A prominent amplified product was detected when immunoprecipitation was carried out with the CDP antibody on cell lysates transfected with the control vector, suggesting specific binding of CDP at the FIH-1 promoter (Fig. 5D). This result also confirms our previously described EMASA result in vivo. Interestingly, overexpression of the dominant-negative mutant of PKC ζ inhibited CDP binding to the FIH-1 promoter, therefore underscoring the importance of PKC ζ function in regulating FIH-1 promoter activity via CDP.

**Defining the site(s) of CDP/Cut that modulates FIH-1 expression.** In order to define the domain or site(s) of CDP that regulates FIH-1 expression in RCC, we overexpressed wild-type and mutant CDP in RCC cells. We then evaluated the phosphorylation status of CDP and its mutant by immunoprecipitating CDP from RCC cell lysates overexpressing the wild-type CDP (CDP-WT) or mutant CDP (CDP-6 mutants; pxm/Cux/6-mutant plasmid contains Ser402, Thr415, Ser789, Thr804, Ser972, and Ser987 and also Ser987 alone [pxm/Cux/CR#-HD/S987A]; these residues were replaced with codons for Ala [4]) and immunoblotted with antiphosphoserine antibodies. Figure 6 showed that the phosphorylation level of CDP was dramatically decreased in the presence of the CDP-6 mutant compared to wild-type CDP (Fig. 6, top panel).

We then questioned whether the CDP-6 mutation modulated FIH-1 expression. The level of FIH protein was measured in whole-cell lysates transfected with either wild-type CDP or the CDP-6 mutant. As shown in Fig. 6, an increased FIH-1 protein level was observed in cells transfected with the

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CDP-6 mutant construct compared to that of the control vector and the level of FIH-1 protein was also decreased in CDP wild-type transfectants (lower panel). These data are in accord with our previous finding that FIH-1 protein increased in the presence of CDP siRNA. Furthermore, we examined whether a CDP mutant containing S987A yielded an effect similar to that of the CDP-6 mutant. Interestingly, a significant decrease in CDP phosphorylation was observed in transfectedants containing a CDP S987A mutant compared to those transfected with a control vector (Fig. 6, top panel). This indicated that this serine is one of the modification sites. Similarly, we observed increased FIH-1 expression in cell extracts from CDP S987A transfectedants compared to wild-type CDP transfectedants. Overall, our results suggest potential CDP sites that modify FIH-1 expression in RCC cells. As we reported earlier, CDP serine phosphorylation depends upon PKC ζ and this controls its interaction with the FIH-1 promoter and its subsequent expression. Taken together, these data suggest a unique control of gene expression by a PKC isoform on the transcription factor CDP, which leads to the modulation of FIH-1 and the potential control of HIF-dependent pathways and, ultimately, tumor angiogenesis.

**DISCUSSION**

In this communication, we addressed the mechanism of PKC ζ-mediated FIH-1 transcriptional control in RCC. With various deletion constructs of the FIH-1 promoter, we were able to identify a specific region of the FIH-1 promoter (−114 to −92 bp) as a regulatory cis element. This region contains the consensus sequence for CDP/Cut/Cux. CDP/Cut is a homeobox protein that usually represses gene transcription. In our FIH-1 gene transcriptional studies, we observed the involvement of PKC ζ, which might facilitate the binding of CDP to the FIH-1.
promoter and thereby repress its transcription. We monitored FIH-1 promoter activity and its endogenous protein level in RCC after eliminating CDP function either by siRNA or by a dominant-negative approach. We observed an up-regulation of FIH-1 in RCC by inhibiting the function of CDP/Cut, suggesting its involvement in this pathway. The confirmation of the connection of CDP/Cut in FIH-1 transcription with its regulation by PKC ζ came from our EMSA and ChIP studies. These results indicated that active PKC ζ can increase CDP binding to the FIH-1 promoter region and lead to an increased inhibition of CDP on FIH-1 transcription. Moreover, we also observed specific serine residues, most likely CDP S987A, that are important for FIH-1 regulation through PKC ζ.

Even under normoxia, the up-regulation of HIF target genes is a common characteristic in cancer cells (3, 9, 42, 43). Clear-cell renal carcinoma is a good example where the vascular endothelial growth factor A gene, a target gene of HIF, is expressed at high levels in the presence of normal oxygen concentrations (6). Activation of oncogenes along with the inactivation of tumor suppressor genes might explain this increased HIF activity under normoxia. Furthermore, in our present study, we have shown an epigenetic regulation of FIH-1 in RCC that may account for this increased HIF activity. Therefore, it highlights an important regulatory mechanism of FIH-1 that leads to high HIF activity in cancer cells and could signify an important target for potential therapies.

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