Molecular Basis for the Cell Type–Specific Induction of SnoN Expression by Hepatocyte Growth Factor

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ABSTRACT
Hepatocyte growth factor (HGF) is a potent antifibrotic cytokine that antagonizes the TGF-β1/Smad signaling in diverse types of kidney cells by different mechanisms. HGF is shown to induce Smad co-repressor Sloan-Kettering Institute proto-oncogene–related novel gene, non–Alu-containing (SnoN) expression in proximal tubular epithelial cells (HKC-8) but not in glomerular mesangial cells and interstitial fibroblasts. This study investigated the molecular mechanisms underlying the cell type–specific induction of SnoN by HGF. Treatment of HKC-8 cells with actinomycin D completely abolished HGF-mediated SnoN induction, suggesting its dependence on gene transcription. Although HGF activated several signal pathways in HKC-8 cells, blockade of extracellular signal–regulated kinase-1 and -2 (Erk-1/2) activation but not Akt and p38 mitogen-activated protein kinase abolished SnoN induction. HGF rapidly activated both upstream and downstream signaling of Erk-1/2, which led to the activation of the cAMP response element–binding protein (CREB). In the promoter region of human SnoN gene, two cAMP response elements were located in close proximity to Sp1 sites. Chromatin immunoprecipitation assay revealed that activated CREB and Sp1 bound to their cognate cis-acting elements in SnoN promoter in response to HGF stimulation. Ectopic expression of wild-type CREB promoted SnoN expression, whereas dominant negative mutant CREB abrogated SnoN induction by HGF. Likewise, chemical blockade of Sp1 binding abolished HGF-mediated SnoN induction. Furthermore, HGF selectively induced CREB phosphorylation in HKC-8 cells but not in mesangial cells and fibroblasts. In vivo, administration of HGF gene induced renal Erk-1/2 phosphorylation, CREB activation, and SnoN expression in obstructive nephropathy. Collectively, these results suggest that CREB activation, in concert with Sp1, constitutes a molecular switch that confers the cell type–specific induction of SnoN in response to HGF stimulation.


Hepatocyte growth factor (HGF) is a unique endogenous protein that possesses potent antifibrotic potential.1,2 Extensive studies in the past several years have demonstrated that administration of exogenous HGF or its gene effectively ameliorates kidney dysfunction and fibrotic lesions in a wide variety of animal models of chronic kidney diseases.3–9 Conversely, blockade of HGF signaling with neutralizing antibody markedly exacerbates the kidney function and accelerates the progression of renal fibrosis in different chronic kidney disease models.10,11 Experimental evidence indicates that HGF is able to antagonize directly the profibrotic TGF-β1/Smad signaling in diverse types of kidney cells by different mechanisms.¹ In kidney tubular epithelial cells, HGF has been shown to block the epithelial-to-mesenchymal transition induced by TGF-β1.¹²,¹³ This action of HGF is primarily mediated by its ability to upregulate Sloan-Kettering In-
stitute proto-oncogene (Ski)-related novel gene, non–Alu-containing (SnoN).\textsuperscript{14} Consistently, HGF induces SnoN expression \textit{in vivo}, and ectopic expression of SnoN impedes TGF-\(\beta\)-1 action in tubular epithelial cells.\textsuperscript{14} SnoN belongs to a family of proteins that are known as Smad transcriptional co-repressors, which include Ski, SnoN, and TG-interacting factor.\textsuperscript{15–17} Through various mechanisms, SnoN can efficiently block TGF-\(\beta\)/Smad signaling. Copious studies have shown that SnoN can interact physically with Smad proteins in the nuclei and sequester the ability of Smads to transactivate gene expression.\textsuperscript{14,15} Furthermore, SnoN is instrumental in recruiting other transcriptional co-repressors, such as the nuclear hormone receptor co-repressor and mammalian Sin3 ortholog, and prevents Smads from binding to the transcriptional co-activator p300/CBP.\textsuperscript{15,16} In normal kidney, SnoN is highly expressed,\textsuperscript{18} which is presumably necessary for keeping the TGF-\(\beta\)/1/Smad signaling tightly constrained. However, the abundance of SnoN protein is progressively reduced in the fibrotic kidney, primarily through an enhanced ubiquitin-dependent proteasomal degradation.\textsuperscript{19,20} Therefore, TGF-\(\beta\)/1/Smad signaling in the pathologic state is dramatically amplified by virtue of losing a crucial negative control mechanism. In this context, restoration of SnoN expression by HGF in the fibrotic kidney could be a primary pathway leading to inhibition of the profibrotic TGF-\(\beta\)/1/Smad signaling.

SnoN induction by HGF seems to occur in a cell type–specific manner. In glomerular mesangial cells and interstitial fibroblasts, HGF is unable to induce SnoN gene expression, although it competently blocks TGF-\(\beta\)/1/Smad signaling \textit{via} other mechanisms.\textsuperscript{21,22} These observations indicate that HGF seems to elicit its antifibrotic effects in different kidney cells through distinctive modes of action. However, the molecular mechanism that governs the cell type–specific induction of SnoN by HGF in tubular epithelial cells remains completely unknown.

In this study, we show that induction of SnoN by HGF is operated at the gene transcriptional level through an extracellular signal–regulated kinase-1 and -2 (Erk-1/2)-dependent pathway. HGF seems to trigger a cascade of signal transduction, which leads to the activation of the cyclic AMP response element (CRE)-binding protein (CREB), a transcription factor that is selectively activated in tubular epithelial cells. Our data suggest that CREB activation, together with Sp1, plays an essential role in mediating the cell type–specific induction of SnoN.

**RESULTS**

**SnoN Induction by HGF Occurs at the Gene Transcriptional Level**

Figure 1, A and B, shows that HGF specifically induced SnoN mRNA expression in human proximal tubular epithelial cells (HKC-8), consistent with a previous report.\textsuperscript{14} To understand the mechanism that governs SnoN induction by HGF in tubular epithelial cells, we first determined whether SnoN induction occurs at the gene transcriptional or posttranscriptional level. To this end, HKC-8 cells were incubated with HGF in the absence or presence of actinomycin D, an inhibitor of gene transcription. As shown in Figure 1, C and D, in the absence of gene transcription after treatment with actinomycin D, HGF failed to induce SnoN mRNA and protein expression. In contrast, HGF was able to induce SnoN expression in HKC-8 cells when the gene transcription was intact. Therefore, it seems clear that SnoN induction by HGF occurs at the gene transcriptional level.

**SnoN Induction Depends on the Activation of Erk-1/2 Signaling**

We next sought to determine which signal pathway triggered by HGF is responsible for mediating SnoN induction. As shown in Figure 2, HGF activated multiple signal pathways in tubular epithelial cells. When HKC-8 cells were treated with HGF for various periods of time, Erk-1/2 mitogen-activated protein kinase (MAPK) was rapidly phosphorylated and activated, as demonstrated by phospho-specific antibody (Figure 2A). Similarly, HGF rapidly induced Akt kinase (Figure 2B) and p38 MAPK phosphorylation (Figure 2C) in HKC-8 cells. We found that blockade of Erk-1/2 activation with PD98059 abolished SnoN induction by HGF (Figure 2E). However, blockade of Akt kinase and p38 MAPK activation had no or
little effect on SnoN induction. Of note, these chemical inhibitors at the concentrations used were able to block Akt and p38 MAPK activation in HKC-8 cells, as previously reported.23,24 These results suggest that HGF induces SnoN expression primarily by an Erk-1/2 MAPK-dependent pathway in tubular epithelial cells.

Consistent with the data on SnoN induction, we found that Erk-1/2 activation was also essential for HGF to elicit its anti–TGF-β1 action. As shown in Figure 3, HGF abolished TGF-β1–induced E-cadherin suppression and α-smooth muscle actin induction in HKC-8 cells; however, blockade of Erk-1/2 activation with PD98059 completely restored the profibrotic TGF-β1 action in tubular epithelial cells. Under the same conditions, blockade of Akt or p38 MAPK signaling had no or little effect on the ability of HGF to antagonize TGF-β1 action.

**HGF Activates CREB and Induces Its Binding to SnoN Promoter**

Figure 4 shows that both upstream and downstream signaling mediators of Erk-1/2 were activated by HGF in tubular epithelial cells. HGF was able to induce a rapid phosphorylation of Raf and Mek1, the upstream kinase of Erk-1/2 in HKC-8 cells. Similarly, the downstream effector kinase of Erk-1/2, p90RSK, was quickly activated. This led to the phosphorylation and activation of CREB, a transcription factor that is activated by Erk-1/2–p90RSK signaling.25 As demonstrated in Figure 4, phospho-specific CREB but not total cellular CREB was dramatically increased shortly after HGF stimulation in HKC-8 cells. The band below phospho-CREB was likely the phosphorylated activating transcription factor-1, a CREB-related protein that is also recognized by this antibody.

To establish the relevance of CREB activation to SnoN induction, we analyzed the promoter region of human SnoN gene. As shown in Figure 5A, there were two putative cAMP response elements (CRE) in the SnoN promoter region. Chromatin immunoprecipitation (ChIP) assay revealed that HGF induced phosphorylated CREB binding to that region in the SnoN promoter in a time-dependent manner (Figure 5B). Quantitative analysis showed that HGF increased the interaction between phospho-CREB and SnoN promoter by approximately 14-fold at 30 min after stimulation (Figure 5C).

**CREB Activation Is Necessary for Mediating SnoN Induction**

To examine the importance of CREB activation in SnoN expression, we sought to investigate whether overexpression of CREB mediates SnoN induction in tubular epithelial cells. To this end, HKC-8 cells were transiently transfected with wild-type CREB expression vector (pCMV-wt-CREB), followed by incubation with HGF for 24 h. As shown in Figure 6A, ectopic expression of CREB induced SnoN expression in HKC-8 cells in the absence of HGF (Figure 6A, lane 1 versus lane 3). SnoN expression was further increased in the CREB-transfected cells after HGF stimulation (Figure 6A, lane 3 versus lane 4).

Figure 6B shows that CREB activation was also required for HGF-mediated SnoN induction in tubular epithelial cells. HKC-8 cells were transfected with dominant negative mutant CREB expression vector (pCMV-dn-CREB) that expresses a variant of the human CREB protein that contains a serine-to-alanine mutation, which blocks phosphorylation of CREB, thereby preventing transcription in a dominant negative manner. We found that overexpression of mutant CREB completely abolished SnoN induction by HGF in HKC-8 cells (Fig-
ure 6B), suggesting that CREB activation is essential for SnoN induction in tubular epithelial cells. Similar results were obtained when using immunofluorescence staining for SnoN (Figure 6C).

HGF Selectively Activates CREB in Tubular Epithelial Cells

Because HGF induces SnoN expression in a cell type–specific manner, we next determined whether there is a variation in CREB activation by HGF in different types of kidney cells. Therefore, HKC-8 cells, human mesangial cells (HMC), and rat kidney interstitial fibroblast (NRK-49F) cells were treated with HGF for various periods of time, and CREB activation was assessed. As shown in Figure 7, HGF induced a dramatic phosphorylation and activation of CREB in HKC-8 cells but not in HMC and NRK-49F cells. However, Erk-1/2 phosphorylation was detected in all three cell types after HGF stimulation (Figure 7). These data suggest that there is a strong correlation between CREB activation and SnoN induction in different kidney cells.

Sp1 Is Indispensable for Mediating the Cell Type–Specific Induction of SnoN by HGF

Sequence analysis also showed that there were two Sp1 binding sites in the proximity of CRE in the SnoN promoter (Figure 5A). This prompted us to examine whether Sp1 transcription factor participates in SnoN regulation. ChIP assay demonstrated that Sp1 could constitutively bind to its cognate sites in the SnoN promoter region (Figure 8A). Furthermore, HGF substantially augmented the interaction between Sp1 and SnoN promoter in a time-dependent manner (Figure 8, A and B). To test the functionality of this Sp1 binding, we examined the effect of chemical blockade of Sp1 binding on SnoN expression. Therefore, HKC-8 cells were pretreated with mithramycin A, a potent inhibitor of Sp1 binding, followed by incubation with HGF for 24 h. As shown in Figure 8, C and D, blockade of Sp1 binding suppressed SnoN expression at basal condition and completely abolished SnoN induction by HGF.
Because Sp1 is predominantly expressed in epithelial cells in the kidney, it seems that Sp1 also plays a critical role in mediating the cell type-specific induction of SnoN by HGF.

HGF Induces Erk-1/2 and CREB Activation In Vivo
Earlier studies showed that HGF can restore SnoN expression that was repressed in the obstructed kidney in vivo. To explore the mechanism by which HGF induces SnoN expression in vivo, we examined the Erk-1/2 and CREB activation in the obstructed kidney after delivery of HGF gene. As shown in Figure 9, A and B, administration of HGF gene induced Erk-1/2 phosphorylation in the obstructed kidney after unilateral ureteral obstruction (UUO). The relative abundance of phosphorylated Erk-1/2 in the obstructed kidney after HGF administration was increased by approximately 12-fold over the controls. Similarly, phospho-CREB was markedly induced by HGF and primarily localized in the nuclei of tubular epithelia (Figure 9C), a pattern that closely resembles SnoN expression in this model. To confirm further the relevance of CREB activation to SnoN induction, we used a double immunostaining approach to examine the co-localization of phosphorylated CREB and SnoN. As shown in Figure 9D, co-localization of phosphorylated CREB and SnoN was clearly observable in the nuclei of the obstructed kidney after administration of HGF gene. Therefore, it seems that HGF also induces SnoN expression in an Erk-1/2–CREB–dependent pathway in vivo.

DISCUSSION
HGF, a potent endogenous antifibrotic cytokine that is capable of antagonizing the profibrotic TGF-β1 signaling, specifically induces SnoN mRNA and protein expression in kidney proximal tubular epithelial cells but not in glomerular mesangial cells and interstitial fibroblasts. However, the molecular basis for this cell type-specific induction was completely unknown. The results presented herein demonstrate that this cell type-specific induction of SnoN is mediated primarily by selective activation of CREB transcription factor in the tubular epithelial cells after HGF stimulation. HGF activates Erk-1/2 MAPK signaling that involves a cascade of signal transduction, which leads to phosphorylation and activation of CREB. Phosphorylated CREB then binds to the cis-acting CRE in the promoter region of human SnoN gene and presumably trans-activates SnoN transcription. Consistently, overexpression of CREB induces SnoN expression in tubular epithelial cells, and a dominant negative mutant CREB abrogates SnoN induction by HGF. These results establish that selective CREB activation may play a crucial role in conferring the cell type-specific induction of SnoN in tubular epithelial cells. Our studies provide significant insights into understanding the mechanism underlying SnoN transcriptional regulation in kidney cells.

CREB is a ubiquitously expressed nuclear transcription factor that contains the basic region/leucine zipper domain. It is originally isolated as a gene regulatory protein that binds to the CRE site of the somatostatin gene promoter region in response to an increase in the cellular cAMP level. CREB forms a homodimer through its leucine zipper domain and binds to DNA through its basic region and is involved in regulating the expression of a wide range of genes that are important to cell proliferation, differentia-
tion, adaptation, and survival. Activation of CREB requires phosphorylation of the serine residue at 133, which is necessary and sufficient for transcriptional activation of target genes. This study demonstrates that HGF activates SnoN expression by an Erk-1/2–CREB–dependent pathway and suggests that SnoN is a direct target of CREB in kidney cells. Because SnoN is intimately implicated in the regulation of TGF-β1/Smad signaling, this suggests that CREB may be an important transcriptional regulator that is capable of controlling the fibrogenic responses of tubular epithelial cells after chronic injury. Of interest, a recent report indicated that CREB activation also plays a critical role in mediating SnoN induction by HGF (Figure 6). Because SnoN gene promoter harbors two CRE and phosphorylated CREB binds to the CRE-containing region of SnoN promoter. Third, ectopic expression of CREB induces SnoN expression and dominant negative CREB abrogates SnoN induction by HGF. Fourth, CREB is selectively activated in tubular epithelial cells but not in mesangial cells and fibroblasts after HGF stimulation, suggesting an intrinsic coupling of SnoN induction to CREB activation. Finally, SnoN induction by HGF in vivo is associated with Erk-1/2 and CREB activation in obstructive nephropathy. Therefore, it seems plausible that selective CREB activation after HGF stimulation is indispensable for conferring the cell type–specific induction of SnoN.

Besides CREB, HGF induction of SnoN is completely dependent on proper binding and function of a ubiquitous transcription factor Sp1, as chemical ablation of Sp1 binding abrogates HGF-induced SnoN expression in tubular epithelial cells (Figure 8). Sp1 is the prototype of a family of proteins that consists of four members with distinct expression pattern and diverse functions in different types of cells. The

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Figure 6. CREB is necessary for mediating SnoN induction in tubular epithelial cells. (A) Ectopic expression of wild-type CREB induced SnoN expression. HKC-8 cells were transiently transfected with empty vector pcDNA3 or wild-type CREB expression vector (pCMV-wt-CREB) as indicated, followed by incubation with 20 ng/ml HGF for 24 h. Whole-cell lysates were immunoblotted with antibodies against SnoN and α-tubulin. (B) Overexpression of mutant CREB abrogated SnoN induction by HGF. HKC-8 cells were transfected with either wild-type (pCMV-wt-CREB) or mutant CREB expression vector (pCMV-dn-CREB), followed by incubation with 20 ng/ml HGF for 24 h. Mutant CREB abrogated SnoN induction in a dominant negative manner. (C) Immunofluorescence staining for SnoN expression in tubular epithelial cells. HKC-8 cells were transfected with either pCMV-wt-CREB or pCMV-dn-CREB expression vectors, followed by incubation with or without HGF (20 ng/ml) for 24 h. Cell nuclear staining was carried out with 4’,6-diamidino-2-phenylindole, HCl.

Figure 7. HGF induces CREB phosphorylation and activation in a cell type–specific manner. HKC-8 cells, human mesangial cells (HMC), and rat kidney interstitial fibroblasts (NRK-49F) cells were treated with 20 ng/ml HGF for various periods of time as indicated. Whole-cell lysates were immunoblotted with antibodies against phosphorylated CREB, phosphorylated Erk-1/2, and actin. Despite Erk-1/2 activation in all three cell types, significant CREB phosphorylation was found only in HKC-8 cells.
that regulates the constitutive expression of many genes, it also participates in controlling either suppressive or inducible expression of a single gene under different circumstances, often through interacting with different partners.\textsuperscript{26,39} For instance, Sp1 mediates HGF receptor (c-met) suppression after oxidative stress by interacting with Egr-1, leading to its sequestration.\textsuperscript{40} Sp1 is also crucial for c-met induction triggered by TGF-\(\beta\)1 through interacting with Smad proteins.\textsuperscript{28} The involvement of both Sp1 and CREB in SnoN induction is also highlighted by their binding to the cognate sites in SnoN promoter in a HGF-dependent manner.

SnoN is an imperative transcriptional co-repressor that makes the TGF-\(\beta\)/Smad signaling tightly controlled in normal conditions. Given its eminent role in constraining TGF-\(\beta\)/Smad signaling, SnoN expression is stringently regulated at both transcriptional and posttranscriptional levels. Earlier studies demonstrated that SnoN protein is progressively reduced in obstructive nephropathy,\textsuperscript{18} and such downregulation of SnoN is primarily mediated by an enhanced ubiquitin-dependent degradation.\textsuperscript{19,20} However, as shown in this study, SnoN induction by HGF occurs at the transcriptional level and is mediated by transcription factors CREB and Sp1. Although more studies are needed, these observations have set a foundation for better understanding of the mechanism that governs the regulation of SnoN under different circumstances.

**CONCISE METHODS**

**Cell Culture and Treatment**

Human proximal tubular epithelial cells (HKC-8) were cultured in DMEM and Ham’s F12 medium (1:1) supplemented with 5% FBS (Invitrogen, Carlsbad, CA), as described previously.\textsuperscript{12,41} HMC were purchased from ScientCell Research Laboratories (San Diego, CA). Normal rat kidney interstitial fibroblast cells (NRK-49F) were obtained from American Type Culture Collection (Manassas, VA). HMC and NRK-49F cells were maintained in DMEM/F12 medium supplemented with 10% FBS. Cells were typically serum-starved for 16 h, followed by incubation with HGF for various periods of time as indicated. In some experiments, cells were pretreated with either various inhibitors at given concentrations or vehicle (0.1% DMSO) 0.5 h before incubation with different cytokines. Human recombinant HGF and TGF-\(\beta\)1 were obtained from R&D Systems (Minneapolis, MN). PD98059 (Mek1 inhibitor), wortmannin (phosphatidylinositol 3-kinase inhibitor), and SC-6376 (p38 MAPK inhibitor) were purchased from Calbiochem (La Jolla, CA). Actinomycin D and mithramycin A were purchased from Sigma (St. Louis, MO). For chemical blockade of Sp1 binding, HKC-8 cells were pretreated with mithramycin A at 10\(^{-7}\) M for 16 h.\textsuperscript{28}

**Animal Model and HGF Treatment**

Animal studies were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and carried out as...
described previously. Briefly, male CD-1 mice that weighed 18 to 22 g were obtained from Harlan Sprague-Dawley (Indianapolis, IN). They were housed in the animal facilities of the University of Pittsburgh Medical Center, with free access to food and water. UUO was performed using an established procedure. On the day of surgery, mice received a single intravenous injection of naked HGF expression plasmid (pCMV-HGF) or empty vector pcDNA3 at 1 mg/kg body wt. At day 7 after UUO, mice were killed and the kidneys were removed. One part of the kidneys was frozen immediately in OCT compound for cryosection to perform immunofluorescence studies. The remaining kidneys were snap-frozen in liquid nitrogen and used for Western blot analyses.

**RNA Isolation and Reverse Transcriptase–PCR**

Total RNA isolation, reverse transcription of the RNA, and PCR amplification were performed as described previously. Briefly, the first-strand cDNA synthesis was carried out by using a Reverse Transcription System kit according to the instructions of the manufacturer (Promega, Madison, WI). PCR amplification was performed using HotStar TaqMaster Mix Kit (Qiagen, Valencia, CA). The primer sequences were as follow: SnoN 5'-TTTCTGCCCCTCCTCACTACCC-3' (sense) and 5'-GACTTGGGGCAACACAGTC-3' (antisense) and β-actin, 5'-TCAAGATCATTGCTCCTGAGC-3' (sense) and 5'-TGGTGTACCTTCCGGTCCAGT-3' (antisense). Relative levels of SnoN mRNA (fold induction over the controls) were calculated after normalization with housekeeping gene actin.

**Western Blot Analysis**

Detection of protein expression by Western blot was carried out according to the established protocols described previously. The primary antibodies used were as follows: Anti-SnoN (sc-9595), anti-Sp1 (sc-59), and anti-actin (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA); anti-α-smooth muscle actin (clone 1A4) and anti-α-tubulin (T-9026; Sigma); anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX); anti-E-cadherin (clone 36; BD Biosciences, San Diego, CA); antibodies against phospho-specific (Thr202/Tyr204) and total Erk-1/2, phospho-specific (Ser473) and total Akt, phospho-specific (Thr183/Tyr182) and total p38 MAPK, phospho-specific (Ser133) and total CREB, phospho-specific Raf, phospho-specific Mek1/2 (Ser217/221), and phospho-specific p90 ribosomal protein S6 kinase (p90RSK; Ser380; Cell Signaling Technology, Beverly, MA). Quantitative analysis of Western blot data was performed by measurement of the intensity of the band signals with the use of National Institutes of Health Image analysis software.

**Figure 9.** HGF induces Erk-1/2 and CREB phosphorylation and SnoN expression in vivo. (A and B) Administration of HGF gene induced Erk-1/2 phosphorylation in the obstructed kidney. Whole-tissue homogenates from the obstructed kidney of different groups as indicated were immunoblotted with antibodies against phosphorylated and total Erk-1/2. Numbers (1, 2, and 3) indicate each individual animal in a given group. Relative abundance of phosphorylated Erk-1/2 after normalization with total Erk-1/2 in each group is shown in B. *P < 0.05 versus the pcDNA3 control. (C) Immunohistochemical staining showed that HGF induced CREB phosphorylation in the obstructed kidney. Phosphorylated CREB was predominantly localized in the nuclei of renal tubules. (D) Double immunofluorescence staining for phosphorylated CREB (red) and SnoN (green) in the obstructed kidneys. Co-localization of phospho-CREB and SnoN (arrowheads) was evident in the obstructed kidney after HGF administration. Nuclear staining in kidney tissue was carried out with 4',6-diamidino-2-phenylindole, HCl.
Immunofluorescence and Immunohistochemical Staining

Indirect immunofluorescence staining was performed using an established procedure. Briefly, HKC-8 cells cultured on coverslips, or kidney cryosections at 5-μm thickness were washed twice with cold PBS and fixed with cold methanol:acetone (1:1) for 10 min at −20°C. After three extensive washes with PBS containing 0.5% BSA, the cells were blocked with 20% normal donkey serum in PBS buffer for 30 min at room temperature and then incubated with specific primary antibodies described previously. For visualization of the primary antibodies, slides were stained with cyanine Cy2- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA). Cells were double-stained with 4′,6-diamidino-2-phenylindole, HCl to visualize the nuclei. As a negative control, the primary antibody was replaced with non-IgG, and no staining occurred. For immunohistochemical staining of kidney sections, paraﬁn-embedded slides were stained with anti–phospho-CREB antibody using the Vector M.O.M. immunodetection kit by the protocol speciﬁed by the manufacturer (Vector Laboratories, Burlingame, CA). Stained slides were mounted with anti-fade mounting medium (Vector Laboratories, Burlingame, CA). Stained slides were mounted with anti-fade mounting medium (Vector Laboratories) and viewed with a Nikon Eclipse E600 Epi-fluorescence microscope (Melville, NY).

ChIP Assay

ChIP assay was performed to analyze in vivo interactions of transcription factors and their cognate cis-acting elements in SnoN promoter. This assay was essentially carried out according to the protocols speciﬁed by the manufacturer (ChIP assay kit; Upstate, Charlottesville, VA). Briefly, after treatment with HGF for various periods of time, HKC-8 cells were cross-linked with 1% formaldehyde for 10 min, washed twice with PBS containing protease inhibitors, and then resuspended in lysis buffer containing protease inhibitors. The chromatin solution was sonicated. After centrifugation, the supernatant was diluted 10-fold in ChIP dilution buffer and preclarred with protein A–agarose containing salmon sperm DNA. The antibodies against phospho-speciﬁc CREB or Sp1 were added and incubated at 4°C overnight, followed by incubation with protein A–agarose for 1 h. The precipitates were washed, and chromatin complexes were eluted. After reversal of the cross-linking at 65°C for 4 h, the DNA was puriﬁed, and 1 μl of input control or ChIP samples were used as a template for PCR using the primer sets for the SnoN promoter regions (from −1383 to −1639) containing two putative CRE and two Sp1 sites. The sequences of primers used for ChIP assay were as follows: forward 5′-GGTGCGGGGCGCAGGGG-3′ and reverse 5′-CGGACCGGGGTCGAG-3′. The relative abundance of the PCR ampliﬁed product was calculated after normalization with the intensities of input DNA.

Plasmid Transfection

For transient transfection, HKC-8 cells were seeded in six-well plates at 5 × 10^5 cells/well. The cells were then transfected with either human wild-type CREB (pCMV-wt-CREB) or dominant negative mutant CREB (pCMV-dn-CREB) expression vectors (Clontech Laboratories, Mountain View, CA) using Lipofectamine 2000 reagent according to the instructions speciﬁed by the manufacturer (Invitrogen). The pCMV-dn-CREB vector expresses a mutant variant of the human CREB protein that contains a serine-to-alanine mutation corresponding to amino acid residue 133. The empty pcDNA3 vector was also transfected as a negative control. After transfection for 24 h, the cells were incubated with 20 ng/ml HGF for an additional 24 h and then subjected to Western blot analyses and immunofluorescence staining.

Statistical Analyses

Statistical analysis was performed using SigmaStat software (Jandel Scientiﬁc Software, San Rafael, CA). Comparisons between groups were made using one-way ANOVA, followed by the Student-Newman-Keuls test. P < 0.05 was considered signiﬁcant.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health grants DK054922, DK061408, DK064005, and DK071040.

DISCLOSURES

None.

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