Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney

Xianghong Zhang,1,2 Yingjian Li,1 Chunsun Dai,1 Junwei Yang,1 Peter Mundel,3 and Youhua Liu1

1Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261; 2Department of Cell Biology, Peking Union Medical College, Beijing 100005, China; and 3Department of Medicine, Albert Einstein College of Medicine, Bronx, New York 10461

Submitted 22 May 2002; accepted in final form 4 August 2002

Zhang, Xianghong, Yingjian Li, Chunsun Dai, Junwei Yang, Peter Mundel, and Youhua Liu. Sp1 and Sp3 transcription factors synergistically regulate HGF receptor gene expression in kidney. Am J Physiol Renal Physiol 284: F82–F94, 2003; 10.1152/ajprenal.00200.2002.—We investigated the expression pattern and underlying mechanism that controls hepatocyte growth factor (HGF) receptor (c-met) expression in normal kidney and a variety of kidney cells. Immunohistochemical staining showed widespread expression of c-met in mouse kidney, a pattern closely correlated with renal expression of Sp1 and Sp3 transcription factors. In vitro, all types of kidney cells tested expressed different levels of c-met, which was tightly proportional to the cellular abundances of Sp1 and Sp3. Both Sp1 and Sp3 bound to the multiple GC boxes in the promoter region of the c-met gene. Coimmunoprecipitation suggested a physical interaction between Sp1 and Sp3. Functionally, Sp1 markedly stimulated c-met promoter activity. Although Sp3 only weakly activated the c-met promoter, its combination with Sp1 synergistically stimulated c-met transcription. Conversely, deprivation of Sp proteins by transfection of decoy Sp1 oligonucleotide or blockade of Sp1 binding with mithramycin A inhibited c-met expression. The c-met receptor in all types of kidney cells was functional and induced protein kinase B/Akt phosphorylation in a distinctly dynamic pattern after HGF stimulation. These results indicate that members of the Sp family of transcription factors play an important role in regulating constitutive expression of the c-met gene in all types of renal cells. Our findings suggest that HGF may have a broader spectrum of target cells and possess wider implications in kidney structure and function than originally thought.

Hepatocyte growth factor; Sp1; Sp3; gene regulation; Akt kinase

Hepatocyte growth factor (HGF) receptor is the product of the c-met protooncogene, which is a membrane-spanning protein that belongs to the receptor tyrosine kinase superfamily (3, 35). The c-met gene was originally isolated from a human osteogenic sarcoma cell line that was treated in vitro with the chemical carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (37). The c-met protein is a 190-kDa, disulfide-linked heterodimer that consists of α- and β-subunits (37). The α-subunit is heavily glycosylated and is completely extracellular. The β-subunit has an extracellular portion that is involved in ligand binding and also has a transmembrane segment and a cytoplasmic tyrosine kinase domain that contains multiple phosphorylation sites. Both subunits are encoded within a single open-reading frame and are produced from the proteolytic cleavage of a 170-kDa precursor (30). On binding to HGF, the c-met receptor undergoes autophosphorylation of the tyrosine residues in its cytoplasmic domain and initiates cascades of signal transduction events that eventually lead to specific cellular responses (5, 31). It has been demonstrated that the HGF/c-met signaling system plays a vital role in cell survival, proliferation, migration, and differentiation in a wide spectrum of target tissues including kidneys (21, 28, 33).

Because all biological activities of HGF are presumably mediated by a single c-met receptor, its expression is likely one of the crucial components that determine cell-type specificity and overall activity of HGF actions. Earlier studies indicated that the c-met gene is predominately expressed in epithelial cells from different organs, whereas its ligand is primarily derived from the mesenchyme (46). This characteristic pattern of expression as well as the pleiotropic nature of its actions makes HGF an important paracrine and/or endocrine mediator for mesenchymal/epithelial interactions, which are critical processes in organ development, tissue regeneration, and tumorigenesis under various physiological and pathological conditions. However, recent studies suggest that the c-met receptor is expressed at different levels in nonepithelial cells as well. For instance, c-met expression is observed in endothelial cells, various types of blood cells, andglomerular mesangial cells (4, 27, 52, 54). Because these cells also express HGF, these observations indicate that the autocrine pathway is another important mode of action for this paired receptor-ligand system, at least in certain types of cells.

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Address for reprint requests and other correspondence: Y. Liu, Dept. of Pathology, Univ. of Pittsburgh School of Medicine, S-405 Biomedical Science Tower, 200 Lothrop St., Pittsburgh, PA 15261 (E-mail: liuy@msx.upmc.edu).

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The kidney is one of the organs in which the c-met receptor is abundantly expressed, although little is known about its function at normal physiological settings (21, 31). Earlier studies (26, 40) revealed that c-met protein is primarily expressed in renal tubular epithelial cells along the entire nephron in normal rat kidney. Little or no c-met protein was observed in other types of cells (such as renal interstitial fibroblasts) aside from renal tubules. However, it remains a question whether these cells truly do not express c-met or their expression level is instead below the detection limits by conventional approaches. Furthermore, the molecular mechanism that governs the constitutive expression of the c-met gene in various types of kidney cells remains largely unknown.

In this study, we examined the expression pattern of the c-met receptor in normal adult kidneys and in a wide variety of kidney cells in vitro. We found that c-met is ubiquitously expressed in normal kidney in a pattern that overlaps with that of the Sp family of transcription factors. Both Sp1 and Sp3 proteins bind to the promoter region of the c-met gene and functionally activate its transcription. All types of kidney cells tested in vitro expressed the functional c-met receptor and induced protein kinase B (PKB)/Akt phosphorylation after HGF stimulation.

MATERIALS AND METHODS

Animals. Male CD-1 mice (body wt 20–24 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). The mice were housed in the animal facilities of the University of Pittsburgh Medical Center and had free access to food and water. Animals were treated humanely using approved procedures in accordance with the guidelines of the Institutional Animal Use and Care Committee of the National Institutes of Health at the University of Pittsburgh School of Medicine. The mice were killed by exsanguination while under general anesthesia. The kidneys were removed and immediately decapsulated. One part of the kidney was frozen in Tissue-Tek optimal cutting-temperature compound in preparation for cryosection. Another part was fixed in 10% neutral-buffered formalin and embedded in paraffin in preparation for histology and immunohistochemical staining.

Cell culture and treatment. Mouse inner medullary collecting duct epithelial cell line 3 (mIMCD-3), rat renal interstitial fibroblasts (NRK-49F), and Drosophila Schneider line 2 (SL-2) cells were obtained from the American Type Culture Collection (Rockville, MD). The human kidney proximal tubular cell line (HKC) was provided by Dr. L. Racusen of Johns Hopkins University. Rat glomerular mesangial cells were a gift of Dr. C. Wu of the University of Pittsburgh. The conditionally immortalized mouse podocyte cell line was established from the transgenic mouse that carries a thermosensitive variant of the simian virus 40 (SV40) promoter as described previously (34). mIMCD-3, HKC, and NRK-49F cells were maintained in a 1:1 DMEM/Ham’s F-12 medium (Life Technologies, Grand Island, NY) mixture supplemented with 10% fetal bovine serum (FBS). Mesangial cells were cultured in RPMI 1640 medium supplemented with 20% FBS. To propagate podocytes, cells were cultured on type I collagen at 33°C in the RPMI 1640 medium supplemented with 10% FBS and 10 U/ml mouse recombinant interferon (IFN)-γ (R & D Systems, Minneapolis, MN) to enhance the expression of a thermosensitive T antigen. To induce differentiation, podocytes were grown at 37°C in the absence of IFN-γ for 14 days under nonpermissive conditions (34). SL-2 cells were grown in Schneider’s medium (Life Technologies) supplemented with 10% FBS. For chemical blockade of Sp binding, mIMCD-3 cells were treated with mithramycin A (Sigma, St. Louis, MO) at different concentrations for various periods of time.

Immunohistochemical staining. Kidney sections from paraffin-embedded tissues were prepared at 4-μm thickness using a routine procedure. Immunohistochemical localization was performed using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) according to procedures described previously (7). The primary antibody against mouse c-met (sc-8057) was obtained from Santa Cruz Biochemical (Santa Cruz, CA). As a negative control, the primary antibody was replaced with nonimmune normal IgG, and no staining occurred.

Frozen section and immunofluorescence staining. Cryosections were prepared at 5-μm thickness in a cryostat and were fixed in a cold 1:1 methanol-acetone mixture for 10 min at −20°C. Immunostaining was performed as described previously (7). Briefly, cryosections were incubated with 20% normal donkey serum in PBS for 30 min at room temperature to reduce background staining. Sections were washed with PBS and incubated with primary antibodies in PBS containing 1% BSA overnight at 4°C. The primary antibodies against mouse c-met, Sp1 (sc-59), and Sp3 (sc-644) were obtained from Santa Cruz Biochemical. Sections were then incubated for 1 h with affinity-purified secondary antibodies (Jackson Immunoresearch Laboratories, West Grove, PA) at a 1:100 dilution in PBS that contained 1% BSA before being washed extensively with PBS. Slides were mounted with anti-fade mounting media and examined on a Nikon Eclipse E600 epifluorescence microscope (Melville, NY) equipped with a digital camera.

Western blot analysis. Various types of kidney cells were lysed with SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). Samples were heated at 100°C for 5–10 min and then loaded and separated on precast 10% SDS-polyacrylamide gels (Bio-Rad, Hercules, CA). The proteins were electrophoresed to a nitrocellulose membrane (Amersham, Arlington Heights, IL) in transfer buffer that contained 48 mM Tris-HCl, 39 mM glycine, 0.057% SDS, and 20% methanol at 4°C for 1 h. Nonpecific binding to the membrane was blocked for 1 h at room temperature with 5% nonfat milk in TBS buffer (20 mM Tris-HCl, 150 mM NaCl, and 0.1% Tween 20). The membranes were then incubated for 16 h at 4°C with various primary antibodies in blocking buffer that contained 5% milk at the dilutions specified by the manufacturers. The phospho-specific Akt antibody (that detects Akt only when it is phosphorylated at specific sites) and the total Akt antibody (that detects Akt independently of phosphorylation state) were obtained from Cell Signaling (Beverly, MA). The antibodies against Sp1, Sp3, c-met, and actin were purchased from Santa Cruz Biochemical. The membranes were washed extensively in TBS buffer and were then incubated with horseradish peroxidase-conjugated secondary antibody (Sigma) at a dilution of 1:10,000 for 1 h at room temperature in 5% nonfat milk dissolved in TBS. Membranes were then washed with TBS buffer, and the signals were visualized using an ECL system (Amersham).

Preparation of nuclear protein extract. For preparation of nuclear protein extracts, mIMCD-3 cells in an exponential growth stage were washed twice with cold PBS and scraped off the plate with a rubber policeman. Cells were collected and the nuclei were isolated according to methods described...
elsewhere (24). Briefly, the pelleted cells were resuspended in 4 volumes of buffer A that contained protease inhibitors: 20 mM MgCl₂, and 3 mM EGTA) buffer that contained protease inhibitors cocktail (Sigma). Whole cell lysates were centrifuged at 12,000 g for 10 min at 4°C, and the supernatants were transferred into fresh tubes. To prepare clear cell lysates, 0.25 μg of normal rabbit IgG and 20 μl of protein A/G PLUS-agarose (Santa Cruz) were added into 1 ml of whole-cell lysates. After incubation for 1 h at 4°C, supernatants were collected by centrifugation at 1,000 g for 5 min at 4°C. Lysates were immunoprecipitated overnight at 4°C with 1 μg each of anti-Sp1, -Sp3, and normal IgG, which was followed by precipitation with 20 μl of protein A/G PLUS-agarose for 3 h at 4°C. After four washes with RIPA buffer, the immunoprecipitates were boiled for 5 min in SDS sample buffer. The resulting precipitated complexes were separated on SDS-polyacrylamide gels and blotted with various antibodies as described.

**Plasmid construction, transfection, and reporter gene assay.** The 0.2met-chloramphenicol acetyltransferase (CAT) and 0.1met-CAT chimeric plasmids, which contain 0.2 and 0.1 kb of the 5'-flanking region of the c-met gene, respectively, and the coding sequence for CAT, have been described elsewhere (23). The Drosophila SL-2 cells, which lack endogenous Sp transcription factors, were used for investigating the effects of Sp proteins on c-met promoter activity (6). At 24 h before transfection, the cells were seeded onto six-well plates at 2 × 10⁵ cells/well. Cells were then transiently cotransfected with a constant amount of 0.2met-CAT or 0.1met-CAT chimeric plasmids and an increasing amount of either pPac-Sp1 or pPac-Sp3 expression vectors under the control of insect actin promoter. The DNA-calcium phosphate method was used according to the instructions of the Cell-Pfect transfection kit (Pharmacia, Piscataway, NJ). Cells were incubated with DNA-calcium phosphate coprecipitation buffer for 1 h at 4°C. Lysates were immunoprecipitated overnight at 4°C with 1 μg each of anti-Sp1, -Sp3, and normal IgG (Santa Cruz). After washing in PBS, the cells were incubated for an additional 24 h before harvest for CAT assays. After being washed in PBS, the cells were pelleted, resuspended in 150 μl of 0.25 M Tris·HCl at pH 7.5, and disrupted by three freeze-thaw cycles. The protein suspension was clarified by centrifugation at 15,000 g for 5 min at 4°C, and the supernatant was collected and assayed for CAT activity by a procedure described previously (23). Because Sp proteins are known to activate the transcription of many internal control reporter vectors driven under the SV-40 early promoter and the thymidine kinase promoter (6, 16), the relative CAT activity in this study was reported after normalization for protein concentration. Protein concentration was determined using a BCA protein assay kit (Sigma). All experiments were repeated at least three times to ensure reproducibility.
cortransfected with CAT reporter plasmids and either 20- or 50-fold molar excess of wild-type or mutant Sp1 oligos. At 36 h after transfection, cells were harvested, and CAT activities were determined.

Statistical analysis. Quantitation of the Western blots was performed by measuring the intensity of the hybridization signals with the use of NIH Image analysis software. Data were expressed as means ± SE. Statistical analyses of the data were carried out by t-test with the use of SigmaStat software (Jandel Scientific, San Rafael, CA). \( P < 0.05 \) was considered significant.

RESULTS

Overlapping expression pattern of c-met and Sp family proteins in normal kidney. The expression of c-met protein in normal mouse kidney was examined by immunohistochemical staining using a specific antibody against c-met. As shown in Fig. 1, the c-met protein was widely expressed in normal mouse kidney. All tubular epithelial cells along the entire nephron were positive for c-met protein, with high levels observed in distal tubules and collecting duct epithelia. Weak staining was also noticeable in the glomeruli, which was most likely present in glomerular visceral epithelial cells (podocytes) and mesangial cells. In contrast, c-met receptor staining in the renal interstitium of normal mouse kidney was extremely weak or non-detectable. When c-met antibody was replaced by normal IgG, no staining occurred (Fig. 1B), suggesting the specificity of c-met staining. These results indicate that c-met protein is constitutively expressed in normal adult kidney in a relatively ubiquitous fashion with different levels in distinct types of kidney cells.

The widespread expression pattern of c-met in normal kidney led us to speculate that there might be ubiquitous trans-acting factor(s) responsible for its transcriptional activation. Earlier studies (23) revealed the presence of multiple GC boxes (Sp1 binding sites) in the promoter region of the c-met gene, which potentially implicates the Sp family of transcription factors in regulating c-met expression in the kidney. Therefore, we examined the expression patterns of Sp1 and Sp3 transcription factors and the relationships with c-met in normal kidney via double immunofluorescence staining for simultaneous detection of both c-met and Sp proteins. As shown in Fig. 2, there was close correlation between the expression patterns of c-met protein and the Sp family of transcription factors in normal adult kidney. In kidney sections, c-met protein expression completely overlapped with the presence of nuclear Sp1 protein (Fig. 2). Similarly, Sp1 and Sp3 proteins were also concomitantly expressed in the nuclei of various kidney cells, suggesting a possibly functional coupling and cooperation with one another. Except on rare occasions in which some cells expressed more Sp1 than Sp3 or vice versa, Sp1 and Sp3 were largely expressed in comparable amounts in many cells of normal adult kidney (Fig. 2).

Correlation of c-met level with Sp family protein abundance in various types of kidney cells in vitro. We next examined c-met protein expression in a wide variety of kidney cells in vitro by using Western blot analysis. As shown in Fig. 3, all types of kidney cells tested, including glomerular mesangial cells, podocytes, proximal tubular epithelial cells, collecting duct epithelial cells, and renal interstitial fibroblasts, expressed different levels of c-met protein. A single band of 145-kDa ß-subunit of c-met protein was observed in all types of kidney cells in polyacrylamide gels under reducing conditions. This observation is consistent with the in vivo data, which demonstrates widespread expression of c-met protein in normal adult kidney as described (see Fig. 1). Western blot analysis also exhibited that all of the kidney cells tested expressed distinctive levels of Sp1 and Sp3 transcription factors. A doublet of Sp1 protein that represented a different phosphorylated status (15) at ~95 kDa was detected in all renal cells. Sp3 displayed two doublets at 124 and 84 kDa, respectively. Presumably, these different isoforms are derived from distinct internal translation initiations (18, 49). Intriguingly, a plot of the abundances of c-met, Sp1, and Sp3 proteins indicates that there is a remarkably tight correlation between the c-met and Sp protein levels in various types of kidney cells in vitro (Fig. 3C). These results establish that c-met receptor levels in diverse types of kidney cells are

Fig. 1. Immunohistochemical localization of c-met protein in normal mouse kidney. A: ubiquitous presence of c-met protein in normal kidney is shown. Specific staining for c-met protein was visible in renal tubules along the entire nephron. Weak staining was also observed in renal glomeruli (arrow). B: as a negative control, no staining was observed in the kidney when c-met primary antibody was replaced with normal IgG. Scale bar, 20 \( \mu \text{m} \).
in proportional to, and are likely dictated by, the endogenous abundance of the Sp family of transcription factors.

Comparison of the c-met receptor levels from podocytes of the differentiated and nondifferentiated states uncovered that c-met receptor expression was repressed in differentiated podocytes (Fig. 3B). This inhibition of c-met expression during podocyte differentiation was accompanied by decreased Sp1 and Sp3 protein levels (Fig. 3B). To further explore the correlation between c-met expression and Sp protein abundances, we examined the kinetics of c-met and Sp protein expression during podocyte differentiation induced by switching podocyte culture to 37°C in the absence of INF-γ (34). As shown in Fig. 4, c-met inhibition occurred in the first 4 days of podocyte differentiation, and levels were sustained beyond this time point. Again, there was a strong association between c-met and Sp protein levels during this period of podocyte differentiation ($r^2 = 0.94$).

**Binding of Sp1 and Sp3 to GC boxes of c-met promoter region.** To examine the possibility of Sp proteins participating in the regulation of the c-met gene, we first determined whether Sp proteins interact with the c-met promoter by performing EMSA using a DNA fragment that contained three cis-acting Sp1 sites (GC boxes) from the c-met gene. When the DNA fragment was incubated with nuclear protein extract derived from mIMCD-3 cells, multiple DNA-protein complexes were formed that had retarded migration, which resulted in three shifted bands in polyacrylamide gels under non-denaturing conditions (C1-C3; see Fig. 5A). These binding complexes were largely abolished by using an unlabeled F1 fragment itself as a competitor in the incubation. Under the same conditions, the complexes were also completely abrogated in the presence of a 100-fold molar excess of the double-stranded oligo that corresponds to the Sp1 site of the c-met gene. The binding complexes were intact when incubated with a 100-fold molar excess of the mutated Sp1 oligo in which two GGs were substituted with two TTs in the Sp1 binding region. Other oligos with unrelated sequences such as activator protein 1 (AP-1), nuclear factor-κB (NF-κB), and cAMP response element did not interrupt the formation of the binding complexes (Fig. 5A), suggesting the specificity of these DNA-protein interactions. Supershift assay with specific antibodies revealed that these binding complexes were contributed by Sp1 and Sp3 transcription factors (Fig. 5B). Incubation with Sp1 antibody caused a further shift of the C2 complex and the formation of supershifted bands (SS1 and SS2), whereas the Sp3 antibody resulted in a supershift of the C1 and C3 complexes and formation of SS3 and SS2 (Fig. 5B). As expected, incubation with normal IgG or other unrelated antibodies such as anti-Egr-1 transcription factor did not cause either a supershift or inhibition of the complex formation.

Careful examination of the supershift data revealed that band SS2 was formed in the reactions involving both anti-Sp1 and anti-Sp3 antibodies (Fig. 5B, lanes 3 and 4). These results implied that SS2 could be attributable to an interacting complex that potentially consisted of Sp1, Sp3, and IgG, which suggests that two members of Sp family proteins (Sp1 and Sp3) may physically interact with each other. To test this hypothesis, we performed additional coimmunoprecipitation experiments to demonstrate a direct interaction between Sp1 and Sp3 in renal epithelial cells. As shown in Fig. 6A, when cell lysate derived from mIMCD-3 cells was immunoprecipitated with Sp1 an-

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**Fig. 2.** Colocalization of c-met protein with Sp family of transcription factors in normal mouse kidney. Representative micrographs demonstrate a close association of c-met protein with Sp1 and Sp3 transcription factors. Immunofluorescence staining for both Sp1 (red) and c-met (green) in cryosections of normal mouse kidney (top). Merging of two micrographs (top right) illustrates the overlapping pattern of c-met and Sp1 localization. Immunofluorescence staining for Sp1 (red) and Sp3 (green) in normal kidney (bottom). Merging of two micrographs (bottom right) demonstrates a largely overlapping pattern of Sp1 and Sp3. Cell nuclei with approximately equal amounts of both Sp1 and Sp3 (yellow) are indicated (arrowheads). Scale bar, 20 μm.
expression, we transfected the 0.2met-CAT reporter construct that contains multiple Sp1 binding sites with expression vector for Sp1 and Sp3 in Drosophila SL-2 cells. Because Drosophila cells lack endogenous Sp activity, the cells provide a sensitive and reliable in vivo assay system for investigating the effects of Sp proteins on gene transcription. As shown in Fig. 7A, Sp1 dramatically activated c-met promoter activity in a dose-dependent manner. An ~18-fold induction in reporter gene activity was observed after cotransfection of SL-2 cells with 0.2met-CAT plasmid and 3 μg of Sp1 expression vector pPac-Sp1. Sp3 alone also induced, to a much less extent, c-met promoter activity. Cotransfection of 3 μg of Sp3 expression vector pPac-Sp3 with 0.2met-CAT plasmid into SL-2 cells resulted in an approximately eightfold induction of reporter activity (Fig. 7A).

To investigate the potentially functional interaction between Sp1 and Sp3 proteins in activating c-met transcription, we cotransfected SL-2 cells with the 0.2met-CAT construct plus both Sp1 and Sp3 expression vectors. As presented in Fig. 7B, the combination of Sp1 and Sp3 resulted in a dramatically synergistic induction of c-met gene transcription. The magnitude of the CAT reporter induction that was elicited by the combined Sp1 and Sp3 was greater than the additive effect obtained by Sp1 and Sp3 individually. Hence, both Sp1 and Sp3 activate c-met transcription in a functionally cooperative manner.

Deprivation of Sp family proteins by decoy oligos in renal epithelial cells inhibits c-met promoter activity. To further confirm the importance of the Sp family proteins in regulating c-met transcription, we cotransfected the 0.2met-CAT reporter construct to mIMCD-3 cells with decoy oligo corresponding to the Sp1 binding site, which competes with c-met promoter Sp1 sites for binding with cellular trans-acting Sp proteins. This strategy presumably leads to a decrease in the availability of the cellular Sp proteins to the c-met promoter. As shown in Fig. 8, introduction of the Sp1 decoy oligo markedly inhibited c-met gene transcription in a dose-dependent fashion. Cotransfection of a 50-fold molar excess of Sp1 decoy oligo together with the 0.2met-CAT plasmid suppressed c-met promoter activity by ~70%. In fact, the CAT reporter gene activity was reduced by Sp1 decoy to a level similar to that elicited by the 0.1met-CAT plasmid in which three Sp1 binding sites were deleted. However, transfection of the mutant Sp1 oligo that failed to bind Sp proteins due to mutations in the Sp1 binding region (see Fig. 5) did not significantly affect 0.2met-CAT reporter activity in renal epithelial cells. Thus the abundance of endogenous cellular Sp family proteins likely dictates the level of c-met expression in kidney cells.

Blockade of Sp protein binding inhibits c-met expression in renal epithelial cells. We next investigated the effects of blockade of Sp binding via chemical antagonist in renal epithelial cells on c-met receptor expression. mIMCD-3 cells were treated with mithramycin A, a potent inhibitor of Sp binding (39, 42), for various periods of time at different concentrations. Because
c-met expression is primarily regulated at the transcriptional level in mIMCD-3 cells (25), the effect of the Sp inhibitor on c-met expression was determined by measuring the protein levels using Western blot analysis after various treatments. As shown in Fig. 9, mithramycin A markedly inhibited c-met expression in mIMCD-3 cells in a dose-dependent manner. At concentrations as low as $10^{-8}$ M, mithramycin A repressed c-met expression by $>80\%$ after a 24-h incubation. The kinetics of c-met inhibition by mithramycin A are presented in Fig. 9B. Blockade of Sp binding significantly inhibited c-met expression as early as 12 h after incubation with the chemical antagonist. Of note, the inhibitory effect of mithramycin A was specific, because expression of other genes such as actin was not blocked by this chemical inhibitor (Fig. 9). These results suggest that the binding of Sp proteins to cognate cis-acting elements is essential for constitutive expression of the c-met gene in renal epithelial cells.

Functionality of c-met receptor in different types of kidney cells in vitro. The ubiquitous expression pattern of c-met in the kidney prompted us to investigate whether the c-met receptor is functional in all types of kidney cells. To this end, we studied the phosphorylation and activation of PKB/Akt kinase, which is a major signaling protein in the pathway leading to cell survival (12, 36), in various types of kidney cells after HGF stimulation. Consistent with a previous report (22), HGF induced marked Akt phosphorylation as early as 5 min after stimulation in proximal tubular epithelial cells, and this induction was sustained to at least 1.5 h after HGF incubation (Fig. 10). In addition to proximal tubular epithelial cells, all other types of kidney cells tested, including glomerular mesangial cells, podocytes, collecting duct epithelial cells, and renal interstitial fibroblasts, responded to HGF stimulation and induced Akt phosphorylation and activation (Fig. 10). Therefore, the c-met receptor is indeed functional in all types of kidney cells tested and responds to HGF stimulation to initiate signal transduction events that lead to cell survival.

We found that the Akt phosphorylation and activation induced by HGF in different types of kidney cells displayed distinctly dynamic patterns (Fig. 10). The activation of Akt kinase by HGF took place in glomerular mesangial cells (RMC) and renal interstitial fibroblasts (NRK-49F) in a similarly dynamic fashion (Fig. 10). The Akt kinase was activated markedly and rapidly (as early as 5 min), but this induction was transient, and the phosphorylated Akt abundance quickly returned toward baseline level after 1 h (Fig. 10). The dynamics of Akt activation in mIMCD-3 cells and

![Fig. 4. Concomitant alterations of c-met expression with Sp1 and Sp3 transcription factors during podocyte differentiation. Mouse podocyte differentiation was initiated by switching the culture from 33 to 37°C in the absence of interferon-γ under nonpermissive conditions. At various time points (in days) as indicated, podocytes were harvested and the cell lysate was immunoblotted with specific antibodies against c-met, Sp1, Sp3, and actin. Identification (right) and molecular sizes (in kDa; left) of the proteins are indicated.](image)

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![Fig. 5. Electrophoresis mobility shift assay (EMSA) demonstrates that both Sp1 and Sp3 proteins bind to the GC boxes in the c-met promoter region. A: $^{32}$P-labeled DNA fragment (F1) corresponding to the nucleotide sequence −217 to −50 of the c-met promoter region was incubated with nuclear protein extract (NPE) from mIMCD-3 cells in EMSA reaction mixture. Three major DNA-protein complexes (designated C1, C2, and C3) were formed between renal epithelial cell nuclear proteins and c-met promoter. Competition for binding was performed by including a 100-fold molar excess of unlabeled F1 itself or oligonucleotides corresponding to Sp1, mutant Sp1 (Sp1m), activator protein 1 (AP-1), nuclear factor-κB (NF-κB), and cAMP response element (CRE) as indicated. Sequences of these oligonucleotides are presented in Table 1. B: Identification of these DNA-protein complexes (C1–C3) was characterized by supershift analysis with specific antibodies and normal rabbit IgG. Supershifted protein-DNA complexes with Sp1 antibody are designated as SS1 and SS2, and those with Sp3 antibody are denoted as SS3 and SS2 (arrows).](image)
differentiated podocytes were comparable to delayed responses starting at 30 and 60 min, respectively, after HGF stimulation. Conversely, HGF induced rapid and sustained Akt phosphorylation in HKC cells and non-differentiated podocytes.

DISCUSSION

HGF and its specific c-met receptor are classically considered as a paired signaling system for mediating signal exchange between mesenchyme and epithelia via paracrine actions (46, 47). This assumption is largely based on observations that the c-met receptor is predominantly expressed in epithelial cells, whereas its ligand is mainly produced by mesenchyme-derived cells (46). In this study, we demonstrate that almost all kidney cells in normal adult animals express different levels of c-met receptor protein. In vitro, all types of kidney cells tested, including glomerular mesangial cells, podocytes, proximal tubular epithelial cells, collecting duct epithelial cells, and interstitial fibroblasts, expressed the functional c-met receptor and responded to HGF stimulation to induce PKB/Akt phosphorylation. Although the present study has not included renal glomerular and vascular endothelial cells, studies elsewhere indicate that endothelial cells also express the functional c-met receptor and respond to HGF stimulation to induce PKB/Akt phosphorylation. Although the present study has not included renal glomerular and vascular endothelial cells, studies elsewhere indicate that endothelial cells also express the functional c-met receptor and respond to HGF stimulation (52). Altogether, these results suggest that c-met receptor expression in the kidney is widespread and ubiquitous. Because it is the receptor that determines the target specificity of HGF actions, our results suggest that HGF may have a broader spectrum of target cells in the kidney and thereby possess wider implications in kidney structure and function than previously envisioned.

Although the c-met receptor has been demonstrated to be expressed in renal tubular epithelial cells, its presence and function in other types of cells such as interstitial fibroblasts are uncertain. In contradiction to the present study, earlier observations often suggested an absence of c-met receptors in renal interstitial cells in normal rats (26). This discrepancy is probably attributable to the low sensitivity of the immunohistochemical staining approach that was employed previously. Consistent with this notion, extremely weak or no staining for the c-met receptor was also observed in the interstitium of mouse kidney in this study (see Fig. 1). The finding of ubiquitous expression of c-met in the kidney is supported by several lines of evidence. First, c-met receptor protein is detectable in all types of homogenous kidney cell popula-
ceptors in these types of cells suggests an autocrine loop formation with simultaneous expression of both the receptor and its ligand in the same cell. These observations expand the modes of HGF action in normal kidney beyond the well-described paracrine and endocrine mechanisms. The physiological significance of HGF autocrine action is largely unknown. It is plausible to speculate that the autocrine signaling of HGF may be one of the pathways essential for the development and maintenance of normal kidney structure and function. In this regard, previous studies indicate that HGF and c-met are coexpressed in early metanephrogenic mesenchyme, which leads to the promotion of mesenchymal-to-epithelial cell transdifferentiation during nephrogenesis (1, 50). Similarly, both c-met and HGF are significantly induced in rat glomerular mesangial cells in response to interleukin-6 stimulation (27).

In light of the widespread expression of the c-met gene in the kidney, it is not surprising to find that c-met is expressed in a pattern that is overlapped with the ubiquitous Sp family of transcription factors. The importance of Sp proteins in the constitutive expression of c-met in the kidney is established by several lines of observations in this study. These include a tight correlation between c-met receptor abundance and the levels of Sp proteins in various types of kidney cells (see Fig. 3). This intrinsic interconnection between c-met and Sp proteins is also evident during podocyte differentiation (see Fig. 4). Hence, cellular endogenous Sp protein level probably is a key molecular determinant for the differential expression of c-met in diverse types of kidney cells. In support of this, Sp proteins are found to bind to the promoter region of the c-met gene and to functionally activate c-met transcription. Conversely, deprivation of Sp proteins by a decoy strategy and blockade of Sp binding by chemical antagonist inhibits c-met expression in renal epithelial cells. Although the present study uses the human c-met promoter, previous studies by Seol and colleagues (44, 45) have demonstrated that the two Sp1 binding sites in the mouse c-met gene are also critical for establishing basal c-met expression as well as for mod-

![Image](https://i.imgur.com/3Q5Q5Q.png)

**Fig. 8.** Deprivation of Sp proteins by cotransfection of decoy oligonucleotides inhibits c-met promoter activity. *A:* schematic representation of chimeric reporter plasmids 0.2met-CAT and 0.1met-CAT. Three Sp1 sites were located at the c-met promoter region between nucleotides −68 and −223 (ovals). *B:* mIMCD-3 cells were cotransfected with 0.2met-CAT plasmid and a 20- or 50-fold molar excess of Sp1 or mutant Sp1 decoy oligos (Sp1mut oligo), respectively, as indicated. Cells were also transfected with 0.1met-CAT construct as a control in which the three Sp1 sites of the c-met promoter region were deleted. Relative CAT activities (with 0.2met-CAT plasmid alone taken as 100) are presented as means ± SE from 3 independent experiments. **P** < 0.01.

![Image](https://i.imgur.com/4Q4Q4Q.png)

**Fig. 9.** Chemical blockade of Sp1 binding with mithramycin A inhibits c-met expression in renal epithelial cells in dose- and time-dependent manners. *A:* mIMCD-3 cells were incubated with various concentrations of mithramycin A for 24 h. *B:* Cells were incubated with 10−6 M of mithramycin A for different periods of time. Cell lysates were separated on polyacrylamide gels and immunoblotted with c-met antibody. The same blots were reprobed with actin to ensure equal loading of the samples.
Fig. 10. Activation of protein kinase B/Akt in various types of kidney cells in response to HGF stimulation. Major types of kidney cells, including RMC cells, NRK-49F cells, mIMCD-3 cells, HKC cells, mouse differentiated glomerular podocytes (D), and nondifferentiated podocytes (ND) were incubated with 20 ng/ml human recombinant HGF for various periods of time as indicated. A: cell lysates were immunoblotted with antibodies against phospho-specific Akt or total Akt, respectively. Molecular size markers (in kDa) are shown. B: dynamic patterns of Akt phosphorylation and activation in various types of kidney cells after HGF stimulation. Quantitative data from densitometric analysis of Western blots were plotted after correction with total Akt.
ulating induced c-met transcription, suggesting that there is little mechanistic difference in the regulation of c-met transcription by Sp proteins in different species. Accordingly, a recent report demonstrates that inhibition of c-met expression in human primary hepatocytes by IFN-α is mediated by decreased binding of Sp1 to the c-met promoter (41). In this context, it should also be noted that Sp1-knockout embryos were severely defective in development and all died around day 11 of gestation (29), a time point precisely before the death of c-met-knockout embryos at days 13 and 14 (2).

The Sp family of transcription factors consists of at least four members with distinct expression patterns and diverse functions in the different types of cells (49). All four proteins share similar structural features, including a highly conserved DNA binding domain that consists of three zinc fingers close to the COOH-terminal region (49). Sp1 is the prototype of this family and is expressed ubiquitously. Sp1 functions as a transcriptional activator for a large number of genes implicated in cell-cycle regulation, hormonal activation, and embryonic development. Sp2 exhibits a significant structural difference from other members of the Sp family and does not bind to the GC box but to a GT-rich element in the promoter region of the T-cell receptor gene (19). Little is known regarding Sp2 tissue distribution and its function. Although Sp3 is also ubiquitously expressed, the expression of Sp4 is tissue specific and largely restricted to the brain (48). Because both Sp3 and Sp1 are often present in the same cell and are indistinguishable in DNA binding specificity, Sp3 is generally considered to be an antagonist for Sp1 by functionally suppressing Sp1-mediated gene activation (10, 11, 17). However, several reports also suggest a positive regulation of gene expression by Sp3 in different circumstances. For instance, Sp3 has been shown to activate the promoter of the human α2(1) collagen gene and the mouse growth-hormone receptor gene (8, 13, 53). In the present study, our data suggest that Sp3 alone activates c-met gene transcription, although to much less extent than Sp1. In addition, Sp3 not only fails to inhibit Sp1-activated c-met gene transcription, but also actually enhances Sp1-induced c-met expression in a synergistic fashion (see Fig. 7). These observations underline that members of the Sp family of transcription factors interact with one another to produce either positive or negative regulation of a particular gene, depending on the context of specific promoter and cellular environments.

An interesting and novel finding in this study is the synergistic action of Sp1 and Sp3 in activating c-met gene transcription. Apart from the cotransfection data presented in Fig. 7B, Western blot analysis also reveals that the differences in c-met levels among diverse types of kidney cells are greater than in either Sp1 or Sp3 alone (see Fig. 3). This suggests a favorable interaction between cellular Sp1 and Sp3 proteins in establishing the constitutive expression of c-met in the kidney. In accordance with this, Sp1 and Sp3 are largely expressed in an identical pattern with comparable abundances in different types of normal kidney cells (see Fig. 2). Although the molecular mechanism that underlies the synergistic activation of the c-met gene by Sp1 and Sp3 remains unknown, physical interaction between two members of the Sp family may be of importance for such functional cooperation. Sp1 is known to be capable of forming homotypic interactions that lead to multimeric complexes (38), which mediate transcriptional synergism among multiple GC boxes. Moreover, many heterotypic interactions of Sp1 with diverse types of transcription factors, such as YY1, E2F, and Smad2/3, just to name a few, have been documented (9, 14, 20, 32, 43). Indeed, a direct interaction between Sp1 and Sp3 in renal epithelial cells has been demonstrated by coimmunoprecipitation with either Sp1 or Sp3 antibodies (see Fig. 6). In addition, supershift experiments using antibodies against either Sp1 or Sp3 (see Fig. 5) exhibit the presence of an additional complex (SS2) with identical size that presumably consists of Sp1, Sp3, and IgG. Therefore, heterotypic interaction between Sp1 and Sp3 occurs in renal epithelial cells and is probably critical for the synergistic activation of c-met gene transcription.

The finding of ubiquitous expression of c-met receptors in various types of renal cells suggests a broader spectrum of target cells for HGF in normal kidney than previously thought. Perhaps more surprisingly, the functional response to HGF by a particular type of cells appears to be unrelated to the cellular abundance of c-met receptors (see Fig. 10). These observations lead one to rethink the HGF biology in the kidney. It now appears certain that HGF signaling possesses important biological activities not only in tubular epithelial cells, where the c-met receptor is robustly expressed, but also in the cells with low abundance of c-met receptors, such as glomerular mesangial cells and interstitial fibroblasts. Undoubtedly, one of the great challenges in the future is to determine the exact function of HGF signaling in a particular type of renal cells in the context of whole kidney in vivo.

In summary, the c-met receptor is expressed in normal adult kidney and in diverse types of kidney cells in a ubiquitous fashion. The constitutive expression of the c-met gene is closely correlated with and primarily mediated by the synergistic actions of transcription factors Sp1 and Sp3. Physiologically, the c-met receptor in all types of kidney cells tested is functional and initiates a cascade of signal transduction events leading to Akt phosphorylation in response to HGF. In view of the fact that the receptor determines the target specificity of the ligand, these results suggest that HGF may have broader implications in kidney structure and function under various physiological and pathological conditions.

The authors thank Drs. R. Tjian and G. Suske for providing the Sp1 and Sp3 expression plasmids.

This work was supported by National Institutes of Health Grants DK-02611, DK-54922, and DK-61408 and by National Science Foundation of China Grant 39825508. C. Dai and J. Yang were supported by postdoctoral fellowships from the American Heart Association Pennsylvania-Delaware Affiliate.
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