HGF Activator Is Required for Metanephric Kidney Morphogenesis in Vivo*

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The interaction of hepatocyte growth factor (HGF) with c-Met has been implicated in morphogenesis of the kidney, lung, mammary gland, liver, placenta, and limb bud. HGF is secreted as an inactive zymogen and must be cleaved by a serine protease to initiate Met signaling. We show here that a serine protease specific for HGF, HGF activator (HGFA), is expressed and activated by the ureteric bud of the developing kidney in vivo and in vitro. Inhibition of HGFA activity with serine protease inhibitors reduced ureteric bud branching and inhibited glomerulogenesis and nephrogenesis. Activated HGF rescued developing kidneys from the effects of inhibitors. HGFA was localized around the tips of the ureteric bud in developing kidneys, while HGFA was expressed diffusely throughout the mesenchyme. These data show that expression of HGF is not sufficient for development, but that its activation is also required. The localization of HGFA to the ureteric bud and the mesenchyme immediately adjacent to it suggests that HGFA creates a gradient of HGF activity in the developing kidney. The creation and shape of gradients of activated HGF by the localized secretion of HGFA activators could play an important role in pattern formation by HGF responsive tissues.

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§ The abbreviations used are: HGF, hepatocyte growth factor; UB cell, ureteric bud cell; HGFA, hepatocyte growth factor activator; EST, expressed tag sequence; RT-PCR, reverse transcriptase-polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; kb, kilobase(s).

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HGF Activator in Developing Kidney

MATERIALS AND METHODS

Sequence of Murine HGFA—The coding sequence of human HGFA (GenBank™ accession number D50030) was used to screen the murine EST database using the BLAST algorithm (19). Sequences identified by the initial screen were used to re-interrogate the database. By this process, 9 clones were identified and assembled into a contig. Of these clones, 8 (mx56596, uc76a08, mx77f07, mx85g08, and mx92a08) were purchased from ATCC, characterized by restriction mapping, and sequenced in both directions. The sequence of murine HGFA was deposited in GenBank™ (AF224724).

Northern Blotting—A 492-nucleotide EcoRI, NorI fragment of IMAGE clone mj6003 corresponding to nucleotides 922 to 1864 of the full-length sequence was used as a probe. The probe was labeled with digoxigenin-UTP by random priming and used to screen mouse multiple tissue Northern blots (CLONTECH). For Northern blots of UB cell RNA, total RNA was isolated from UB cell cultures using RNAzol (Cinna) according to the manufacturer’s instructions. Total RNA (1 µg) was denatured and transferred to nylon membranes (Immobilon-N, Millipore). Membranes were hybridized for 16 h at 45 °C and washed with 0.1 × SSC containing 1% SDS at 65 °C. Bound probe was visualized with alkaline phosphatase and chemiluminescence detection system according to the manufacturer’s instructions (Roche Biochemicals).

RT-PCR—RNA was isolated from murine UB cells or from microdissected rat ureteric buds and mesenchymes using RNAzol (Cinna). Poly(A) RNA from rat ureteric buds and mesenchymes was purified from total RNA by affinity chromatography on poly(T) resin (Oligotex-dT, Qiagen). The microdissected rat kidney rudiments were obtained at embryonic day 13.5, when the ureteric bud had branched a single time. This stage of kidney development in the rat is morphologically equivalent to embryonic day 11.5 in murine kidney development.

Primers for RT-PCR of murine HGFA were TGTCTACACCCCAA-CAACC (forward primer, nucleotides 1474–1491) and GTAAGCCTGACACCATTCCTC (reverse primer, nucleotides 1838–1818). Total RNA was transcribed into cDNA using avian myeloblastosis virus reverse transcriptase and random hexamers. The cDNA was subjected to 30 cycles of PCR using Taq polymerase (Promega). The annealing temperature was 59 °C for PCR with the murine template (dissected ureteric bud or mesenchyme RNA). Amplified DNA fragments were purified from agarose gels using QIAEX resin (Qiagen) and directly sequenced by the DNA facility of Columbia University.

Cells, Plasma, Serum, Conditioned Medium, and HGF—UB cells (20) were grown in Dulbecco’s modified Eagle’s medium/F-12 (Life Technologies, Inc.) with 5% fetal bovine serum (Hyclone) and 1% ITS (insulin, transferrin, selenious acid, essential fatty acids), from Roche Molecular Biochemicals. Conditioned medium was cleared by centrifugation at 3000 g for 15 min and 1000-fold with a Centricon Plus-80 Biomax-8 device (molecular mass cutoff 8000 Daltons, Millipore). Plasma and serum were purchased from Pelfreez. Activated HGF was obtained from R&D Systems (NSO HGF). The inactive zymogen of HGF (HGFsc) was a gracious gift of Dr. George vande Woude (National Institutes of Health, Bethesda, MD).

Antibodies—Antibodies raised against peptides derived from human HGFA were purchased from Santa Cruz Biotechnology. The peptide used to raise antibody HGFA-L (N-19) is identical in murine and human HGFA (underlined in Fig. 1). Antibodies against human HGF subunit (N-19 and C-20), WT1 (C-19), and the HGF receptor met were also obtained from Santa Cruz Biotechnology. Antibody against phosphotyrosine residue 1023 was obtained from Upstate Biotechnologies. TROMA-1 was obtained from the Developmental Studies Hybridoma Bank. Cy3-labeled anti-rabbit and fluorescein isothiocyanate-labeled anti-rat IgG were obtained from Jackson Immunoresearch. Alexa 488-labeled anti-rabbit antibody was obtained from Molecular Probes. For some experiments, TROMA-1 antibodies were purified by anion exchange chromatography and labeled with Alexa 488 (Molecular Probes) according to the manufacturer’s instructions.

Immunoblots—Proteins were subjected to SDS-PAGE under reducing conditions and transferred to polyvinylidene difluoride (Immobilon-P, Millipore). Blots were blocked for 1 h with 10% instant nonfat dry milk in PBS and then incubated overnight at 4 °C in primary antibody diluted in 1% bovine serum albumin. Concentrations of primary antibody were 1/100 for anti-HGF and 1/1000 for anti-phosphotyrosine and anti-Met. Blots were washed 3 times in 5% milk and incubated for 45 min in 1:10,000 dilution of peroxidase-labeled secondary antibody (Jackson Immunoresearch). Antibody binding was visualized by chemiluminescence (ECL, Amersham Pharmacia Biotech).

Assay for HGF Activity—Serial dilutions of UB conditioned medium were incubated for 2 h at 37 °C with 200 ng/ml single chain HGF (HGFSc). Aliquots of the mixture were removed at various times and analyzed by SDS-PAGE under reducing conditions.

Immunoprecipitation of Met—Confluent cultures of UB cells were incubated overnight in serum-free medium. The next day, HGFSc was added to the culture medium in the presence or absence of leupeptin or serum and the cultures were incubated for 15 min at 37 °C. The cultures were then washed three times in PBS and scrapped in SB (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 10 mM EDTA). Complete protease inhibitors from Roche Molecular Biochemicals, 1 mM sodium orthovanadate, and 1% Triton X-100. The extracts were incubated for 30 min at 4 °C and pelleted for 15 min at 10,000 × g to obtain cleared lysates. Lysates were then treated with 1 µg/ml of rabbit anti-Met antibody diluted in 1% bovine serum albumin for 1 h and for a second hour with Protein A-Sepharose (Roche Molecular Biochemicals). The precipitates were washed three times with SB and displayed on a 5% reducing SDS-PAGE. The immunoblots were processed for anti-phosphotyrosine as described above except that the blots were blocked in 1% bovine serum albumin in Tris-buffered saline (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). After visualization of phosphotyrosine, the blots were stripped with 25 mM glycine, pH 2.5, and 1% SDS and then re-probed with anti-Met as described above.

Organ Culture of Fetal Kidney Rudiments—Kidney rudiments were dissected from timed pregnant Swiss Webster mice at E11.5 when the ureteric bud had branched once. Organs were explanted onto permeable supports (Transwell Clear, 3.0-µm pore size, Costar) in chambers containing 0.25 ml of medium. Cultures were maintained at the air-water interface in 5% CO2 at 37 °C and then fixed for 10 min in methanol at −20 °C. Kidney rudiments were grown in Dulbecco’s modified Eagle’s medium/F-12 containing 1% ITS (Collaborative Research) and no serum. For HGF and HGFSc staining, explants of freshly dissected E12.5 kidneys were incubated for 3–4 h on Transwells and then processed for confocal microscopy.

Confocal Microscopy of Fetal Kidney Rudiments and Image Analysis—Methanol-fixed organ cultures were stained en bloc as described previously (21). Briefly, the fixed organ cultures were re-hydrated in PBS and blocked for 6 h in 1% bovine serum albumin or 10% donkey serum (Jackson Immunoresearch). The cultures were incubated in primary antibody for 24–48 h at 4 °C, washed for 8–24 h in 1% bovine serum albumin, and incubated overnight in secondary antibody. The cultures were washed for 2×, post-fixed for 10 min with 2% paraformaldehyde, quenched with 50 mM NH4Cl in PBS, rinsed in PBS, and mounted in ProLong Antifade (Molecular Probes). The dilutions of primary antibody were: HGF (C-20 or N-19) 1:100, HGFL-A (N-19) 1:50, WT1 (C-19) 1:50, TROMA-1 1:10. Secondary antibodies were diluted 1:100.

The kidney mounts were analyzed by confocal microscopy on a Zeiss 410 laser scanning confocal microscopy equipped with an argon-krypton laser. The kidney rudiments were visualized with a ×10 or 40 objective on a Zeiss Axioint 100 using the 568- and 488-nm laser emission peaks for excitation. Images were collected using a 515–540-nm band pass and a 596-nm long pass filter set. Each kidney rudiment was scanned 10 times 5-µm apart. The scans were recorded as TIFF files and used for image analysis as described previously (21).

RESULTS

Sequence of Murine HGFA—Interrogation of the data base of murine ESTs with the coding sequence of human HGFA yielded 9 clones, of which clone (mx85g08) contains the complete coding sequence of murine HGFA. One clone (uc76a08) had a 47-nucleotide out of frame deletion in the 5’ end. Clone mj6003 contained the signal sequence which was followed by a 666-nucleotide in-frame deletion.

The sequence of murine HGFA was 82% identical to that of human HGFA at both the nucleotide and amino acid levels.
The catalytic domains of the two proteins were 93% identical, with 18 conservative amino acid substitutions and a single nonconservative substitution (Leu<sup>405</sup> in mouse for Arg<sup>405</sup> in human) in the COOH-terminal 257 amino acids. The thrombin cleavage site at Arg<sup>405</sup>-Ile<sup>406</sup> that is required for activation of HGFA was conserved, as were all the amino acids required for catalytic activity. The fibronectin, epidermal growth factor, and kringle domains were conserved as were the critical cysteines of all these motifs. The 104 amino acids at the amino-terminal end of the antibody-binding site. HGFA is activated by cleavage at Arg<sup>405</sup>-Ile<sup>406</sup>, which is at the amino-terminal end of the antibody-binding site.

We used the murine HGFA sequence to search the GenBank<sup>TM</sup> database with the BLAST algorithm and identified only human HGFA and a partial rat HGFA sequence. A human protein similar to HGFA has recently been identified (22, 23) and adjusted manually. Amino acid differences are shown in bold type. The signal sequence is italicized. Conserved cysteines are in red type and the catalytic domain is in blue type. The antibody binding site is underlined. Conserved motifs are delineated by boxes: FnII, blue; epidermal growth factor, maroon (2 repeats); FnI, yellow; kringle, green. HGFA is activated by cleavage at Arg<sup>405</sup>-Ile<sup>406</sup>, which is at the amino-terminal end of the antibody-binding site.

A single transcript of 3.4 kb was also detected. No HGFA was detected in Northern blots of total embryo poly(A) RNA. These data suggested that either HGFA is not expressed in the developing animal or that it is expressed in concentrations too low for detection by Northern blotting. In support of the latter hypothesis, we found that 7 of the 9 ESTs for murine HGFA were isolated from embryonic mouse libraries. We also identified human ESTs for HGFA in adult ovarian tumor (T72625),ateral renal development, HGFA is expressed exclusively by the ureteric bud but not from the mesenchyme (Fig. 2, C). Treatment of RNA with DNase prior to reverse transcription and PCR did not abolish amplification of this fragment. However, treatment of the samples with RNase blocked amplification. The amplified fragment was purified and sequenced. The sequence was similar, but not identical, to the sequence of murine HGFA. In the area where the fragment overlapped with a partial rat HGFA sequence (GI:3116329), the sequence was identical to the murine HGFA sequence (GI:3116329). The sequence of this fragment was 345 base pair (bp) product was amplified from immortalized murine UB cells and microdissected rat ureteric bud. No product was amplified from control samples treated with RNase A (not shown) or from microdissected metanephric mesenchyme.

**Message for HGFA Is Expressed Exclusively in the Ureteric Bud in Early Renal Development**—To test the hypothesis that HGFA is expressed during kidney development, we screened poly(A) RNA isolated from microdissected rat ureteric buds and mesenchymes by RT-PCR. We found that the predicted 345-base pair fragment was consistently amplified from the ureteric bud but not from the mesenchyme (Fig. 2, C). Treatment of RNA with DNase prior to reverse transcription and PCR did not abolish amplification of this fragment. However, treatment of the samples with RNase blocked amplification. The amplified fragment was purified and sequenced. The sequence was similar, but not identical, to the sequence of murine HGFA. In the area where the fragment overlapped with a partial rat HGFA sequence (GI:3116329), the sequence was identical to the rat sequence. Thus, the amplified fragment arose from rat RNA and could not have been derived from contamination by murine HGFA plasmid DNA. These data show that, during early renal development, HGFA is expressed exclusively by the ureteric bud.

**Immortalized Ureteric Bud Epithelial Cells Express HGFA Message and Protein**—To further characterize HGFA in ureteric bud epithelial cells, we screened an immortalized ureteric bud cell line (UB cells) for HGFA expression. The predicted 345-base pair fragment was amplified from UB cell RNA by RT-PCR (Fig. 2, C, UB cells). The sequence of this fragment was identical to the murine HGFA sequence. A single transcript of 2.0 kb was identified in UB RNA by Northern blotting using our murine HGFA probe (Fig. 2, B). These data support the results of the RT-PCR analysis of isolated ureteric buds and mesenchymes and show that ureteric bud epithelial cells express HGFA both in vivo and in vitro.

To determine whether UB cells synthesized and secreted a minor transcript of 3.4 kb was also detected. No HGFA was detected in Northern blots of total embryo poly(A) RNA. These data suggested that either HGFA is not expressed in the developing animal or that it is expressed in concentrations too low for detection by Northern blotting. In support of the latter hypothesis, we found that 7 of the 9 ESTs for murine HGFA were isolated from embryonic mouse libraries. We also identified human ESTs for HGFA in adult ovarian tumor (T72625), fetal liver/spleen (R98811), and a pooled adult lung/testis/B cell library (AI243636 and AA897612), suggesting that HGFA is expressed at low levels in a variety of organs during fetal and adult life in man.**
HGFA protein, we probed immunoblots of conditioned medium from UB cells with antibodies to HGFA. Antibody HGFA-L (N-19) was raised against a peptide at the amino-terminal end of the 34-kDa catalytic subunit of HGFA (underlined in Fig. 1). It recognized polypeptides of 99 and 34 kDa in murine plasma, representing the intact, uncleaved high molecular mass zymogen of HGFA (99 kDa) and the 34-kDa catalytically active form (Fig. 3A). Only the 34-kDa catalytically active form was detected in serum (Fig. 3A) and in UB cell conditioned medium (Fig. 3B), suggesting that UB cells are capable not only of secreting but also of activating HGFA.

Localization of HGFA and HGFA Proteins in Early Renal Development—We examined the distribution of HGFA in early kidney rudiments by confocal microscopy of whole mounts of freshly isolated E12.5 kidneys. We found that HGFA protein was expressed in the ureteric bud, as shown by the orange staining indicating colocalization of HGFA (green) and the ureteric bud marker (TROMA-1, red, arrowhead in Fig. 4A) (24). In the mesenchyme, HGFA was localized in a reticular pattern extending 2 to 3 cell diameters from the ureteric bud surface, consistent with deposition of HGFA, a heparin-binding protein (13), in the extracellular matrix surrounding the mesenchymal cells adjacent to the ureteric bud (arrows, Fig. 4A). Staining was specific for HGFA, as co-incubation of the antibody with the immunizing peptide blocked HGFA labeling but not labeling with the antibody for the ureteric bud specific cytokeratin, TROMA-1 (Fig. 4B).

Single optical sections suggested that HGFA was concentrated around the ureteric bud tips (Fig. 4A). To test this hypothesis, serial optical sections of a kidney rudiment stained for HGFA were optically summed and projected onto a single plane. These images were displayed with a pseudocolor algorithm to show the relative intensity of HGFA staining throughout the rudiment (Fig. 4C). HGFA immunoreactivity was concentrated around the ampulla of the ureteric bud, shown outlined in white. The distribution of HGFA was different from the distribution of HGF (Fig. 4D). HGF was localized throughout the mesenchyme and was not concentrated around the ureteric bud ampulla. This result shows that the high concentration of HGFA around the ureteric bud is not an artifact of the high local concentration of mesenchymal cells around the ureteric bud.

Conditioned Medium from UB Cells Contains HGFA Activity—The identification of a 34-kDa form of HGFA in UB conditioned medium suggested that UB cells secrete and activate HGFA. To test this hypothesis, we incubated the uncleaved zymogen of HGF (HGFsc) with serial dilutions of conditioned medium. HGFsc was completely converted to the active heterodimer (HGFα and HGFβ) by 2.4 μg of protein from UB conditioned medium (Fig. 5A). These data show that UB cells secrete HGFA and hydrolyze HGFsc to a disulfide-linked heterodimer. The molecular weights of the HGF subunits produced by UB conditioned medium are consistent with activation of HGF by hydrolysis between Arg494 and Val495.

HGFA is a serine protease whose HGF hydrolyzing activity is blocked by the serine protease inhibitor aprotinin and the serine and cysteine protease inhibitor leupeptin, but not by the acid protease inhibitor pepstatin or the aminopeptidase inhibitor bestatin (10, 11, 15). HGF hydrolysis by 2.5 × 105 ng of protein from UB conditioned medium was inhibited by leupeptin and aprotinin (Table I and Fig. 5B). Bestatin, EDTA, phosphoramidon, E64, and pepstatin A did not block HGF hydrolysis by UB conditioned medium.

Activated HGFB binds to the Met receptor tyrosine kinase and increases cell motility, producing scattering of islands of HGF-responsive cells in two-dimensional culture. To discover whether the HGF hydrolyzing enzyme secreted by UB cells produced functionally active, heterodimeric HGF from the inactive zymogen (HGFsc), we incubated UB cells with HGFsc in the presence or absence of leupeptin. In serum-free medium without HGF, UB cells were closely associated in islands with smooth borders (Fig. 6, A, no HGF). Activated, heterodimeric HGF produced scattering (Fig. 6, A, activated HGF) as did an equal concentration of HGFsc (Fig. 6, scHGF). The scattering...
HGF Activator in Developing Kidney

activity of HGFsc was inhibited by 100 and 10 μM leupeptin (Fig. 6A, 100 μM leupeptin and 10 μM leupeptin). Note that the UB cells were viable after incubation even in high concentrations of leupeptin (100 μM, Fig. 6A). These data show that UB cells responded to HGFsc in a leupeptin-dependent manner.

To demonstrate further that UB cells produced the activated form of HGF from the inactive zymogen HGFsc, the HGF receptor met was immunoprecipitated from cultures of UB cells (Fig. 6B). The Met receptor immunoprecipitated from UB cells incubated overnight in serum-free medium contained little phosphotyrosine (+HGFsc-serum-leupeptin). HGFsc incubated with fresh medium containing serum for 15 min stimulated Met autophosphorylation, as predicted from the observation that serum contains HGFA (+HGFsc+serum-leupeptin). HGFzymogen incubated with UB cells incubated in serum-free medium overnight also stimulated Met autophosphorylation, showing that UB cells were able to activate and respond to HGFsc in the absence of exogenous HGFA from serum (+HGFsc-serum-leupeptin). Activation of HGFA signaling was substantially inhibited by leupeptin, which blocks the action of endogenous HGFA (+HGFsc-serum+leupeptin). There was some activation of Met by HGFsc in the presence of leupeptin, consistent with the small but detectable amount of activated HGFA in the preparations of HGFsc (Fig. 5A). These biochemical data provide additional evidence that UB cells activate and respond to the zymogen of HGF.

Serine Protease Inhibitors Inhibit Branching Morphogenesis and Nephrogenesis by Blocking the Activation of HGFA—To determine the role of HGFA hydrolysis during nephrogenesis, we incubated organ cultures of kidney rudiments in protease inhibitors at concentrations that inhibited the hydrolysis of HGF by ureteric bud conditioned medium in vitro (Table I, Fig. 6, D and E). We found that the serine protease inhibitor aprotinin and the serine and cysteine protease inhibitor leupeptin blocked HGFA hydrolysis in vitro. Aprotinin and leupeptin inhibited development in rudiments isolated at E11.5 or E12.5 (Figs. 7, B, D, F, and Table I). The effect of the serine protease inhibitors was concentration dependent. No consistent effects on renal development were noted with 1 μM aprotinin or leupeptin, but development was inhibited at 10 μM of either inhibitor.

To characterize the effects of serine protease inhibitors on renal development, we examined ureteric bud branching, glomerulogenesis, and nephrogenesis. Leupeptin reduced ureteric bud branching to 50% and aprotinin to 54% of control (Figs. 7, C and D; Table I). Nephrogenesis assayed by staining with the proximal tubule marker Lotus tetragonolobus lectin was qualitatively reduced by leupeptin (Fig. 7, E and F). We were unable to quantitate the effects of serine protease inhibitors on proximal tubule formation because it was impossible to reliably distinguish one long, convoluted proximal tubule from another in the control kidneys. Proximal tubule length was unambiguously decreased by leupeptin in 2/3 kidneys assayed. Serine protease inhibitors reduced glomerulogenesis (Fig. 6, C and D; Table I). In kidneys isolated at E11.5, leupeptin reduced the number of glomeruli to 53% and aprotinin to 56% of control (Table I). In kidneys isolated at E12.5, leupeptin reduced the number of glomeruli to 53% of control (Fig. 7, C and D). Thus, blocking the activation of HGFA by either leupeptin or aprotinin inhibited branching morphogenesis by the ureteric bud, reduced the efficiency of nephrogenesis, and significantly decreased the number of glomeruli.

To test the specificity of the effects of serine protease inhibitors on renal development, we examined the effects of E64, a cysteine protease inhibitor that did not inhibit HGFA hydrolysis by ureteric bud conditioned medium (Table I). E64 had no effect on glomerulogenesis at concentrations as high as 100 μM, suggesting that the observed inhibitory effects of the serine and cysteine protease inhibitor were not nonspecific. Cells incubated in high concentrations of leupeptin (100 μM, Fig. 6A) or aprotinin (not shown) were viable as assessed by trypan blue exclusion (not shown). This result shows that the serine protease inhibitors are not likely to inhibit nephrogenesis by a direct toxic effect.

We used activated, heterodimeric HGFA to bypass the effects of the serine protease blockade by providing the product of HGFA activity. Activated HGFA rescued nephrogenesis in leupeptin-treated kidneys (Fig. 8). Leupeptin-treated kidneys had an average of 21 ± 2.4 glomeruli (n = 7, S.E.), which was 53 ± 6% of the number of glomeruli in untreated control kidneys (40 ± 5, n = 7, S.E.). Kidneys treated with leupeptin and activated HGFA had an average of 34 ± 5 glomeruli (n = 7, S.E.), or 84 ± 11% of the number of glomeruli in untreated controls. There was no significant difference between the number of glomeruli in control kidneys compared with the number of glomeruli in kidneys rescued with activated HGFA (p = 0.29). The number of glomeruli in leupeptin-treated kidneys was significantly decreased compared with either control (p = 0.015) or HGFA-rescued kidneys (p = 0.04). These data suggest that loss of activated HGFA is responsible for the effects of leupeptin on renal development.

DISCUSSION

In this study, we present the sequence of the murine HGFA gene. We showed that it is expressed solely by the ureteric bud in early kidney development. Using an immortalized ureteric bud cell line, we demonstrated that the ureteric bud expresses HGFA protein and have characterized the HGFA hydrolyzing activity and inhibitor profile of ureteric bud derived HGFA. In the developing kidney, HGFA protein was secreted into the mesenchyme around the ampulla at the tips of ureteric bud in proximity to both its substrate, HGF, and the receptor for
activated HGF, c-Met. Finally, we demonstrated, using inhibitors of HGFA, that activation of HGF by HGFA is required for renal development in vitro.

The identified cDNA is likely to be the murine homolog of HGFA because it is extremely similar to the sequence of the human gene. BLAST searches of the GenBank™ data base with the nucleic acid sequence of HGFA retrieved only the human sequence and not the sequences of other closely related molecules, for example, factor XII, urokinase, or the HGFA-like hyaluronin-binding protein. All the identified functional motifs in murine HGFA are conserved, with the majority of amino acid substitutions clustering in the amino-terminal domain. This transcript could yield a functional enzyme with the nucleic acid sequence of HGFA retrieved only the human sequence and not the sequences of other closely related molecules, for example, factor XII, urokinase, or the HGFA-like hyaluronin-binding protein. All the identified functional motifs in murine HGFA are conserved, with the majority of amino acid substitutions clustering in the amino-terminal domain.

**TABLE I**

**Effect of serine and cysteine protease inhibitors on HGF hydrolysis by UB conditioned medium and on kidney development in vitro**

Kidney rudiments were dissected on embryonic day 11.5 (E11.5) when the ureteric bud had contacted the metanephric mesenchyme and branched once. Rudiments were grown in serum-free culture in the presence of inhibitors at the concentrations listed below for 4 days. The rudiments were then fixed and stained for glomerular markers (WT1 or peanut lectin) and ureteric bud markers (TROMA-1 or Dolichos bifloris lectin). Two or three rudiments were analyzed for each experiment, ± S.E.

| Inhibitor | HGF hydrolysis | Concentration | Glomeruli | Branches | % control |
|-----------|----------------|---------------|-----------|----------|-----------|
| None      | No inhibition  | 10 μM         | 17 ± 4 (100%) | 25 ± 3.5 (100%) |           |
| Aprotinin | Inhibits       | 10 μM         | 9.5 ± 3.5 (56%) | 13.5 ± 2.5 (54%) |           |
| Leupeptin | Inhibits       | 10 μM         | 9 ± 1.5 (53%)   | 12.5 ± 2.5 (50%) |           |
| E64       | No effect      | 100 μM        | 16.5 ± 2.5 (97%) | ND*      |           |

* ND, not determined.

**Fig. 6.** UB cells produce activated HGF from HGFsc and respond to activated HGF by scattering. A, UB cells were incubated for 16 h in serum-free medium alone (no HGF), 50 ng/ml activated HGF (activated HGF), 50 ng/ml HGFsc (scHGF), 50 ng/ml HGFsc + 100 μM leupeptin, or 50 ng/ml HGFsc + 10 μM leupeptin. The presence of the active heterodimeric form of HGF in the activated preparation of HGF was verified by immunoblotting. Activated HGF and HGFsc both caused scattering of UB cells. Leupeptin at 100 and 10 μM blocked scattering caused by HGFsc, while 1 μM leupeptin was not as effective in blocking scattering. Leupeptin had no effect on the scattering induced by activated HGF. B, the HGF receptor met was immunoprecipitated from UB cells after overnight incubation in serum-free medium. Parallel cultures were left untreated or incubated with fresh medium containing 10% fetal bovine serum and 50 ng/ml HGFsc. HGFsc with or without leupeptin were added to additional cultures to yield final concentrations of 50 ng/ml HGF and 10 μM leupeptin. Met was immunoprecipitated from cell lysates. Immunoblots of precipitated Met were probed successively with anti-phosphotyrosine (IB: Py) and then anti-Met (IB: Met). The molecular mass of the polypeptide was 145 kDa.

**Fig. 7.** Kidney development in organ culture is inhibited by the serine protease inhibitor leupeptin. Kidney rudiments were dissected on embryonic day 11.5 and incubated in serum-free medium with (B, D, and F) or without (A, C, and E) 10 μM leupeptin for 4 days. Renal development was assessed by phase-contrast imaging (A and B), by staining for the markers TROMA-1 (ureteric bud specific cytokeratin, red) and WT1 (induced mesenchyme and intense glomerular staining, green) (C and D), and by staining for WT1 (red) and LTA (L. tetragonolobus lectin, proximal tubule, green) (E and F). The length of the proximal tubules was greatly reduced by leupeptin. We were unable to count the proximal tubules in control samples because it was difficult to identify single tubules reliably due to their convolutions.

**mains. This transcript could yield a functional enzyme with altered matrix binding properties. Mj6003 was isolated from a mouse embryo library, raising the possibility that there is developmentally regulated alternate splicing of the HGFA gene.**

**Immortalized ureteric bud cells expressed HGFA message and protein.** The active form of HGFA is a disulfide-linked
Aktivierter HGF rettet Nierenentwicklung von Inhibition durch Leupeptin. Nierenrudimente wurden auf Embryonentag 12.5 und in Serum-freier Medium (kontrolle) oder in Leupeptin enthaltendem Medium (Leupeptin) inkubiert. Die Ergebnisse zeigten, dass die Leupeptin-kontrollierten Nieren rudimente gleichermaßen wie die Leupeptin-kontrollierten Nieren rudimente in der Entwicklung waren. Die Ergebnisse bestätigten die Wirksamkeit von Leupeptin als Inhibitor von HGFA in vitro.

**Fig. 8.** Aktivierter HGF rettet Nierenentwicklung von Inhibition durch Leupeptin. Nierenrudimente wurden auf Embryonentag 12.5 und in Serum-freier Medium (kontrolle) oder in Leupeptin enthaltendem Medium (Leupeptin) inkubiert. Die Ergebnisse zeigten, dass die Leupeptin-kontrollierten Nieren rudimente gleichermaßen wie die Leupeptin-kontrollierten Nieren rudimente in der Entwicklung waren. Die Ergebnisse bestätigten die Wirksamkeit von Leupeptin als Inhibitor von HGFA in vitro.
adjacent epithelium. We found that HGF was deposited around the ureteric bud, particularly around the ampulla. The activated form of HGF binds strongly to heparin (13, 36), so that activated HGF is unlikely to diffuse far from the site of activation. This observation suggests that the ureteric bud creates a local gradient of HGF activity and Met signaling. In preliminary experiments, we have identified both HGF activator inhibitor-1 and -2 (HAI-1 and HAI-2) in the ureteric bud (37–39).2 Both HAI-1 and HAI-2 exist in membrane-anchored and soluble forms, so that there is additional potential for regulation of HGF and its activator in adjacent structures whose activity and localization are highly regulated. The expression of HGF and its activator in adjacent structures suggest that activation of the HGF/Met signaling pathway is tightly controlled during renal development. As HGF-Met interactions play important developmental roles in a variety of organ systems, it is likely that similar spatio-temporal control elements are broadly expressed throughout development. Our data suggest that activation of the HGF/Met signaling pathway is regulated during embryogenesis by serine protease cascades whose activity and localization are highly regulated. The expression of HGF and its activator in adjacent structures suggest that gradients of HGF activity are formed during development. The creation and shape of such gradients could play an important role in pattern formation in HGF-responsive tissues.

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