**Activation Mechanisms of the Urokinase-type Plasminogen Activator Promoter by Hepatocyte Growth Factor/Scatter Factor**

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Hepatocyte growth factor/scatter factor (HGF/SF) is a pleiotropic effector inducing invasion and metastasis of tumor cells that express the Met tyrosine kinase receptor. One of the effectors of HGF/SF is the urokinase-type plasminogen activator, a serine protease that facilitates tumor progression and metastasis by controlling the synthesis of the extracellular matrix degrading plasmin. Stimulation of NIH 3T3 cells that were stably transfected with the human Met receptor (NIH 3T3-Methuman) with HGF/SF induced a trans-activation of the urokinase promoter and urokinase secretion. Induction of the urokinase promoter by HGF/SF via the Met receptor was blocked by co-expression of a dominant-negative Grb2 and Sos1 expression construct. Further, the expression of the catalytically inactive mutants of Ha-Ras, RhoA, c-Raf, and Erk2 or addition of the Mek1-specific inhibitor PD 098059 abrogated the stimulation of the urokinase promoter by HGF/SF. A sequence residing between −2109 and −1870 base pairs (bp) was critical for stimulation of the urokinase gene by HGF/SF. Mobility shift assays with oligonucleotides spanning an AP-1 site at −1880 bp or a combined PEA3/AP-1 site at −1967 bp showed binding of nuclear factors from NIH 3T3-Methuman cells. Expression of an expression plasmid that inhibits DNA binding of AP-1 proteins (A-Fos) abrogated inducible and basal activation of the urokinase promoter. Nuclear extract from unstimulated NIH 3T3-Methuman cells contained more JunD and showed a stronger JunD supershift with the AP-1 oligonucleotides, compared with HGF/SF-stimulated cells. Consistent with the levels of JunD expression being functionally important for basal expression of the urokinase promoter, we found that overexpression of wild type JunD inhibited the induction of the urokinase promoter by HGF/SF. These data suggest that the induction of urokinase by HGF/SF is regulated by a Grb2/Sos1/Ha-Ras/c-Raf/RhoA/Mek1/Erk2/c-Jun-dependent mitogen-activated protein kinase pathway.

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Hepatocyte growth factor/scatter factor (HGF/SF) is a multipotent growth factor affecting motility, morphogenesis, growth, and angiogenesis. Aberrant expression of HGF/SF is associated with enhanced tumor invasion and metastasis and has been shown to be a strong negative prognostic factor in human breast cancer. The receptor for HGF/SF encoded by the c-Met protooncogene is synthesized as a single polypeptide 170-kDa precursor. After glycosylation and proteolytic cleavage, the resulting dimer has a 50-kDa α-chain exposed on the cell surface. The 145-kDa β-chain spans the plasma membrane and has a kinase domain within the cytoplasmatic region. Upon HGF/SF binding, the Met receptor dimerizes and becomes phosphorylated on tyrosines located in the cytoplasmatic region of the β-chain. These tyrosine residues then act as specific binding sites for adaptor proteins and Src homology 2 domains, which transmit signals intracellularly, one effector being Ras. The exact targets of HGF/SF-Met signaling downstream of Ras have not been elucidated, and it is unclear which signal transduction pathways mediate the diverse biological signals of HGF/SF.

One signal transduction pathway involved in signaling through Ras or small G-proteins of the Rho family is the mitogen-activated protein kinase (MAPK) cascade. The best known pathway, the Ras → Raf → Mek → Erk-MAPK cascade, is typically stimulated by growth factors and mitogens such as HGF/SF, EGF, and fibroblast growth factor. Two other MAPK pathways (p38- and Jnk-MAPK) are stimulated primarily by stress, cytokines, or hormones and require activation of a member of the Rho family, although Ras can participate. Phosphorylation of MAPKs by both tyrosine and threonine residues leads to activation of various transcription factors, including members of the AP-1 family encompassing Jun and Fos family members that can homodimerize and heterodimerize after activation. Fine tuning of the transcriptional response is dependent upon which member of the AP-1 family is activated. Although c-Jun has been shown to have activating properties on gene expression, binding of JunD inhibits promoter activity.

Because HGF/SF has been shown to be involved in tumor cell invasion and metastasis, it has been hypothesized that tumor-associated proteases might be one target of HGF/SF-Met signaling. Indeed, two groups of proteases, matrix metalloprotei-
ases (12) and the serine protease urokinase (13, 14), are up-regulated by HGF/SF in different cell lines. Urokinase (15) converts plasminogen into plasmin, a serine protease with broad substrate specificity toward components of the extracellular matrix including laminin, vitronectin, and fibronectin (16–18). Together, these proteolytic functions facilitate the migration of tumor cells through the extracellular matrix and basement membrane barriers. Membrane attachment of urokinase to the urokinase receptor increases the rate of plasmin formation at the plasma membrane (19) and focuses proteolytic activity at the leading edge of the tumor (20). High urokinase expression is correlated with a poor prognosis of patients suffering from a variety of different types of cancer including that of the breast, ovary, and lung (21, 22).

Studies on the regulation of urokinase expression have shown that the urokinase gene is regulated at the transcriptional level (23–25). The urokinase promoter contains functional binding sites for the transcription factors AP-1, PEA3, and NF-kB (24, 25), which are important for both constitutive and regulated urokinase expression (26). An additional important enhancer sequence of the urokinase promoter is a combined PEA3/AP-1 element (24) homologous to the polyoma virus enhancer A element (27) that has been shown to be a target for transcriptional activation by oncogenes in several promoters (28).

Because the HGF/SF precursor protein shares a very high sequence homology with the kringle and serine protease domains of plasminogen, it was discussed whether urokinase is a putative activator of the precursor molecule HGF/SF precursor protein. Indeed, Naldini et al. (29) showed that urokinase activates HGF/SF precursor protein to the active form, which is a disulfide-linked heterodimer consisting of a 69-kDa α-subunit and a 34-kDa β-subunit. Activation involves the formation of a complex between HGF/SF precursor protein and urokinase binding to the Met receptor (30). Considering that urokinase has been previously shown to be up-regulated by HGF/SF (13, 14), we determined the signal transduction pathway connecting the Met receptor with the transcription factors regulating urokinase gene expression.

MATERIALS AND METHODS

Cell Culture—NIH 3T3 cell lines were obtained from the American Type Culture Collection and stably transfected with the human Met cDNA or the empty vector (pMB1) that contains the long terminal repeat promoter from Moloney murine sarcoma virus and the polyadenylation signal of simian virus 40 by the calcium phosphate method (31). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10 mM HEPES, 272 mM asparagine, 550 mM arginine, penicillin-streptomycin, and 10 mM fetal calf serum (all from Life Technologies, Inc.). Collection of conditioned medium was performed using the same medium without fetal calf serum (serum-free medium). Purified human HGF/SF (kindly provided by R. Schwall, Genentech, South San Francisco, CA) represents the activated, heterodimeric form and was used, if not otherwise specified, at a final concentration of 200 ng/ml.

Vectors—The dominant-negative Erk1 and Erk2 constructs contain the coding region of these MAP kinases in which the conserved codon 71 and 52 of Erk1 and Erk2 involved in phosphate transfer was mutated from lysine to arginine (32), thus impairing catalytic activity. The dominant-negative form of Sos1 (33, 34) lacks the guanine nucleotide exchange domain. Grb2ΔN is an amino-terminal Src homology 3 domain deletion mutant of Grb2 (35). RasN17 is a dominant-negative Ha-Ras construct in which amino acid 17 is substituted by asparagine (36). The dominant-negative RheN19 mutant displays a mutation at amino acid 19 where serine is replaced by asparagine (37). The RafC4 expression plasmid encodes the c-Raf protein lacking the carboxy-terminal kinase domain. RafBXX is an in-frame deletion of amino acids 26–302 of c-Raf rendering the serine-threonine kinase constitutively active (38). The TAM 67 vector encodes a C-Jun protein lacking the trans-activation domain between amino acid 3–122 but retains the leucine zipper and DNA-binding domains (10). The A-Fos construct has an amphipathic acidic extension appended to the amino terminus of the Fos leucine zipper that binds the basic region of Jun, thus preventing binding of the basic region of Jun/Fos to DNA (39). In the Jun/D/EB1 vector, the dimerization domain of wt JunD is replaced by the heterologous homodimerization domain of the Epstein-Barr virus transcription factor EB1, and binds DNA exclusively as a homodimer (40).

Zymography—Conditioned media collected from equal numbers of cells were denatured and electrophoresed in a 10% SDS-PAGE gel containing 0.2% (w/v) casein with or without 5 μg/ml plasminogen. The gel was incubated at room temperature for 2 h in the presence of 2.5% Triton X-100 and subsequently overnight at 37 °C in a buffer containing 10 mM CaCl2, 0.15 mM NaCl and 100 mM Tris-HCl, pH 7.5. The gel was stained for protein with 0.25% Coomassie. Plasminogen-dependent proteolysis was detected as a white zone in a dark field. As a control, the same samples were run in a gel without plasminogen.

Western Blot Analysis—Cells were lysed in buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (buffer A), cleared by centrifugation, and electrophoresed in a 10% SDS-PAGE gel under reducing condition. The resolved proteins were transferred to a nitrocellulose membrane (BA-S85, Schleicher & Schuell). The filter was subjected to a buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.25% gelatin (w/v), and 0.5% Triton X-100 and incubated sequentially with a rabbit polyclonal antibody against the human Met receptor (SC-161, Santa Cruz Biotechnology, Santa Cruz, CA) followed by a horseradish peroxidase-conjugated anti-rabbit IgG. Reactive proteins were visualized by ECL according to the manufacturer (Amer sham Pharmacia Biotech).

MAPK Assays—Cells were stimulated with indicated amounts of HGF/SF for 24 h, lysed in buffer A, and cleared by centrifugation. After normalization for protein, extracts were incubated for 3 h at 4 °C with protein A-agarose and antibody to Erk (C16, Santa Cruz Biotechnology, Santa Cruz, CA). The beads were washed three times in HNTG buffer (20 mM HEPES, 150 mM NaCl, 10% glycerol, 10 mM sodium pyrophosphate) and then incubated in kinase buffer (1 μg/ml myelin basic protein, 50 μM ATP, 1 μC of [γ-32P]ATP) for 15 min at 30 °C. The reaction was terminated by adding 2× reducing sample buffer and heating to 95 °C for 3 min. The beads were removed by centrifugation, and the supernatant was separated in a 15% SDS-PAGE gel. The gel was dried and autoradiographed for 6 h at −80 °C.

Transfections—Cells were transfected by the calcium phosphate method (41, 42) with chloramphenicol acetyltransferase (CAT) reporter constructs fused to the wild type or deleted fragments of the human urokinase promoter (23). To correct for transfection efficiency, all transient transfections were performed in the presence of 4 μg of a β-galactosidase expression vector. A solution containing 24 mM calcium chloride, 15 mM HEPES, pH 7.1, 280 mM NaCl, 1.5 mM Na2HPO4, was added dropwise to plasmid DNA with continuous stirring and added to 60% confluent cells. After 6 h the cells were rinsed twice with phosphate-buffered saline, changed to fresh 10% fetal bovine serum-containing medium, and cultured for an additional 42 h. The cells were harvested and lysed by repeated freeze-thaw cycles in 0.25× Tris-HCl, pH 7.8. Transfection efficiencies were determined by assaying for β-galactosidase activity. After normalization for transfection efficiency, CAT activity was measured by incubating cell lysates at 37 °C with 4 μM [14C]chloramphenicol and 1 mg/ml acetyl coenzyme A. The mixture was separated by extraction with ethyl acetate, and acetylated products were separated on thin layer chromatography plates using chloroform/methanol as the mobile phase. Reactions were visualized by autoradiography, and radioactivity was quantified using a Molecular Dynamics 445 SI PhosphorImager.

Mobility Shift Assays—Cells at 80% confluency were stimulated with 100 ng/ml HGF/SF for 12 h, and nuclear extracts were prepared as described by Digiam et al. (43). NIH 3T3-MetΔ-TAM (7.5 μg) were incubated in a buffer containing 20 mM HEPES, 0.2 mM EDTA, 0.25 mM diithithioreitol, 50 mM NaCl, 10% glycerol, and 1 μg poly(dI-dC). To each reaction 5 fmol of a Klenow end-labeled [α-32P]ATP oligonucleotide were added in the absence or presence of a 100-fold excess of the wild type or mutated competitor sequence, and binding was allowed for 15 min. Subsequently, 1 μg of the indicated antibody was added, and incubation was continued for 1 h at 4 °C. The reaction mixture was electrophoresed in a 5% polyacrylamide gel using 0.5× TBE (89 mM Tris, 89 mM boric acid, 1 mM EDTA) running buffer. The gel was dried and exposed to x-ray film overnight at −80 °C. The sequences are shown in Fig. 1.
RESULTS

Met-HGF/SF Signaling Activates the Urokinase Promoter and Stimulates Urokinase Secretion—To study the regulation of the urokinase-type plasminogen activator by HGF/SF, we used a cell clone (called NIH 3T3-Methum) that is derived from an NIH 3T3 cell line stably transfected with the human Met receptor (31). The NIH 3T3-neo cell clone is stably transfected with the empty vector and was used as a control. NIH 3T3-Methum cells overexpress the human Met receptor as shown by Western blotting with an antibody recognizing human Met (Fig. 2A) that does not interact with the mouse Met receptor. Transient transfection of a CAT reporter driven by the wild type urokinase promoter in NIH 3T3-Met cells shows basal activity (Fig. 2B) compared with NIH 3T3-neo cells, which show almost no basal activity (data not shown). Addition of increasing amounts of human HGF/SF to the NIH 3T3-Methum cells 24 h prior to harvesting induces the urokinase promoter in a dose-dependent manner (Fig. 2, B and C). Human HGF/SF does not activate the endogenous mouse Met receptor. To determine whether NIH 3T3-Methum cells can be stimulated to secrete urokinase, we collected conditioned media from HGF/SF-stimulated NIH 3T3-Methum cells and performed plasminogen zymography. A weak activity (Mr = ~55 kDa), which is absent from NIH 3T3-neo cells, is present in NIH 3T3-Methum cells, which could be stimulated substantially after addition of human HGF/SF (Fig. 2D). Addition of an antibody against urokinase prior to zymography abolished the induction of proteolytic activity by HGF/SF (data not shown). Deletion of plasminogen from the gel abolished the band, which indicates that the proteolytic activity could be ascribed to a plasminogen activator (Fig. 2E). These data are in accordance with previous results showing that HGF/SF-Met signaling up-regulates urokinase (2, 20, 44) and additionally suggest that the increased synthesis of urokinase after stimulation with HGF/SF is more likely a reflection of trans-activation of the urokinase promoter.

The HGF/SF-dependent Stimulation of Urokinase Promoter Activity Is Inhibited by the Co-expression of Plasmids Encoding Dominant-negative Grb2 and Sox1—we were interested in following the signaling pathway involved in activation of the urokinase promoter by HGF/SF from the Met receptor to the nucleus and for this purpose made use of dominant-negative forms of key signal transduction proteins. In general, after activation of receptor tyrosine kinase receptors, adaptor proteins like Grb2 bind to the cytoplasmatic part of the protein and recruit Sox1 to the plasma membrane (35). This places Sox1 in the vicinity of Ras-GDP, leading to the exchange of GDP to GTP and the activation of Ras signaling. NIH 3T3-Met cells were co-transfected with the urokinase promoter and an expression vector encoding a dominant-negative Grb2 (32). Expression of the mutated Grb2 (Grb2C2AN) antagonized the induction of the urokinase promoter by HGF/SF as well as basal promoter activity, whereas the Grb2 (pCGN) vector does not show an effect (Fig. 3). Overexpression of a dominant-negative Sox (33) also reduced inducible and constitutive activation of the urokinase promoter by HGF/SF (Fig. 3). In contrast, a dominant-negative PI 3-kinase (p110Δkin) (45) did not show any effect on basal or inducible urokinase gene regulation (data not shown), and wortmannin, a known inhibitor of PI 3-kinase, showed no inhibition of urokinase promoter activity (see Fig. 5A). Together these data indicate a role for Grb2/Sos1 but not for PI 3-kinase in the regulation of urokinase expression by HGF/SF-Met signaling.

Involvement of Ha-Ras, RhoA, and c-Raf in Regulation of the Urokinase Promoter by Human HGF/SF—To determine whether Ha-Ras is also involved in the induction of urokinase by Met-HGF/SF signaling, NIH 3T3-Methum cells were cotransfected with a dominant-negative Ha-Ras protein, RasN17, and the wild type urokinase promoter. The inducible activation of the urokinase promoter by HGF/SF was completely abrogated by co-expression of the RasN17 construct, whereas the vector control (pSV2neo) had no effect (Fig. 4A).

Because c-Raf binds to activated Ha-Ras (8), we determined the sensitivity of urokinase expression to a dominant-negative c-Raf expression vector ( RafC4) (38). NIH 3T3-Methum cells were transiently transfected with the urokinase promoter-driven CAT reporter and RafC4 or the empty vector control (pBG4C) and stimulated with 200 ng/ml of human HGF/SF. Expression of RafC4 led to a reduction in the ability of HGF/SF to stimulate the urokinase promoter (Fig. 4A). The empty vector control failed to repress the induction of the CAT reporter by HGF/SF. Similarly co-expression of a dominant-negative RhoA (RhoN19) (37) inhibited basal and HGF/SF-mediated activation of the urokinase promoter (Fig. 4B).

RhoA can be part of c-Raf-dependent and -independent signal transduction pathways (46, 47). To determine the localization of RhoA in the c-Raf-Erk signaling cascade, a constitutively activated c-Raf (Raf BXB) (38) and the dominant-negative RhoA was co-transfected with the urokinase promoter in NIH 3T3-Met cells. Expression of the constitutively activated serine-threonine kinase caused an activation of the urokinase promoter that could be abolished by the dominant-negative RhoN19 construct (Fig. 4C). Thus, regulation of urokinase by RhoA is an event downstream of c-Raf.

Repression of the Urokinase Promoter by a MEK1-specific Inhibitor (PD 098059) and by a Dominant-negative Erk2 Expression Construct—Because Mek1 is stimulated by the serine-threonine kinase c-Raf (9), we tested the possibility that the urokinase promoter was regulated through this MAPK activator. A specific inhibitor of Mek1, PD 098059, (48) was added to NIH 3T3-Met cells immediately after transfection with the urokinase promoter, and the cells were stimulated with HGF/SF. The inhibitor abrogated the induction of the urokinase promoter by HGF/SF (Fig. 5A).

We were interested in determining which MAPK is involved in the regulation of the urokinase promoter by HGF/SF and did three sets of experiments in this regard. First, we cotransfected a dual activity phosphatase (CL 100) that inactivates multiple MAPK members (49) and found that it down-regulated basal as well as HGF/SF-induced activation of the urokinase promoter (data not shown). We therefore concluded that member(s) of the MAPK family are involved in regulation of the urokinase promoter. We next tested each MAPK family member individually. NIH 3T3-Met cells were transiently cotransfected with the urokinase promoter-driven CAT reporter and expression vectors encoding either a dominant-negative Erk1 or Erk2 (32). Expression of the dominant-negative Erk2 but not Erk1 (data not shown) caused a repression of HGF/SF-mediated urokinase promoter activation (Fig. 5B). Cotransfection of a dominant-negative Jnk1 expression vector (data not shown) or incubation of the NIH 3T3-Met cells with the highly specific p38 inhibitor SB 203580 (Fig. 5A) did not affect induction of the urokinase promoter by HGF/SF. If Erk2 is involved in the regulation of the urokinase promoter by HGF/SF, then this ligand should be able to stimulate Erk activity in NIH 3T3-Met cells. To test this hypothesis we performed an in vitro kinase assay to...
measure Erk-MAPK activation. After addition of increasing amounts of HGF/SF to NIH 3T3-Met\textsuperscript{bun} cells, we found a sustained activation of Erk activity after the 24-h stimulation period, a time at which EGF had lost its activation potential (Fig. 5C). Stimulation of NIH 3T3-Met\textsuperscript{bun} cells with EGF for 20 min strongly induced Erk-MAPK activity (data not shown). In contrast HGF/SF did not affect Jnk or p38 activity (data not shown).

Induction of AP-1 DNA Binding Activity by HGF/SF—The previous experiments were performed with the urokinase promoter containing 2109 nucleotides upstream of the translation start site. To determine the region of the urokinase promoter required for its stimulation by HGF/SF, NIH 3T3-Met\textsuperscript{bun} cells were transfected with CAT reporters driven by different 5’-flanking regions (–2345, –2109, and –1870 bp) of the urokinase promoter, and the cells were stimulated for 24 h with HGF/SF (Fig. 6A and B). A strong activation was evident with 2109 bp of 5’-flanking sequence, which was not further augmented with longer stretches of the urokinase promoter (–2345 bp). The –1870 bp urokinase CAT construct showed only a low activity and could not be stimulated by HGF/SF. We have previously shown that an AP-1-binding site at –1880 bp (AP-1B) and a combined PEA3/AP-1 site at –1967 (AP-1A) residing in the region between –2109 and –1870 is critical for its stimulation by HGF/SF. NIH 3T3-Met\textsuperscript{bun} and NIH 3T3-Met\textsuperscript{Hum} cells, were grown to 80% confluency in the presence of 10% fetal calf serum and 2 h later stimulated with the indicated amounts of HGF/SF for 24 h. The cells were lysed and assayed for CAT activity (data not shown). Because mobility shift assays employing an oligonucleotide spanning the AP-1A site showed two different retarded complexes (Fig. 6C, asterisk), whereas nuclear extract from HGF/SF-induced NIH 3T3-Met\textsuperscript{bun} cells showed two different retarded complexes (Fig. 6C, arrows), the specificity of the AP-1 binding interaction was indicated by the ability of an excess of unlabeled oligonucleotide to compete for the binding (Fig. 6C). Because the AP-1A site is a combined PEA3/AP-1 site, we were interested in determining which part of this combined site is important for binding of nuclear factors from HGF/SF-induced NIH 3T3-Met\textsuperscript{bun} nuclear extracts (Fig. 6C, lanes 2 and 7). The mobility of the AP-1A containing oligonucleotide was reduced and showed one retarded complex in the presence of NIH 3T3-Met\textsuperscript{bun} nuclear extracts (Fig. 6C, asterisk), whereas nuclear extract from HGF/SF-induced NIH 3T3-Met\textsuperscript{bun} cells showed two different retarded complexes (Fig. 6C, arrows). The specificity of the AP-1 binding interaction was indicated by the ability of an excess of unlabeled oligonucleotide to compete for the binding (Fig. 6C). Because the AP-1A site is a combined PEA3/AP-1 site, we were interested in determining which part of this combined site is important for binding of nuclear factors from HGF/SF-induced NIH 3T3-Met\textsuperscript{bun} cells. The results indicate that nuclear factors from HGF/SF-stimulated and unstimulated NIH 3T3-Met\textsuperscript{bun} cells have specific DNA binding activity toward the AP-1 part but not to the PEA3 part (data not shown). Because mobility shift assays indicate specific binding of NIH 3T3-Met\textsuperscript{bun} nuclear extract to AP-1 sites in the urokinase

Fig. 2. HGF/SF induces the urokinase promoter and urokinase secretion in NIH 3T3-Met\textsuperscript{Hum} cells. A, cell lysates were resolved on a 10% PAGE and human Met (h-Met) detected by Western blot analysis using an antibody to human Met. The band at ~140 kDa represents the single chain precursor form of Met, whereas that at ~140 kDa represents the β-chain of the mature Met heterodimer. The experiment was repeated twice. B, NIH 3T3-Met\textsuperscript{Hum} cells at 70% confluency were transiently transfected with 5 μg of a CAT reporter driven by the wild type urokinase promoter (uPA CAT). After 6 h, the medium was changed, and cells were cultured for 16 h, after which they were stimulated with the indicated amounts of HGF/SF for 24 h. The cells were lysed and assayed for CAT activity (data not shown). C, NIH 3T3-Met\textsuperscript{Hum} and NIH 3T3-Met\textsuperscript{Hum} cells have specific DNA binding activity toward the AP-1 part but not to the PEA3 part (data not shown). The bands at ~140 kDa represents the β-chain of the mature Met heterodimer. The experiment was repeated twice.
Grb2 (Grb2 encoding dominant-negative Grb2 and Sos1. promoter activity is inhibited by the co-expression of plasmids involving Ha-Ras (Fig. 4(11). Because the induction of the urokinase promoter by antagonize transformation by activated Ha-Ras in mouse cells family that has been shown to negatively regulate growth and Down-regulation of JunD—
icity. The data shown represent the average values and standard deviations of four independent experiments.

promoter, we determined the functional role of the AP-1 family of transcription factors in regulating urokinase expression. With this objective, NIH 3T3-Met(b) cells were transiently transfected with an expression vector encoding a dominant-negative protein termed A-Fos that inhibits DNA binding of AP-1 proteins in an equimolar concentration (39). NIH 3T3-Met(b) cells were transiently cotransfected with the urokinase promoter and 2.5 μg of A-Fos (Fig. 6D). Transfection of the expression vector encoding A-Fos reduced HGF/SF-inducible activity of the urokinase promoter. By contrast, co-transfection of the urokinase construct with equivalent amounts of empty vector (pCMV 500) did not reduce urokinase promoter activity. The AP-1 family member c-Jun has been shown to be involved in the mitogenic response of various growth factors, cytokines, and tumor promoters (51), so we questioned whether it is also involved in the induction of the urokinase promoter by HGF/SF. Indeed, cotransfection of a dominant-negative c-Jun mutant (10) in NIH 3T3-Met(b) cells prevented the induction of the promoter by HGF/SF (data not shown), suggesting that c-Jun is one of the transcription factors involved in the activation of the urokinase promoter by HGF/SF. These results suggest that in NIH 3T3-Met(b) cells, basal and HGF/SF-inducible activity of the urokinase promoter requires trans-acting factor(s) that bind to AP-1 sites.

**Induction of the Urokinase Promoter by HGF/SF Involves Down-regulation of JunD—**JunD is a member of the AP-1 family that has been shown to negatively regulate growth and angiogenic transformation by activated Ha-Ras in mouse cells (11). Because the induction of the urokinase promoter by HGF/SF involves Ha-Ras (Fig. 4A), we explored the possibility of whether JunD is affected by HGF/SF in NIH 3T3-Met(b) cells and whether it would affect urokinase gene regulation. To investigate this possibility, we carried out Western blotting with an antibody against JunD employing nuclear extracts from stimulated and unstimulated NIH 3T3-Met(b) cells. After stimulation with HGF/SF, the content of JunD protein in nuclear extract from NIH 3T3-Met(b) cells was lower com-
HGF/SF-mediated Activation of Urokinase Gene Expression

FIG. 5. Abrogation of the HGF/SF-dependent induction of urokinase promoter activity by a MEK1-specific inhibitor (PD 098059) and a dominant-negative Erk2 expression vector. A, NIH 3T3-Methum cells were transiently transfected with the urokinase CAT reporter (uPA-CAT) and stimulated with 200 ng/ml HGF/SF. Concomitantly the inhibitors PD 098059 (20 μM), SB 203580 (20 μM), and wortmannin (100 nM) dissolved in MeSO were added, or an equivalent amount of MeSO without inhibitor was applied. B, NIH 3T3-Methum cells were co-transfected with the urokinase CAT reporter, a β-galactosidase-expressing vector, and 5 μg of an expression vector encoding a dominant-negative Erk2 (mt Erk2). For both A and B, the cell extracts, corrected for differences in transfection efficiency, were assayed for CAT activity. The conversion of [14C]chloramphenicol to acetylated derivatives was determined using a PhosphorImager and expressed as fold induction relative to basal urokinase promoter activity. The data shown represent the average values and standard deviations of three independent experiments. C, effect of HGF/SF and EGF on Erk-MAPK activity. Cells were stimulated with the indicated amounts of HGF/SF and EGF for 24 h, and equal amounts of protein were subjected to immunoprecipitation with antibody to Erk, and in vitro kinase assays were performed using myelin basic protein as the substrate. Transfer of the upper gel part to nitrocellulose and Western blotting with an Erk antibody demonstrated the same level of Erk in all samples (data not shown). The results shown are representative of three experiments.

that this protein is a component of the DNA-binding complex. However, the intensity of the retarded band was greater for unstimulated (Fig. 7B, lanes 4 and 11) than for stimulated (Fig. 7B, lanes 5 and 12) NIH 3T3-Methum cells, and binding of the JunD antibody to nuclear extracts from unstimulated cells blocked DNA-protein complex formation markedly. This is consistent with the higher JunD protein content found in nuclear extracts from unstimulated NIH 3T3-Methum cells (Fig. 7A). Overexpression of a dominant-negative JunD, which lacks amino acids 1–162 corresponding to the trans-activation domain, reduced basal activity of the urokinase promoter in uninduced NIH 3T3-Methum cells (Fig. 7C), indicating that JunD is required for basal urokinase activity in NIH 3T3-Methum cells.

Because mobility shift assays indicated that JunD binding to both AP-1 sites in the urokinase promoter is lower after HGF/SF stimulation, we determined the function of this transcription factor in regulating urokinase expression. We reasoned that up-regulation of JunD prior to stimulation of NIH 3T3-Methum cells with HGF/SF should abrogate the induction of the urokinase promoter by HGF/SF. To this end, NIH 3T3-Methum cells were transfected with the urokinase promoter and an expression vector encoding wt JunD (Fig. 8A) and then stimulated with HGF/SF. Indeed, expression of 2.5 μg of wt JunD reduced the induction of the urokinase promoter by 50% relative to the empty expression vectors. However, the repression of HGF/SF induction of urokinase promoter activity was reduced in a concentration-dependent manner at higher JunD concentrations (10 μg). To exclude the possibility that the observed effect of wt JunD on the urokinase promoter is not exclusively an indirect effect caused by heterodimerization with another member of the AP-1 family of transcription factors, we repeated the experiment with an artificial derivative of JunD, denoted JunD/EB1 (Fig. 8B). In the JunD/EB1 construct the naturally occurring dimerization domain has been replaced by an heterologous homodimerization domain from the Ebstain-Barr virus transcripton factor EB1 and forms only JunD/EB1 homodimers in the cell (40). The inductive effect of HGF/SF on the urokinase promoter could also be repressed with JunD/EB1, confirming that JunD is involved in the regulation of urokinase by HGF/SF. All together these results suggest that the induction of urokinase by HGF/SF can be inhibited by overexpression of JunD.

DISCUSSION

HGF/SF is a pleiotropic growth factor that promotes cell proliferation and survival and stimulates cell motility. Increased invasiveness of cancer cells after HGF/SF stimulation is accompanied by up-regulation of various proteases (2, 52). Although we and others (13, 14, 53) showed that HGF/SF up-regulates one important invasion-associated protease, urokinase, the signal transduction pathways, and transcriptional regulatory mechanisms are still unknown. Using dominant-negative expression vectors of signal transduction proteins, we show here that up-regulation of the urokinase promoter by HGF/SF is mediated via activation of a Grb2/Sos1/ Ha-Ras/c-Raf/RhoA/Mek1/Erk2/c-Jun pathway. Moreover JunD is overexpressed in unstimulated cells and down-regulated after stimulation of the urokinase promoter with HGF/SF.

Much attention has been given to identifying the adaptor proteins associated with the Met receptor following stimulation by HGF/SF. In this context, Grb2 (34), Gab1 (54), PI 3-kinase (55), and Cbl (6) have been identified as being activated by HGF/SF stimulation involving two phosphorylation sites, Tyr1349 and Tyr1356, located at the carboxyl terminus of Met. Rahimi et al. (55) used dominant-negative constructs of p85...
and wortmannin to show that PI 3-kinase mediates the proliferative effect of HGF/SF. These authors show that the inhibitory effect of PI 3-kinase on proliferation is mediated by S6 kinase and does not involve c-Jun expression. In our experiments we did not observe any involvement of PI 3-kinase in regulation of the urokinase promoter. Neither the PI 3-kinase inhibitor wortmannin nor a dominant-negative PI 3-kinase (45) inhibited the induction of the promoter by HGF/SF, whereas Grb2/Sos1 and c-Jun were important for the basal and inducible activity of the urokinase promoter. Therefore binding of different adaptor proteins mediate distinct biological activities of HGF/SF. Binding of Grb2/Sos1 to the Met receptor mediates effects like branching morphogenesis (52), transformation (6), and as shown here, urokinase regulation, whereas binding of PI 3-kinase induces S6 kinase and proliferation (55).

Our previous work (42) showed that Ha-Ras regulates the urokinase promoter, but at that time we did not look at Rho family members, because their role in transformation was not recognized then (56). The results presented here show that Ha-Ras and RhoA, both members of a superfamily whose activities are controlled by GDP/GTP cycling, are necessary for the induction of the urokinase promoter by HGF/SF. RhoA, a Rho family member, is involved in control of cell shape but also cooperates with Ras and Raf in oncogenic cell transformation and anchorage-independent growth (37, 47). The general role of RhoA in regulation of HGF/SF responses is not clear because inhibition of RhoA does not prevent HGF/SF-induced membrane ruffling and cell spreading (57). Our results indicate a role for Rho in HGF/SF signaling by affecting the regulation of urokinase. The effect of RhoA on urokinase is not general to all transforming Rho family members, because a dominant-negative Rac1 mutant had no effect on urokinase promoter activity (data not shown). Although we showed involvement of RhoA in the regulatory response by HGF/SF-Met receptor signaling, this effect might not be restricted to HGF/SF alone. RhoA might regulate urokinase expression in general, because a constitutive active RhoA mutant transforms NIH 3T3 cells to grow and invade the surrounding tissue in nude mice (47). This would suggest that the invasive phenotype of RhoA transformed cells is mediated at least in part through up-regulation of urokinase.

The observation that dominant-negative mutants of Ha-Ras, RhoA, and c-Raf inhibit inducible and basal activation of the urokinase promoter by HGF/SF suggests a role for RhoA in the Erk-MAPK pathway. Involvement of RhoA in the Erk-MAPK pathway is in agreement with the inhibitory effect of C3 transferase, a RhoA inhibitor, on Erk2 activation (58). Although RhoA can activate the Jnk-MAPK pathway (59), in the case of HGF/SF-induced urokinase regulation we did not observe any effect of a dominant-negative Jnk expression plasmid, nor could HGF induce Jnk activity in NIH 3T3-Methum cells. This argues against an involvement of Jnk downstream of RhoA for

Fig. 6. Induction of AP-1 DNA binding activity by HGF/SF. A, NIH 3T3-Met\textsuperscript{tum} cells were transiently transfected with 5 \mu g of a CAT reporter driven by the indicated 5'-deleted fragment of the urokinase promoter and a vector encoding the \beta-galactosidase gene, and the cells were stimulated with 200 ng/ml HGF/SF, lysed, and assayed for CAT activity. B, the conversion of \textsuperscript{14}C chloramphenicol to acetylated derivatives was determined using a PhosphorImager and expressed as fold induction relative to the \textsuperscript{2109} bp urokinase promoter. The data shown represent the average values and standard deviations. C, nuclear extracts (n.e.) from NIH 3T3-Met\textsuperscript{tum} cells treated with or without 100 ng/ml of HGF/SF for 12 h were incubated with 2 \times 10^9 cpm of a Klenow \alpha-\textsuperscript{32}P-end-labeled oligonucleotide spanning the AP-1A site (combined PEA3/AP-1 site at –1967) in the absence or presence of a 100-fold excess of the indicated competitors, and electrophoresed in a 5% polyacrylamide gel. The AP-1A mt competitor oligonucleotide has point mutations in the AP-1 part, whereas the Ets mt competitor has point mutations in the PEA3 part of the combined PEA3/AP-1 site, respectively (for sequences see Fig. 1). Arrows indicate retarded complex of HGF/SF-stimulated NIH 3T3-Met\textsuperscript{tum} cells, and the asterisk indicates unstimulated cells. D, NIH 3T3-Met\textsuperscript{tum} cells were transiently transfected with the urokinase CAT reporter (\textit{uPA-CAT}) and stimulated with 200 ng/ml HGF/SF, and the cells were lysed and assayed for CAT activity. The data shown represent the average values and standard deviations. For A–D the results shown are representative of three or more separate experiments.
urokinase regulation. Additionally we identified Erk2 as the MAPK involved in up-regulation of urokinase by HGF/SF. RhoA is located downstream of c-Raf in the signal transduction pathway from the Met receptor to the urokinase promoter. This is consistent with previous reports (37, 59) showing that trans-formation by c-Raf or Ha-Ras can be inhibited by co-expression of a dominant-negative RhoA (RhoN19), suggesting involvement of RhoA somewhere between c-Raf and Erk.

The NIH 3T3 cell clone used in this study, NIH 3T3-Methum, is stably transfected with the Met receptor and has a higher basal urokinase promoter activity, compared with the vector transfected NIH 3T3-neo cell clone, which has no detectable promoter activity. Therefore, even without ligand stimulation, overexpression of human Met activates the urokinase promoter and the MAPK signaling pathway. Overexpression of dominant-negative components of the MAPK pathway in NIH 3T3-Methum repressed constitutive urokinase promoter activity, which indicates that the same signaling mechanism is used as in HGF/SF-Met-induced urokinase expression.

On the basis of the results reported in this paper, we propose a model (Fig. 9) addressing different signaling molecules important in the regulation of the urokinase promoter by HGF/SF-Met signaling, providing insights regarding the mechanisms by which a protein-tyrosine kinase regulates a certain cellular gene. Activation of the Met receptor by HGF/SF leads to recruitment of Grb2 and Sos1 to the receptor, thereby activating Ha-Ras and c-Raf. The signal is transduced to the nucleus through a c-Raf/Mek1/Erk2-dependent cascade and activates AP-1 family members. PI 3-kinase, Rac, Jnk, p38, S6-kinase, and ATF-2 are not involved in this response. Despite the apparent linear nature of the Ras → Raf → Mek → Erk MAPK signaling cascade, there is evidence that it represents a subset of a complex array of signaling interactions at several levels (46). This might also apply for the regulation of the urokinase regulation. Additionally we identified Erk2 as the MAPK involved in up-regulation of urokinase by HGF/SF. RhoA is located downstream of c-Raf in the signal transduction pathway from the Met receptor to the urokinase promoter. This is consistent with previous reports (37, 59) showing that transformation by c-Raf or Ha-Ras can be inhibited by co-expression of a dominant-negative RhoA (RhoN19), suggesting involvement of RhoA somewhere between c-Raf and Erk.

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**Fig. 7.** Stimulation of NIH 3T3-Methum cells with HGF/SF reduces nuclear JunD content. A, nuclear extracts (n.e.) from NIH 3T3-Methum cells treated with or without 100 ng/ml of HGF/SF were resolved on a 10% SDS-PAGE and analyzed by Western blot analysis using an antibody to JunD in the presence (+) or the absence (−) of the immunizing peptide. The product at ~39 kDa represents JunD. B, nuclear extracts were prepared from the NIH 3T3-Methum cell clone treated with or without 100 ng/ml of HGF/SF and incubated with Klenow a-32P-end-labeled oligonucleotides spanning the AP-1B (lanes 2–7) or the AP-1A (lanes 9–14) sites of the urokinase promoter. After 15 min, antibody to JunD or IgG as control (1 μg) was added, and the reaction mixture was subsequently subjected to gel electrophoresis. The data are representative of three experiments. C, NIH 3T3-Methum cells were co-transfected with a CAT reporter driven by the wild type urokinase promoter and 3 μg of an expression vector encoding a trans-activation domain lacking JunD (RVPD ΔJunD) or the empty expression vector (pLMVP) and stimulated with 200 ng/ml HGF/SF, and the cells lysed and assayed for CAT activity. The data shown represent the average values and standard deviations of three independent experiments.

**Fig. 8.** Induction of the urokinase promoter by HGF/SF involves down-regulation of JunD. A, NIH 3T3-Methum cells were transiently transfected using 5 μg of a CAT reporter driven by the wild type urokinase promoter (uPA-CAT) and varying amounts of wt JunD cDNA (vector pLMVP) or 5 μg of the expression vector JunD/EB1 (B), an artificial JunD construct that can form only JunD homodimers, and stimulated with HGF/SF. For A and B, the cell extracts, corrected for differences in transfection efficiency, were assayed for CAT activity. [14C]Chloramphenicol conversions were determined with a PhosphorImager and expressed as fold induction relative to basal urokinase promoter activity. The data shown represent the average values and standard deviations of three or more separate experiments.
urokinase promoter. Each component of the signaling cascade identified here as being involved in the regulation of the urokinase promoter by HGF/SF may be activated by other than the identified upstream components.

The transcriptional requirements for urokinase promoter stimulation by HGF/SF, 12-O-tetradecanoylphorbol-13-acetate, or Ha-Ras are similar (42, 60). Mutation of both AP-1 sites at –1967 and –1880 bp in the urokinase promoter substantially impaired the activity of HGF/SF as well as 12-O-tetradecanoylphorbol-13-acetate (60) to stimulate the promoter. The AP-1 site at –1967 bp is a combined PEA3/AP-1 site, and a remarkable difference between 12-O-tetradecanoylphorbol-13-acetate or Ha-Ras (42) and HGF/SF induction of the urokinase promoter is that the PEA3 part of this site is not required for HGF/SF induction. Addition of HGF/SF to the NIH 3T3-Met′′ cell clone shows a change from one complex (containing JunD) that had bound to the AP-1 sites to two complexes. Although we did not identify every single protein in these complexes, we are certain that they involve AP-1 family members. Transient overexpression of A-Fos, a hybrid protein that inhibits DNA binding of all endogenous AP-1 family members (39), completely down-regulated inducible urokinase expression, and a dominant-negative c-Jun construct inhibited the induction by 70%. Thus the induction of the urokinase promoter is mediated at least partially by c-Jun.

Several of our observations are consistent with a role for JunD in the constitutive activity of the urokinase promoter. Unstimulated NIH 3T3-Met′′ cells have a higher JunD content than HGF/SF-stimulated cells, and the amount of JunD bound to oligonucleotides spanning the AP-1A and AP-1B sites of the urokinase promoter was higher for untreated cells. The high JunD expression of untreated NIH 3T3-Met′′ cells accounts for part of the constitutive activity of the urokinase promoter, because a dominant-negative JunD reduced the basal activity of the urokinase promoter by 60%. When stimulated with HGF/SF, the JunD content of the cell decreases, and less JunD is bound to the urokinase promoter so that other AP-1 family members, including c-Jun can mediate the activation of the urokinase promoter by HGF/SF. Similarly, Pfarr et al. (11) showed a high JunD level in resting fibroblasts and a degradation of JunD following serum stimulation, whereas in parallel the level of c-Jun increased. Overexpression of JunD partially reversed Ha-Ras transformation and growth in soft agar. Together with other studies (11, 40), this lead to the assumption that JunD has an inhibitory effect on cell growth and anti-mitogenic effects. To test this idea further, we overexpressed wt JunD in NIH 3T3-Met′′ cells and showed that the induction of the urokinase promoter by HGF/SF could be reduced by 50%. This indicates that JunD can inhibit growth factor-induced activation of a tumor-associated protease. The inhibition of HGF/SF-mediated urokinase activation by JunD could result from antagonizing the c-Jun function, by forming JunD/c-Jun heterodimers. Interestingly, higher amounts of wt JunD caused a decrease in the repression of HGF/SF induction of urokinase promoter activity. It is thus possible that the ratio of JunD to c-Jun is an important feature for the regulation of urokinase by HGF/SF. Alternatively, high level expression of JunD could result in titration of corepressors for JunD from the urokinase promoter and impair transcriptional regulation.

In conclusion, using an NIH 3T3 cell line that has been stably transfected with the Met receptor, we have demonstrated that HGF/SF induces the urokinase promoter by a Grb2/Sos1/Ha-Ras-c-Raf/RhoA/Mek1/Erk2/c-Jun-mediated signal transduction pathway. Considering the strong evidence implicating urokinase (20, 21, 61) and HGF/SF-Met signaling (2, 3) in tumor cell invasion and metastasis, these findings raise the possibility that interfering with this signaling cascade may reduce HGF/SF-induced urokinase synthesis and extracellular matrix degradation in cancer cells.

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![FIG. 9. Proposed model of urokinase promoter activation by HGF/SF.](image-url)
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