Tissue-type plasminogen activator (tPA), a serine protease well known for generating plasmin, has been demonstrated to induce matrix metalloproteinase-9 (MMP-9) gene expression and protein secretion in renal interstitial fibroblasts. However, exactly how tPA transduces its signal into the nucleus to control gene expression is unknown. This study investigated the mechanism by which tPA induces MMP-9 gene expression. Both wild-type and non-enzymatic mutant tPA were found to induce MMP-9 expression in rat kidney interstitial fibroblasts (NRK-49F), indicating that the actions of tPA are independent of its proteolytic activity. tPA bound to the low density lipoprotein receptor-related protein-1 (LRP-1) in NRK-49F cells, and this binding was competitively abrogated by the LRP-1 antagonist, the receptor-associated protein. In mouse embryonic fibroblasts (PEA-13) lacking LRP-1, tPA failed to induce MMP-9 expression. Furthermore, tPA induced rapid tyrosine phosphorylation on the β subunit of LRP-1, which was followed by the activation of Mek1 and its downstream Erk-1 and -2. Blockade of Erk-1/2 activation by the Mek1 inhibitor abolished MMP-9 induction by tPA in NRK-49F cells. Conversely, overexpression of constitutively activated Mek1 induced Erk-1/2 phosphorylation and MMP-9 expression. In mouse obstructed kidney, tPA, LRP-1, and MMP-9 were concomitantly induced in the renal interstitium. Collectively, these results suggest that besides its classical proteolytic activity, tPA acts as a cytokine that binds to the cell membrane receptor LRP-1, induces its tyrosine phosphorylation, and triggers intracellular signal transduction, thereby inducing specific gene expression in renal interstitial fibroblasts.

Tissue-type plasminogen activator (tPA) is a serine protease that plays a pivotal role in blood coagulation and fibrinolysis. In the kidney, the main function of tPA is to convert plasminogen into the biologically active plasmin, which in turn participates in regulating the catabolism of extracellular matrix and tissue fibrosis. In light of its proteolytic potential, tPA has generally been considered to be beneficial in the pathogenesis of fibrotic lesions, leading to increased matrix degradation and decreased matrix accumulation (2). However, previous studies from our laboratory demonstrate that genetic ablation of tPA actually protects the kidney from development of interstitial fibrosis in obstructive nephropathy (3), suggesting that increased tPA is detrimental in certain pathological situations. The deleterious effect of tPA is attributed to its ability to induce matrix metalloproteinase-9 (MMP-9) production, which leads to the destruction of the tubular basement membrane and subsequent generation of the matrix-producing myofibroblasts via tubular epithelial to mesenchymal transition (4, 5). In vitro, tPA directly induces MMP-9 gene expression and protein secretion in renal interstitial fibroblast cells (3). However, how tPA, a well-known serine protease, transduces its signal into the nucleus to control specific gene expression is poorly understood.

In vivo evidence suggests that MMP-9 induction by tPA is likely operated through a plasmin-independent mechanism because plasmin activity is not altered in tPA−/− kidneys (3). These observations led us to hypothesize that tPA may function as a cytokine that binds to a cell membrane receptor, triggering a cascade of intracellular signal transduction that ultimately leads to the transactivation of specific genes in the nucleus. This speculation is consistent with growing evidence that the function of tPA can go beyond its proteolytic activity in a variety of other physiological and pathological settings (6, 7). For instance, tPA has been shown to increase endothelial cell proliferation (8), induce opening of the blood-brain barrier (9), regulate seizure spreading (10), modulate neuron apoptosis (11, 12), and amplify neurotoxicity after hemoglobin exposure (13). This wide spectrum of biological actions suggests that tPA has the ability to activate cellular signaling that can lead to the modulation of specific gene expression. In support of this notion, the studies herein have revealed that tPA is capable of inducing the tyrosine phosphorylation of the low density lipoprotein receptor-related protein-1 (LRP-1), a multifunctional receptor present in renal interstitial fibroblasts.

LRP-1 is a member of the low density lipoprotein receptor family that is implicated in lipoprotein metabolism and in the homeostasis of proteases and protease inhibitors (14). After being synthesized as a single chain molecule, LRP-1 is subsequently processed by furin into an external 515-kDa α subunit and an 85-kDa β subunit that contains a transmembrane segment and cytoplasmic tail with two NPYX motifs and numerous tyrosine residues (14–16). The α and β subunits are noncovalently associated with each other on the cell surface. LRP-1 is present in multiple cells and recognizes more than 30 structurally distinct ligands with high affinity, including lipoproteins, proteases, proteinase-inhibitor complexes, matrix proteins, and growth factors. Although...
generally considered a scavenger receptor, increasing evidence indicates that LRP-1 has the potential to mediate cellular signaling (14, 16).

In this study, we demonstrated that tPA binds to the cell membrane receptor LRP-1, induces its tyrosine phosphorylation, triggers phosphorylation of the intracellular signal mediators Mek1 and Erk-1/2, and finally results in MMP-9 gene up-regulation in renal interstitial fibroblasts. Furthermore, ablation of LRP-1 or inhibition of Erk-1/2 activation completely abolishes MMP-9 induction by tPA, whereas constitutive activation of Erk-1/2 imitates tPA and induces MMP-9 expression. These findings establish a novel mode of action elicited by tPA, in which it functions as a cytokine that triggers intracellular signal transduction and controls specific gene expression.

**MATERIALS AND METHODS**

**Cell Culture and Treatments**—Normal rat renal interstitial fibroblasts (NRK-49F), mouse homozygous LRP-deficient embryo fibroblasts (PEA-13), and their wild-type counterpart (MEF-1) were purchased from the ATCC (Manassas, VA). NRK-49F cells were cultured in Dulbecco’s modified Eagle’s medium/Ham’s F12 (1:1) supplemented with 10% fetal bovine serum (Invitrogen), whereas PEA-13 and MEF-1 cells were incubated in Dulbecco’s modified Eagle’s medium containing 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1.0 mM sodium pyruvate, and 10% fetal bovine serum. After growth to 70% confluence, cells were changed to serum-free medium for 24 h. Human single chain tPA was reconstituted according to the manufacturer’s instructions (Sigma) and added to the culture medium at different concentrations for various periods of time as indicated. For control experiments, cells were treated with vehicle. Human recombinant tPA was obtained from American Diagnostica Inc. (Greenwich, CT), whereas mouse recombinant wild-type and non-enzymatic mutant tPA were purchased from Molecular Innovations Inc. (Southfield, MI). Of note, tPA from three different sources (Sigma, American Diagnostica, and Molecular Innovations) was able to induce MMP-9 expression, regardless of whether they were natural or recombinant proteins or originated from human or mouse. In some experiments, cells were pretreated for 30 min with various chemical inhibitors at the concentration specified, followed by subsequent incubation with tPA. The serine protease inhibitor aprotinin and Mek1 inhibitor PD98059 were obtained from Calbiochem-Novabiochem Corporation.

**RT-PCR**—Cells were incubated with or without tPA for 24 h at specified concentrations. Total RNA was extracted using TRIzol reagent, according to the manufacturer’s instructions (Invitrogen). Two μg of total RNA was reverse-transcribed into cDNA in a 20-μl reaction buffer using AMV reverse transcriptase (Promega) with random primers at 42 °C for 30 min. MMP-9 mRNA expression in rat NRK-49F cells was determined by RT-PCR according to the protocols described previously (3). For detecting MMP-9 expression in mouse MEF-1 and PEA-13 cells, the primers were as follows: 5'-CCATGAGTCCCTGGCAG (sense) and 5'-AGATGTGTGTTATGATG (antisense) (17), and the PCR protocol consisted of 45 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR products were size fractionated on a 1.0% agarose gel and detected by ethidium bromide staining. The relative abundance of mRNA was determined by the intensity ratio of MMP-9 to β-actin.

**Gelatin Zymography**—Gelatin zymographic analysis of MMP-9 activity was performed according to the method described previously (3, 18). Briefly, after cells were treated with tPA, supernatants of the culture were collected and centrifuged to remove the cellular debris. The protein concentration was determined by the bichenchoninic acid protein assay kit (Sigma). A constant amount of protein (15 μg) was loaded onto a 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin (Bio-Rad Laboratories Inc.). After electrophoresis, the gel was incubated in developing buffer and then stained with a solution of 30% methanol, 10% glacial acetic acid, and 0.5% Coomassie Blue G250, followed by destaining in the same solution without dye. The clear bands on a blue background indicate the proteolytic activity of the MMPs.

**Western Blot Analysis**—Western blot analysis for specific protein expression was performed essentially according to an established procedure (19). The primary antibodies used were as follows: rabbit anti-MMP-9 (Sigma); antibodies against phosphospecific and total Erk-1/2, as well as phosphospecific Mek1, phosphospecific and total Akt, and phosphospecific and total p38 MAPK (Cell Signaling Technology, Beverly, MA); mouse anti-Mek1 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse anti-human tPA monoclonal antibody (Oxford Biomedical Research Inc., Oxford, MI); anti-phosphotyrosine antibody PY20 (BD biosciences, San Diego, CA); mouse monoclonal anti-LRP (clone 11H4) (ATCC, Manassas, VA); mouse anti-actin (Chemicon, Temecula, CA). The secondary horseradish peroxidase-conjugated antibodies were obtained from Sigma and Chemicon, respectively.

**Co-immunoprecipitation**—The binding of tPA to LRP-1 was assessed according to a modified co-immunoprecipitation method described elsewhere (20). NRK-49F cells were incubated at 4 °C with or without 10−7 M recombinant human single-chain tPA, in the presence or absence of RAP (3.5 × 10−7 M) for 1 h. The cells were lysed in CHAPS buffer (10 mM CHAPS, 20 mM HEPES, pH7.4, 150 mM NaCl, 2 mM CaCl2), and the resultant lysates were incubated with the monoclonal antibody against LRP-1 at 4 °C overnight. Protein A/G PLUS-agarose beads (Santa Cruz) were added and incubated at 4 °C for 3 h. The beads were then washed with CHAPS buffer, and the proteins were extracted using a reducing sampling buffer, separated on 10% SDS-polyacrylamide gel, and analyzed by Western blot analyses using anti-tPA or anti-LRP-1 antibodies, respectively. For negative control, identical co-immunoprecipitation experiments were carried out with an irrelevant anti-glyceraldehyde-3-phosphate dehydrogenase antibody in place of anti-LRP-1. For the detection of tyrosine phosphorylation on the LRP-1 β subunit, NRK-49F cells were treated with 10−8 M tPA for various periods of time, and the proteins from the immunoprecipitated complexes were analyzed using anti-phosphotyrosine and anti-LRP-1 antibodies, respectively.

**Adenovirus Infection**—NRK-49F cells were seeded to 90% confluence in complete medium on 6-well culture plates. After an overnight incubation, the cultures were washed twice and changed to serum-free medium. The recombinant adenovirus harboring constitutively active Mek1 (Ad-Mek1-CA) was described previously (21). The adenovirus containing β-galactosidase (Ad LacZ) was provided by Dr. X. Wang of the University of Pittsburgh (Pittsburgh, PA). The adenovirus was added to the cultures at a concentration of 2 or 4 × 109 particles/ml. Cells were incubated for 24 and 48 h, and then supernatants and cell lysates were collected for gelatin zymographic assay and Western blot analysis, respectively.

**Animal Model**—Male CD-1 mice weighing 20–22 g were obtained from Harlan Sprague-Dawley Inc. (Indianapolis, IN). Unilateral ureteral obstruction was performed using established procedures as described elsewhere (3). Mice subjected to sham operation served as controls. At day 7 after the surgery, mice were sacrificed, and kidneys were removed and then snap frozen in Tissue-Tek OCT compound (Sakura Finetek Inc., Torrance, CA) for cryosection.

**Indirect Immunofluorescence Staining**—Kidney cryosections were prepared at 5-μm thickness and fixed in phosphate-buffered saline containing 3% paraformaldehyde and 0.2% Triton X-100 for 10 min. After
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FIGURE 1. tPA induces MMP-9 gene expression and protein secretion by a mechanism independent of its proteolytic activity. A, RT-PCR analysis demonstrated that tPA induced MMP-9 mRNA expression. Rat renal interstitial fibroblast (NRK-49F) cells were treated with human tPA for 24 h. Western blot analyses showed that tPA induced MMP-9 secretion. Cells were incubated with tPA for 48 h. Supernatants of the cultures were collected for Western blot analyses, and whole cell lysates were immunoblotted with actin antibody for the normalization of cell numbers. B, time-dependent MMP-9 induction by tPA. NRK-49F cells were incubated with 10^{-8} M tPA for various periods of time as indicated. Supernatants were immunoblotted with MMP-9 antibody, whereas cell lysates were probed for actin. D, protease inhibitor aprotinin (50 units/ml) did not affect MMP-9 induction by tPA. Zymography (top) and Western blot (bottom) were performed. E, graphical presentation of the relative abundance of MMP-9 (fold induction over the controls) following various treatments as indicated. Data are presented as mean ± S.E. of three independent experiments. *, p < 0.01 versus the controls. F–I, both mouse recombinant wild-type (F and G) and non-enzymatic mutant tPA (H and I) induced MMP-9 mRNA expression. A representative picture (F and H) and graphical presentation (G and I) are shown. *, p < 0.01 versus the controls (n = 4).

blocking with 10% normal donkey serum in phosphate-buffered saline for 30 min, the sections were incubated overnight at 4°C with rabbit polyclonal anti-LRP 2629 antibody (kindly provided by Dr. Dudley Strickland of the University of Maryland, Rockville, MD), anti-tPA, and anti-MMP-9, respectively, followed by incubating for 1 h with cyanine Cy3-conjugated secondary antibodies (Jackson Immunoresearch Laboratories Inc., West Grove, PA). Slides were then stained with the proximal tubular marker, fluorescein-conjugated lectin from *Tetragonolobus purpureas* (Sigma), to localize the proximal tubules. Non-immune IgG served as a negative control, and no staining was observed. Slides were mounted with Vectashield anti-fade mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories Inc., Burlingame, CA) and viewed using an Eclipse E600 epifluorescence microscope equipped with a digital camera (Nikon Inc., Melville, NY).

Statistical Analysis—All data examined were expressed as mean ± S.E. Statistical analysis of the data were performed using SigmaStat software (Jandel Scientific Software, San Rafael, CA). Comparison between groups was made using one-way analysis of variance followed by the Student's *t* test. A *p* value of less than 0.05 was considered statistically significant.

RESULTS

tPA Induces MMP-9 Gene Expression by a Mechanism Independent of Its Proteolytic Activity—Previous studies from our laboratory have shown that ablation of tPA results in diminished intrarenal MMP-9 induction in obstructive nephropathy in vivo (3). Because renal plasmin activity is not altered in tPA−/− mice, this raised the possibility that tPA may modulate MMP-9 gene expression through a mechanism independent of plasmin generation (3). To validate this speculation, we investigated the effect of the plasmin inhibitor on tPA-mediated MMP-9 induction, using normal rat kidney interstitial fibroblast (NRK-49F) cells as a model system. As shown in Fig. 1, incubation of NRK-49F cells with tPA induced MMP-9 mRNA expression and protein secretion in a dose-dependent manner, as previously described (3). However, in the presence of aprotinin, a potent plasmin inhibitor, tPA was still able to induce MMP-9 expression in NRK-49F cells (Fig. 1, D and E), indicating that, similar to the *in vivo* situation, tPA action is irrelevant to plasminogen activation.

To further determine whether tPA action is dependent on its proteolytic activity, we examined MMP-9 induction in NRK-49F cells using non-enzymatic mutant tPA. The non-enzymatic tPA has the serine residue within the active site of the enzyme mutated to alanine, which renders it catalytically inactive (22), without altering its binding properties (23). As shown in Fig. 1 (H and I), non-enzymatic mutant tPA was also able to induce MMP-9 mRNA expression in a dose-dependent manner, similar to its wild-type counterpart (Fig. 1F). Thus it appears clear that tPA exerts its gene regulatory activity by a mechanism independent of its proteolytic activity in renal fibroblasts.

tPA Specifically Binds to Membrane Receptor LRP-1 in Renal Interstitial Fibroblasts—The fact that tPA may directly induce MMP-9 gene expression prompted us to hypothesize that tPA transduces its signal to the nucleus through a cell membrane receptor and subsequent intracellular signaling mediators, the mode of action utilized by all cytokines. We reasoned that if that is the case, we might be able to detect the cellular proteins that undergo tyrosine phosphorylation in response to tPA stimulation. To this end, NRK-49F cells were incubated with 10^{-8} M tPA for various periods of time as indicated. Supernatants were immunoblotted with MMP-9 antibody, whereas cell lysates were probed for actin. D, protease inhibitor aprotinin (50 units/ml) did not affect MMP-9 induction by tPA. Zymography (top) and Western blot (bottom) were performed. E, graphical presentation of the relative abundance of MMP-9 (fold induction over the controls) following various treatments as indicated. Data are presented as mean ± S.E. of three independent experiments. *, *p* < 0.01 versus the controls. F–I, both mouse recombinant wild-type (F and G) and non-enzymatic mutant tPA (H and I) induced MMP-9 mRNA expression. A representative picture (F and H) and graphical presentation (G and I) are shown. *, *p* < 0.01 versus the controls (n = 4).
m tPA, and the cell lysates were immunoblotted with specific anti-phosphotyrosine antibody. As shown in Fig. 2A, tPA markedly induced the tyrosine phosphorylation of an 85-kDa protein in renal interstitial fibroblasts.

To unveil the identity of this 85-kDa protein, we surveyed possible candidates that could at least meet two criteria: 1) the protein or its subunit is 85 kDa in size; 2) the protein has an intracellular domain in which tyrosine residues reside that can be phosphorylated. This selection process allowed us to predict that the 85-kDa protein might be the β subunit of LRP-1, the prototype of the low density lipoprotein receptor-related proteins. To provide evidence that LRP-1 functions as a tPA cell membrane receptor that transduces its signal, we examined the potential binding of tPA to LRP-1 in renal fibroblasts using a co-immunoprecipitation approach. As presented Fig. 2B, tPA was clearly detectable in the anti-LRP-1 antibody-precipitated complexes, suggesting the physical binding of tPA to LRP-1 in renal fibroblasts. Under the identical conditions, no tPA was detected in the complexes precipitated by an irrelevant anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Fig. 2D). Furthermore, binding of tPA to LRP-1 was competitively abrogated by the receptor-associated protein, a molecule that binds to LRP-1 and blocks its interactions with all known ligands.

Ablation of LRP-1 Abolished tPA-mediated MMP-9 Induction—To unravel the functionality of LRP-1 in tPA-mediated MMP-9 induction, we next assessed the effect of LRP-1 ablation on MMP-9 induction by tPA in fibroblasts. For this purpose, we utilized mouse homozygous LRP-1-deficient embryonic fibroblasts PEA-13 and their wild-type counterpart MEF-1. As shown in Fig. 3A, Western blot analysis confirmed the absence of LRP-1 in PEA-13 cells, whereas abundant LRP-1 protein was detectable in the wild-type MEF-1 cells and NRK-49F fibroblasts. RT-PCR analysis demonstrated that deficiency of LRP-1 abolished MMP-9 mRNA induction by tPA in fibroblasts. MEF-1 and PEA-13 fibroblasts were incubated without or with 10^{-7} m tPA for 24 h. C, graphic presentation of the relative abundance of MMP-9 mRNA among different groups. Data are presented as mean ± S.E. of six independent experiments. *, p < 0.01 versus the control group in MEF-1 cells.

FIGURE 2. tPA induces cellular protein tyrosine phosphorylation and specifically binds to the LRP-1 in NRK-49F cells. A, Western blot analyses demonstrated that tPA induced tyrosine phosphorylation of an 85-kDa protein in renal interstitial fibroblasts. NRK-49F cells were incubated with 10^{-8} m tPA for 2 min, and whole cell lysates were immunoblotted with anti-phosphotyrosine antibody. An 85-kDa protein was phosphorylated upon tPA stimulation. B and C, co-immunoprecipitation assay revealed the specific binding of tPA to the cell membrane protein, LRP-1. NRK-49F cells were treated with 10^{-7} m tPA in the presence or absence of the receptor-associated protein (RAP) (3.5 × 10^{-7} M) at 4 °C for 1 h and then lysed in CHAPS buffer. The cell lysates were immunoprecipitated with LRP-1 antibody, followed by immunoblotting with either tPA antibody (B) or LRP-1 antibody (C). No tPA was detected in the complexes precipitated by an irrelevant anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (D and E). Identical immunoprecipitation experiments were carried out by using anti-LRP-1 and anti-glyceraldehyde-3-phosphate dehydrogenase antibodies, respectively, followed by immunoblotting with anti-tPA (D) or anti-LRP-1 antibodies (E).

FIGURE 3. LRP-1 deficiency abrogated tPA-mediated MMP-9 induction in fibroblast cells. A, Western blot analysis demonstrated the expression of LRP-1 in different cell lines. Whole cell lysates from rat renal interstitial fibroblasts (NRK-49F), LRP-1-deficient mouse embryonic fibroblasts (PEA-13), and their wild-type counterparts (MEF-1) were immunoblotted with antibodies against LRP-1 and actin, respectively. B, RT-PCR analysis demonstrated that deficiency of LRP-1 abolished MMP-9 mRNA induction by tPA in fibroblasts. MEF-1 and PEA-13 fibroblasts were incubated without or with 10^{-7} m tPA for 24 h. C, graphic presentation of the relative abundance of MMP-9 mRNA among different groups. Data are presented as mean ± S.E. of six independent experiments. *, p < 0.01 versus the control group in MEF-1 cells.
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**FIGURE 4.** tPA induces rapid phosphorylation of the LRP-1 β subunit on tyrosine residues and triggers intracellular MAPK signaling. *A*, tPA induced tyrosine phosphorylation of the LRP-1 β subunit in renal interstitial fibroblasts. NRK-49F cells were incubated with 10⁻⁸ M tPA for different periods as indicated and then subjected to immunoprecipitation with anti-LRP-1 β subunit antibody, followed by immunoblotting with either anti-phosphotyrosine (PY20) or anti-LRP-1 antibodies. B and C, tPA triggered Mek1 and downstream Erk-1/2 phosphorylation in renal interstitial fibroblasts. NRK-49F cells were incubated with 10⁻⁸ M tPA for various periods of time as indicated. Whole cell lysates were immunoblotted with antibodies against phosphospecific and total Mek1 (B) or phosphospecific and total Erk-1/2 (C).

**FIGURE 5.** Erk-1/2 activation is required for tPA-mediated MMP-9 induction in renal interstitial fibroblasts. NRK-49F cells were pretreated with PD98059, a specific inhibitor of Mek1, at the concentrations of 10⁻⁸ M (lanes 3 and 4) or 20 µM (lanes 5 and 6) for 30 min, respectively, followed by incubation with 10⁻⁸ M tPA for an additional 48 h. Supernatants of the cultures were collected for Western blot analysis of MMP-9. Whole cell lysates were immunoblotted with actin antibody for normalization of cell numbers.

**FIGURE 6.** Erk-1/2 activation is sufficient for MMP-9 induction in renal interstitial fibroblasts. *A*, infection with recombinant adenovirus harboring constitutively active Mek1 (Ad-Mek1-CA) induced Erk-1/2 activation in NRK-49F cells. The cells were infected with different dosages of Ad-Mek1-CA adenovirus (lane 2, 2 × 10⁸ particles/ml; lane 3, 4 × 10⁸ particles/ml) for 24 h. Whole cell lysates were immunoblotted with antibodies against phosphospecific and total Erk-1/2. Cells infected with recombinant adenoviruses containing β-galactosidase (Ad-LacZ) (lane 1, 4 × 10⁸ particles/ml) served as control. B and C, zymographic (B) and Western blot (C) analyses demonstrated an increased MMP-9 expression after Erk-1/2 activation. Supernatants of the cultures were collected at 24 and 48 h, respectively, after infection with either Ad-LacZ or Ad-Mek1-CA adenovirus, and subjected to gelatin zymographic analysis or Western blotting for MMP-9.

tPA Induces Rapid Tyrosine Phosphorylation of LRP-1 and Triggers Intracellular Signal Transduction—Given that LRP-1 is essential for MMP-9 induction by tPA, we further explored the downstream signal pathway that leads to MMP-9 gene expression. First we examined the kinetics of tyrosine phosphorylation on the LRP-1 subunit, as early as 0.5 min after stimulation. This induction of the tyrosine phosphorylation of LRP-1 was transient and quickly returned to baseline level (Fig. 4A).

To define the signal pathway that is downstream of LRP-1 phosphorylation after tPA stimulation, we examined the activation of several major signal transduction pathways in NRK-49F cells. tPA was found to induce a rapid MAPK kinase Mek1 phosphorylation in renal fibroblasts. Phospho-Mek1 began to increase as early as 0–2 min and was sustained to at least 5 min, after treatment with tPA (Fig. 4B). Furthermore, Erk-1 and -2, the downstream effector kinases of Mek1, were also rapidly activated by tPA in renal fibroblasts, as demonstrated by a significant increase in phospho-Erk-1/2, which started at 2 min and was sustained to at least 10 min after tPA treatment (Fig. 4C). Under the same conditions, tPA did not activate protein kinase B/Akt and p38 MAPK in NRK-49F cells (data not shown), indicating the specificity of Mek1/Erk-1/2 activation by tPA.

Activation of Erk-1/2 Is Required for tPA-mediated MMP-9 Induction—To establish the relevance of Erk-1/2 activation by tPA to MMP-9 induction, we assessed the functional consequence of inhibiting Erk-1/2 activation in renal fibroblasts. As shown in Fig. 5, pretreatment of NRK-49F cells with PD98059, a specific inhibitor for Mek1, completely blocked MMP-9 expression induced by tPA (Fig. 5). PD98059 at 10 µM significantly blocked tPA-mediated MMP-9 induction in NRK-49F cells, whereas the phosphoinositide 3-kinase inhibitor wortmannin and the p38 MAPK inhibitor SC68376 had little influence on MMP-9 induction by tPA (data not shown). These observations are consistent with the notion that Erk-1/2 activation is required for tPA-mediated MMP-9 gene expression.

Erk-1/2 Activation Is Sufficient for MMP-9 Induction in Renal Interstitial Fibroblasts—To further investigate the role of Erk-1/2 activation in the induction of MMP-9 by tPA, we employed an opposite strategy by overexpressing constitutively active Mek1 in renal fibroblasts. NRK-49F cells were infected with a recombinant adenoviral vector carrying constitutively active Mek1 (Ad-Mek1-CA) or control adenovirus (Ad-LacZ). As shown in Fig. 6A, overexpression of the constitutively active Mek1 induced Erk-1/2 phosphorylation in NRK-49F cells. Zymographic analysis exhibited that activation of Erk-1/2 induced by ectopic expression of the constitutively active Mek1 induced MMP-9 secretion.
in NRK-49F cells (Fig. 6b). The baseline levels of MMP-9 also increased as the incubation time was extended, which could result from an accumulation of secreted MMP-9 in the supernatants. Similar results were obtained using Western blot analysis (Fig. 6C). Therefore, Erk-1/2 activation is not only necessary but also sufficient for mediating MMP-9 induction by tPA.

Concomitant Induction of tPA, LRP-1, and MMP-9 in the Fibrotic Kidney after Ureteral Obstruction—We examined the expression and localization of tPA, LRP-1, and MMP-9 in fibrotic kidneys induced by ureteral obstruction. By using immunofluorescent staining, we found that tPA was markedly induced in the obstructed kidney at 7 days after unilateral ureteral obstruction, compared with sham controls. This is consistent with an increase in tPA mRNA in the same model (3). Furthermore, tPA induction in the fibrotic kidney was exclusively localized to renal interstitium (Fig. 7, top panel) but not tubular epithelial cells. This induction of tPA was accompanied by an up-regulation of LRP-1 and MMP-9. As shown in Fig. 7, LRP-1 and MMP-9 were also markedly induced in the obstructed kidney. Moreover, the induction of LRP-1 and MMP-9 was also localized to renal interstitium. Hence, tPA, LRP-1, and MMP-9 are induced in the interstitial cells of the fibrotic kidney in a coordinated fashion.

DISCUSSION

Tissue plasminogen activator (tPA) is classically viewed as a simple serine protease whose function is to cleave plasminogen to plasmin. However, our present studies demonstrated that tPA binds to the cell membrane receptor LRP-1, induces its phosphorylation on tyrosine residues, triggers intracellular signal transduction, and trans-activates MMP-9 gene expression in renal interstitial fibroblasts. This mode of action elicited by tPA resembles that of a cytokine, a signaling protein that transduces an extracellular signal into a cellular response across the plasma membrane in an autocrine and/or paracrine fashion (25, 26). Similar to other classic cytokines, such as hepatocyte growth factor (HGF), tPA signaling is composed of several essential components of the typical cytokine signaling circuit, including a secreted ligand (tPA), a transmembrane receptor that contains domains on both sides of the plasma membrane (LRP-1), intracellular signaling mediators (Mek1 and Erk-1/2), and target genes (MMP-9) (Fig. 8). These readily fulfill the basic principles of cytokine signaling. Therefore, our present studies add tPA to a long list of signaling proteins that mediate intercellular communications in an ever-changing environment. Needless to say, the notion that tPA is a cytokine provides a conceptual advance in our understanding of the function and biology of tPA and helps to reconcile the bewildering actions of tPA often observed in different physiological and pathological conditions.

Structurally, tPA belongs to a family of the so-called kringle-containing proteins that include uPA, plasminogen, prothrombin, HGF, and macrophage-stimulating protein (27–29). All proteins in this family are produced as a single precursor and then proteolytically processed into the α and β chains. Although the β chain harbors the serine protease catalytic domain, the α chain contains the characteristic kringle domain, a looped, disulfide-linked structure consisting of ~78 amino acid residues. The kringle domain is present in varying numbers ranging from one in uPA to five in plasminogen and is thought to play a critical role in protein-protein interactions necessary for the biological functions of their resident proteins. At least two kringle-containing proteins, namely HGF and macrophage-stimulating protein, are well characterized cytokines that play a crucial role in such diverse cellular processes as cell proliferation, survival, and differentiation (30, 31). In this context, it does not seem surprising that tPA, a kringle-containing protein that is structurally related to HGF and macrophage-stimulating protein, is also capable of functioning as a cytokine, transducing an extracellular signal to the nucleus and modulating specific gene expression.

The identification of tPA as a cytokine by no means suggests that it cannot retain its proteolytic ability. In fact, we envision that the proteo-

FIGURE 7. Concomitant induction of tPA, LRP-1, and MMP-9 in the fibrotic kidney induced by unilateral ureteral obstruction. At 7 days after unilateral ureteral obstruction (UuO) or sham operation, the obstructed kidneys were processed for cryosection. The sections were then immunostained with specific antibodies against tPA, LRP-1, and MMP-9, respectively. The slides were counterstained with renal proximal tubule marker, fluorescein-conjugated lectin from T. purpurea (green). Top panel, staining with tPA; middle panel, staining with LRP-1; bottom panel, staining with MMP-9.

FIGURE 8. Schematic illustration of the signal transduction pathway leading to MMP-9 gene expression by tPA. We propose that tPA acts as a cytokine that binds to the cell membrane receptor LRP-1, induces its tyrosine phosphorylation, activates signal transducers such as Mek1/Erk-1/2, and ultimately leads to expression of specific genes. The signaling scheme of a classic cytokine, HGF, is also presented in parallel for comparison. Purple solid circles denote the kringle domains in tPA and HGF. Red asterisks indicate the tyrosine phosphorylation sites in LRP-1 and the HGF receptor, c-met.
tPA Acts as a Cytokine

lytic activity and cytokine function of tPA is likely to co-exist in the single molecule in a non-mutually exclusive manner. As a serine protease, tPA is known to cleave plasminogen into its biologically active form, plasmin, a protease that in turn can activate some matrix metalloproteinases (including MMP-9) from their zymogen to active forms. In addition, tPA modulates the post-translational activation of several growth factors such as HGF (directly) or TGF-β1 (indirectly via plasmin), by virtue of its proteolytic capacity (32, 33). Some members of the kringle-containing protein family, such as HGF, possess no serine protease activity because of the substitution of two residues in the active catalytic triads of the serine protease domain. Site-directed mutation to restore the functional catalytic triad in HGF does not affect its ability to promote cell growth or any other cytokine function (34). These observations are in harmony with the idea that both proteolytic activity and cytokine function can co-exist in a single protein. It is of interest to note that in the unilateral ureteral obstruction model, the proteolytic activity of tPA appears to be functionally redundant, because ablation of tPA in mice does not result in an alteration of plasmin, HGF, or TGF-β1 (3). However, tPA deficiency largely abolishes MMP-9 induction, indicating that the cytokine function of tPA in vivo is irreparable.

LRP-1 is apparently the membrane receptor that transmits tPA cytokine signaling. This is corroborated by the fact that tPA specifically binds to it and induces it to undergo a rapid phosphorylation on tyrosine residues. Unlike its functional cousin uPA, which has a dedicated cell membrane receptor uPAR, thus far there is no so-called specific tPA receptor. Although it is known that tPA can bind to LRP-1, such binding is thought to result in tPA endocytosis, thereby providing a primary clearance mechanism (35–38). In fact, LRP-1 is capable of binding to more than 30 structurally distinct ligands, including proteases such as tPA and uPA and protease-inhibitor complexes, and is thought to be essential for the clearance of these proteases (14, 16). Nevertheless, accumulating evidence has indicated a cellular signaling potential for LRP-1, in which LRP-1 transmits extracellular signals into a cellular response. In particular, LRP-1 has been identified as the specific receptor for connective tissue growth factor (CTGF), mediating the biological actions of CTGF (39–41).

The cytokine function of tPA drastically changes our view on the biology of this protein, once thought to be a simple serine protease. The ability of tPA to modulate specific gene expression renders it a signaling protein that has a much broader spectrum of biological function than previously envisioned. Not only does tPA induce MMP-9 gene expression in renal interstitial fibroblasts, but it also stimulates MMP-9 production in human cerebral microvascular endothelial cells (42). tPA has also been demonstrated to regulate endothelial cell proliferation and modulate neuron apoptosis (8, 11–13, 43). Such diverse activities almost certainly require an intracellular signal transduction followed by specific gene expression and can hardly be explained by its proteolytic ability alone. Through activating cellular signaling, tPA is capable of exerting a wide variety of biological actions. This possibility becomes even greater when the potential interaction and integration between signaling pathways initiated by other signal stimuli is considered.

tPA and other kringle-containing proteins such as uPA and plasmin are the critical components of the matrix-degrading proteolytic network. In view of their proteolytic ability, these proteins are historically associated with matrix accumulation, leading to a reduced fibrosis after chronic injury. However, gene knock-out studies have painted a different and complex picture on the function of these proteins in relation to fibrotic lesions in vivo (3, 44, 45). Ablation of tPA has been linked to a dramatic renoprotection in obstructive nephropathy; and more interestingly, this seems to have little to do with its proteolytic activity. Rather, the pathogenetic effect of tPA in obstructive nephropathy primarily depends upon its cytokine function that results in MMP-9 gene expression (3). In accordance with this, concomitant induction of tPA, LRP-1, and MMP-9 is observed in the interstitial cells of fibrotic kidney (Fig. 7).

The notion that tPA is a cytokine that transmits an extracellular signal into the nucleus leaves many questions unanswered. For instance, it remains elusive how the LRP-1 receptor is phosphorylated on the tyrosine residues following tPA stimulation. Because LRP-1 lacks intrinsic tyrosine kinase activity, its phosphorylation may require the participation of separate cytoplasmic, non-receptor tyrosine kinases. Second, it remains to be determined how LRP-1 phosphorylation leads to intracellular signal transduction, such as Mek1 and Erk1/2 activation. Previous studies have shown that in Src-transformed cells, activated LRP-1 associates with Shc, an adaptor protein that contains a carboxyl-terminal Src homology-2 (SH2) domain and an amino-terminal phosphotyrosine binding domain that is implicated in signal transduction by protein tyrosine kinases (46–48). It is therefore possible that Shc links LRP-1 activation to the Ras-Mek1-Erk1/2 pathway in renal interstitial fibroblasts. Third, we know little about whether or not tPA signaling involves interactions with other signaling molecules such as another tyrosine kinase receptor or an integrin receptor; and if so, how these signals are integrated (48–50). Further studies are undoubtedly needed to address these issues and to provide a better understanding on the biology of tPA signaling.

In summary, we have presented herein evidence that tPA, a serine protease in nature, acts as a typical cytokine that binds to the cell membrane receptor LRP-1, induces its phosphorylation, triggers intracellular signal transduction, and modulates specific gene expression. These findings will dramatically change our conception of tPA function in the setting of chronic renal fibrosis. We can no longer view tPA as a simple serine protease. Instead, as a cytokine, tPA categorically activates cellular signaling and mediates intercellular communications, thereby having the potential to elicit a wide range of cellular activities.

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