Mitogenic Signaling of Urokinase Receptor–Deficient Kidney Fibroblasts: Actions of an Alternative Urokinase Receptor and LDL Receptor–Related Protein

GUOQIANG ZHANG, XIAOHE CAI, JESÚS M. LÓPEZ-GUISA, SARAH J. COLLINS, and ALLISON A. EDDY

University of Washington, Children’s Hospital and Regional Medical Center, Division of Nephrology, Seattle, Washington

Abstract. The urokinase receptor (uPAR) attenuates myofibroblast recruitment and fibrosis in the kidney. This study examined the role of uPAR and its co-receptor LDL receptor-related protein (LRP) in the regulation of kidney fibroblast proliferation and extracellular signal-regulated kinase (ERK) signaling. Compared with uPAR+/+ cells, uPAR−/− kidney fibroblasts were hyperproliferative. UPAR−/− fibroblast proliferation was 60% inhibited by an ERK kinase inhibitor. LRP protein was reduced and extracellular accumulation of urokinase-type plasminogen activator (uPA) and plasminogen activator inhibitor type 1 (PAI-1) proteins were greater in uPAR−/− cultures. Addition of functional uPA protein or LRP antisense RNA significantly increased ERK signaling and cell mitosis in both genotypes. Enhanced uPAR−/− fibroblast proliferation was reversed by a recombinant nonfunctional uPA peptide. The density of cell-bound fluor-uPA was similar between uPAR−/− and uPAR+/+ fibroblasts (78 ± 6 versus 92 ± 16 units). These data suggest that uPAR-deficient kidney fibroblasts express lower levels of its scavenger co-receptor LRP, resulting in greater extracellular accumulation of uPA and PAI-1. Enhanced proliferation of uPAR−/− fibroblasts seems to be mediated by uPA-dependent ERK signaling via an alternative urokinase receptor.

Progressive destruction of the kidney is a consequence of excessive extracellular matrix accumulation (1). During renal fibrogenesis, resident interstitial fibroblasts typically proliferate and undergo a phenotypic transformation associated with de novo expression of smooth muscle specific α-actin (α-SMA) (2). These myofibroblasts are the primary source of the interstitial matrix proteins that accumulate and ultimately destroy nephron integrity and function (3). The molecular signals that regulate renal fibroblast activation and proliferation remain incompletely delineated. TGF-β and plasminogen activator inhibitor type 1 (PAI-1) proteins were known to play important roles (4,5).

The urokinase receptor (uPAR) is a glycosylated 50- to 60-kD protein that serves to concentrate serine protease activity in pericellular regions (6). The serine proteases, urokinase-type and tissue-type plasminogen activators (uPA, tPA), have been implicated in diverse biologic processes, including cell proliferation and migration, tissue remodeling, wound healing, morphogenesis, tumor development and metastasis, and angiogenesis. uPA acts primarily at extravascular sites, often localizing to cell surfaces as a result of high-affinity binding to uPAR, whereas tPA is typically active at intravascular sites of fibrin deposition and binds to annexin II but not to uPAR. Along with uPA, the other known uPAR ligands are vitronectin and kininogen.

In addition to enhancing pericellular uPA activity, although uPAR itself is a nonsignaling glycosylphosphatidylinositol-linked receptor, it serves multiple roles as a result of interactions with cellular co-receptors that include α(v)β3, α(v)β5, and β1 integrins (7). Another important binding partner is the LDL receptor–related protein (LRP), a multifunctional scavenger and signaling receptor (8). PAI-1, the major uPA inhibitor, interacts with uPAR-bound uPA, leading to internalization and degradation of PAI-1 and uPA via a process that requires LRP. In addition to PAI-1, several other LRP ligands have been implicated in fibrogenesis, including protease inhibitors, matrix proteins, and connective tissue growth factor.

Both uPAR and LRP have been reported to be involved in cellular mitogenic signaling pathways. Several studies have documented mitogenic effects of uPA, but the role of uPAR has often been inferred and not directly examined. Interactions between endogenous uPA, and uPAR and one of its co-receptors may stimulate basal levels of activated extracellular signal-regulated kinases (ERK), thereby regulating cellular behavior (9,10). Through interactions with adapter and scaffold proteins, LRP may modify mitogen-activated protein kinase (MAPK)/ERK signaling initiated by other receptor(s), thereby regulating cell proliferation and differentiation (8).

LRP and uPAR are often coordinately expressed (11). In our
recent studies, both receptors were expressed by renal interstitial cells during fibrogenesis, and uPAR expression had important fibrosis-attenuating effects (12). An important observation was the presence of significantly fewer interstitial myofibroblasts in the kidneys of wild-type mice compared with uPAR-deficient mice. Given the presumed promitotic effects of uPAR and evidence that resident interstitial fibroblasts proliferate and differentiate into myofibroblasts during the early phase of chronic kidney damage (13), it seemed paradoxical that uPAR deficiency was associated with a greater number of myofibroblasts and worse fibrosis. Therefore, the present study was designed to determine whether uPAR deficiency alters kidney fibroblast proliferation using a series of in vitro experiments with primary cultures of kidney fibroblasts isolated from C57BL/6 wild-type and uPAR-deficient mice. The results identify three important mechanisms: (1) increased expression of LRP receptors by uPAR+/+ fibroblasts leads to enhanced clearance of uPA from the extracellular environment, (2) uPA is a fibroblast mitogen that seems to bind to an alternative receptor on uPAR/−/− fibroblasts, and (3) the ERK signaling pathway is involved in the mitogenic response of the alternative uPA receptor.

Materials and Methods

Cell Cultures

Primary renal cortical fibroblast cultures were established as described previously (14) using uPAR-deficient (uPAR−/−) and wild-type (uPAR+/+) mice on an identical C57Bl/6 background, provided by Dr. Peter Carmeliet (Leuven, Belgium) (15). In brief, the renal cortex was separated from the medulla, minced, and differentially sieved to isolate tubular-rich interstitial fragments devoid of glomeruli. The tubulointerstitial fragments were cultured at 37°C with 5% CO₂ in DMEM/F12 (1:1) supplemented with 5% FCS (vol/vol; pH 7.35 to 7.45), hereafter referred to as full medium (FM), until a fibroblast monolayer reached confluence. Early passage uPAR+/+ and uPAR−/− fibroblasts were frozen in multiple aliquots, and all experiments were performed with cells at three to five passages. Fibroblast identity was based on a typical fibroblastic spindle-like shape; pan-cytokeratin negativity; and expression of vimentin, and collagen. All initial descriptive studies were initiated at ~20% confluence and continued for 48 h, when cells were ~90% confluent. Cells were trypsinized and counted using a hemocytometer. Culture conditions using the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (MTS assay; Promega, Madison, WI) according to the manufacturer’s instructions. The MTS Assay is a colorimetric method for determining the total number of viable cells. Our preliminary study confirmed a significant correlation between counted cell numbers and OD values at 490 nm (r = 0.982, R² = 0.965). Each experiment was typically performed with n = 12 separate wells of fibroblasts in 96-well plates under identical conditions. Nuclear mitotic activity was assessed as levels of PCNA protein measured by Western blotting. Culture medium was decanted, and the cells were treated with lysis buffer. Cell lysates and extracellular matrix were collected by scraping and sonication. Protein samples (20 µg) were separated by 7.5 to 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane, and the immunoreactive protein was visualized using ECL enhanced chemiluminescence (reagents from Amersham Pharmacia Biotech, Piscataway, NJ). Amidoblack staining of the blots was used to determine loading equality. In our preliminary studies, protein loads between 5 and 30 µg were within a linear range using amidoblack staining (R² = 0.9754). Band densities were quantified using the NIH Image Analysis Program, and data are expressed in arbitrary densitometric units.

Cellular Proliferation and ERK Activation

Fibroblast mitotic activity was evaluated under several different culture conditions using the CellTiter 96 Aqueous One Solution Cell Proliferation Kit (MTS assay; Promega, Madison, WI) according to the manufacturer’s instructions. ERK activation was measured by Western blotting as levels of phosphorylated ERK (p-ERK) and phosphorylated retinoblastoma protein (p-Rb) protein using mouse anti–phospho-p44/42 MAPK (Thr202/ Tyr204) E10 monoclonal antibody, rabbit anti-total p44/42 MAPK polyclonal antibody, and rabbit anti–phospho-retinoblastoma protein (p-Rb, Ser795; Cell Signaling Technology, Beverly, MA). Expression of cytoskeletal protein α-SMA was also evaluated by Western blotting. Fibroblast mRNA levels for different cyclin-dependent kinases, including cdk1, cdk2, cdk4, cdk6, and cdk9, were determined using a nonradioactive RiboQuant Multi-Probe RNase Protection Assay System (Pharmingen, San Diego, CA) according to the manufacturer’s instructions.

Antibodies and cDNA Probes

Primary antibodies that were used for immunohistochemical staining, Western blotting, and selected antibody neutralization studies were FITC-conjugated anti-pan cytokeratin antibody (Sigma Chemical, St. Louis, MO); goat anti-mouse uPAR1 neutralizing antibody (R&D Systems, Minneapolis, MN); horseradish peroxidase (HRP)-conjugated mouse monoclonal antibodies for cow vimentin, human α-SMA, and proliferating cell nuclear antigen (PCNA) (Dako, Carpinteria, CA); goat anti–type I collagen antiserum (Southern Bio-technology Ass., Birmingham, AL); rabbit anti-LRP antiserum from Dr. D.K. Strickland (American Red Cross, Rockville, MD) (16); mouse anti-murine PAI-1 neutralizing monoclonal antibody (MA-33H1F7) from Dr. P. Declerck (Katholieke University, Leuven, Belgium) (17); and rabbit anti-mouse uPA polyclonal antibody (American Diagnostica, Greenwich, CT). Secondary antibodies were FITC-conjugated goat anti-rabbit IgG (Organon Teknika; West Chester, PA), rabbit anti-goat IgG (Southern Biotechnology Ass.), HRP-conjugated goat anti-rabbit IgG (Chemicon International), and goat anti-mouse IgG (Sigma Chemical). cDNA probes used were rat PAI-1 from Dr. T.D. Gelehrter (University of Michigan, Ann Arbor, MI) (18), rat uPA from Dr. J. Degen (Children’s Hospital Research Foundation, University of Cincinnati, Cincinnati, OH) (19), human LRP from Dr. D.K. Strickland (American Red Cross) (20), and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH; BD Biosciences Clontech, Palo Alto, CA).
Phenotypic Comparison of uPAR+/+ and uPAR−/− Kidney Fibroblasts

LRP mRNA levels were measured by RT-PCR using primer sequences 5’-GTATCTCAAGGGCTGGCGGTG-3’ (1F) and 5’-TG-CACCCAGCATAGGTCCTC-3’ (2R) with a predicted PCR product size of 619 bp (22). Primers for GAPDH were included in the PCR reaction as an internal control for RNA quantity and integrity (PCR product 339 bp). Protein levels were measured by Western blotting, and expression was confirmed in situ by immunostaining.

Confocal microscopy was performed with fibroblasts in DMEM/F12 supplemented with 1% FCS and 5.8 × 10⁻⁹ M mouse recombinant PAI-1 (American Diagnostica). After a 5-min incubation at 37°C, the cells were fixed in 0.5% paraformaldehyde, permeabilized with 0.05% Triton X-100, and stained with anti-LRP antibody followed by goat anti-rabbit IgG-FITC. Cells were examined with an ACAS Ultima Laser Cytometer (Meridian Instruments, Okemos, MI). Fluorescence excitation was at 488 nm, and FITC emission was detected with a 530/20 band pass filter. A ×60 oil immersion objective was used for scanning cells with a step (pixel) size of 0.2 μm in the x-y plane and a step size of 0.4 μm for serial optical sections in the z axis. Photographs were taken using a computerized SPOT camera.

Levels of uPA and PAI-1 protein were examined by Western blotting, and steady-state mRNA levels were examined by Northern blotting as we previously described (12,23). For the Northern blots, total cellular RNA (15 μg/lane) was separated by 1% agarose gel electrophoresis, and the results of GAPDH probing were obtained to evaluate RNA loading equality. The density of each hybridization band was quantified using the NIH Image program.

ERK kinase, LRP, and uPA Inhibition Studies

The MEK1/2 (ERK1/2 kinases) inhibitor UO126 (1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene; 20 μM; Cell Signaling Technology) was added to 48-h cultures to suppress ERK phosphorylation. Trypan blue stain (0.4%; GIBCO BRL Life Technologies, Grand Island, NY) indicated no cellular toxic effects when the cells were cultured in the presence of 20 μM UO126 for up to 72 h.

An antisense RNA strategy was used to evaluate the role of LRP in fibroblast proliferation. Antisense oligonucleotide transfection experiments were performed using previously published sequences (24). Preliminary experiments determined that sense or antisense ODN (25 μM) in DMEM/F12 plus 1% FCS premixed with lipofectin (5 μg/ml) for 30 min before transfection were effective without toxicity up to 96 h. LRP expression was measured by RT-PCR to determine transfection efficiency.

To determine the role of uPA as a mitogen for uPAR−/− kidney fibroblasts, we performed studies using the full-length nonfunctional recombinant mouse uPA peptide (from American Diagnostica; cat. No. 119PC) (25). UPAR+/+ and uPAR−/− fibroblasts were cultured in SFM with or without uPA119 (5 × 10⁻⁸ M) for 72 h, because this dosage demonstrated maximal inhibitory effect in the growth of these cells in our preliminary pilot studies.

uPA Stimulation Studies

Parallel cell culture studies were performed to determine whether a full-length functional uPA peptide (ProuPA from Dr. Jack Henkin; Abbott Laboratories, Abbott Park, IL) (26) could modulate fibroblast proliferation rates. Experiments were performed in SFM.

Figure 1. Urokinase receptor (uPAR) and LDL receptor-related protein (LRP) expression. Fibroblasts were cultured at 37°C for 48 h in DMEM/F12 supplemented with 5% FCS (full medium [FM]). uPAR was expressed exclusively by uPAR+/+ renal fibroblasts as demonstrated by reverse transcription–PCR (RT-PCR; A) and immunofluorescence staining (B). Expression of LRP was downregulated on uPAR−/− fibroblasts as shown by immunostaining (C), RT-PCR (D), and Western blot analysis (E; n = 4, P < 0.05, uPAR−/− versus uPAR+/+). Protein loading was evaluated by amidoblack staining. The figures are representative data from one of two independent experiments. The ability of plasminogen activator inhibitor type 1 (PAI-1) to induce LRP clustering on kidney fibroblasts was examined by confocal laser scanning microscopy. Within 5 min of addition of PAI-1 (5.8 × 10⁻⁹ M), LRP capping was observed on uPAR+/+ cells but not on uPAR−/− cells (F). Magnifications: ×400 in B and C, ×600 in F.
**uPA Binding Study**

The recombinant mouse uPA was labeled with Alexa Fluor 546 protein labeling kit (Molecular Probes) per the manufacturer’s instructions. uPAR+/+ and uPAR−/− fibroblasts were incubated in binding buffer (SFM containing 0.5% BSA) alone, binding buffer plus Alexa Fluor–labeled uPA119 (10−7 M), or binding buffer plus Alexa Fluor–labeled uPA and a 50-fold excess of unlabeled uPA119 at 37°C for 60 min (22). After washes, the cells were examined by computerized fluorescence microscopy and the luminance of the fluorescence was analyzed with the Image-Pro Plus image analysis software (Media Cybernetics, Silver Spring, MD). To exclude intracellular fluorescence as a result of endocytosis of the Alexa Fluor–labeled uPA, we repeated binding studies at 4°C for 2 h and measured fluorescence intensity in situ by scanning the cells using a Typhoon 9410 Variable Mode Imager (Amersham Biosciences).

**In Vivo Studies**

Staining for p-ERK and double staining for α-SMA and bromodeoxyuridine (brdU) were performed on kidney paraffin sections of uPAR+/+ and uPAR−/− mice (n = 5 each) with obstructive nephropathy of 7 d duration (12,23). Cytoplasmic α-SMA and nuclear brdU were labeled using the HRP substrate DAB+ nickel (dark bluish/black color) and the AKP substrate Fast red/Naphthol (red color), respectively. Proliferating fibroblasts were blindly counted in 10 consecutive cortical high-power fields (×750) by a method previously reported (13).

**Statistical Analyses**

All data were expressed as mean ± 1 SD unless otherwise stated. Results were analyzed by the t test or the Mann Whitney U test (binding studies and histology data) using the SPSS or Excel software. P < 0.05 was considered statistically significant. All in vitro experiments were dual, and data were not combined for statistics unless otherwise stated.

**Results**

**uPAR-Deficient Kidney Fibroblasts Express Lower LRP Levels**

The uPAR genotype of the primary kidney fibroblast cultures was verified by RT-PCR and immunostaining (Figure 1,
A and B). LRP, the important scavenger co-receptor of uPAR (8), was significantly downregulated in uPAR⁺⁻ cells compared with the uPAR⁺⁺ cells as detected by Western blotting (0.5 ± 0.2 versus 1 ± 0.2 units, −/− versus +/+; P < 0.05) and RT-PCR (Figure 1, C through E). Confocal microscopy demonstrated that PAI-1 (5.8 ± 10⁻⁹ M) in 1% FCS medium stimulated LRP surface clustering on uPAR⁺⁺ cells but not uPAR−/− cells (Figure 1F), indicating the involvement of uPAR in PAI-1/LRP interactions.

UPAR-Deficient Fibroblasts Manifest a Hyperproliferative Phenotype and Increased ERK Activation

During our initial studies, we observed that primary cultures of uPAR⁻⁻ fibroblasts in DMEM/F12 with 5% FCS (full medium [FM]) grew quickly, reaching ~90% confluence by 48 h. Cell number increased to a greater extent in uPAR⁻⁻ than the +/+ cells after 16, 40, and 48 h (all P < 0.05; Figure 2A). Cell cycle entry, measured as PCNA expression by Western blot analysis, further documented the hyperproliferation of uPAR−− cells (3.9 ± 0.6 versus 1 ± 0.7 units, −/− versus +/+; P < 0.05 after 48-h culture; Figure 2, B and C). Addition of anti-uPAR neutralizing antibody to the culture medium (1% FCS) of the uPAR⁺⁺ fibroblasts increased proliferation rates compared with cells cultured with control nonimmune goat Ig (Figure 2, D through F).

Given the importance of ERK signaling pathways in cellular proliferation (27,28), levels of ERK phosphorylation were evaluated. The basal levels of ERK phosphorylation, expressed as p-ERK/total ERK after 48-h culture in FM, were threefold higher in the uPAR−− cells than in the +/+ cells (Figure 3, A and B). Phosphorylated retinoblastoma protein (pRb), the key cell-cycle regulator that promotes passage through the G1-S restriction site, was significantly increased (37-fold) in uPAR−− fibroblasts compared with the +/+ cells after culture in FM for 48 h (Figure 3C). Evaluation of multiple cell-cycle regulatory kinases using a multiprobe RNAase protection assay determined that PITALRE (cdk9) was the predominant cyclin-dependent ki-
nase mRNA expressed by the renal fibroblasts. Expression levels were similar between uPAR+/+ and −/− cells (137 ± 5.1 versus 134 ± 10.4 units; P > 0.05).

The α-SMA cytoskeletal protein has been regarded as a marker of the “activated” or “transformed” state of renal fibroblasts (myofibroblasts) and may be linked with ERK signal transduction and cell proliferation (2). α-SMA protein levels were significantly higher in uPAR-deficient kidney fibroblasts (6.5 ± 1.8 versus 1 ± 0.2 units, −/− versus +/+ after 48 h in culture; Figure 3D).

**Excessive Extracellular Accumulation of uPA and PAI-1 Protein in uPAR-Deficient Fibroblast Cultures**

The primary uPAR ligand is uPA. PAI-1 binds to uPAR indirectly via interactions with uPA. The entire uPA–uPAR–PAI-1 complex undergoes endocytosis and degradation via a process that requires the LRP receptor. When cultured in 5% FCS medium for 48 h, the fibroblast uPA protein level (cellular + extracellular protein) was twofold higher in uPAR−/− cells (2.1 ± 0.7 versus 1 ± 0.6 units; P < 0.05) than in uPAR+/+ cells, despite similar mRNA levels (1.2 ± 0.6 versus 1 ± 0.4 units; P > 0.05; Figure 4A). Similarly, significantly more PAI-1 protein accumulated in the uPAR−/− cells (5.3 ± 1.5 versus 1 ± 1.1 units; P < 0.05), despite lower PAI-1 mRNA levels (0.41 ± 0.07 versus 1 ± 0.15 units; P < 0.05) when compared with the +/+ cells (Figure 4B).

**ERK Inhibition Attenuates Proliferation of uPAR-Deficient Kidney Fibroblasts**

Because ERK phosphorylation levels were significantly elevated in uPAR−/− fibroblasts, the contribution of ERK activation to the hyperproliferative phenotype of these cells was investigated using the ERK kinase inhibitor UO126. Preliminary dose–response studies demonstrated complete inhibition of fibroblast ERK activation with 20 μM UO126. After a 48-h incubation, UO126 (20 μM in medium containing 1% FCS) completely suppressed the phosphorylation of ERK and was associated with significantly lower PCNA protein levels (29 ± 17 versus 70 ± 7 units, UO126 versus control) and α-SMA protein levels (7 ± 2 versus 20 ± 11 units, UO126 versus control).
control; Figure 5, A and B). Significantly fewer fibroblasts were observed in the UO126-treated groups of both uPAR−/− and +/+ cells after 16 and 48 h (Figure 5C).

**LRP Deficiency Promotes Fibroblast Proliferation and ERK Phosphorylation**

To evaluate whether the decreased expression of LRP on uPAR−/− kidney fibroblasts played a functional role in fibroblast proliferation, we performed studies using an LRP antisense oligonucleotide (ODN) (24). Antisense ODN transfection completely inhibited LRP expression in both uPAR−/− and +/+ cells as demonstrated by RT-PCR at 48 h (Figure 6A). LRP inhibition significantly enhanced proliferation of both uPAR−/− and +/+ kidney fibroblasts compared with the sense ODN-treated control groups of the same uPAR genotype as measured by the MTS assay and by PCNA Western blot analysis (Figure 6, B through D). Concurrently, p-ERK levels significantly increased by 1.4- and 1.6-fold in uPAR−/− and +/+ cells, respectively, when compared with sense ODN-treated groups (Figure 6, E and F).

**uPA Stimulates Proliferation and ERK Activation in uPAR+/+ and uPAR−/− Cells**

Given that uPA protein levels were higher in cultures of uPAR−/− kidney fibroblasts, we investigated whether uPA modulated fibroblast proliferation. Even when cultured without serum (SFM) for 72 h, uPAR−/− cells slowly proliferated, whereas uPAR+/+ cells remained quiescent (Figure 7, A and B). However, when cells were co-cultured in SFM in the presence of uPA119 (5 × 10⁻⁹ M), a full-length, nonfunctional recombinant mouse uPA (25), the low-level ERK phosphorylation and proliferation of uPAR−/− cells were completely neutralized as measured by the MTS assay and Western blot analysis for PCNA and p-ERK (both P < 0.05, n = 12 and 4, respectively; Figure 7, A through C). The uPAR+/+ fibroblasts were quiescent in SFM; their PCNA expression level was not altered by co-culture in the presence of uPA119 (Figure 7, A and B).

Addition of functional recombinant ProuPA peptide (5 × 10⁻⁹ M) to the culture medium initiated early (initial 120 min) ERK phosphorylation in uPAR+/+ fibroblasts but not in the uPAR−/− cells (Figure 7D). However, after 48 h, ProuPA increased p-ERK levels as much as sevenfold in uPAR−/− compared with nonstimulated fibroblasts (7.6 ± 0.5 versus 1 ± 0.3 in uPAR−/− cells; 1.3 ± 0.1 versus 1 ± 0.1 in uPAR+/+ cells; both P < 0.05; Figure 7C). After a 48-h incubation, ProuPA stimulated PCNA expression 7.8- and 3.6-fold in uPAR−/− and +/+ fibroblasts, respectively, compared with unstimulated cells of the same genotype (Figure 7E). The mitogenic effect of ProuPA in renal fibroblasts was further confirmed by the changes in viable cell numbers by the MTS assay and Western blot analysis for PCNA and p-ERK (both P < 0.05, n = 12 and 4, respectively; Figure 7, A through C). The uPAR+/+ fibroblasts were quiescent in SFM; their PCNA expression level was not altered by co-culture in the presence of uPA119 (Figure 7, A and B).

Addition of functional recombinant ProuPA peptide (5 × 10⁻⁹ M) to the culture medium initiated early (initial 120 min) ERK phosphorylation in uPAR+/+ fibroblasts but not in the uPAR−/− cells (Figure 7D). However, after 48 h, ProuPA increased p-ERK levels as much as sevenfold in uPAR−/− compared with nonstimulated fibroblasts (7.6 ± 0.5 versus 1 ± 0.3 in uPAR−/− cells; 1.3 ± 0.1 versus 1 ± 0.1 in uPAR+/+ cells; both P < 0.05; Figure 7C). After a 48-h incubation, ProuPA stimulated PCNA expression 7.8- and 3.6-fold in uPAR−/− and +/+ fibroblasts, respectively, compared with unstimulated cells of the same genotype (Figure 7E). The mitogenic effect of ProuPA in renal fibroblasts was further confirmed by the changes in viable cell numbers by the MTS assay and Western blot analysis for PCNA and p-ERK (both P < 0.05, n = 12 and 4, respectively; Figure 7, A through C). The uPAR+/+ fibroblasts were quiescent in SFM; their PCNA expression level was not altered by co-culture in the presence of uPA119 (Figure 7, A and B).
cell proliferation assay (Figure 7F). The ATF of uPA is reported to stimulate melanoma cell proliferation independent of uPAR (25). The ATF fragment containing the growth factor–like domain and kringle domain induced a modest 1.3-fold increased proliferation of uPAR+/H11002/ and not uPAR+/H11001/ fibroblasts at the dose of 5 × 10⁻⁷ M (Figure 7G).

**uPA Binds to Kidney Fibroblasts that Lack uPAR**

Previous studies have suggested the existence of cellular uPA binding sites in addition to the high-affinity receptor uPAR, although this putative receptor(s) has not yet been identified (25,29,30). uPA binding was evaluated by fluorescence microscopy (37°C study) or by a Typhoon 9410 Imager (4°C study) using fluorochrome-labeled recombinant mouse uPA. The mean fluorescence luminance of kidney fibroblasts (after subtraction of background fluorescence luminance of buffer-incubated control cells) was similar between uPAR+/H11001/ and −/− cells when measured by PCNA protein expression by Western blot analysis at 48 h (A) and by the MTS proliferation assay performed at both 16 and 48 h (C and D; n = 12 per group, *P < 0.05, antisense versus sense of the same uPAR genotype). The suppression of fibroblast LRP was also associated with enhanced ERK signal activation. p-ERK1/2 Western blot analysis demonstrates significantly higher levels in antisense (AS) LRP-treated fibroblasts compared with sense (S) LRP-treated cells of the same uPAR genotype after culture for 48 h (E and F). Densitometric data of p-ERF1/2 bands are expressed as mean ± 1 SD arbitrary units, n = 3 each (F). *P < 0.05, AS versus S of same genotype.

Figure 6. LRP effects on cell proliferation. Kidney fibroblast LRP mRNA expression measured by RT-PCR is silenced after treatment with antisense oligonucleotides (AS; 25 μM) in media with 1% FCS for 48 h (A). LRP antisense augmented cell-cycle entry rates of both uPAR+/+ and −/− cells when measured by PCNA protein expression by Western blot analysis at 48 h (B) and by the MTS proliferation assay performed at both 16 and 48 h (C and D; n = 12 per group, *P < 0.05, antisense versus sense of the same uPAR genotype). The suppression of fibroblast LRP was also associated with enhanced ERK signal activation. p-ERK1/2 Western blot analysis demonstrates significantly higher levels in antisense (AS) LRP-treated fibroblasts compared with sense (S) LRP-treated cells of the same uPAR genotype after culture for 48 h (E and F). Densitometric data of p-ERF1/2 bands are expressed as mean ± 1 SD arbitrary units, n = 3 each (F). *P < 0.05, AS versus S of same genotype.
ERK Phosphorylation and Fibroblast Proliferation in Damaged Kidneys Is Enhanced in uPAR-Deficient Mice

To determine whether these in vitro observations occur in vivo, we examined kidneys from uPAR+/+ and uPAR−/− mice after 7 d of ureteral obstruction. Numerous α-SMA–positive activated fibroblasts were present in the interstitium of both kidneys, although they were more numerous in uPAR−/− mice (Figure 9, A and B). Double immunostaining demonstrated significantly more proliferating α-SMA + fibroblasts in uPAR−/− mice (fivefold increase) compared with the uPAR+/+, as assessed by in situ incorporated brdU (Figure 9, A through C). Western blot analysis and immunostaining also showed higher levels of phosphorylated ERK in the tubulointerstitium of the uPAR-deficient mice (Figure 9, D through G).

Discussion

Kidney tubes are a major source of uPA; its activity level increases after injury caused by obstruction (5,12). Through its proteolytic effects on extracellular matrix, uPA is predicted to attenuate fibrosis severity. More recently, it has become evident that uPA also has important uPAR-dependent cellular activities that dampen the fibrogenic response (23). The present study provides two important new insights. First, uPAR serves to maintain renal fibroblasts in an "inactive" phenotype by promoting the clearance of uPA–PAI-1 ligands via the uPAR-LRP degradation pathway. The uPAR-deficient kidney fibroblasts also expressed lower levels of the LRP scavenger co-receptor, resulting in greater extracellular accumulation of uPA and PAI-1 and higher levels of activated ERK. Second, in the absence of uPAR, kidney fibroblast proliferation is en-
hanced and seems to be mediated at least in part by uPA-dependent ERK signaling via a currently uncharacterized alternative urokinase receptor.

Downregulation of LRP on uPAR-deficient fibroblasts seems to promote their hyperproliferative phenotype by at least two mechanisms: extracellular accumulation of mitogens and altered basal ERK activity. Receptor-bound uPA–PAI-1 complexes undergo endocytotic degradation with recycling of the receptors (uPAR and LRP) to the cell membrane (31). The failure of PAI-1 to initiate LRP membrane clustering in uPAR−/− fibroblasts illustrates the dysfunctional PAI-1 scavenging pathway in uPAR−/− cells. This pathway, together with a newly identified cell surface 200-kD protein (p200), is also responsible for the clearance of uPA (32). Levels of uPA protein were higher in cultures of uPAR−/− fibroblasts, where it seemed to function as a fibroblast mitogen.

LRP is linked through its intracellular domain to a group of recently identified adapter and scaffold proteins that are key organizers and regulators of the actin cytoskeleton and integrin-mediated MAPK/ERK1/2 signaling (8). MAPK/ERK1/2 signal transduction has been reported to initiate diverse cellular events, including proliferation, cytoskeletal reorganization, epithelial-mesenchymal transition, and LRP expression (27,28,33). The present study suggests that the hyperproliferative phenotype of the uPAR-deficient fibroblasts was mediated by ERK activation because the increased mitotic activity was reversed by the ERK1/2 kinase inhibitor, UO126. Decreased LRP expression by uPAR null fibroblasts seems to be associated with their ERK activity, given that fibroblast LRP inhibition alone enhanced cell proliferation and increased basal p-ERK levels, whether uPAR was expressed or not. However, unlike uPAR+/+ fibroblasts stimulated with exogenous uPA, ERK phosphorylation was delayed until at least 120 min in the uPAR−/− cells. The absence of this very initial phase of the uPAR-mediated MAPK/ERK1/2 signaling suggests one plausible explanation for the downregulation of LRP in the uPAR−/− cells (34–36). This time lag before ERK activation in the uPAR−/− fibroblasts suggests that cross-talk with other intracellular signaling pathways precedes ERK activation after ligand binding to the alternative uPA receptor.

In addition to high-affinity binding of the growth factor–like uPA domain to uPAR, previous studies reported specific uPA binding to an alternative, still unidentified low-affinity receptor (25,29,30,34). Whereas the mitogenic effects of uPA are widely known (25), the role of uPAR has not been as clearly delineated. In fact, some previous uPA blocking studies using a specific monoclonal antibody or uPAR antisense reported no effect on uPA-induced proliferation, consistent with our current findings that uPA-induced kidney fibroblast proliferation is independent of uPAR expression (25,30,34). In fact, in the present study, the opposite response was observed, with either genetic uPAR deficiency or pretreatment with a uPA-blocking antibody associated with higher rates of uPA-induced proliferation than with control wild-type fibroblasts. The augmented mitotic activity of the uPAR−/− cells was completely reversed by the nonfunctional uPA119 blocking peptide, suggesting that the hyperproliferative state of uPAR-deficient kid-

![Figure 8. Alexa Fluor–uPA binding to kidney fibroblasts. Immunofluorescence photomicrographs illustrate uPA binding to kidney fibroblasts using Alexa Fluor–labeled uPA as a probe in a study performed at 37°C (A). Control fibroblasts cultured on coverslips in FM fail to fluoresce after incubation in binding buffer alone. After a 60-min incubation with Alexa Fluor–uPA (1 × 10^{-7} M) in binding buffer, both uPAR+/+ and uPAR−/− fibroblasts demonstrate uPA binding. Competition experiments with an excess of nonlabeled uPA (5 × 10^{-6} M) for 60 min blocks Alexa Fluor–uPA binding. A binding study repeated at 4°C demonstrates similar binding results when cellular fluorescence scanning was examined in situ using a Typhoon 9410 Variable Mode Imager (B). *P < 0.05, Alexa Fluor–labeled uPA versus binding buffer alone or Alexa Fluor–uPA with excessive uPA119, n = 4 per group. Magnification, ×400 in A.](image)
response induced by the full single-chain uPA peptide (1.3-versus 7.8-fold increase). Perhaps preservation of the intramolecular structure of uPA by internal disulfide bonds, known to be essential for its proteolytic activity, is necessary to maximize its mitogenic function as well (38, 39). A recent phage display study identified a putative uPA kringle domain-binding consensus sequence that is expressed by at least 100 proteins, including 12 cell surface receptors, some of which are known uPAR co-receptors: LRP, integrin α(v), membrane glycoprotein 130 (IL-6 receptor), insulin-like growth factor II receptor, and uPAR-associated protein uPARAP (Endo 180), suggesting potential candidates for the alternative urokinase mitogenic receptor(s) (40). Ongoing microarray studies in our laboratory also support the presence of an alternative uPA-binding site on kidney fibroblasts. After stimulation of uPAR-deficient fibroblasts with pro-uPA for 48 h, 133 genes with an annotated biologic function were significantly upregulated compared with nonstimulated cells, including five genes involved in cell proliferation and 24 involved in signal transduction (unpublished data). What is also becoming apparent from these stud-
ies is that the mitotic response represents only one of several biologic processes that are activated when uPAR fibroblasts are stimulated with uPA, and many of these have been implicated directly in fibrogenesis. The _de novo_ expression of α-SMA by kidney fibroblasts not only is a marker of cellular activation and transition to a myofibroblastic phenotype, but also may serve as an alternative signal transduction pathway. This phenotypic change often occurs in association with fibroblast proliferation and synthesis of extracellular matrix proteins. It therefore is noteworthy that genetic uPAR deficiency was associated with enhanced α-SMA expression and filamentous actin disassembly (data not shown), illustrating a functional connection between the uPAR/uPA/PAI-1/LRP membrane complex and the actin cytoskeleton. It has previously been reported that inhibition of the formation of uPAR–uPA–PAI-1 tripartite complexes significantly alters cytoskeletal signal transduction (41).

The origin of kidney interstitial myofibroblasts during fibrogenesis is a topic of great interest given their pivotal role in kidney parenchymal destruction by fibrosis. Evidence currently suggests at least four possible sources of these cells: activation and proliferation of resident interstitial fibroblasts, migrated perivascular cells, circulating mesenchymal cells, and transdifferentiated tubular epithelial cells. _In vivo_ studies have documented that _in situ_ proliferation of interstitial fibroblasts does occur after injury (13). Data from the present study suggest that enhanced mitotic activity accounts at least in part for greater number of interstitial myofibroblasts and more severe fibrosis observed in mice that lack uPAR. The uPAR-deficient kidney fibroblasts express lower levels of their scavenger co-receptor LRP, resulting in greater extracellular accumulation of uPA and PAI-1. Enhanced proliferation of uPAR−/− fibroblasts seems to be mediated by uPA-dependent ERK signaling _via_ a currently uncharacterized novel urokinase receptor that is predicted to promote fibrosis.

**Acknowledgments**

This work was supported by National Institutes of Health Grants DK54500 and DK44757 (A.A.E.). Part of this work was published as an abstract in _J Am Soc Nephrol_ 14: 20A, 2003.

Our sincere thanks to Dr. Peter Carmeliet for providing breeding pairs of uPAR-deficient and wild-type mice, to Jack Henkin for ProuPA, and to Dr. Graham Parry for the purified ATF peptide.

**References**


protein suggests that this molecule is a multifunctional receptor. J Biol Chem 265: 17401–17404, 1990
41. Fincham VJ, James M, Frame MC, Winder SJ: Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. EMBO J 19: 2911–2923, 2000