Urokinase Anchors uPAR to the Actin Cytoskeleton

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PURPOSE. To investigate the expression and localization of urokinase plasminogen activator (uPA) and its receptor (uPAR) and their interaction with the actin cytoskeleton in human corneal fibroblasts.

METHODS. Primary cultured human corneal fibroblasts were exposed to exogenous uPA to investigate its effect on the distribution of uPAR under resting conditions and in a scrape-wound model. Fluorescence microscopy, immunolocalization, immunoprecipitation, and the actin depolymerizing drug cytochalasin D were used to evaluate uPAR’s interaction with the actin cytoskeleton.

RESULTS. uPA/uPAR was immunodetected in large (200 μm²) aggregates devoid of detectable F-actin. However, when uPA was added to corneal fibroblasts before fixation, a dynamic association between uPAR and the actin cytoskeleton was revealed: the uPA/uPAR complex was immunodetected throughout the surface of the plasma membrane in the form of dispersed small aggregates (0.05 μm²). Association of uPAR with actin stress fibers was visualized when FITC-labeled uPA was added to the cells. This codistribution of uPA/uPAR and actin was not detected when the cells were pretreated with the actin-depolymerizing drug cytochalasin D. uPAR was found associated with actin but was also in focal adhesions, the termination points of F-actin, where it colocalized with the integrin αvβ3 in cells migrating into a scrape wound. Immunoprecipitation experiments confirmed the physical association of uPAR with αvβ3 in fibroblasts.

CONCLUSIONS. The authors propose that uPA/uPAR ligation anchors the complex to the actin cytoskeleton and is a part of the mechanism responsible for uPA-induced cell migration in fibroblasts. (Invest Ophtalmol Vis Sci. 2004;45:2967–2977) DOI:10.1167/iovs.04-0030

After corneal wounding, activated fibroblasts migrate into the stroma to repair and remodel the damaged tissue.1,2 The recent discovery of plasminogen in wounded cornea3 led us to investigate the expression and localization of urokinase plasminogen activator (uPA) and its receptor (uPAR) in corneal fibroblasts. uPA is an extracellular serine protease that cleaves plasminogen to plasmin. The protease activity of uPA is enhanced by binding to uPAR. Plasmin, a broad-spectrum protease, promotes cell migration in a variety of tissues by activating matrix-sequestered metalloproteinases and growth factors.4–5

The purpose of the present study was to examine the subcellular localizations of the uPA/uPAR complex to gain a better understanding of uPA’s mechanism of action in eliciting its downstream effects on the actin cytoskeleton, especially in the repair corneal fibroblasts. It is known in other cell types that binding of uPA to uPAR initiates activation of intracellular signaling molecules such as focal adhesion kinase (FAK), mitogen-activated protein kinase (MAPK), and the Jak/Stat pathway, promoting rearrangements of the actin cytoskeleton and cell movement.6–8 Because uPAR is a glycosyl phosphatidylinositol (GPI)–linked protein and therefore lacks a transmembrane domain,9 it must interact with another plasma membrane resident molecule to initiate intracellular signaling. In this regard, uPAR’s association with αv, β2, or β3 integrins in several noncorneal cell types has been demonstrated.9–10 We reasoned that knowledge of uPA/uPAR subcellular distribution would provide insights into our understanding of cell movement, since uPA/uPAR plays a role both as a protease system and as a signaling complex during cell migration.

The in vivo activated fibroblast phenotype can be reproduced in vitro by extracting quiescent human corneal fibroblasts (keratocytes) from the corneal stroma and culturing them in serum-containing medium. In the present study, we exploited this in vitro model to investigate the expression and distribution of uPA/uPAR in corneal fibroblasts. We observed that activated motile fibroblasts express both uPA and its receptor as opposed to quiescent fibroblasts (in agreement with previous studies by Tripathi et al.15) and to corneal myofibroblasts, which express none or little uPA/uPAR (Bernstein et al., manuscript in preparation). We confirmed by immunodetection that uPA and uPAR were in focal adhesions and lamellipodia. Herein, we report the new finding that when uPA binds to uPAR, the uPA/uPAR complex becomes widely distributed over the entire cell surface. Furthermore, the uPA/uPAR complex colocalizes with F-actin, not simply at focal adhesion sites where these fibers terminate, but along the length of cortical actin fibers. This suggests that both cell attachment to the matrix and urokinase protease activity is regulated at many sites on the cell surface. The implications of these findings provide new insights into uPA/uPAR’s role in wound healing.

Materials and Methods

Antibodies and Reagents

The following antibodies were used for immunofluorescence: uPAR mouse monoclonal (R2; Keld Danø, The Finsen Center, Copenhagen, Denmark), uPA rabbit polyclonal (R1; Lilliana Ossowski, Mount Sinai School of Medicine, New York, NY), αvβ1 mouse monoclonal (VIF4; Ralph Isberg, Tufts Medical School, Boston, MA), β3 rabbit polyclonal (B36; Barry Coller Rockefeller University, New York, NY), and α-tubulin mouse monoclonal (B512; Sigma, St. Louis, MO).

Urokinase was from American Diagnostica (Greenwich, CT). For direct visualization of uPA bound to uPAR, we used uPA-FITC (American Diagnostica). Diisopropyl fluorophosphate–inhibited uPA (DFP-uPA)16 was provided by Steven Gonias, University of Virginia School of Medicine.
Preparation of Human Corneal Fibroblasts

Human corneas, from donor eyes aged 30 to 65 years and not suitable for transplantation, were obtained from the Lion’s Eye Bank (Manhas-set, NY) and the National Disease Research Interchange (NDRI; Philadelphia, PA). After the limbus was discarded, corneas were cut into small pieces and epithelium released by incubating the corneal pieces overnight at 4°C in 5 mg/mL Dispase II (Roche Diagnostics, Mannheim, Germany) in DMEM-F12 without sodium bicarbonate (Sigma) followed by shaking at 37°C for 1 hour. The remaining stromal pieces were incubated in two sequential collagenase incubations of 500 U/mL (Sigma) in the same medium. Keratocytes were collected by centrifugation and resuspended in DMEM-F12, 10% fetal bovine serum (FBS; Invitrogen-Gibco, Rockville, MD) and antibiotics and placed in culture. The cultured fibroblasts were studied up to seven passages.

Immunocytochemistry

Immunodetection was performed on cells after fixation with 3% p-formaldehyde (Fisher Scientific, Fair Lawn, NJ) in PBS (pH 7.4) for 15 minutes at room temperature (RT) followed by quenching with 0.1 M glycine (pH 7.5). After nonspecific binding was blocked with 3% normal goat serum (Jackson Immunoresearch, West Grove, PA), cells were incubated with primary antibodies and then with secondary antibodies, either anti-mouse or anti-rabbit IgG AlexaFluor dyes 488 and 568 (Molecular Probes), respectively, at RT. Cells were routinely not Triton-permeabilized before staining for uPA/uPAR to avoid extraction of GPI-linked uPAR.

To assess the impact of uPA binding to uPAR, cells were incubated with 4.0 nM uPA diluted in PBSA (PBS, 1% BSA, 1.0 mM MgCl2, 0.5 mM CaCl2) at RT for 10 minutes. Coverslips were washed in PBS twice and then fixed in 3% p-formaldehyde before indirect immunodetection (described earlier). Concentrations of uPA from 0.4 nM and higher were as effective as 4.0 nM.

Incubation of cells with fluorescent-tagged uPA (uPA-FITC) provided direct visualization of uPAR rather than through immunodetection. uPA-FITC had been used previously to detect cell-surface uPAR by flow cytometry (American Diagnostics protocol). However, we did not use this reagent for flow cytometry analysis. Rather, we used the same protocol, but the final readout was by fluorescence microscopy, not flow cytometry. Specifically, before addition of uPA-FITC, fibroblasts on coverslips were acid-stripped of surface uPA by incubation with 0.05 M glycine, 0.1 M NaCl (pH 3.0; 2 minutes, RT), neutralized with 0.5 M HEPES, 0.1 M NaCl (pH 7.5) and washed with PBS twice (PBS, 1.0 mM MgCl2, 0.5 mM CaCl2). Non-specific protein binding was blocked with PBSA (PBS, 100 nM uPA-FITC (16.0 nM in PBSA) was applied to the cells for 10 minutes at RT terminated by washing in PBS. We followed this by fixing with 3% p-formaldehyde, quenching with 0.1 M glycine (pH 7.5), and mounting the coverslip for fluorescence microscopy. In some experiments, cells were also immunodetected after fixation for dual-localization studies as described earlier. Because of the relatively faint fluorescent signal of FITC (compared with indirect immunofluorescence with AlexaFluor-tagged antibodies) we required 16 nM uPA-FITC to yield a detectable signal. However, the same pattern of uPA/uPAR distribution was produced if the stripping protocol was followed by addition of 4.0 nM uPA before fixation and immunodetection for uPA/uPAR.

To visualize uPA/uPAR in focal adhesions, 4 nM uPA diluted in PBSA was added to the cells for 10 minutes at RT before fixation and permeabilization with either p-formaldehyde (15 minutes at RT), 0.1% Triton X-100 (1 minute at RT), or methanol (5 minutes at −20°C) and indirect immunodetection was performed as described earlier.

All cells were viewed with one of three microscopes (Axioskop or Axiovert; Carl Zeiss Meditec, Thornwood, NY; or a laser scanning confocal microscope; Leica, Wetzlar, Germany), as indicated. For the Axiovert, images were captured with a charge-coupled device (CCD) camera (SPOT-2; Diagnostic Instruments, Sterling Heights, MI; processed by PhotoShop; Adobe Systems, Mountain View, CA). For the Axiovert, images were captured with another CCD camera (Axiocam MRm; Carl Zeiss Meditec). Optical sections were generated by inserting a slider (ApoTome; Carl Zeiss Meditec) into the illumination path. The images were subsequently combined and the out-of-focus haze removed (Axiovision software; Carl Zeiss Meditec). The size of the uPA/uPAR aggregates was measured with Image J software (available by ftp at zippy.nimh.nih.gov/ or at http://rsb.info.nih.gov/ij/image; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD).

Wounding Studies

Confluent cultures in DMEM-F12 and 10% FBS were scrape-wounded with a pipette tip. The medium was replaced, and the coverslips were fixed after incubation at 37°C for 4 hours.

Cytoskeletal Drug Studies

Based on preliminary studies with concentrations of 10 to 1000 nM cytochalasin, we found that we could depolymerize the actin cytoskeleton while retaining the flattened fibroblast phenotype and cell attachment if human corneal fibroblasts were incubated with 100 nM cytochalasin D in serum-free DMEM-F12 plus 0.2% BSA for 30 minutes at 37°C. To depolymerize the microtubule cytoskeleton, cells were incubated with 10 μM nocodazole in serum-free DMEM-F12 plus 0.2% BSA for 30 minutes at 37°C. These inhibitors were present during uPA treatment before fixation.

uPA-FITC–Binding Studies

To assay uPA binding to uPAR, we modified the protocol of Estreicher et al.19 by substituting uPA-FITC for 125I-uPA (see Fig. 3B). Human corneal fibroblasts in 100-mm dishes were washed with PBS twice and 4 nM uPA-FITC in PBSA was added for 10 minutes at RT. Cells were then washed with PBS twice and cross-linked with 2 mM DTSSP (Pierce, Rockford, IL) in PBS for 30 minutes at RT. Cells were detached, and the cross-linker quenched with 20 mM Tris (pH 7.5), 250 mM sucrose, and 1 mM EDTA (for 20 minutes, at 4°C). The cells were pelleted at 3000 rpm at 4°C and the pellet lysed in RIPA (50 mM Tris [pH 7.5], 150 mM NaCl, 1% Triton X-100, and 0.5% deoxycholate, 0.1% SDS) plus protease inhibitor tablet (EDTA-free; Roche Diagnostics), 20 minutes, 0°C to 4°C and centrifuged at 14,000 rpm. Twenty micrograms of detergent-soluble protein was separated on a 8% SDS-PAGE gel and transferred to nitrocellulose (Protran; Schleicher & Schuell, Keene, NH), and Western blotted with anti-FITC antibody followed by an anti-rabbit IgG secondary coupled to HRP (Jackson ImmunoResearch). Bands were visualized with chemiluminescent substrate (Pierce). Specificity of binding was determined by competition: 4 nM of unlabeled uPA was incubated with the cells for 10 minutes at RT before addition of 4 nM uPA-FITC (see Fig. 3B, lane 2).

Immunoprecipitation

To identify cell-surface proteins associated with uPAR, we washed human corneal fibroblasts with PBS and cell surface proteins were biotinylated with sulfo-NHS-LC-biotin (EZ-Link; Pierce) for 15 minutes on ice. Cultures were washed twice with PBS and lysed in 0.5% Triton X-100, 150 mM NaCl, 10 mM Tris, 1 mM MgCl2, 1 mM CaCl2, 3 mM NaN3, 3 mM NaF, EDTA-free, and phenylmethylsulfonyl fluoride.
**RESULTS**

**uPA Stimulates uPA/uPAR Distribution over the Fibroblast Cell Surface**

uPA and uPAR were immunodetected in corneal fibroblasts. When we fixed cells with p-formaldehyde without prior uPA addition and immunodetected for uPA and uPAR, large aggregates (∼200 μm²) of uPA/uPAR were detected at the tips of cells and occasionally in more central locations (Figs. 1A, 1D; arrows), with small aggregates (∼0.05 μm²) also visible on the cell surface (Fig. 1A, arrowhead). In contrast, when we added uPA before p-formaldehyde fixation (Figs. 1B, 1E), uPA or uPAR were immunodetected only in small aggregates dispersed over the cell’s surface. The small aggregates also delineated the cell’s border. Figure 1 illustrates the great variety of size and shape that human corneal fibroblasts assume in culture and these differences, are not dependent on addition of uPA.

**uPA-Induced uPAR Immunostaining Pattern Does Not Require Protease Activity**

To determine whether uPAR’s dispersal into small aggregates required the protease activity of uPA in addition to ligand binding, we used DFP-uPA, which binds to uPAR but lacks protease activity. Like uPA, addition of the enzymatically inactive DFP-uPA yielded small uPA/uPAR aggregates (Fig. 1C). Because small aggregates still form in the absence of protease activity, we conclude that it is uPA/uPAR ligation, not uPA’s protease activity, that leads to their formation.

**Fixation Protocols that Reveal uPAR’s Cytoskeletal Association**

Fixation and permeabilization with methanol precipitates structurally bound proteins, while releasing membrane lipid-associated proteins and peripheral membrane proteins. p-Formaldehyde fixation followed by Triton X-100 has a similar effect, because Triton permeabilization solubilizes membrane lipids and membrane proteins that are not cross-linked to the cytoskeleton by p-formaldehyde. Of note, methanol fixation and p-formaldehyde fixation followed by Triton permeabilization resulted in the absence of large immunodetectable uPA/uPAR aggregates (compare Fig. 1A with Figs. 2A, 2C). Instead, small dots were visualized throughout the cytoplasm, and some were aligned in stress-fiber-like patterns. These data support the contention that the large aggregates seen in Figure 1A were uPA/uPAR complexes not tethered to cytoskeletal components. The absence of cytoskeletal anchorage may explain why these uPA/uPAR complexes could be aggregated by antibody, even after fixation with p-formaldehyde. Treatment with uPA before fixation and permeabilization increased the amount of uPA (and uPAR) seen in focal adhesions, as previously described (Figs. 2B, 2D). The focal adhesion is a site in which a molecule such as uPAR, which is limited to the extracellular leaflet of the membrane bilayer, is connected to the intracellular actin cytoskeleton by integrin mediation (described later).

**uPAR Localization after Stripping Cell Surface Endogenous uPA**

We developed a protocol to visualize newly occupied uPAR that is independent of immunocytochemical antibody detec-
tion, thereby avoiding antibody-induced cross-linking of uPA/ uPAR molecules. Cells were incubated with low-pH glycine (see the Methods section) to strip endogenously produced uPA from its receptor.\textsuperscript{17,22} To these stripped cells, we added uPA conjugated to FITC. We directly visualized the distribution of uPA-FITC and observed that the addition of uPA produced a dramatic effect on cell morphology. uPA-FITC addition to stripped cells produced uPA-containing fingerlike projections at the cell’s perimeter rather than typical lamellipodia (Fig. 3A). The arrows in Figure 3 indicate the cell’s extensions, and the arrowheads indicate the border of the “retracted” plasma membrane. Low-pH glycine alone did not cause cell border retraction or filopodial extension; this motility required the addition of uPA (with or without FITC). By live-cell confocal microscopy, we confirmed that the filopodial projections were the result of greatly stimulated motility as shown by both cell border extension and retraction (data not shown). Antibody to uPA immunodetected the same linear distribution as was visualized with uPA-FITC (Fig. 3D). This indicates that the uPAR bound by uPA-FITC was immobilized, thereby preventing the antibodies from cross-linking the complex into large aggregates (Figs. 3C, 3D).

We used Western blot detection to confirm that uPA-FITC bound to uPAR. Cells were incubated with uPA-FITC, followed by cross-linking, detergent solubilization, separation of proteins by gel electrophoresis, and transfer to nitrocellulose. uPA-FITC bound to the cell surface was detected with an anti-FITC antibody (Fig. 3B, lane 1). The most prominent bands were observed at 100- and 70-kDa and correspond to uPAR bound to uPA, and uPAR bound to the A chain of uPA, respectively, as determined by Estreicher \textit{et al}.\textsuperscript{18} using \textsuperscript{125}I-uPA. Minor bands were also observed, suggesting that uPA binds to other cell surface proteins as well as to uPAR. Unlabeled uPA competitively inhibited the uPA-FITC binding (lane 2), confirming the authenticity of the uPA-FITC-labeled bands.

\textbf{Immobilized uPAR Is Anchored to the Actin Cytoskeleton}

F-actin stress fibers in filopodial extensions of stripped cells colocalized with uPA-FITC in both the retracted cell border and the extensions (Figs. 4A, 4B, overlay). To confirm that the extensions included cytoplasm confined by the plasma membrane, fibroblasts were incubated with CMFDA, a vital dye that labels the cytosol of living cells. Indeed, CMFDA was visualized in the cell body as well as in the filopodial extensions (Fig. 4C, and enlargement). We also asked whether uPA/uPAR was associated with microtubules. We detected both uPA and tubulin in the retracted cell body, but only uPA in the extensions (Figs. 4D, 4E). Because the extensions represent the actively motile part of the cell, uPA induced alignment of uPAR with F-actin in these extensions supports the hypothesis that uPAR is anchored to the actin cytoskeleton during uPA-induced cell migration.

We further examined the interaction of uPA with actin by disrupting the cytoskeleton with 100 nM cytochalasin D. This concentration was chosen after preliminary experiments with 10 to 1000 nM indicated that at 100 nM, cell attachment and spreading were maintained, but few organized F-actin stress fibers were detectable by rhodamine-phalloidin staining (Fig. 5D). In noncytochalasin-treated cells, as expected, uPA dispersed the uPA/uPAR complex into small aggregates (Fig. 5A). However, when cells were pretreated with cytochalasin D, uPA addition did not yield small aggregates. Only large aggregates were visualized (Figs. 5B, 5C) that were identical with those observed when cells were immunodetected without uPA treatment (Figs. 1A, 1D). These correspond to large regions lacking F-actin (Figs. 1A, 1D, arrows). Actin treadmills between polymerized F-actin and depolymerized G-actin. Cytochalasin D binds to G-actin and prevents the assembly of F-actin. Thus, in the presence of cytochalasin D, the potential for uPA/uPAR to anchor to F-actin is greatly reduced. These observations are...
uPA-FITC binding provides direct visualization of activated uPAR. (A) Fibroblasts were stripped of cellsurface uPA with glycine (pH 3.0) followed by neutralization and incubation with uPA-FITC for 10 minutes before p-formaldehyde fixation. The uPA-FITC was visualized in linear arrays (arrow). Arrowhead: retracted plasma membrane. Wide-field fluorescence microscopy. (B) uPA uPA-FITC was added to unfixed fibroblasts. After cross-linking, lysis, and separation by SDS-PAGE, the proteins were transferred to nitrocellulose and detected with anti-FITC (lane 1). uPA-FITC bound to fibroblasts was detected at 100 kDa, representing uPA-FITC bound to uPA and at 70 kDa, representing the A domain of uPA-FITC bound to uPAR (arrows). Pretreatment of cells with unlabeled uPA inhibited uPAR binding by uPA-FITC (lane 2), as indicated by the greatly diminished detection by anti-FITC. Control for anti-FITC antibody (lane 3). uPA-FITC (1.5 μg) diluted in sample buffer was subjected to SDS-PAGE and was detected at 55 kDa, as expected (arrowhead). (C, D) uPA/uPAR localization as visualized by uPA-FITC and immunodetection on the same cell. (C) uPA-FITC was visualized in small aggregates in linear arrays as in (A) after stripping and incubation with uPA-FITC before p-formaldehyde fixation. (D) The distribution of uPA immunodetected with anti-uPA colocalized with uPA-FITC. Arrows, linear arrays; arrowheads, retracted cytosol. Confocal microscopy. Bar, 20 μm.

When we added exogenous uPA to migrating cells before fixation, in contrast to the detection of large aggregates, the uPA/uPAR complex was dispersed in small aggregates over the entire cell including the lamellipodia (Fig. 6C, arrow). Furthermore, stress fibers were readily detected in the lamellipodia of the uPA-treated migrating cells (Fig. 6D, arrow), compared with the barely visible stress fibers in Figure 6B. Our findings demonstrate that in corneal fibroblasts, uPA/uPAR can polarize to the front of a migrating cell, but that the binding of new, exogenous uPA distributes the complex over the entire cell surface. In situ, this distribution would localize protease activity throughout the fibroblast-matrix interface. Furthermore, uPA addition promotes F-actin assembly in the extending lamellipodia, thereby enhancing the contractile apparatus used in migration.

**Integrin αvβ3 and uPAR Colocalize in Focal Adhesions**

Because GPI-linked proteins lack cytoplasmic domains capable of directly binding to cytosolic molecules, including actin, GPI-linked proteins must associate with transmembrane proteins, such as integrins, to initiate intracellular signaling. When we examined the distribution of integrins previously found to associate with uPAR in other cell types. For these studies, we used cells migrating into a scrape wound. First, we confirmed that uPA could be colocalized with vinculin, a focal adhesion protein (Fig. 7A). In focal adhesions at the front of the cell, we found that uPAR and β3 were colocalized (Figs. 7B, 7C, inset). In contrast, α5β1 appeared to elongated focal adhesions that were not in the lamellipodia and did not colocalize with uPA/uPAR (Fig. 7D). These divergent localizations suggest that αvβ3 plays a role in uPA-associated corneal fibroblast migration and α5β1 does not.
A similar pattern of \( \alpha v \beta 3 \) segregating into focal contacts and \( \alpha 5 \beta 1 \) into linear streaks was described for smooth muscle cells migrating into a scrape wound. Because uPAR colocalized with \( \alpha v \beta 3 \), next we determined whether they are physically associated. Fibroblast surface proteins were labeled with membrane impermeable biotin, the cells were solubilized, and the lysate immunoprecipitated with antibodies to \( \beta 3 \), uPAR, or \( \alpha 5 \beta 1 \). The coimmunoprecipitated surface proteins were detected with streptavidin-HRP, and the results are shown in Figure 7E. An antibody to \( \beta 3 \) (lane 1) immunoprecipitated \( \alpha v \beta 3 \) and a complex banding pattern of surface proteins. Immunoprecipitation with an antibody to uPAR revealed a similar pattern of biotinylated proteins. uPAR is a glycosylated protein, detected as bands between 50 to 65 kDa. Other molecular weight uPAR-containing species are detectable also, such as uPAR bound to uPA or to the uPA inhibitor PAI-1/2. This uPAR banding pattern was corroborated independently by Western blot detection (Fig. 7F), using a different antibody from the one used for immunoprecipitation. Thus, the similar banding patterns in both the \( \beta 3 \) and uPAR immunoprecipitation suggest that these proteins are physically associated in the plasma membrane. However, it was surprising that bands representing \( \alpha v \beta 3 \) were not observed (Fig. 7E, lane 2), whereas two other high molecular weight bands were identified (Fig. 7E, lane 2, stars). It was conceivable that the anti-uPAR antibody did not recognize uPAR that was bound to \( \beta 3 \). In such a \( \beta 3 \)-uPAR complex, the anti-uPAR recognition site may be sequestered. To test this, we performed sequential immunoprecipitation. Lysates were incubated with anti-uPAR antibody to remove uPAR that readily bound to anti-uPAR, and then, the remaining supernatant was immunoprecipitated with anti-\( \beta 3 \) in hopes that this would bring down the \( \beta 3 \)-uPAR complex. A 5-minute chemiluminescence exposure revealed a fraction of uPAR that coimmunoprecipitated with \( \beta 3 \) even after uPAR was removed from the lysate with anti-uPAR antibody (Fig. 7E, lane 3). We therefore
conclude that the uPAR antibody (lane 2) did not immunoprecipitate uPAR that was bound to β3, whereas the β3 antibody recognized β3 that was bound to uPAR (Fig. 7E, lanes 1 and 3). The sensitivity of the biotin-streptavidin system was necessary to demonstrate that uPAR immunoprecipitated with integrin, and the final identification was by characteristic molecular weight. This is in contrast to reports of cancer cells in which both uPAR and integrins are pathologically overexpressed\(^8\) and in which the immunoprecipitated proteins can be identified by Western blot. Finally, as predicted by the lack of colocalization of α5β1 and uPA, anti-α5β1 did not immunoprecipitate uPAR (Fig 7E, lane 4).

**DISCUSSION**

Here we report that in corneal fibroblasts, activated uPA/uPAR complex is anchored to and aligns with F-actin. Thus, in addition to localization at focal adhesions, as has been reported in other cells,\(^{20}\) we found that uPA/uPAR was dispersed extensively over the cell’s surface. These observations change the paradigm for uPA’s enzymatic activity: Whereas it was previously viewed that uPA/uPAR was restricted to focal adhesions and to the front of the cell, we propose that uPA/uPAR is distributed over much of the cell’s surface where uPA interfaces with the extracellular matrix as the fibroblast migrates through the stroma. This uPA/uPAR distribution localizes uPA’s proteolytic activity at the extensive cell-matrix interface where it can promote degradation of matrix and activation of latent growth factors and of MMPs sequestered in the matrix.

Although wounding has been known to upregulate uPA in the epithelium of the cornea,\(^{26–28}\) our current report provides the first information regarding the expression of uPA/uPAR in the stromal cells which is crucial for developing models of...
uPA/uPAR functions in cell migration, proteolysis, and signal transduction during wound healing.

Exogenous uPA Leads to uPAR-Cytoskeletal Interaction

GPI-linked proteins like uPAR, are typically found in glycolipid-cholesterol-enriched lipid rafts of plasma membranes. Similarly, uPAR and β3 were immunodetected and colocalized (yellow, arrows) in the inset (uPAR, red and β3, green; arrows). (A) uPA (green) and vinculin (red) were immunodetected and were found to colocalize (yellow, arrow). Similarly, (B) uPAR and (C) β3 were immunodetected and colocalized (yellow, arrowheads) in the inset (uPAR, red and β3, green; arrows). (D) In contrast to β3, α5β1 did not localize to the lamellipodia where we had detected β3 with uPA/uPAR. Arrowheads: uPA (green); arrows: α5β1 (red), which were detected in different focal adhesions. Wide-field fluorescence microscopy. Physical association of uPAR and integrin β3 was detected by coimmunoprecipitation. (E) Fibroblast surface protein was biotinylated. Triton X-100 soluble protein was immunoprecipitated with anti-β3 (lane 1), anti-uPAR (R2 Ab; lane 2), anti-α5β1 (lane 4). The supernatant from the anti-uPAR immunoprecipitation was subsequently immunoprecipitated by anti-β3 (lane 3). Immunoprecipitated biotin-labeled proteins were detected with streptavidin-HRP. Lanes 1, 2, and 3: uPAR was visualized as two bands between molecular masses 40 and 60 kDa (arrows). Lane 4: α5β1 only. (F) uPAR was immunodetected (using 399RAb) in a Western blot of 50 μg of Triton-X-100 soluble lysate. Bar (A): 20 μm.

uPA/uPAR functions in cell migration, proteolysis, and signal transduction during wound healing.

Figure 7. uPA/uPAR colocalized in focal adhesions with vinculin and β3 integrin. uPA was added for 10 minutes before methanol fixation (as in Fig. 2B) of confluent fibroblasts that were scrape-wounded and incubated for 4 hours. (A) uPA (green) and vinculin (red) were immunodetected and were found to colocalize (yellow, arrow). Similarly, (B) uPAR and (C) β3 were immunodetected and colocalized (yellow, arrowheads) in the inset (uPAR, red and β3, green; arrows). (D) In contrast to β3, α5β1 did not localize to the lamellipodia where we had detected β3 with uPA/uPAR. Arrowheads: uPA (green); arrows: α5β1 (red), which were detected in different focal adhesions. Wide-field fluorescence microscopy. Physical association of uPAR and integrin β3 was detected by coimmunoprecipitation. (E) Fibroblast surface protein was biotinylated. Triton X-100 soluble protein was immunoprecipitated with anti-β3 (lane 1), anti-uPAR (R2 Ab; lane 2), anti-α5β1 (lane 4). The supernatant from the anti-uPAR immunoprecipitation was subsequently immunoprecipitated by anti-β3 (lane 3). Immunoprecipitated biotin-labeled proteins were detected with streptavidin-HRP. Lanes 1, 2, and 3: uPAR was visualized as two bands between molecular masses 40 and 60 kDa (arrows). Lane 4: α5β1 only. (F) uPAR was immunodetected (using 399RAb) in a Western blot of 50 μg of Triton-X-100 soluble lysate. Bar (A): 20 μm.
uPA that generated the small aggregates was identical with the $K_d$ for specific binding of uPA to uPAR on HeLa cells (0.4 nM uPA),18 whereas a lower concentration (0.1 nM uPA) did not. Second, the same small aggregates were observed along the length of the F-actin fibers in cells exposed to uPA whether visualized by FITC bound to uPA or by immunodetection.

At first glance, there seems to be a paradox, because endogenous uPA is immunodetected in large aggregates, but uPA added exogenously at a concentration of 0.4 nM or greater, favors a shift of uPA/uPAR from large to small aggregates. One possibility is that the higher concentration of uPA represents the threshold required for actin-based signaling. Alternatively, the large aggregates may represent a form of uPAR that is no longer in signaling mode, as when PAI-1 binds to uPA that is bound to uPAR. PAI-1–binding precedes endocytosis of the uPA/uPAR complex.54,55; thus, antibodies could cross-link these untethered uPA/uPAR/PAI-1 complexes into large aggregates. Last, it is possible that at this concentration, uPA binds not only to uPAR but also to other distinct cell-surface receptors. In this regard, a lower-affinity second binding site for uPA has been identified on the surface of smooth muscle cells, melanoma cells, and leukocytes.45–50 These may be the additional bands detected by uPA–FITC binding (Fig. 5B).

The Role of Integrins in uPAR Signaling
Binding of uPA to uPAR initiates a signal-transduction cascade including activation of FAK and the MAPK pathway in cancer and endothelial cells,6,8 and the Jak/Stat pathway in vascular endothelial and smooth muscle cells.7,59,60 Because uPAR is a GPI-linked protein, it lacks a transmembrane domain and therefore cannot interact directly with intracellular proteins to generate intracellular signaling. In contrast, integrins are transmembrane proteins, binding both extracellular matrices and intracellular cytosolic molecules that connect integrins to the actin cytoskeleton.41 In various cell types, uPAR has been found to associate with $\beta_1$, $\beta_2$, or $\beta_3$ integrins, as demonstrated by resonance energy transfer,10 immunoprecipitation,8 and uPAR/integrin binding and adhesion studies.11–14 These reports have led to the hypothesis that uPA binding to uPAR generates an intracellular signal through an integrin-mediated pathway resulting in cell movement.

We investigated whether the transmembrane integrin plays a role in associating the GPI-linked uPAR to the actin cytoskeleton in the human corneal fibroblasts (see model, Fig. 8C). By immunocytochemistry, we colocalized uPAR with $\beta_3$ in focal adhesions and by immunoprecipitation showed that they are associated after lysis. Thus, $\beta_3$ integrins are likely to be at least one of the transmembrane proteins connecting uPAR to the actin cytoskeleton. A study by Fujiwar et al.42 reported that transmembrane proteins may act as the “pickets” in the actin-based membrane-cytoskeletal “fence” that demarcate compartments in the plasma membrane.62 These compartments are rich in membrane phospholipids and GPI-linked proteins like uPAR.43 By measuring single molecule diffusion rates, they determined that the median distance between pickets is ~750 nm.62 The average diameter we measured for small aggregates was approximately 240 nm, with a clear ring of empty space around each (ref to Fig. 1B, enlargement) and is thus consistent with uPAR being cross-linked to nearby transmembrane pickets.

uPA in Signal Transduction
uPA/uPAR ligation activates a variety of intracellular signaling molecules in different cell types. We used chemical inhibitors to many of these pathways and expected to inhibit the ability of uPAR to engage the actin cytoskeleton (data not shown). Only cytochalasin D, which disrupts F-actin assembly, prevented the uPA-induced attachment of uPAR to the cytoskeleton. In endothelial cells, cytochalasin D abolishes the uPA-stimulated phosphorylation of FAK and decreases uPA-stimulated ERK1/2 activation.6 This finding is consistent with Tang’s suggestion that a functional link between the cytoskeleton and uPAR is required for downstream uPA-induced signaling.

CONCLUSION
Previous studies showed that uPA promotes an increase in uPAR-mediated cellular adhesion by binding to vitronectin an extracellular matrix protein.24,44–46 Thus, uPA and uPAR are involved in both cell adhesion and, as previously discussed, cell movement. These apparently contradictory actions may indicate that uPA and uPAR participate in the full cycle of matrix attachment and release that is required for migration. Future studies will define coordination of the pathways that regulate uPA’s multiple actions. For example, based on recent reports that small guanosine triphosphatases (GTPases; RhoA or Rac) were activated by adding uPA or overexpressing uPAR,47,48 it is plausible that RhoA, which is involved in stress fiber reorganization and cell migration, may regulate the cycle of adhesion and release. Finally, understanding the association between uPA and its impact on actin bundling and severing proteins will
uncover more about uPA’s control over cell adhesion and migration.

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References
46. Sidenius N, Blasi F. Domain 1 of the urokinase receptor (uPAR) is required for uPAR-mediated cell binding to vitronectin. FEBS Lett. 2000;470:40–46.