Direct Binding of Occupied Urokinase Receptor (uPAR) to LDL Receptor-related Protein Is Required for Endocytosis of uPAR and Regulation of Cell Surface Urokinase Activity

Ralf-Peter Czekay,^{*‡} Thomas A. Kuemmel,^{*} Robert A. Orlando,[†] and Marilyn Gist Farquhar^{*‡}

Departments of ^{*}Cellular and Molecular Medicine and [†]Pathology University of California, San Diego, La Jolla, California 92093-0651

Submitted September 11, 2000; Revised December 27, 2000; Accepted March 1, 2001
Monitoring Editor: Monty Krieger

Low-density lipoprotein receptor-related protein (LRP) mediates internalization of urokinase: plasminogen activator inhibitor complexes (uPA:PAI-1) and the urokinase receptor (uPAR). Here we investigated whether direct interaction between uPAR, a glycosyl-phosphatidylinositol-anchored protein, and LRP, a transmembrane receptor, is required for clearance of uPA:PAI-1, regeneration of unoccupied uPAR, activation of plasminogen, and the ability of HT1080 cells to invade extracellular matrix. We found that in the absence of uPA:PAI-1, uPAR is randomly distributed along the plasma membrane, whereas uPA:PAI-1 promotes formation of uPAR-LRP complexes and initiates redistribution of occupied uPAR to clathrin-coated pits. uPAR-LRP complexes are endocytosed via clathrin-coated vesicles and traffic together to early endosomes (EE) because they can be coimmunoprecipitated from immunoisolated EE, and internalization is blocked by depletion of intracellular K⁺. Direct binding of domain 3 (D3) of uPAR to LRP is required for clearance of uPA:PAI-1-occupied uPAR because internalization is blocked by incubation with recombinant D3. Moreover, uPA-dependent plasmin generation and the ability of HT1080 cells to migrate through Matrigel-coated invasion chambers are also inhibited in the presence of D3. These results demonstrate that GPI-anchored uPAR is endocytosed by piggy-backing on LRP and that direct binding of occupied uPAR to LRP is essential for internalization of occupied uPAR, regeneration of unoccupied uPAR, plasmin generation, and invasion and migration through extracellular matrix.

INTRODUCTION

The urokinase plasminogen activator (uPA):plasmin system is involved in enhancing cell migration and invasion (Cohen *et al.*, 1991; Reiter *et al.*, 1993) during embryogenesis, wound healing, and metastasis (reviewed by Andreasen *et al.*, 1994; Dano *et al.*, 1985). Our current understanding of the uPA system is that uPA is activated upon binding to its cell surface receptor, uPAR (Cubellis *et al.*, 1989), a glycosyl-phosphatidylinositol (GPI)-anchored membrane protein

(Nielsen *et al.*, 1988; Ploug *et al.*, 1991) with three homologous extracellular domains (D1, 2, and 3) (Behrendt *et al.*, 1991). After activation, uPA catalyzes the conversion of plasminogen to plasmin (Ellis *et al.*, 1989), which has a broad substrate specificity and is able to degrade many extracellular matrix proteins (Moser *et al.*, 1993). uPA-dependent plasminogen activation is inhibited by specific uPA inhibitors with the best studied being plasminogen activator inhibitor type-1 (PAI-1) (Blasi *et al.*, 1987; Saksela and Rifkin, 1988). Inactive uPA:PAI-1 complexes are rapidly internalized by the LDL receptor-related protein (LRP) (Herz *et al.*, 1988, 1992) and degraded in lysosomes (Olson *et al.*, 1992; Conese *et al.*, 1995). Clearance of uPA:PAI-1 by LRP is accompanied by decreased uPAR at the cell surface, suggesting that uPAR is also internalized by LRP (Herz *et al.*, 1992; Olson *et al.*, 1992; Williams *et al.*, 1994; Conese *et al.*, 1995). It has also been demonstrated that when binding of uPA:PAI-1 to LRP is prevented with antibodies against LRP, cell surface plas-

[‡] Corresponding author. E-mail address: rpczekay@scripps.edu.
Abbreviations used: α 2M, α 2-macroglobulin; D2, domain 2 of uPAR; D3, domain 3 of uPAR; EE, early endosomes; LDL, low density lipoprotein; LRP, LDL receptor-related protein; PAI-1, plasminogen activator inhibitor type-1; PM, plasma membrane; Tf, transferrin; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor.

minogen activity is reduced, presumably by delaying internalization of uPAR and preventing regeneration of unoccupied uPAR capable of binding uPA (Zhang *et al.*, 1998).

Although this is the widely accepted model for regulation of the uPA system, the detailed mechanisms of how uPAR and LRP cooperate in clearance of uPA:PAI-1 as well as the trafficking itinerary of uPAR are not well understood. For example, it is not clear whether uPAR directly interacts with LRP or whether uPA:PAI-1 bridges uPAR and LRP. Moreover, the pathway by which uPAR is internalized has not yet been established.

LRP, like other members of the LDL receptor family, is internalized via clathrin-coated pits (Herz *et al.*, 1988; Chen *et al.*, 1990; Krieger and Herz, 1994), whereas uPAR, a GPI-anchored protein, has been shown to be distributed randomly along the plasma membrane (PM) under steady-state conditions (Conese *et al.*, 1995; Maxfield and Mayor, 1997; Nykjaer *et al.*, 1997).

To achieve a better understanding of the interplay between uPAR and LRP in regulation of cell surface plasminogen activator activity, we have investigated the interaction between uPAR and LRP and the trafficking of uPAR and LRP in HT1080 cells, a human fibrosarcoma cell line. We demonstrate here that uPA:PAI-1 initiates direct binding of domain 3 (D3) of uPAR to LRP on the PM and redistribution of uPAR to clathrin-coated pits and that this interaction is necessary for cointernalization of the two receptors into early endosomes (EE). We also show that binding of uPAR to LRP is essential for clearance of uPA:PAI, regeneration of unoccupied uPAR, and plasmin generation at the cell surface.

MATERIALS AND METHODS

Reagents

Human two-chain-uPA was purchased from American Diagnostica (Greenwich, CT) and human PAI-1 from Calbiochem (San Diego, CA). Holo-transferrin (Tf) and CHAPS were obtained from Sigma (St. Louis, MO), protein G-agarose from Amersham Pharmacia Biotech (Piscataway, NJ), and the thiol cleavable cross-linker 3,3'-di-thiobis [sulfo succinimidyl-propionate] (DTSSP) from Pierce (Rockford, IL). Chromozym PL was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Antibodies

Mouse mAb against human uPAR was provided by Dr. Marc Shuman (University of California, San Francisco). A rabbit polyclonal antibody against recombinant soluble human uPAR₁₋₂₇₄ (465) was generated as previously described (Orlando and Farquhar, 1993). Anti-LRP (456) was raised in rabbits against human LRP (Orlando and Farquhar, 1994), and anti-LRP (1073) was raised against a 14 amino acid peptide from the cytoplasmic tail of human LRP (Czekay *et al.*, 1995). Mouse mAb against the ectodomain of human LRP (8G1) was obtained from Dr. Dudley Strickland (Holland Laboratory, American Red Cross, Rockville, MD). A hybridoma cell line expressing mAb 11H4 (recognizes the cytoplasmic tail of human LRP) was obtained from American Type Culture Collection. Polyclonal antisera against the cytoplasmic tail of human transferrin (TF) receptor and human uPA were obtained from Dr. Ian Trowbridge (Salk Institute) and Oncogene (Cambridge, MA), respectively. Affinity-purified chicken polyclonal antibodies against a peptide from the N terminus of human caveolin-1 (Stan *et al.*, 1997) were provided

by Dr. Radu V. Stan (University of California, San Diego). Affinity-purified mAb against AP-2 α -adaptin (Beck *et al.*, 1992) was provided by Dr. Sandra Schmid (Scripps Research Institute). HRP-coupled donkey anti-rabbit or anti-mouse (H+L) IgG, depleted of cross-reactivity, was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell Culture

Normal rat kidney (NRK) cells and human fibrosarcoma HT1080 cells were purchased from American Type Culture Collection and cultured according to their instructions. Cells were grown to confluency over 3–6 d.

Expression and Purification of Recombinant Soluble uPAR and Domains 2 and 3 of Human uPAR

uPAR cDNA (IMAGE Consortium, accession number T75241) encoding amino acids 1–274 (lacking the GPI-anchor site), D2 (encoding amino acids 92–174), and D3 cDNAs (encoding amino acids 192–274) were amplified by PCR (primers available on request) and subcloned into pET28b (*EcoRI/HindIII*; Novagen, Madison, WI). *Escherichia coli* strain BL21-DE3 expressing soluble uPAR₁₋₂₇₄ and the D2 and D3 domains of uPAR were grown and induced as described (Orlando and Farquhar, 1994). Bacteria were pelleted by centrifugation ($2500 \times g$, 10 min, 4°C), resuspended in TBS (20 mM Tris, pH 7.4, 150 mM NaCl) containing 100 μ g/ml lysozyme, and lysed in TBS containing 3% *N*-lauroyl sarcosine. Lysates were cleared by centrifugation ($15,000 \times g$, 20 min, 4°C) and supernatants were incubated with Ni²⁺-affinity resin (ProBond; Invitrogen, Carlsbad, CA). Bound uPAR₁₋₂₇₄, D2, and D3 were eluted with TBS, 0.1% *N*-lauroyl sarcosine, 500 mM imidazole, and dialyzed against TBS, 0.1% *N*-lauroyl sarcosine.

Immunofluorescence

For immunofluorescence cells were grown to ~75% confluence on glass coverslips, fixed for 20 min with 2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, quenched in PBS containing 0.1% BSA and 10 mM glycine for 30 min as described (McCaffery and Farquhar, 1995), and permeabilized with 0.05% Triton X-100 in PBS for 3 min. Cells were then incubated with primary rabbit, mouse, or chicken antibodies for 1 h followed by incubation with appropriate Alexa 594- or Alexa 488-conjugated goat anti-rabbit, anti-mouse, or anti-chicken IgG (Molecular Probes, Eugene, OR). In some cases anti-uPAR antibodies were bound to the surface at 4°C in the presence or absence of D3 before fixation (Myohanen *et al.*, 1993). Cells were examined by deconvolution microscopy with the Applied Precision DeltaVision imaging system coupled to an inverted Nikon SE 200 fluorescence microscope. For cross-sectional images of cells, stacks of at least 50 sections (150-nm step width) of raw image data were obtained to optimize reconstruction of the center plane image. Deconvolution was done on a SGI workstation (UNIX) using DeltaVision reconstruction software, and images were processed as TIFF files, pseudocolored, and superimposed using Photoshop 5.0 (Adobe Systems, Mountain View, CA).

Immunoblotting and Immunoprecipitation

For immunoblotting, cell lysates were prepared in 10 mM CHAPS in buffer A (20 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM CaCl₂) and processed for SDS-PAGE (Laemmli, 1970), immunoblotting, and detection by enhanced chemiluminescence (Pierce) as described (Czekay *et al.*, 1997). Biotinylated proteins were detected using HRP-avidin (ABC-Biotinylation kit; Vector Laboratories, Burlingame, CA).

For immunoprecipitation, cell lysates were incubated with mAbs against either uPAR (3.9 μ g) or LRP (2.6 μ g), mixed with protein

G-agarose beads (20 μ l/1 ml of cell lysate) for 16 h at 4°C, and processed for SDS-PAGE, followed by autoradiography or immunoblotting and densitometry.

Radioiodination

Holo Tf, α 2M, uPAR₁₋₂₇₄, and D3 were radioiodinated with Na-¹²⁵I (PerkinElmer Life Science Products, Boston, MA) using Iodo-Beads (Pierce). Specific activities were 4994, 1861, 3550, and 9821 cpm/ng, respectively. Cell surface proteins were radioiodinated using the lactoperoxidase method (Czekay *et al.*, 1995).

Purification, Activation, and Degradation of α 2M

α 2-Macroglobulin (α 2M) was purified from pooled citrated human plasma (San Diego Blood Bank, San Diego, CA) as described (Kurecki *et al.*, 1979) except that elution buffer was modified to 0.01 M Na-acetate and 0.15 M NaCl, pH 5. Purified α 2M was activated for receptor binding with 0.4 M methylamine in 0.1 M Tris-HCl, pH 8, for 2 h at room temperature and unbound methylamine removed by passage over a PD-10 column (Amersham Pharmacia Biotech).

Cells were incubated at 37°C with ¹²⁵I-labeled α 2M (2 nM) for 8 h in the presence or absence of unlabeled α 2M (40 nM) in DMEM, 1 mg/ml BSA, 10 mM HEPES, pH 7.4. Medium was removed after 0, 2, 4, and 8 h, microfuged for 2 min, and supernatants were processed for trichloroacetic acid (TCA) precipitation and gamma counting (Czekay *et al.*, 1997). Degradation was calculated as TCA-soluble cpm divided by specific activity of the radioiodinated ligand (Czekay *et al.*, 1997) normalized to total cellular protein (BCA Protein Assay; Pierce).

Cell Surface Biotinylation

Cell monolayers were acid-washed in isoosmolar buffer B (50 mM glycine-HCl, pH 3.0, 100 mM NaCl) for 3 min at 4°C to release endogenous bound uPA from surface uPAR (Cubellis *et al.*, 1989). After washing (twice in buffer B and twice in ice-cold PBS), surface proteins were biotinylated with 400 μ M Biotin-XX (Molecular Probes) on ice for 35 min, and the reaction was quenched with 20 mM glycine in PBS, pH 7.4, for 15 min.

Cell Surface Binding and Uptake of Radioiodinated Tf and α 2M

Cells were incubated with ¹²⁵I-labeled holo-Tf (200 ng/ml) at 4°C (to radiolabel the cell surface pool of Tf receptor) and 18°C (to allow uptake of ¹²⁵I-labeled Tf into EE) (Czekay *et al.*, 1997) after which the cells were acid-washed to release ¹²⁵I-labeled Tf bound to Tf receptors at the cell surface (Czekay *et al.*, 1997).

¹²⁵I-labeled α 2M (2 nM) was bound to cells for 4 h at 4°C in the presence or absence of varying amounts of unlabeled α 2M (0.06–40 nM), rinsed, and cell-associated radioactivity was quantified by gamma counting and normalized to total cellular protein (BCA Protein Assay; Pierce). Binding affinities (apparent K_D) for ¹²⁵I-labeled α 2M were determined as the concentration of unlabeled α 2M which resulted in 50% inhibition of binding. Specificity was determined as the difference between total binding (without competition) and nonspecific binding (noncompetable). The amount of bound ligand was calculated as cpm divided by the specific activity of ¹²⁵I-labeled α 2M.

Cross-linking Experiments

¹²⁵I-labeled, recombinant uPAR₁₋₂₇₄ (25 nM) was bound to HT1080 cells in serum-free DMEM (containing 2% BSA, 20 mM HEPES, pH 7.4) for 2 h at 4°C in the presence or absence of unlabeled recom-

binant uPAR₁₋₂₇₄ (2.5 μ M) or the D2 (1.25 μ M) or D3 (1.25 μ M) domains of uPAR. Cells were washed and bound proteins were cross-linked using the thiol cleavable cross-linker DTSSP (1 mM) (Pierce) for 20 min at 4°C. The reaction was quenched with TBS for 10 min at 4°C, cell lysates were prepared in 10 mM CHAPS, and immunoprecipitation was carried out with anti-LRP mAb (11H4).

Binding of ¹²⁵I-uPAR₁₋₂₇₄ or ¹²⁵I-D3 to Purified LRP

Placental tissue (Scripps Clinic, La Jolla, CA) was finely minced, homogenized in buffer A (containing 10 μ g/ml aprotinin, 0.5 mM PMSF) by using a TissueMizer (Tekmar, Cincinnati, OH) for 3 min, and centrifuged (15,300 $\times$ g, 30 min) at 4°C. Placental proteins were extracted with 10 mM CHAPS in buffer A for 2 h, and LRP was affinity purified on mAb 11H4 (5 mg) prebound to protein G-Sepharose beads (Oncogene). The bound material contained only LRP when analyzed by SDS-PAGE and silver staining.

Affinity-purified immobilized LRP was incubated with either ¹²⁵I-uPAR₁₋₂₇₄ (100 nM) or ¹²⁵I-D3 (50 nM) in the presence or absence of unlabeled uPAR₁₋₂₇₄ (4 μ M), D2 (1.5 μ M), or D3 (1.5 μ M), and radioiodinated proteins were visualized by autoradiography followed by densitometry or PhosphorImager analysis (Czekay *et al.*, 1995).

Internalization of uPAR after Binding uPA:PAI-1 and Cell Fractionation

uPA:PAI complexes were formed by incubation of PAI-1 (5 μ M) with two-chain uPA (100 nM) for 1 h at room temperature as described (Cubellis *et al.*, 1989). Cells were acid-washed, biotinylated (to specifically label the surface pool of uPAR), and incubated with uPA:PAI-1 (50 nM) for 1 h at 4°C or sequentially at 4 and 18°C, 1 h each, and cell fractionation was carried out as described (Czekay *et al.*, 1997). In brief, postnuclear supernatants (1 ml) were top loaded onto isoosmolar 20%-Percoll (Amersham Pharmacia Biotech, Alameda, CA), and gradients were established by centrifugation (20,000 $\times$ g, 52 min) at 4°C. Fractions (1 ml) were collected from the top of the gradient, and the distribution of biotinylated uPAR was determined by immunoprecipitation with anti-uPAR mAb followed by blotting with HRP-coupled avidin and detection by chemiluminescence. In some cases, binding and internalization were done in the presence of either 200 μ g/ml protein A-purified anti-LRP IgG (456), which blocks ligand binding to LRP (Orlando, unpublished observations), or 2.5 μ M D3 to block uPAR binding to LRP. All solutions contained Ca²⁺, which is required for ligand binding to LRP (Moestrup *et al.*, 1990). For K⁺ depletion, cells were incubated in 5 ml of hypotonic medium (1:1, DMEM/water) before uPA:PAI-1 binding followed by incubation for 30 min in isotonic K⁺-free buffer (Larkin *et al.*, 1983) at room temperature.

Binding of ¹²⁵I-uPA:PAI-1 to uPAR₁₋₂₇₄

Recombinant purified human uPAR₁₋₂₇₄ (2 nM) was incubated in PBS for 1 h at 4°C with ¹²⁵I-uPA:PAI-1 (10 nM) complexes in the presence or absence of D3 (100 nM), and samples were mixed with anti-uPAR (465) and protein G-Sepharose beads (Oncogene) for 16 h at 4°C. Bound proteins were processed for SDS-PAGE, autoradiography, and densitometry.

Immunoisolation of Early Endosomes

Postnuclear supernatants were fractionated on Percoll gradients as described above and gradient fractions 8–10 (containing EE) were pooled. mAb 11H4, specific for the C terminus of human LRP, was prebound to protein G-agarose beads in buffer B and subsequently incubated with pooled fractions 8–10 for 2 h at room temperature.

After washing, immunisolates were incubated for 1 h on ice in buffer B containing 10 mM CHAPS, beads were separated from supernatant by centrifugation, and Percoll in the supernatant was pelleted (Czekay *et al.*, 1997). Proteins released into the supernatant and those remaining bound to the agarose beads were processed for immunoblotting.

uPA Activity at the Cell Surface

Cells were acid-washed as described above, incubated with uPA: PAI-1 (10 nM) at 4°C for 1 h, and, after removing unbound ligand, at 37°C for 1 h in the presence or absence of D3 (5 µg/ml). Subsequently, active uPA (2 nM) was bound to HT1080 cells at 4°C for 1 h in the presence of anti-LRP (456), 200 µg/ml. After washing, cells were incubated in 50 mM Tris-HCl, pH 7.4, 0.1 M NaCl with 0.2 µM human plasminogen (Calbiochem) and 0.5 mM Chromozym PL (Tosyl-Gly-Pro-Lys-4-nitranilide-acetate; Roche Molecular Biochemicals), a chromogenic substrate for plasmin, according to the manufacturer's instructions. Hydrolysis of the substrate was measured at 405 nm, and absorbance was used to calculate the generation of plasmin (U/ml).

Transwell Invasion Assays

Cells were detached from tissue culture plates with PBS, pH 7.4, containing 10 mM EDTA, and resuspended in DMEM (10% fetal calf serum). Cells ($5 \times 10^5/500 \mu\text{l}$) were added to the upper compartment of Matrigel-coated invasion chambers, 8-µm pore size (Becton Dickinson, Bedford, MA) in the presence or absence of soluble uPAR₁₋₂₇₄ (2.5 µM), D3 (1.25 µM), or D3 and active uPA (8 nM). Culture medium alone (750 µl) was added to the lower part of the invasion chamber. After incubation at 37°C for 36 h, cells were removed from the upper side of the filter membrane by using cotton swabs. Filters were fixed, stained, and mounted according to the manufacturer's instructions. Cells that invaded through the Matrigel and migrated to the under side of the filter were counted. The values obtained were calculated by averaging the total number of cells from three filters per condition.

RESULTS

LRP Expressed on the Surface of HT1080 Cells Is Functionally Active

To study the interactions between uPAR and LRP we used HT1080 cells, a human fibrosarcoma cell line, known to express functional uPAR on the PM (Xue *et al.*, 1997). Because LRP expression has not been previously studied in this cell line, we first verified that it is expressed and functionally active at the cell surface. LRP was clearly detected after cell surface radioiodination of HT1080 cells followed by immunoprecipitation with anti-LRP mAb (11H4), and it comigrated with LRP from NRK cells (Herz *et al.*, 1990) (Figure 1A, inset). We then tested whether HT1080 cells are capable of binding and internalizing α2M, a well-established specific ligand for LRP. ¹²⁵I-labeled α2M bound to HT1080 cells with an apparent K_D of 0.75 nM (Figure 1A), and 42 ng of ¹²⁵I-labeled α2M/mg of total cell protein was degraded in a linear manner over 8 h (Figure 1B). Thus, the ability of HT1080 cells to bind and degrade ligand is comparable to noncancer cells (Moestrup and Gliemann, 1989).

Figure 1. Expression of LRP by HT1080 cells. (A) LRP on HT1080 cells shows a high affinity for α2M (apparent $K_D = 0.75$ nM). HT1080 cells were incubated with ¹²⁵I-α2M (2 nM), an LRP-specific ligand, at 4°C in the presence or absence of varying amounts of unlabeled α2M (0.06–40.0 nM). Cell-associated radioactivity was quantitated by gamma counting, normalized to total cell protein, and the affinity was calculated as described in MATERIALS AND METHODS. (Inset) LRP immunoprecipitated from HT1080 cells comigrates with LRP from NRK cells. HT1080 cells and NRK cells (used as control) and cell lysates were radioiodinated and processed for immunoprecipitation with anti-LRP mAb (11H4). (B) HT1080 cells take up and degrade ¹²⁵I-α2M linearly up to 8 h. Cells were incubated at 37°C with ¹²⁵I-α2M (2 nM) for 8 h. TCA-soluble radioactivity released into the incubation medium was used to quantify the amount of ligand degraded as described in MATERIALS AND METHODS.

LRP Is Located in Clathrin-coated Pits on the Plasma Membrane of HT1080 Cells

Next, we investigated the distribution of LRP on the PM of HT1080 cells under steady-state conditions by immunofluorescence. Because it has been assumed that LRP is localized in clathrin-coated pits (Herz *et al.*, 1988; Krieger and Herz, 1994), we carried out double labeling for LRP and AP-2, a well-established marker for clathrin-coated pits and vesicles associated with the PM (Brodsky, 1997). Staining for LRP (Figure 2A) was concentrated along the PM where it showed a striking overlap with AP-2 (Figure 2B), confirming its location in clathrin-coated pits.

Distribution of uPAR on the Plasma Membrane of HT1080 Cells

To determine the distribution of uPAR we carried out double labeling for uPAR and either AP-2 or caveolin-1, a marker for caveolae (Rothberg *et al.*, 1992). Staining for uPAR (Figure 2, D and G), caveolin-1 (Figure 2E), and AP-2 (Figure 2H) was seen on the PM, but there was little or no overlap between uPAR and either caveolin or AP-2. We conclude that under steady-state conditions LRP is associated with clathrin-coated pits, whereas uPAR is not associated with either clathrin-coated pits or caveolin-enriched microdomains.

Figure 2. Distribution of LRP and uPAR on the surface of HT-1080 cells at steady state. (A–C) Double labeling showing significant overlap of LRP (A) and AP-2 (B) by immunofluorescence. (D–F) Little or no overlap is seen between uPAR (D) and caveolin (E). (G–I) Similarly, there is little or no overlap between uPAR (G) and AP-2 (H). Cells were incubated with polyclonal anti-uPAR (3936) or anti-LRP mAb (456) at 4°C, fixed with 2% formaldehyde, permeabilized with Triton X-100 and subsequently incubated with anti-AP-2 (mAb) or anti-caveolin (chicken), followed by detection with appropriate secondary antibodies.

Redistribution of uPAR on the PM in the Presence of uPA:PAI-1 Complexes

Binding of exogenous uPA:PAI-1 complexes to uPAR has been shown to initiate internalization of both uPA:PAI-1 and uPAR (Olson *et al.*, 1992). To investigate the fate of uPAR after uPA:PAI-1 binding we carried out immunofluorescence on HT1080 cells that had been acid-washed to release endogenous bound ligand followed by incubation in the presence or absence of uPA:PAI-1 complexes at 4°C, and double labeling for uPAR and LRP. We found that in the absence of uPA:PAI-1, there was little overlap in staining for uPAR and LRP (Figure 3, A–C). However, in the presence of uPA:PAI-1, the overlap in staining for uPAR with both LRP (Figure 3, D–F) and AP-2

(Figure 3, G–I) was strikingly increased. These data indicate that binding of uPA:PAI-1 to uPAR induces redistribution of occupied uPAR on the PM to clathrin-coated pits, suggesting that uPA:PAI-1 may bridge uPAR and LRP.

uPAR Coprecipitates with LRP after uPA:PAI-1 Binding to the Cell Surface

To find out whether uPAR and LRP form a molecular complex after binding uPA and PAI-1, we carried out immunoprecipitation with anti-LRP mAb (11H4) on lysates of HT1080 cells incubated at 4°C in the presence or absence of uPA:PAI-1 followed by immunoblotting for uPAR. We found considerable uPAR in precipitates obtained with anti-

Figure 3. Distribution of uPAR in the presence of uPA:PAI-1 complexes and D3. (A–C) In HT1080 cells incubated in the absence of uPA:PAI-1 complexes there is little overlap in staining for LRP (A) and uPAR (B). (D–E) In the presence of uPA:PAI-1 overlap in the staining for uPAR (D) and LRP (E) is dramatically increased. There is also increased overlap in staining for uPAR (G) and AP-2 (H), indicating redistribution of uPAR to clathrin-coated pits. (J and K) Redistribution of uPAR to clathrin-coated pits in the presence of uPA:PAI-1 is not affected by the presence of D3. HT1080 cells were acid-washed, incubated at 4°C in the absence or presence of uPA:PAI-1 complexes and D3 (J–L), and processed for immunofluorescence as described in Figure 2 legend.

LRP from cells incubated in the presence of uPA:PAI-1 (Figure 4, lane 2), whereas only traces of uPAR could be detected in precipitates from controls incubated in the ab-

sence of uPA:PAI-1 (Figure 4, lane 1). These data indicate that LRP and uPAR form immunoprecipitable complexes at the surface of HT1080 cells in the presence of uPA:PAI-1.

Figure 4. LRP and uPAR form detergent-stable complexes at the surface of HT1080 cells in the presence of uPA:PAI-1. In the absence of uPA:PAI-1 (lane 1), only traces of uPAR are detected in immunoprecipitates obtained with anti-LRP, whereas in the presence of uPA:PAI-1 immunoprecipitates contain considerable uPAR (lane 2). HT1080 cells were surface radioiodinated, incubated in the presence or absence of uPA:PAI-1, and processed for immunoprecipitation with anti-LRP mAb (11H4). Precipitated proteins were subsequently immunoblotted with anti-uPAR (465).

Coimmunoprecipitation of uPAR₁₋₂₇₄ with Endogenous LRP

Next, we tested whether uPAR binds to endogenous LRP on the PM by carrying out cross-linking experiments in the presence of recombinant, soluble (lacking a GPI-anchor) uPAR. When ¹²⁵I-uPAR₁₋₂₇₄ was bound to the cell surface of acid-washed cells at 4°C followed by cross-linking and immunoprecipitation with anti-LRP mAb (11H4), considerable ¹²⁵I-uPAR₁₋₂₇₄ coprecipitated with LRP (Figure 5A, lane 2). The amount of ¹²⁵I-uPAR that coprecipitated was greatly reduced (>85%) in the presence of unlabeled uPAR₁₋₂₇₄ (Figure 5A, lane 3). When similar experiments were carried out in the presence of recombinant D3 or D2, D3 greatly reduced (>90%) uPAR₁₋₂₇₄ binding (Figure 5A, lane 5), whereas D2 had no significant effect (Figure 5A, lane 4). These findings suggest that uPAR binds to LRP through D3.

uPAR₁₋₂₇₄ Binds Directly to Affinity-purified LRP through Domain 3

Next, we incubated affinity-purified, immobilized LRP with ¹²⁵I-uPAR₁₋₂₇₄ in the presence or absence of unlabeled uPAR₁₋₂₇₄, D2, or D3. We found that ¹²⁵I-uPAR₁₋₂₇₄ bound directly to LRP (Figure 5B, lane 1), and the amount bound was greatly reduced (>85%) in the presence of unlabeled uPAR₁₋₂₇₄ (Figure 5B, lane 2) or D3 (Figure 5B, lane 4). D2 had no effect on the binding (Figure 5B, lane 3). Furthermore, we found that ¹²⁵I-D3 also bound to immobilized LRP

(Figure 5B, lane 5), and binding was reduced (>90%) in the presence of unlabeled D3. Collectively, these results demonstrate that 1) uPAR binds directly to LRP on the surface of HT1080 cells, and 2) the binding site is located in D3.

uPAR with Bound uPA:PAI-1 and LRP Are Internalized and Delivered to Early Endosomes

To investigate the trafficking of uPAR:LRP complexes in the presence of uPA:PAI-1, we carried out subcellular fractionation by using ¹²⁵I-Tf as a marker to identify PM and EE fractions. When HT1080 cells were incubated with ¹²⁵I-Tf for 1 h at 4°C and homogenates were fractionated on isoosmotic Percoll gradients, the majority of the ¹²⁵I-Tf sedimented in gradient fractions 4–6 (Figure 6A, open squares), indicating the location of PM. After incubation of cells at 18°C for 1 h to allow uptake into EE, the majority of the ¹²⁵I-Tf sedimented in fractions 8–10 (Figure 6A, closed squares), defining fractions containing EE.

To follow the fate of uPAR after uPA:PAI-1 binding, HT1080 cells were surfaced biotinylated, incubated with uPA:PAI-1, and fractionated on Percoll gradients after incubation at 4°C or binding at 4°C followed by incubation at 18°C for 1 h. After uPA:PAI-1 binding at 4°C, the majority of the biotinylated uPAR cosedimented with ¹²⁵I-Tf in PM fractions (4–6) (Figure 6B). However, when cells were subsequently incubated at 18°C after binding the peak of the biotinylated uPAR shifted to EE fractions (8–10) (Figure 6C). When similar experiments were carried out on cells incubated in the presence of anti-LRP (456) (Figure 6E) at 18°C, uPAR remained largely associated with PM-containing fractions, confirming previous reports that anti-LRP blocks internalization of uPAR (Zhang *et al.*, 1998). From these results we conclude that uPAR is internalized into an EE compartment after uPA:PAI-1 binding, and internalization occurs via LRP.

uPAR Is Internalized via Clathrin-coated Vesicles

LRP is known to be internalized via clathrin-coated vesicles (Chen *et al.*, 1990), but the route of internalization of uPAR has not yet been established. To obtain information on this point we used a procedure (i.e., hypotonic shock and depletion of intracellular K⁺) that selectively blocks clathrin-mediated endocytosis (Larkin *et al.*, 1983) but has no effect on internalization via caveolae (Roettger *et al.*, 1995). When cells were K⁺ depleted and incubated at 18°C as described above, uptake of both ¹²⁵I-Tf (Figure 6A, open triangles) and uPAR (Figure 6D) was completely abolished. These results together with our immunocytochemical data demonstrate that internalization of occupied uPAR is clathrin mediated.

D3 Blocks Internalization of uPAR but Does Not Affect Binding of uPA:PAI-1

Next, we tested the effects of D3 on internalization of uPAR. We found that internalization of uPAR was blocked in the presence of D3, because uPAR remained largely associated with PM-containing fractions (Figure 6F).

Because previous studies have suggested that regions within D3 are important for high-affinity binding of uPA to uPAR (Ploug, 1998), it was necessary to determine whether D3 blocks binding of uPA:PAI-1 to uPAR. Thus, we carried out experiments in which we incubated ¹²⁵I-uPA:PAI-1 complexes (10 nM) with recombinant purified human uPAR₁₋₂₇₄

Figure 5. Direct binding of soluble uPAR to LRP via D3 of uPAR. (A) Considerable ^{125}I -uPAR₁₋₂₇₄ can be detected in anti-LRP immunoprecipitates (lane 2) after cross-linking. The amount of cross-linked ^{125}I -uPAR₁₋₂₇₄ is reduced (80–90%) in the presence of unlabeled uPAR₁₋₂₇₄ (lane 3) and D3 (lane 5). D2 (lane 4) has no significant effect on uPAR₁₋₂₇₄ binding to LRP. Acid-washed cells were incubated with 25 nM recombinant ^{125}I -uPAR₁₋₂₇₄ at 4°C in the presence (+) or absence (–) of 2.5 μM unlabeled uPAR₁₋₂₇₄, or 1.25 μM recombinant D2, or D3 followed by cross-linking with DTSSP (lanes 2–5) and immunoprecipitation with anti-LRP mAb (11H4). Immunoprecipitated proteins were processed for autoradiography and densitometry. (B) Binding of ^{125}I -uPAR₁₋₂₇₄ to affinity-purified LRP (lane 1) is reduced by >85% in the presence of unlabeled uPAR₁₋₂₇₄ (lane 2) or D3 (lane 4). D2 (lane 3) has no effect on ^{125}I -uPAR₁₋₂₇₄ binding to LRP. In addition, ^{125}I -D3 binds to affinity-purified LRP (lane 5) and the binding is reduced by >95% in the presence of unlabeled D3 (lane 6). Purified LRP was incubated with ^{125}I -uPAR₁₋₂₇₄ (100 nM) or ^{125}I -D3 (50 nM) at 4°C in the absence or presence of unlabeled uPAR₁₋₂₇₄ (4 μM), D2 (1.5 μM), or D3 (1.5 μM). Immunoprecipitated proteins were processed for autoradiography and densitometry. (C) Binding of ^{125}I -uPA:PAI-1 to recombinant uPAR₁₋₂₇₄ (lane 1) is not affected by D3 (lane 2). uPAR₁₋₂₇₄ (2 nM) was incubated with ^{125}I -uPA:PAI-1 (10 nM) in PBS in the presence or absence of D3 (100 nM) and processed for immunoprecipitation with anti-uPAR (465). Samples were processed for autoradiography and densitometry.

(2 nM) in the presence or absence of D3 (100 nM). We found that D3 had no effect on the binding of uPA:PAI-1 to uPAR₁₋₂₇₄ (Figure 5C).

We also found that D3 does not block redistribution of uPAR induced by uPA:PAI-1 complexes (Figure 3, J–L). We conclude that D3 blocks internalization of uPAR but has no effect on the binding of uPA:PAI-1 to uPAR or on the redistribution of uPAR with bound uPA:PAI-1 to clathrin-coated pits.

Presence of uPAR:LRP Complexes in Early Endosomes

Next, we investigated whether uPAR and LRP remain associated during internalization into EE. We prepared EE fractions (8–10) from surface biotinylated cells incubated at 18°C in the presence of uPA:PAI-1 and used them as starting material for immunoprecipitation of EE on mAb 11H4 (specific for the cytoplasmic tail of LRP), which had been preadsorbed to protein G-agarose. Vesicles that had been immunoadsorbed to the beads were then detergent solubilized (to release non-LRP-associated proteins), and the beads with bound LRP were pelleted and analyzed by immunoblotting for LRP-associated proteins. As shown in Figure 7, most (>90%) of the uPA:PAI-1 (lane 3) and Tf receptor (lane 4) were released into the supernatant after detergent treatment, whereas most (>90%) of the LRP (lane 5) and uPAR (lane 6) remained bound to the beads. These results show that uPAR and LRP are internalized together and remain associated in EE, whereas most of the uPA:PAI-1 is no longer bound to uPAR in EE.

D3 Blocks Cell Surface uPA Activity

Previously, it was shown that blocking uPA:PAI-1 binding to LRP with RAP impairs regeneration of unoccupied uPAR and reduces uPA activity at the cell surface, presumably by blocking endocytosis of uPAR (Zhang *et al.*, 1998). Thus, we reasoned that blocking uPAR binding to LRP should also lead to decreased cell surface uPA activity and subsequent generation of plasmin in HT1080 cells. To determine whether this is the case we quantified uPA-dependent plasmin activation in the presence and absence of D3 using a chromogenic assay. After incubation with uPA:PAI-1 in the presence of recombinant D3 at 4°C (1 h) or 37°C (1 h), there was a ~60% reduction in surface-bound uPA activity (0.5 ± 0.02 mU/ml plasmin activity) compared with controls (1.5 ± 0.1 mU/ml) over 60 min (Figure 8). These results suggest that preventing the binding of uPAR to LRP with recombinant D3 results in decreased uPA activity at the cell surface, presumably by preventing clearance of occupied uPAR and regeneration of unoccupied uPAR capable of binding activated uPA at the cell surface.

D3 Inhibits Migration of HT1080 Cells through Matrigel-coated Transwell Chambers

Because blocking LRP:uPAR interaction reduces cell surface uPA activity it might also be expected to slow or prevent the ability of cells to migrate through the extracellular matrix. To determine whether this is the case, we carried out a well-established cell invasion assay (Kramer *et al.*, 1986;

A **B** **C** **D** **E** **F**

Figure 6. Internalization of uPAR requires binding to LRP. (A) In cells incubated with ^{125}I -Tf at 4°C ($\square$), Tf peaks in fractions 4–6, indicating the location of PM. After incubation at 18°C ($\blacksquare$), ^{125}I -Tf peaks in fractions 8–10 where it defines the location of EE. In cells subjected to K^+ depletion ($\triangle$), uptake of ^{125}I -Tf is blocked, because no ^{125}I -Tf cosedimented with EE. (B) In cells incubated at 4°C with uPA:PAI-1, biotinylated uPAR sediments in PM fractions 4–6. (C) After 60-min incubation at 18°C, the distribution of a substantial fraction of the biotinylated uPAR shifts to EE fractions (8–10). (D) After K^+ depletion and incubation at 18°C the majority of the biotinylated uPAR is found in PM fractions (4–6), and little or no uPAR is detected in EE fractions (8–10), indicating that internalization of uPAR is blocked. After incubation for 60 min at 18°C in the presence of 200 $\mu\text{g}/\text{ml}$ anti-LRP (456) (E), the majority of the biotinylated uPAR remains associated with PM fractions. (F) In the presence of 1.25 μM D3, virtually all detectable, biotinylated uPAR

Figure 7. Presence of LRP and uPAR complexes in EE. The majority of the uPAR (lane 6) coprecipitates with LRP (lane 5) and is found in the pellet, which contains only trace amounts of uPA:PAI-1 (lane 7). The majority of the uPA:PAI-1 complexes (lane 3) and Tf receptor (lane 4) do not coprecipitate with LRP and are found in the supernatant, which contains only traces of LRP (lane 1) and uPAR (lane 2). Thus, uPAR and LRP form a complex that can be coprecipitated from EE. uPA:PAI-1 was bound to biotinylated HT1080 cells at 4°C, and the cells were fractionated as in Figure 6. EE fractions were pooled and LRP-containing vesicles were immunoprecipitated on mAb 11H4 (against the cytoplasmic tail of LRP) bound to protein G-agarose beads. Beads were treated with 10 mM CHAPS, and the proteins released into the supernatant and those remaining bound to the beads were analyzed by immunoblotting with anti-LRP (1073), anti-uPAR (465), anti-uPA, and anti-Tf receptor.

Albini *et al.*, 1987; Chen *et al.*, 1997) in which we analyzed the ability of HT1080 cells to migrate through Matrigel-coated Transwell invasion chambers in the presence or absence of soluble uPAR₁₋₂₇₄ or D3. In the absence of uPAR₁₋₂₇₄ or D3 (Figure 9) 115 ($\pm$ 12) cells (100%) migrated through the Matrigel layer. However, in the presence of recombinant uPAR₁₋₂₇₄ or D3 the number of cells that migrated through the filter was reduced to 54 ($\pm$ 3) cells (47%) and 9 ($\pm$ 3) cells (8%), respectively. Addition of proteolytically active uPA to cells incubated in the presence of D3 restored the number of HT1080 cells able to migrate through the Matrigel-coated filters to control levels (105 [$\pm$ 7] cells; 91%), indicating that the effect can be attributed in large part to uPA. No significant affect on the number migrating through the Matrigel layer was detectable when D2 was added to the assay. These

(~95%) is found in PM fractions after incubation at 18°C. HT1080 cells were acid-washed, surface biotinylated, and incubated with either ^{125}I -Tf (200 ng/ml) or uPA:PAI-1 complexes (50 nM) at 4°C (60 min) or for 4°C (60 min) followed by 18°C (60 min). In some cases D3 (1.25 μM) or anti-LRP antibodies (456; 200 $\mu\text{M}/\text{ml}$) were added or intracellular K^+ was depleted as described in MATERIALS AND METHODS. Postnuclear supernatants were fractionated on Percoll gradients, and fractions were processed for gamma counting of ^{125}I -Tf or for immunoprecipitation of uPAR and by detection of the biotinylated, cell surface pool of uPAR by blotting with HRP-coupled avidin. In E and F, PM fractions (4–6) and EE fractions (8–10), were pooled before analysis.

Figure 8. Inhibition of uPAR/LRP interaction reduces plasminogen activation. Plasmin activity at the cell surface of HT1080 cells is reduced from 1.5 mU (control) to 0.5 mU/ml/60 min when cells are incubated in the presence of D3. Acid-washed cells were incubated sequentially for 1 h at 4°C and 1.5 h at 37°C with uPA:PAI-1 (10 nM) in the presence or absence of D3 (5 μ g/ml) and subsequently incubated at 4°C with proteolytically active uPA (2 nM) for 1 h in the presence of anti-LRP antibodies (456; 200 μ g/ml). Plasminogen (0.2 μ M) was bound to the cell surface at 4°C, and uPA-dependent plasmin generation was calculated as absorbance change at 405 nm over 60 min using Chromozym PL as a plasmin substrate.

data indicate that inhibiting uPAR's interaction with LRP and preventing endocytosis of uPAR significantly reduces the ability of HT1080 cells to invade and to migrate through extracellular matrix.

DISCUSSION

It has been established that LRP mediates the clearance of uPA:PAI-1 from the extracellular environment and that this process is accompanied by internalization of uPAR (Conese *et al.*, 1995), but the clearance mechanism and the intracellular trafficking itinerary of uPAR have remained unknown. In this article we investigated the nature of the interaction between uPAR and LRP during clearance of uPA:PAI-1 and their endocytic trafficking. We have shown that in HT1080 cells, uPAR directly interacts with LRP in the presence of uPA:PAI-1 and that LRP and occupied uPAR form stable complexes that are cointernalized into EE via clathrin-coated vesicles. We have further demonstrated that blocking the interaction between the two receptors results in decreased clearance of uPA:PAI-1 and decreased plasmin generation at the cell surface and significantly reduces the ability of the cells to migrate through extracellular matrix in a standard cell invasion assay.

Our immunofluorescence results indicate that at steady-state LRP and uPAR are localized in different microdomains of the PM. LRP, like other members of the LDL receptor gene family, is concentrated in clathrin-coated pits, whereas uPAR is not associated with either clathrin-coated vesicles or caveolae because it did not colocalize with either caveolin or with AP-2,

Figure 9. Blocking uPAR/LRP interaction inhibits migration of HT1080 cells through Matrigel-coated filters. Cells (5×10^5) were seeded onto the top of Matrigel-coated filter membranes and incubated at 37°C in the presence or absence of uPAR₁₋₂₇₄ (2.5 μ M), D3 (1.25 μ M), or D3 in combination with active uPA (8 nM). Cells that migrated through the filters and appeared at the under side of the filters were counted after 36 h of incubation. In the presence of uPAR₁₋₂₇₄ and D3 the number of invading cells is decreased to 46.5 and 7.5% of control, respectively. Coincubation of cells with D3 and active uPA restores the ability of the cells to migrate to near control levels.

Figure 10. Model depicting proposed events in the interaction and trafficking of uPAR and LRP. (A) Active uPA binds to uPAR on the PM. 1) PAI-1 binds to and inhibits uPA. 2) uPA:PAI-1 bridges occupied uPAR and LRP, which leads to relocation of occupied uPAR into clathrin-coated pits and promotes a stable interaction between D3 of uPAR and LRP. 3) These quaternary complexes are internalized via clathrin-coated pits and delivered to EE. 4) uPA:PAI-1 dissociates from uPAR and LRP in EE and traffics through late endosomes (LE) to lysosomes for degradation. 5) Unoccupied uPAR and LRP also dissociate and 6) return to the cell surface via recycling vesicles (RV). This makes available unoccupied uPAR capable of binding pro-uPA. 7) Presence of active uPA at the cell surface catalyzes the generation of plasmin, which can degrade extracellular matrix. (B) When binding of occupied uPAR to LRP is prevented, uPA:PAI-1 bridges uPAR and LRP and occupied uPAR moves into clathrin-coated pits. However, clearance of occupied uPAR is blocked (8) and occupied inactive uPAR accumulates on the cell surface. As a consequence, generation of plasmin and degradation and invasion of extracellular matrix are decreased.

markers for caveolae and clathrin-coated pits, respectively (Brodsky, 1997). The latter finding is in keeping with previous reports indicating that at steady-state uPAR as well as other GPI-anchored proteins are rather broadly distributed at the cell surface (Conese *et al.*, 1995; Maxfield and Mayor, 1997; Nykjaer *et al.*, 1997). However, we found that when cells are incubated in the presence of uPA:PAI, which binds to uPAR, uPAR forms a complex with LRP and moves into clathrin-coated pits. That binding is direct and occurs at the D3 domain of uPAR was suggested by cross-linking experiments and the fact that addition of recombinant D3 completely blocked both the binding and internalization of occupied uPAR. This was confirmed by demonstrating that recombinant uPAR binds to affinity-purified LRP. The demonstration of direct interaction between uPAR and LRP is a novel finding, because it has been assumed or implied that uPA-PAI-1 bridges the two receptors and bridging is sufficient for endocytosis of the complexes (Andreassen *et al.*, 1994). There has been no previous work showing a requirement for direct interaction between uPAR and LRP.

It has been shown previously (Behrendt *et al.*, 1991) that uPAR is composed of three homologous domains (designated D1, D2, and D3 from the N terminus) of which D1 contains the ligand-binding domain. Currently, little is known about the physiological functions of D2 and D3. However, it has been hypothesized that they might increase the specificity of uPAR for its ligand and facilitate uPAR interaction with adhesion receptors at the sites of focal con-

tacts (Dear and Medcalf, 1998). Our findings demonstrate D3 contains a binding site for LRP and provide novel insights into the function of D3.

We also established that when occupied by uPA:PAI-1 the GPI-anchored protein uPAR is internalized into EE via clathrin-coated vesicles, because internalization does not occur when clathrin-mediated endocytosis is blocked by K^+ depletion. Furthermore, uPAR remains associated with LRP during internalization to EE even after dissociation of uPA:PAI-1 as demonstrated by our immunoprecipitation analysis on immunoprecipitated EE. LRP is known to internalize ligands through a clathrin-dependent pathway mediated by the NPXY motifs in its cytoplasmic tail, whereas GPI-anchored proteins such as uPAR lack this motif and are usually assumed to be internalized via caveolae (Lisanti *et al.*, 1993; Anderson, 1998). To our knowledge, this is the first demonstration of a GPI-anchored protein piggybacking with another receptor to be internalized via clathrin-coated vesicles. Indirect evidence suggests that the same might apply to uPAR and certain integrins (Memmo and McKeown-Longo, 1998; Simon *et al.*, 2000) and to prions, which have been postulated to interact with unknown transmembrane receptors (Shyng *et al.*, 1994).

Taken together, our results provide evidence for the model of uPA:PAI-1 clearance and uPAR internalization shown in Figure 10. According to this model, formation of uPA:PAI-1 complexes on uPAR bridges occupied uPAR and

LRP at the cell surface, promotes relocation of occupied uPAR into clathrin-coated pits, and initiates binding of LRP to the D3 domain of uPAR. This results in generation of a quaternary complex, uPA:PAI-1/uPAR:LRP, which is then internalized into EE via clathrin-coated vesicles. uPA:PAI-1 is released from the receptors in EE and traffics through late endosomes to lysosomes for degradation (Cubellis *et al.*, 1990). Unoccupied uPAR (Nykjaer *et al.*, 1997) and LRP recycle to the cell surface. Unoccupied uPAR is available for binding and activation of pro-uPA, which catalyzes the generation of plasmin, promoting degradation of extracellular matrix and cell invasion. When binding of occupied uPAR to LRP is prevented (i.e., in the presence of recombinant D3), uPA:PAI-1 is still capable of bridging the two receptors and uPAR redistributes into clathrin-coated pits. However, internalization of occupied uPAR is prevented, leading to accumulation of occupied uPAR in clathrin-coated pits on the PM. Accumulation of redistributed uPAR occupied by inactive uPA at the cell surface decreases plasmin generation and degradation and invasion of extracellular matrix.

Because activation of secreted pro-uPA requires its binding to cell surface uPAR (Ellis *et al.*, 1989; Stephens *et al.*, 1989) and unoccupied uPAR constitutes the sole binding and activation site for pro-uPA at the cell surface (Vassalli *et al.*, 1985), it follows that the number of unoccupied uPAR at the cell surface is a limiting factor in uPA activation and activity.

The net effect of the interaction of between occupied uPAR and LRP is to remove uPAR with inactive protease (uPA) activity from the cell surface and replace it with unoccupied uPAR capable of binding pro-uPA and initiating a new cycle of plasminogen activation. The importance of LRP in regeneration of cell surface protease activity and cell migration has previously been demonstrated by the finding that RAP and anti-LRP antibody impair the regeneration of unoccupied uPAR and decrease uPA activity of human trophoblast cells (Zhang *et al.*, 1998). Moreover, it was reported that LRP-deficient cultured fibroblasts (Weaver *et al.*, 1997) and cells in which LRP expression has been down-regulated by tumor-promoting phorbol esters (Picone *et al.*, 1989) have increased amounts of uPAR on their surfaces compared with normal or untreated cells (Picone *et al.*, 1989; Weaver *et al.*, 1997). Our data validate the unique role of LRP in regulation of extracellular plasminogen activation and further demonstrate that uPAR binding to LRP is a mandatory step in the internalization of these receptors and in the regeneration of unoccupied uPAR.

ACKNOWLEDGMENTS

We thank Ileana Popa for preparation of activated α 2M. This work was supported by a Department of Defense Breast Cancer Research Program Grant DAMD-96-1-6317 to M.G.F., Fellowship DAMD17-96-1-6318 to R.-P.C., and a Peter Lippincola Fellowship to T.K.

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