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Direct interaction of the kringle domain of urokinase-type plasminogen activator (uPA) and integrin α β3 induces signal transduction and enhances plasminogen activation.

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Abbreviations: CHO, Chinese hamster ovary; GFD, growth factor domain; GPI, glycosylphosphatidylinositol; PBS, phosphate-buffered saline; LMW, low-molecular-weight; PIPLC, Phosphatidylinositol-specific phospholipase C; uPA, urokinase-type plasminogen activator; uPAR, urokinase-type plasminogen activator receptor;

ABSTRACT

It has been questioned whether there are receptors for urokinase-type plasminogen activator (uPA) that facilitate plasminogen activation other than the high affinity uPA receptor (uPAR/CD87) since studies of uPAR knockout mice did not support a major role of uPAR in plasminogen activation. uPA also promotes cell adhesion, chemotaxis, and proliferation besides plasminogen activation. These uPA-induced signaling events are not mediated by uPAR, but mediated by unidentified, lower-affinity receptors for the uPA kringle. We found that uPA binds specifically to integrin αvβ3 on CHO cells depleted of uPAR. The binding of uPA to αvβ3 required the uPA kringle domain. The isolated uPA kringle domain binds specifically to purified, recombinant soluble, and cell surface αvβ3, and other integrins (α4β1 and α9β1), and induced migration of CHO cells in an αvβ3-dependent manner. The binding of the uPA kringle to αvβ3 and uPA kringle-induced αvβ3-dependent cell migration were blocked by homologous plasminogen kringles 1-3 or 1-4 (angiostatin), a known integrin antagonist. We studied whether the binding of uPA to integrin αvβ3 through the kringle domain plays a role in plasminogen activation. On CHO cell depleted of uPAR uPA enhanced plasminogen activation in a kringle and αvβ3-dependent manner. Endothelial cells bound to and migrate on uPA and uPA kringle in an αvβ3-dependent manner. These results suggest that uPA binding to integrins through the kringle domain plays an important role in both plasminogen activation and uPA-induced intracellular signaling. The uPA kringle-integrin interaction may represent a novel therapeutic target for cancer, inflammation, and vascular remodeling.

BACKGROUND

Urokinase-type plasminogen activator (uPA) is a highly restricted serine protease that converts the zymogen plasminogen to active plasmin. uPA binds with high affinity to a cell-surface uPA receptor (uPAR) that has been identified in many cell types. uPAR is a glycosylphosphatidylinositol (GPI)-anchored 35-55kDa glycoprotein. This system mediates pericellular proteolysis of extracellular matrix proteins including fibrin degradation (fibrinolysis) and plays an important role in cancer, inflammation, and immune responses (1-4). The

single chain form of uPA (sc-uPA) has three independently folded domains: the growth factor domain (GFD) (residue 1-46), kringle (residue 47-135) domains, and serine protease domain (residue 159-411). Enzymatic digestion of sc-uPA yields an amino terminal fragment (ATF), which consists of the GFD and kringle domains, and the low molecular weight fragment (LMW-uPA), which consists of the serine protease domain. The uPAR-binding site of uPA is located in the GFD domain (5); this binding is stabilized by the kringle (6). It has generally been accepted that uPA signaling involves its binding to uPAR through its GFD (7).

uPA binding to uPAR on the cell surface facilitates activation of plasminogen to plasmin in vitro by increasing the rate of pro-uPA activation by plasmin, by decreasing the apparent K_m of uPA to plasmin, and by increasing the K_{cat}/K_m of uPA to plasmin (8). It is interesting that uPA-knockout mice do not have major thrombotic disorders (9). This is probably because of the redundant fibrinolytic function by tissue-type plasminogen activator (tPA). Indeed, combined uPA and tPA knockout mice show extensive thrombotic disorders very similar to those observed in plasminogen-knockout mice, but these are rarely detected in animals lacking uPA or tPA alone (10). In contrast to uPA, studies performed in uPAR-knockout mice do not really support a major role of uPAR in fibrinolysis. Fibrin deposits are found within the livers of mice with a combined deficiency in uPAR and tPA, but not in uPAR-knockout mice, indicating a minor role for uPAR in plasminogen activation (10). The extraordinarily mild consequences of combined uPAR and tPA deficiency raised the question of whether there are other receptors for uPA that might facilitate plasminogen activation (4, 10).

Beside plasminogen activation, uPA has been shown to induce the adhesion and chemotactic movement of myeloid cells (11, 12), to induce cell migration in human epithelial cells (13) and bovine endothelial cells (14), and to promote cell growth (15-17). Notably these signaling functions of uPA do not require its proteolytic activity. Several studies suggest that uPA has additional, unidentified cell-surface receptor(s) other than uPAR that are involved in signaling events. For example, in one study it was observed that blocking of uPA binding to uPAR using a monoclonal antibody (mAb) or by depletion of cell surface uPAR with phosphatidylinositol-specific phospholipase C (PIPLC) did not inhibit uPA-induced mitogenic effects in smooth muscle cells

(18). Koopman et al. reported that uPA-induced mitogenic effects in melanoma cells are independent of high-affinity binding to uPAR, and suggested the existence of a low-affinity binding site on this cell type based on the kinetic data (19). The chemotactic action of uPA on smooth muscle cells was shown to depend on its kringle domain, and kinetic evidence was presented to indicate that these cells express a lower-affinity kringle receptor distinct from uPAR (20). We have previously reported that the isolated uPA kringle augments vascular smooth muscle cell constriction in vitro (21) and in vivo (22). Taken together these observations all suggest that cells express uPA-binding proteins (other than uPAR) that mediate signaling from uPA.

In the present study, we sought to identify the uPA binding protein(s) involved in plasminogen activation and uPA signaling other than uPAR. In this paper, we report that uPA binds directly to integrin $\alpha v\beta 3$ through its kringle domain, independent of uPAR, and that the uPA kringle mediates signaling events by acting as an integrin agonist through a pathway that is distinct from that described for uPAR. We present evidence that uPA binding to integrin $\alpha v\beta 3$ on the cell surface through the kringle domain enhances plasminogen activation besides inducing integrin-mediated signaling. We show that endothelial cells bind to and migrate on uPA and uPA kringle in an $\alpha v\beta 3$-dependent manner. Our findings suggest that the uPA-integrin interaction is a novel therapeutic target in inflammation and cancer.

METHODS
Materials
    Monoclonal antibody (mAb) SG73 (anti-$\alpha 4$) (23) and KH72 (anti-$\alpha 5$) were provided by K. Miyake (University of Tokyo, Japan). MAb Y9A2 (anti-$\alpha 9$) (24) was obtained from D. Sheppard (University of California San Francisco, CA). MAbs 7E3 (anti-$\beta 3$) (25) and 16N7C2 (anti-$\beta 3$) (26) were provided by B. S. Coller (Rockefeller University, NY) and H. Deckmyn (Center for Molecular and Vascular Biology, Leuven, Belgium), respectively. MAb LM609 (anti-$\alpha v\beta 3$) (27) was a gift from D. Cheresh (The Scripps Research Institute, La Jolla, CA). Anti-uPAR mAb (3B10) (28) was provided by R. F. Todd (University of Michigan Medical Center, Ann Arbor, MI). The anti-uPA kringle antibody (Ab 963) was a gift from J. Henkin (Abbott Laboratories, Abbott Park, IL). MAb UNG-5 (anti-LMW uPA) was obtained from IKTEK Ltd. (Moscow, Russia). Anti-uPA kringle and anti-LMW uPA mAbs linked to

Sepharose 4B were from IKTEK Ltd. GRGDS and GRGES peptide were purchased from Advanced ChemTech (Louisville, KY). Phosphatidylinositol-specific phospholipase C (PIPLC) was obtained from Glyko, Inc. (Novato, CA).

Recombinant uPA and uPAR fragments were prepared as previously described (29). Recombinant soluble $\alpha v\beta 3$ was generated as previously described (30). Purified human $\alpha v\beta 3$ was obtained from Chemicon International (Temecula, CA). Cells expressing different integrin α and/or β subunits (wild type and mutant) have been described (31). CHO cells expressing human $\alpha 9$ ($\alpha 9$-CHO) were provided by D. Sheppard. These cells express human α/hamster $\beta 1$ ($\alpha 2$-, $\alpha 3$-, $\alpha 4$-, and $\alpha 9$-CHO cells) or hamster αv/human $\beta 3$ hybrid ($\beta 3$-CHO cells). Chinese hamster ovary (CHO) cells were obtained from the American Type Culture Collection (Manassas, VA). CHO cells expressing the three-domain form of human uPAR (designated uPAR-CHO) have been described (32). The $\alpha 5$-deficient B2 variant of CHO cells (33) was provided by R. Juliano (University of North Carolina, Chapel Hill, NC). Calf pulmonary endothelial (CPAE) cells were obtained from ATCC.

Adhesion assays

Adhesion assays were performed as previously described (31). Briefly, wells within 96-well Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA), were coated with 100 μl of PBS (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4) containing substrates at concentrations of up to 500 nM and were incubated overnight at 4°C. Remaining protein binding sites were blocked by addition of 0.2% bovine serum albumin (BSA; Calbiochem) for 1 h at room temperature. CHO cells (10^5cells/well) in 100 μl of Hepes-Tyrode buffer (10 mM HEPES, 150 mM NaCl, 12 mM NaHCO$_3$, 0.4 mM NaH$_2$PO$_4$, 2.5 mM KCl, 0.1% glucose, 0.02% BSA) supplemented with 2 mM MgCl$_2$ were added to the wells and incubated at 37°C for 1 h. After non-bound cells were removed by rinsing the wells with the same buffer, bound cells were quantified by measuring endogenous phosphatase activity (34).

Migration assays.

Cell migration was analyzed using tissue culture-treated 24-well Transwell plates (Costar, Cambridge, MA) with polycarbonate membranes of pore size 8 μm. The lower side of the filter was coated with various concentrations (20-200 nM) of substrates. Coated filters were placed into serum-free migration buffer (Dulbecco's modified Eagle's medium supplemented with 10 mM Hepes, 0.5% BSA, and 1 x penicillin-streptomycin), and cells (100 μl) suspended in the same buffer (8 x 10^5cells/ml) were added to the upper chamber. The cells were incubated at 37°C in 5% CO$_2$ for 12-16 h. Cells in the upper chamber were removed by wiping and cells that migrated to the lower surface of the filters were fixed and stained with 0.5% crystal violet in 20% ethanol, and counted. The result for each well is the mean cell number of 4 randomly selected high magnification microscopic fields in triplicate experiments. In order to verify quantification of mean number of migrated cells, we took a picture of the stained membrane and determined the number of cells on the entire lower surface of the membrane using ImageJ software (http://rsb.info.nih.gov/ij/). We obtained essentially identical results using either method. In some studies, anti-integrin antibodies (10 μg/ml) were incubated with cells for 15 min prior to the assay. For CPAE cells we added 1% FCS to the migrating buffer.

Direct binding of recombinant soluble $\alpha v\beta 3$ to the uPA kringle.

Wells within 96-well microtiter plates were coated with 10 μg/ml ligand (50 μg/ml for the uPA kringle, 10 μg/ml for vitronectin), blocked with 1% BSA for 1 h at room temperature, and integrins (5 μg/ml) were incubated in binding buffer (10 mM Tris/HCl, 0.15 M NaCl supplemented with 1 mM CaCl$_2$ and MgCl$_2$, or 1 mM MnCl$_2$ with various reagents at room temperature for 2 h. After washing with the binding buffer once, biotinylated anti-6His tag (Velcro) antibody was added, incubated for 30 min, and then washed three times with the same buffer (35). The $\beta 3$ chain has a 6His tag at the C-terminus (35). Bound integrins were detected by horseradish peroxidase-streptavidin and a substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS).

Binding of uPA kringle to purified $\alpha v\beta 3$

Wells of 96-well microtiter plates (Dynex, Immulon 4 HBX) were coated overnight at 4°C with 45 μl of 5 μg/ml purified human $\alpha v\beta 3$ integrin in HEPES-Tyrode buffer. The wells were washed twice with 200 μl of 0.2% BSA/HEPES-Tyrode buffer containing 2 mM MgCl$_2$ and blocked by incubating with the buffer for 2 h at room temperature. The wells were washed once with 200 μl of 0.1% BSA/HEPES-Tyrode buffer containing 2 mM MgCl$_2$, and incubated for 1 h at 37°C with a 50 μl solution

of 0.1% BSA/HEPES-Tyrode buffer containing increasing concentrations of ^{125}I-labeled uPA kringle (up to 400 nM). After incubation, the wells were washed three times with 200 μl of HEPES-Tyrode buffer containing 2 mM $MgCl_2$. The wells were incubated with 150 μl of 1 M sodium hydroxide for 20 min at room temperature, and the radioactivity in the solution was measured. In some experiments, cation-independent binding was measured in the presence of EDTA instead of cations, and BSA instead of purified αvβ3 was used to measure non-specific binding. For binding inhibition studies, increasing concentrations of either non-labeled uPA kringle or angiostatin (K1-4) were added to the fixed concentration (400 nM) of ^{125}I labeled uPA kringle.

Plasminogen activation on the cell surface.
Parental CHO cells and β3-CHO cells were plated into 96-well plate at $1x10^5$ cells/well. Sixteen h later, the cells were treated with 1 unit/ml PIPLC in 1% BSA/PBS with Ca^{2+}, Mg^{2+} (50 μl/well) for 1 h at 37°C. Then the plate was incubated for 30 min at 4°C, and washed two times with the cold binding buffer 1% BSA/HEPES-Tyrode containing 2 mM $MgCl_2$. Wild type (wt) or delta kringle (ΔK) uPA dilutions in the cold binding buffer were applied to the cells at 0, 10, 15, 25, 30, and 50 nM concentrations and incubated for 1 h at 4°C. The cells were washed three times with the binding buffer, then 200 nM Glu-plasminogen and 0.3 mM SpectrozymePL chromogenic substrate (American Diagnostica, Inc.) dilution in the binding buffer (100 μl/well) was added. Hydrolysis of the chromogenic substrate was monitored continuously at 37°C over 4 h over each 2-min interval at 405 nm using ThermoMax microplate reader (Molecular Devices). The amounts of wt and ΔK uPA bound to the cells were determined by reference to linear standard curves for Vmax prepared with wt and ΔK uPA. Kd was calculated using GraphPad Prism Version 3.02.

Other Methods. Flow cytometric analysis and transfections were performed as described (36).

RESULTS

Effect of uPAR depletion on uPA binding to cells.
As it has been reported that uPA transduces signals in an uPAR-independent manner (see Introduction), we searched for other uPA binding proteins by depleting GPI-linked cell surface uPAR using PIPLC. The expression of human uPAR on the surface of transfected CHO cells (designated uPAR-CHO cells) (32) was monitored by flow cytometric analysis using the anti-human uPAR mAb 3B10. PIPLC removed greater than 95% of the cell surface uPAR (37). We have previously reported that uPAR-CHO cells adhere to immobilized uPA in an uPAR-dependent manner (37). Human uPA binds to hamster uPAR on parent CHO cells as well (37). Accordingly, PIPLC treatment reduced adhesion of uPAR-CHO cells to immobilized uPA to the level of mock-transfected CHO cells. In contrast, PIPLC treatment of β3-CHO cells did not significantly affect adhesion to uPA (Fig. 1a, b). We have further tested the specificity of the adhesion of β3-CHO cells to uPA at the coating uPA concentration of 200 nM, at which mock-CHO cells do not significantly bind to uPA. We found that RGD peptide and mAb 16N7C2 (anti-β3) blocked binding of αvβ3-expressing CHO to uPA, whereas the control RGE peptide or control mouse IgG did not (Fig. 1c). These results suggest that uPA is able to interact with cell-associated αvβ3 as a ligand in an uPAR-independent manner.

Identification of the uPA domain that interacts with αvβ3.
We found that anti-kringle mAb (mAb 963) blocked, but anti-LMW mAb (UNG-5) did not block, adhesion of β3-CHO cells to uPA, suggesting that the kringle domain may be, but the serine protease domain may not be, critically involved in the uPA-αvβ3 interaction (Fig. 1c). To verify the role of uPA domains in binding to integrins, we examined the ability of several recombinant uPA fragments (Fig. 2a) to support the adhesion of β3-CHO cells and uPAR-CHO cells (as controls). We found that β3-CHO adhered to the immobilized uPA fragment that lacks the GFD domain containing the uPAR-binding site (ΔGFD-uPA), but mock-CHO or uPAR-CHO cells did not (Fig. 2b). We also found that ΔGFD-uPA is able to bind directly to the purified αvβ3 with a half maximal binding of approximately 50 nM, but did not bind to BSA as a negative control (Fig. 2c). These results suggest that uPA binds to isolated αvβ3 in a GFD/uPAR-independent manner, consistent with the behavior of cell-associated αvβ3 described above.

We also found that β3-CHO and uPAR-CHO cells adhered to the amino-terminal fragment (ATF) of uPA that contains the GFD and the kringle domain, while parental CHO cells adhered less well to the ATF (Fig. 2d). The isolated kringles (residues 47-143 or K143; and residues 47-135 or K135)

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supported the adhesion of β3-CHO cells, but did not support the adhesion of mock-CHO cells or uPAR-CHO cells (Figs. 2e and 2f). Although both kringles supported the adhesion of β3-CHO cells, we observed more binding to K143 than to K135 (Fig. 2f). These results suggest that 1) GFD is critical for uPA binding to uPAR, consistent with previous reports, but is not critical for binding to αvβ3, and that 2) the isolated kringle domain supports uPA binding to αvβ3, but not to uPAR.

Direct binding of the uPA kringle to αvβ3

We tested whether the isolated uPA kringle fragment binds directly to isolated αvβ3. To do so, we first measured the binding of recombinant soluble αvβ3 to immobilized uPA kringle (Fig. 3a). We found that recombinant soluble αvβ3 directly bound to the kringle in an activation-dependent manner. Binding of recombinant soluble αvβ3 to the uPA kringle was inhibited by EDTA, cyclic RGD, and the ligand-blocking anti-αvβ3 mAbs LM609 and anti-β3 mAb 7E3, indicating that the binding is RGD- and αvβ3-dependent (Fig. 3b).

We also determined the binding of labeled uPA kringle to immobilized purified αvβ3. We found that the binding of uPA kringle to αvβ3 was cation-dependent, activation-dependent, dose-dependent and saturable (half-maximal binding ~125 nM; Fig. 3c) and roughly parallel to binding of β3-CHO to immobilized kringle (Fig. 2e). There was no specific binding of ^{125}I-kringle to immobilized albumin measured in parallel (not shown). The binding of labeled uPA kringle to immobilized purified αvβ3 was effectively blocked by unlabeled uPA kringle (Fig. 3d). Also, RGD peptide and EDTA blocked, but RGE peptide did not block, binding of labeled uPA kringle to immobilized purified αvβ3 (60.5%, 46%, and 10.8% inhibition, respectively). These results clearly indicate that the interaction between uPA kringle and αvβ3 is specific, and that the uPA kringle binds directly to αvβ3 as a ligand.

We have reported that angiostatin (a plasminogen fragment that contains N-terminal 3 or 4 plasminogen kringle domains) is a ligand for αvβ3 (38). We therefore tested whether angiostatin competes for binding to αvβ3. We found that the binding of ^{125}I-labeled uPA kringle was blocked by angiostatin (Fig. 3d), indicating that the uPA kringle and angiostatin share common binding-sites on αvβ3.

The uPA kringle domain induces αvβ3-mediated cell migration.

It has been reported that uPA induces cell migration (13-17). We tested whether the uPA kringle domain induces cell migration upon binding to αvβ3. We found that uPA, ATF, and the isolated kringle (K143) all induced the migration of β3-CHO cells to a similar extent, but did not affect the migration of CHO cells (Fig. 4a). RGD peptide, anti-β3 blocking mAb 16N7C2, and anti-uPA kringle mAb 963 inhibited the uPA kringle-induced cell migration, whereas RGE peptide and control antibodies did not (Fig. 4b), suggesting that the migration is specific to αvβ3 and the uPA kringle. These results suggest that the uPA kringle may induce migration of β3-CHO cells in an αvβ3-dependent manner. We have previously shown that angiostatin, unlike other integrin ligands, does not induce stress-fiber formation or spreading on αvβ3 binding (38) or migration of β3-CHO cells (39), suggesting that angiostatin may be an αvβ3 antagonist. In accordance with this concept, angiostatin blocked uPA kringle-induced migration of β3-CHO cells (Fig. 4c). These results suggest that angiostatin acts as an αvβ3 antagonist by blocking the binding of the uPA-kringle to the integrin.

Binding specificity of the isolated kringle domain to integrins other than αvβ3.

We then asked whether αvβ3 antagonists totally inhibit uPA kringle-mediated signaling in other cells or situations. To begin to address this question, we tested the ability of other integrins to bind to the isolated kringle domain using CHO cells expressing different integrins (Fig. 5). We found that the kringle domain supports adhesion of α4- and α9-CHO cells, but did not support adhesion of α2-, α3-, and mock-transfected CHO cells, suggesting that α2β1 and α3β1 are not important for binding to the uPA kringle. Antibodies to α4β1 and α9β1 integrins blocked adhesion to the kringle domain, suggesting that the adhesion is specific. Adhesion of mock-transfected CHO cells to the uPA kringle was no greater than that of the α5-deficient B2 variant of CHO cells (33), suggesting that α5β1 is also not important for binding to the uPA kringle. These results indicate that the uPA kringle may interact with several β1 integrins in addition to αvβ3, and that these integrins may contribute to uPA-induced signaling through binding to the uPA kringle.

Plasminogen activation on the cell surface in a uPA kringle and integrin-dependent manner.

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We have tested whether the interaction between integrins and uPA kringle involved in plasminogen activation. We treated β3-CHO and control CHO cells with PIPLC as described to remove cell surface GPI-linked proteins including uPAR, and then incubated with uPA or ΔK-uPA. We then measured the ability of the cells to activate plasminogen by using a plasmin-specific substrate. We found that β3-CHO cells showed much higher ability to activate plasminogen in a manner dependent on the uPA added (Fig. 6). Deletion of the kringle domain (with ΔK-uPA) markedly reduced the plasminogen activation on β3-CHO, indicating that αvβ3 and uPA-dependent plasminogen activation required the kringle domain of uPA. These results suggest that the binding of uPA kringle to integrin αvβ3 induces plasminogen activation.

The binding of uPA and uPA kringle to integrin αvβ3 in the presence of serum.
Since the body fluid contains several major adhesion molecules (e.g., fibrinogen and vitronectin), it is possible that these proteins block the binding of uPA or uPA kringle to integrins and thus the binding of uPA and uPA kringle to integrins may not occur in the presence of these adhesion molecules. We found that β3-CHO cells adhered to immobilized uPA and uPA kringle at much higher levels than mock-CHO cells in the presence of 10% FCS (Fig. 7), suggesting that uPA and uPA kringle can bind to αvβ3 under the conditions used. This is consistent with the observations that the RGD motifs are cryptic in soluble fibronectin, fibrinogen, vitronectin and other adhesion molecules in the body fluid (40, 41).

Endothelial cell adhesion to and migration on uPA and uPA kringle.
We have used CHO cells that express recombinant integrins to define integrin specificity of uPA in this study. We tested whether endothelial cells bind to uPA and uPA kringle in an integrin-dependent manner. αvβ3 is a major integrin in calf pulmonary artery endothelial (CPAE) cells (data not shown) and their integrin expression profile is similar to that of bovine aorta endothelial (BAE) cells (32). We found that CPAE cells adhered to uPA and uPA kringle and anti-αvβ3 mAb LM609 effectively blocked the adhesion (Fig. 8a). Also CPAE cells migrated on uPA and uPA kringle and LM609 effectively blocked the migration (Fig. 8b). These results suggest that integrin-dependent cell

adhesion to and migration on uPA and uPA kringle are not limited to CHO cells.

DISCUSSION

Specific uPA kringle-integrin interaction.
In the present study we demonstrated that 1) uPA binds specifically to the integrin αvβ3 on the surface of cells depleted of uPAR, 2) uPA lacking the GFD (that is required for uPA binding to uPAR) can still bind to the cell surface through αvβ3, and 3) the uPA kringle is responsible for αvβ3-dependent cell adhesion. Antibodies against αvβ3 and RGD peptide blocked these interactions, indicating that uPA kringle binds to αvβ3 in a manner similar to that of other known αvβ3 ligands. In addition, we showed that β1 integrins (α4β1 and α9β1) interact with the uPA kringle to a similar extent as αvβ3. Taken together, these results suggest that the uPA kringle-integrin interaction mediates uPA binding to the cell surface in a uPAR-independent manner, and that anti-integrin and anti-uPA kringle agents will block uPAR-independent uPA binding to the cell surface. These results do not, however, exclude the possibility that other domains of uPA or other non-integrin cell surface receptors may be involved in uPAR-independent uPA binding to cells (22).

It has been reported that cells express high and low affinity uPA binding sites that are distinguishable based on affinity and the requirements for ligand binding. The high affinity-binding sites (Kd ~1 nM) correspond to uPAR that binds to uPA through the GFD (19, 20), but the low affinity-binding sites (Kd 20-90 nM) have not been identified. The present results suggest that the low affinity-binding sites may include integrins such as αvβ3. The αvβ3 integrin is a promiscuous receptor for many extracellular matrix ligands, including vitronectin, fibronectin, and fibrinogen. Its expression is greatly up-regulated on endothelial cells exposed to growth factors or undergoing angiogenesis in tumors, wounds, and inflammatory tissue (42) and blockade of αvβ3 with mAb or low-molecular weight antagonists inhibits blood vessel formation in a variety of in vivo models (43), including tumor angiogenesis and neovascularization during oxygen-induced retinopathy. Thus, expression of this integrin on activated endothelial cells may be important in angiogenesis and neovascularization during development. During this paper is under review it has also been reported the kringle domain of uPA potentiates LPS-induced neutrophil

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activation through interaction with αvβ3 integrins (44). We propose that uPA binding to αvβ3 facilitate plasminogen activation on the cell surface and transduces signals through integrin pathways. This is consistent with the potential roles of αvβ3 under such conditions. It is intriguing that anti-integrin agents may be a potent means to modulate uPA-mediated signaling.

We showed that uPA and uPA kringle bind to αvβ3 in the presence of 10% FCS. This is consistent with the previous reports that RGD adhesive signals are cryptic or poorly exposed in several integrin ligands, including fibrinogen (40), vitronectin (41), and fibronectin (45). Thus, we conclude that uPA and uPA kringle can bind to integrins in the presence of the adhesion molecules described above in the body fluid, and thus the binding of uPA and uPA kringle to integrins is biologically relevant.

It has been reported that the uPA kringle facilitates the binding of uPA to uPAR (6). An uPA variant lacking the kringle bound to soluble uPAR with the similar "on-rate" but with a faster "off-rate" than wild type, demonstrating an important role for the kringle in stabilizing the binding of uPA to uPAR. The present study demonstrates that the uPA kringle specifically interacts with several integrins. It is, however, unclear whether the interaction between uPA kringle and integrins further stabilizes uPA binding to uPAR at this point. If this is the case, it is expected that blocking the kringle-integrin interaction may block uPA/uPAR signaling (e.g., with the isolated uPA kringle and angiostatin). It would be interesting to address this hypothesis in future experiments using uPA mutants in which integrin-binding sites in the kringle domain are mutated.

Integrin αvβ3-uPA kringle interaction contributes to plasminogen activation.
Pluskota et al. reported that neutrophil integrin αMβ2 recognizes the kringle domain of uPA, and proposed that αMβ2, uPAR, and uPA generate a trimolecular complex (46). These results suggest that direct interactions between the uPA kringle and integrins play a critical role in uPA binding to cell surface. However, the expression of αMβ2 is limited to leukocytes, and thus it was unclear whether such integrin-dependent plasminogen activation occurs in other cell types. We showed that the binding of uPA to integrin αvβ3 through the kringle domain contributes to plasminogen activation besides integrin-mediated signaling.

While αMβ2 directly binds to both plasminogen and uPA (through the kringle domain) (47), we have previously reported that integrin αvβ3 does not bind plasminogen while it binds to plasmin and angiostatin (38, 39). Thus αvβ3 and uPA-dependent plasminogen activation probably requires other plasminogen receptors, notably annexin II (48) and α-enolase (49). Since αMβ2 is limited to neutrophils and monocytes, the non-αMβ2- dependent plasminogen activation is potentially important in other cell types expressing αvβ3 (e.g., activated endothelial cells, osteoclasts, and cancer cells). Indeed we showed that endothelial cells adhere to and migrate on uPA and uPA kringle in an αvβ3-dependent manner. Also other uPA kringle binding integrins (e.g., α4β1 and α9β1) are expected to mediate plasminogen activation as well (e.g., on lymphocytes and neutrophils).

The uPA kringle is an αvβ3 agonist, while angiostatin (plasminogen kringles 1-4) is an αvβ3 antagonist
There is extensive in vitro and in vivo data to indicate that uPA contributes to tumor cell migration and that its expression correlates inversely with prognosis in human cancers (16, 50). It is also clear that angiostatin is a potent inhibitor of tumor-induced angiogenesis in animal models. However, the mechanism by which uPA promotes tumor proliferation and migration and the mechanism of action of angiostatin are both incompletely understood. We have previously reported that angiostatin is a ligand for integrin αvβ3, but does not induce stress fiber formation upon integrin binding, suggesting that angiostatin is a potential integrin antagonist (38). We have shown that plasmin also interacts with αvβ3 through its kringle domains, and that angiostatin blocks plasmin-induced signaling (39), suggesting that plasmin signaling may be a target for angiostatin. In the present study, we have shown that the uPA kringle induces αvβ3-mediated cell migration, suggesting that the uPA kringle is an αvβ3 agonist. Angiostatin does not induce migration of β3-CHO cells (39). Interestingly, angiostatin blocked binding of the uPA kringle to purified αvβ3, suggesting that angiostatin and uPA kringle compete for binding to αvβ3. We have also shown that angiostatin blocked the uPA kringle-induced cell migration, suggesting that uPA kringle-induced signaling may be another potential target for angiostatin activity. It would be interesting to address whether angiostatin blocks integrin- and uPA kringle-dependent plasminogen

activation in future studies. Interestingly, the uPA kringle and plasminogen kringle are homologous and each of the disulfide linkages is conserved (amino acid identity is 35-41%). The plasminogen kringles (e.g., K5) and uPA kringle are structurally very similar and they are superposable with a root mean square distance about 1.5 Å. It would be interesting to study in future experiments how the two homologous molecules induce apparently opposite effects upon integrin binding.

CONCLUSIONS

uPA binds specifically to the integrin $\alpha v\beta 3$ on the surface of cells depleted of uPAR. The kringle domain within full-length uPA is required for $\alpha v\beta 3$ binding. The isolated uPA kringle domain binds specifically to purified, recombinant soluble, and cell surface $\alpha v\beta 3$, as well as to certain other integrins ($\alpha 4\beta 1$ and $\alpha 9\beta 1$), and induced migration of CHO cells in an $\alpha v\beta 3$-dependent manner. These results suggest that certain integrins can function as receptors for uPA through binding to uPA kringle. Besides integrin-mediated signal transduction the binding of uPA to $\alpha v\beta 3$ was shown to enhance plasminogen activation on the cell surface. uPA kringle-integrin interactions may represent a novel therapeutic target for cancer, inflammation, and vascular remodeling.

REFERENCES
1.   Chapman HA. Plasminogen activators, integrins, and the coordinated regulation of cell adhesion and migration. Curr Opin Cell Biol 1997;9(5):714-24.
2.   Andreasen PA, Kjoller L, Christensen L, Duffy MJ. The urokinase-type plasminogen activator system in cancer metastasis: a review. Int J Cancer 1997;72(1):1-22.
3.   Dano K, Romer J, Nielsen BS, Bjorn S, Pyke C, Rygaard J, et al. Cancer invasion and tissue remodeling--cooperation of protease systems and cell types. Apmis 1999;107(1):120-7.
4.   Mondino A, Blasi F. uPA and uPAR in fibrinolysis, immunity and pathology. Trends Immunol 2004;25(8):450-5.
5.   Appella E, Blasi F. The growth factor module of urokinase is the binding sequence for its receptor. Ann N Y Acad Sci 1987;511:192-5.
6.   Bdeir K, Kuo A, Sachais BS, Rux AH, Bdeir Y, Mazar A, et al. The kringle stabilizes urokinase binding to the urokinase receptor. Blood 2003.
7.   Waltz DA, Fujita RM, Yang X, Natkin L, Zhuo S, Gerard CJ, et al. Nonproteolytic role for the urokinase receptor in cellular migration in vivo. Am J Respir Cell Mol Biol 2000;22(3):316-22.
8.   Ellis V, Pyke C, Eriksen J, Solberg H, Dano K. The urokinase receptor: involvement in cell surface proteolysis and cancer invasion. Ann N Y Acad Sci 1992;667:13-31.
9.   Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, et al. Physiological consequences of loss of plasminogen activator gene function in mice. 1994;368(6470):419-424.
10.   Bugge TH, Flick MJ, Danton MJ, Daugherty CC, Romer J, Dano K, et al. Urokinase-type plasminogen activator is effective in fibrin clearance in the absence of its receptor or tissue-type plasminogen activator. Proc Natl Acad Sci U S A 1996;93(12):5899-904.
11.   Waltz DA, Sailor LZ, Chapman HA. Cytokines induce urokinase-dependent adhesion of human myeloid cells. A regulatory role for plasminogen activator inhibitors. J Clin Invest 1993;91(4):1541-52.
12.   Gyetko MR, Todd RF, 3rd, Wilkinson CC, Sitrin RG. The urokinase receptor is required for human monocyte chemotaxis in vitro. J Clin Invest 1994;93(4):1380-7.
13.   Busso N, Masur SK, Lazega D, Waxman S, Ossowski L. Induction of cell migration by pro-urokinase binding to its receptor: possible mechanism for signal transduction in human epithelial cells. J Cell Biol 1994;126(1):259-70.
14.   Odekon LE, Sato Y, Rifkin DB. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. J Cell Physiol 1992;150(2):258-63.
15.   Rabbani SA, Mazar AP, Bernier SM, Haq M, Bolivar I, Henkin J, et al. Structural requirements for the growth factor activity of the amino-terminal domain of urokinase. J Biol Chem 1992;267(20):14151-6.
16.   Aguirre Ghiso JA, Kovalski K, Ossowski L. Tumor dormancy induced by downregulation of urokinase receptor in human carcinoma involves integrin and MAPK signaling. J Cell Biol 1999;147(1):89-104.
17.   Fischer K, Lutz V, Wilhelm O, Schmitt M, Graeff H, Heiss P, et al. Urokinase induces proliferation of human ovarian cancer cells: characterization of structural elements required for growth factor function. FEBS Lett 1998;438(1-2):101-5.
18.   Kanse SM, Benzakour O, Kanthou C, Kost C, Lijnen HR, Preissner KT. Induction of vascular SMC proliferation by urokinase indicates a novel mechanism

of action in vasoproliferative disorders. Arterioscler Thromb Vasc Biol 1997;17(11):2848-54.

19.   Koopman JL, Slomp J, de Bart AC, Quax PH, Verheijen JH. Mitogenic effects of urokinase on melanoma cells are independent of high affinity binding to the urokinase receptor. J Biol Chem 1998;273(50):33267-72.

20.   Mukhina S, Stepanova V, Traktouev D, Poliakov A, Beabealashvilly R, Gursky Y, et al. The chemotactic action of urokinase on smooth muscle cells is dependent on its kringle domain; Characterization of interactions and contribution to chemotaxis. J Biol Chem 2000;275:16450-16458.

21.   Haj-Yehia A, Nassar T, Sachais BS, Kuo A, Bdeir K, Al-Mehdi AB, et al. Urokinase-derived peptides regulate vascular smooth muscle contraction in vitro and in vivo. Faseb J 2000;14(10):1411-1422.

22.   Nassar T, Haj-Yehia A, Akkawi S, Kuo A, Bdeir K, Mazar A, et al. Binding of urokinase to low density lipoprotein-related receptor (LRP) regulates vascular smooth muscle cell contraction. J Biol Chem 2002;277(43):40499-504.

23.   Miyake K, Hasunuma Y, Yagita H, Kimoto M. Requirement for VLA-4 and VLA-5 integrins in lymphoma cells binding to and migration beneath stromal cells in culture. J. Cell Biol. 1992;119:653-662.

24.   Wang A, Yokosaki Y, Ferrando R, Balmes J, Sheppard D. Differential regulation of airway epithelial integrins by growth factors. Am J Respir Cell Mol Biol 1996;15(5):664-72.

25.   Coller BS. A new murine monoclonal antibody reports an activation-dependent change in the conformation and/or microenvironment of the platelet glycoprotein IIb/IIIa complex. J Clin Invest 1985;76(1):101-8.

26.   Deckmyn H, Stanssens P, Hoet B, Declerck PJ, Lauwereys M, Gansemans Y, et al. An echistatin-like Arg-Gly-Asp (RGD)-containing sequence in the heavy chain CDR3 of a murine monoclonal antibody that inhibits human platelet glycoprotein IIb/IIIa function. Br J Haematol 1994;87(3):562-71.

27.   Cheresh DA, Smith JW, Cooper HM, Quaranta V. A novel vitronectin receptor integrin $(\alpha_v\beta_x)$ is responsible for distinct adhesive properties of carcinoma cells. Cell 1989;57:59-69.

28.   Min HY, Semnani R, Mizukami IF, Watt K, Todd RFd, Liu DY. cDNA for Mo3, a monocyte activation antigen, encodes the human receptor for urokinase plasminogen activator. J Immunol 1992;148(11):3636-42.

29.   Higazi AAR, Mazar A, Wang J, Quan N, Griffin R, Reilly R, et al. Soluble human urokinase receptor is composed of two active units. J Biol Chem 1997;272(8):5348-53.

30.   Takagi J, DeBottis DP, Erickson HP, Springer TA. The role of the specificity-determining loop of the integrin beta subunit I-like domain in autonomous expression, association with the alpha subunit, and ligand binding. Biochemistry 2002;41(13):4339-47.

31.   Zhang XP, Kamata T, Yokoyama K, Puzon-McLaughlin W, Takada Y. Specific interaction of the recombinant disintegrin-like domain of MDC- 15 (metargidin, ADAM-15) with integrin alphavbeta3. J Biol Chem 1998;273(13):7345-50.

32.   Tarui T, Mazar AP, Cines DB, Takada Y. Urokinase-type plasminogen activator receptor (CD87) is a ligand for integrins and mediates cell-cell interaction. J Biol Chem 2001;276(6):3983-90.

33.   Schreiner CL, Bauer JS, Danilov YN, Hussein S, Sczekan M, Juliano RL. Isolation and characterization of chinese hamster ovary cell variants deficient in the expression of fibronectin receptor. J. Cell Biol. 1989;109:3157-3167.

34.   Prater CA, Plotkin J, Jaye D, Frazier WA. The properdin-like type I repeats of human thrombospondin contain a cell attachment site. J. Cell Biol. 1991;112:1031-1040.

35.   Takagi J, Erickson HP, Springer TA. C-terminal opening mimics 'inside-out' activation of integrin alpha5beta1. Nat Struct Biol 2001;8(5):412-6.

36.   Takada Y, Puzon W. Identification of a regulatory region of integrin β1 subunit using activating and inhibiting antibodies. J. Biol. Chem. 1993;268:17597-17601.

37.   Tarui T, Andronicos N, Czekay RP, Mazar AP, Bdeir K, Parry GC, et al. Critical role of integrin alpha 5 beta 1 in urokinase (uPA)/urokinase receptor (uPAR, CD87) signaling. J Biol Chem 2003;278(32):29863-72.

38.   Tarui T, Miles LA, Takada Y. Specific interaction of angiostatin with integrin {alpha}v{beta}3 in endothelial cells. J Biol Chem 2001;276:39562-39568.

39.   Tarui T, Majumdar M, Miles LA, Ruf W, Takada Y. Plasmin-induced migration of endothelial cells: A potential target for the anti-angiogenic action of angiostatin. J Biol Chem 2002;277:33564-33570.

40.   Ugarova T, Budzynski A, Shattil S, Ruggeri Z, Ginsberg M, Plow E. Conformational changes in fibrinogen elicited by its interaction with platelet membrane glycoprotein GPIIb-IIIa. Journal of Biological Chemistry 1993;268(28):21080-7.

41.   Seiffert D, Smith JW. The cell adhesion domain in plasma vitronectin is cryptic. J Biol Chem 1997;272(21):13705-10.

42.   Eliceiri BP, Cheresh DA. The role of alphav integrins during angiogenesis: insights into potential mechanisms of action and clinical development. J Clin Invest 1999;103(9):1227-30.

43.	Eliceiri BP. Integrin and growth factor receptor crosstalk. Circ Res 2001;89(12):1104-10.

44.	Kwak S-H, Mitra S, Bdeir K, Strassheim D, Park J, Kim J, et al. The kringle domain of urokinase-type plasminogen activator potentiates LPS-induced neutrophil activation through interaction with avb3 integrins. J. Leukoc. Biol. 2005;78.

45.	Ugarova TP, Zamarron C, Veklich Y, Bowditch RD, Ginsberg MH, Weisel JW, et al. Conformational transitions in the cell binding domain of fibronectin. Biochemistry 1995;34(13):4457-66.

46.	Pluskota E, Soloviev DA, Plow EF. Convergence of the adhesive and fibrinolytic systems: recognition of urokinase by integrin alpha Mbeta 2 as well as by the urokinase receptor regulates cell adhesion and migration. Blood 2003;101(4):1582-90.

47.	Pluskota E, Soloviev DA, Bdeir K, Cines DB, Plow EF. Integrin alphaMbeta2 orchestrates and accelerates plasminogen activation and fibrinolysis by neutrophils. J Biol Chem 2004;279(17):18063-72.

48.	Celikel R. Crystallization of the OP-G2 Fab fragment: a fibrinogen mimic with specificity for the platelet glycoprotein IIb/IIIa. Acta Crystallogr D Biol Crystallogr 1993;49(Pt 4):421-2.

49.	Miao H, Li S, Hu YL, Yuan S, Zhao Y, Chen BP, et al. Differential regulation of Rho GTPases by beta1 and beta3 integrins: the role of an extracellular domain of integrin in intracellular signaling. J Cell Sci 2002;115(Pt 10):2199-2206.

50.	Ossowski L, Aguirre-Ghiso JA. Urokinase receptor and integrin partnership: coordination of signaling for cell adhesion, migration and growth. Curr Opin Cell Biol 2000;12(5):613-20.

Fig. 1. uPA binding to the cell surface in an integrin αvβ3-dependent and uPAR-independent manner.

(Panels a and b) Depletion of uPAR from the cell surface blocked uPA binding to uPAR-CHO cells, but did not affect uPA binding to β3-CHO cells. To deplete GPI-linked uPAR on the cell surface, β3-CHO, uPAR-CHO, or control mock-transfected CHO cells were treated with PIPLC. The treatment removed more than 95% of human uPAR from uPAR-CHO cells as determined by flow cytometry with anti-uPAR mAb 3B10 (data not shown). uPA was immobilized to wells of 96-well microtiter plates at the indicated coating concentrations, and incubated with cells without (a) or with (b) pre-treatment with PI-PLC. Bound cells were quantified as described (39). (Panel c) uPA binding to β3-CHO cells is specific to αvβ3 and the kringle domain. uPA (200 nM coating concentration) was immobilized to wells of 96-well microtiter plates and incubated with β3-CHO cells in the presence of mAb 16N7C2 (anti-β3), Ab 963 (anti-kringle), mAb UNG-5 (anti-LMW-uPA), or RGD or RGE peptides (100 μM). Data are shown as means +/- S.D. of triplicate experiments.

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Fig. 2. The kringle domain of uPA mediates binding to αvβ3.
(Panel a) The uPA fragments or mutants used in the experiments. (Panels b, d, e) uPA fragments were immobilized onto wells of 96-well microtiter plates at the indicated coating concentrations and incubated with β3-CHO, uPAR-CHO, or mock-CHO cells. The ability of the uPA fragments to support adhesion of these cells was determined as described in the text (b. ΔGFD-uPA; d, ATF; and e, kringle 47-143 (K143)). (Panel c) Binding of labeled ΔGFD-uPA to immobilized purified αvβ3 was performed as described in the text in the presence of 2 mM $MnCl_2$. BSA instead of purified αvβ3 was used as a negative control. Specific binding is calculated by subtracting the binding of labeled ΔGFD-uPA to BSA from the binding to purified αvβ3. Note that specific binding of ΔGFD-uPA to αvβ3 is saturable. (Panel f) uPA kringle 47-143 (K143) supports adhesion of β3-CHO cells better than uPA kringle 47-135 (K135). K143 and K135 were immobilized onto wells of 96-well microtiter plate at a coating concentration of 500 nM and incubated with β3-CHO and mock-transfected CHO cells. Data are shown as means +/- S.D. of triplicate experiments.

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Fig. 3. Binding of recombinant soluble αvβ3 to uPA kringle.
(Panel a). Binding of recombinant soluble αvβ3 to various ligands was measured. Wells of 96-well microtiter plates were coated with vitronectin (10 μg/ml), or uPA kringle (K135)(50 μg/ml), blocked with BSA, and incubated with recombinant soluble αvβ3 (5 μg/ml) at room temperature for 2h. Bound integrins were quantified using biotinylated anti-tag antibody, horseradish peroxidase-conjugated streptavidin and peroxidase substrate 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS). Absorbance at 415 nm was measured. Data is shown as an average of two independent experiments. (Panel b). Binding of recombinant soluble αvβ3 to uPA kringle was measured in the presence of various inhibitors. The inhibitors and the concentrations used are EDTA (2 mM), cRGDfV (48 μM), LM609 (anti-αvβ3, at 50x dilution of ascites), 7E3 (anti-β3, 10 μg/ml), and control IgG (purified mouse IgG, 10 μg/ml, Sigma). (Panel c). Cation-dependent binding of uPA kringle to αvβ3. Wells of 96-well microtiter plates were coated with 45 μl of 5 μg/ml purified human αvβ3, and blocked with BSA. The wells were incubated for 1 h at 37°C with ^{125}I-labeled uPA kringle at the indicated concentrations in the presence of 2 mM EDTA instead of 2 mM MgCl$_2$. The bound radioactivity was then measured. Specific binding is calculated by subtracting the binding in the presence of EDTA from the total binding. (Panel d). Binding of ^{125}I-labeled uPA kringle to immobilized αvβ3 was measured in the presence of increasing concentrations of either unlabeled uPA kringle or angiostatin (K1-4). The data suggest that the binding of uPA kringle to αvβ3 is specific and that uPA kringle and angiostatin compete for binding to αvβ3. Data are shown as means +/- S.D. of triplicate experiments.

Fig. 4. uPA kringle induces cell migration in an αvβ3-dependent manner.

(Panel a) Cell migration was analyzed using tissue culture-treated 24-well Transwell plates. The lower side of the filter was coated with uPA, ATF, or kringle (K143). Coated filters were placed into serum-free migration buffer, and β3-CHO cells (closed column) or mock-CHO cells (open column) were added to the upper chamber. The cells were incubated at 37°C in 5% CO_2 for 12-16 h. Cells that migrated to the lower surface of the filters were counted. Data are shown as the number of migrating cells relative to those of β3-CHO cells with no coating. uPA, ATF, and K143 were used at a coating concentration of 500 nM each. (Panel b) The effect of various inhibitors on migration of β3-CHO cells on uPA kringles was determined. Inhibitors and their concentrations are RGD and RGE peptides at 100 μM; 16N7C2 (anti-β3) and Ab 963 (anti-uPA kringle) at 5 μg/ml. (Panel c). Migration of β3-CHO cells on uPA kringles (K143 and K135) at the coating concentration of 500 nM was measured in the presence and absence of angiostatin (AS, K1-3 at 4 μM). Angiostatin was added to the upper chamber at 4 μM. Data are shown as means +/- S.D. of triplicate experiments.

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Fig. 5. uPA kringle interacts specifically with several integrins.
uPA kringle (K143) was immobilized onto wells of 96-well microtiter plates at a coating concentrations of 300 nM, and incubated with CHO cells expressing different integrins. Cells adhered to immobilized uPA kringle were quantified. B2 (α5-deficient CHO variant), mock-CHO, uPAR-CHO cells, and BSA were used as controls. MAbs to α4 (SG/72), α9 (Y9A2), and αvβ3 (LM609) were used at 10 μg/ml to explore the specificity of binding. Data are shown as means +/- S.D. of triplicate experiments.

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Fig. 6. Integrin-dependent plasminogen activation on the cell surface.
Parental CHO cells and β3-CHO cells in wells of 96-well plate were treated with PIPLC to deplete uPAR, and incubated with wt or delta kringle (ΔK) uPA in the cold binding buffer for 1 h at 4°C. The cells were washed with the binding buffer, and plasminogen activation was determined using Glu-plasminogen and SpectrozymePL chromogenic substrate at 37°C. Data are shown as means +/- S.D. of triplicate experiments.

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Fig. 7. Adhesion of β3-CHO cells to uPA and uPA kringle in the presence of 10% FCS.
Adhesion of β3-CHO and mock CHO cells was tested in Hepes-Tyrode buffer containing 2 mM Mg^{2+} as described in the Method section except that the buffer contains 10% FCS. The coating concentration of uPA and uPA kringle is 100 nM. Data are shown as means +/- S.D. of triplicate experiments.

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Fig8. Interaction of bovine pulmonary artery endothelial (CPAE) cells with uPA and uPA kringle.
a) Adhesion of CPAE cells to immobilized uPA and uPA kringle (at the 100 nM coating concentration) was measured as described in the method section. Anti-integrin $\alpha v\beta 3$ LM609 was used at 10 μg/ml. Data are shown as means +/- S.D. of triplicate experiments. The results suggest that CPAE cells adhere to uPA and uPA kringle in an $\alpha v\beta 3$-dependent manner.
b) Migration of CPAE cells on uPA and uPA kringle (100 nM coating concentrations) was determined as described in the method section using Transwell modified Boyden chambers. Anti-integrin $\alpha v\beta 3$ LM609 was used at 10 μg/ml. 1% FCS was included in the migration buffer. The number of migrating cells to the bottom of the membrane was counted. Data are shown as means +/- S.D. of triplicate experiments. The results suggest that CPAE cells migrate on uPA and uPA kringle in an $\alpha v\beta 3$-dependent manner.

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