Critical Role of Integrin $\alpha_5\beta_1$ in Urokinase (uPA)/Urokinase Receptor (uPAR, CD87) Signaling*

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Urokinase-type plasminogen activator (uPA) induces cell adhesion and chemotactic movement. uPA signaling requires its binding to uPA receptor (uPAR/CD87), but how glycosylphosphatidylinositol-anchored uPAR mediates signaling is unclear. uPAR is a ligand for several integrins (e.g. $\alpha_5\beta_1$) and supports cell-cell interaction by binding to integrins on apposing cells (in trans). We studied whether binding of uPAR to $\alpha_5\beta_1$ in cis is involved in adhesion and migration of Chinese hamster ovary cells in response to immobilized uPA. This process was temperature-sensitive and required mitogen-activated protein kinase activation. Anti-uPAR antibody or depletion of uPAR blocked, whereas overexpression of uPAR enhanced, cell adhesion to uPA. Adhesion to uPA was also blocked by deletion of the growth factor domain (GFD) of uPA and by anti-GFD antibody, whereas neither the isolated uPA kringle nor serine protease domain supported adhesion directly. Interestingly, anti-$\alpha_5$ antibody, RGD peptide, and function-blocking mutations in $\alpha_5\beta_1$ blocked adhesion to uPA. uPA-induced cell migration also required GFD, uPA, and $\alpha_5\beta_1$, but $\alpha_5\beta_1$ alone did not support uPA-induced adhesion and migration. Thus, binding of uPAR causes uPAR to act as a ligand for $\alpha_5\beta_1$ to induce cell adhesion, intracellular signaling, and cell migration. We demonstrated that uPA induced RGD-dependent binding of uPAR to $\alpha_5\beta_1$ in solution. These results suggest that uPA-induced adhesion and migration of Chinese hamster ovary cells occurs as a consequence of (a) uPA binding to uPAR through GFD, (b) the subsequent binding of a uPA-uPAR complex to $\alpha_5\beta_1$ via uPAR, and (c) signal transduction by $\alpha_5\beta_1$.

Urokinase-type plasminogen activator (uPA) is a highly restricted serine protease that converts the zymogen plasminogen to the active plasmin. Plasmin, in turn, mediates pericellular proteolysis of extracellular matrix proteins in the path of cellular invasion (1, 2). uPA has also been shown to induce adhesion and chemotactic movement of myeloid cells (3, 4), to induce cell migration in human epithelial cells (5) and bovine endothelial cells (6), and to promote cell growth (7–9). These signaling functions of uPA do not require its proteolytic activity.

uPA is composed of three independently folded domain structures, growth factor domain (GFD) (residue 1–43), kringle domain (residue 50–131), and serine protease domain (residue 159–411). Enzymatic digestion of uPA by plasmin generates an amino-terminal fragment (ATF) that consists of the GFD and kringle domains and the low molecular weight fragment (LMW-uPA), possessing serine protease activity. uPA binds with high affinity through GFD (10) to a cell-surface receptor (uPAR/CD87) that has been identified in many cell types (1). uPAR is a glycosylphosphatidylinositol-anchored 35–55-kDa glycoprotein. It is generally accepted that uPA-mediated signaling requires prior binding to uPAR. However, the mechanism by which uPAR mediates signaling events is still to be fully elucidated. A major problem in understanding how uPA signals derives from the fact that uPAR has no transmembrane structure, leading to the proposal that hypothetical transmembrane adapters may be involved in this process (11).

Among the candidate transmembrane adapters are the integrins, a family of cell adhesion receptor heterodimers that interact with many extracellular matrix and cell-surface ligands (12). At least 18 $\alpha$ and 8 $\beta$ subunits have been identified. Integrin-ligand interaction is involved in many biological and pathological situations, including cell anchorage and migration, cell-cell interaction during immune response, development, wound healing, vascular remodeling, and cancer metastasis and invasion (13). Integrins transduce signals from outside cells through their interaction with specific ligands. uPAR has been shown to associate with integrins by co-immunoprecipitation, immunocolocalization, and resonance energy transfer approaches (14–16). However, it has not been established whether the association of uPAR with integrins is responsible for uPA-mediated signaling. We have recently reported that recombinant soluble uPAR is a ligand for several $\beta_1$ and $\beta_2$ integrins (17), and we postulated that uPAR can transduce signals through the integrin signaling pathway upon binding to integrin in trans. However, it is still unclear whether uPAR binds to integrins as a ligand when both are present on the same membrane (in cis). It has been proposed that integrins “laterally associate” with uPAR (for review, see Ref. 2) and play a role in uPA-uPAR-initiated signaling events. However, the role of the integrin itself in the uPA-uPAR signaling is unclear, since in the current models other integrin
FIG. 1. Cell adhesion to uPA in a uPAR-dependent manner. a and b, expression of uPAR on mock-transfected CHO (a) and uPAR-CHO (b) cells. Cells were stained with rabbit polyclonal anti-uPAR or control rabbit IgG followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Stained cells were analyzed by flow cytometry. c, adhesion of uPAR- and mock-transfected CHO cells to immobilized uPA. Wells in 96-well Immulon-2 microtiter plates were coated with 100 µl of phosphate-buffered saline containing uPA at a concentration of 50–1000 nM. Cells (10^5 cells/well) were added to the wells and incubated at 37 °C for 1 h. Bound cells were quantified by measuring endogenous phosphatase activity.
ligands (e.g. fibronectin) appear to be essential for uPA-uPAR signaling (8).

In this study we designed experiments to identify the role of integrin α5β1 in uPA-uPAR signaling using recombinant uPA fragments and Chinese hamster ovary (CHO) cells overexpressing uPAR or mutant α5β1. We found that cells adhered to immobilized uPA in a signaling-dependent manner. Anti-uPAR antibody, depletion of uPAR, and deletion of the GFD of uPA effectively blocked cell adhesion to uPA, suggesting that binding of uPA to uPAR through GFD is critical for cell adhesion to uPA. Interestingly, anti-α5 antibody, RGD peptide, and function-blocking α5β1 mutations blocked cell adhesion to uPA, suggesting that α5β1 is critical to this process as well. uPA-induced migration of CHO cells also required GFD of uPA, uPAR, and α5β1. We demonstrated that uPA-induced RGD-dependent binding of uPAR to α5β1 in solution. These results suggest that uPA-induced signaling in CHO cells involves a process in which (a) uPA binds to uPAR, (b) the uPA-uPAR complex binds to α5β1 as a ligand in cis, and (c) signal transmission is initiated through α5β1.

**EXPERIMENTAL PROCEDURES**

**Materials**

Monoclonal antibody (mAb) KH72 (anti-α5) was a kind gift of K. Miyake (University of Tokyo, Tokyo, Japan). mAb 15-13C (anti-α5/α2) (18) was a kind gift of S. J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN). mAbs P1F6 (anti-α5β1), HA5 (anti-α5), and VC5 (anti-α5) were purchased from Chemicon (Temecula, CA). Anti-uPAR monoclonal antibody (3B10) (19) was kindly provided by R. F. Todd III (University of Michigan Medical Center, Ann Arbor, MI). The polyclonal anti-uPA has been described previously (20). The anti-uPA kringle antibody (AB863) was a kind gift from J. Henkin (Abbott Laboratories, Abbott Park, IL). Anti-uPA kringle and anti-LMW uPA mAbs linked to Sepharose 4B were from IKTEK Ltd. (Moscow, Russia). A mAb against soluble uPAR (clone D2D3–813, IgG1) was raised against the soluble uPAR D2D3 fragment. Strategic Biosolutions (Newark, DE) generated the ascsites and purified the antibody using a 50-ml Amersham Biosciences protein A-Sepharose fast flow column. GRGDS and GRGES peptides were purchased from Advanced ChemTech (Louisville, KY). Phosphatidylinositol-specific phospholipase C was obtained from Glyko, Inc. (Nevato, CA). PD98059 was purchased from Calbiochem. Protein G-agarose was from Amersham Biosciences. Na235I was purchased from PerkinElmer Life Sciences, and Iodo beads were from Pierce. CHO cells were obtained from the American Type Culture Collection (Manassas, VA). CHO cells expressing the three domain forms of human uPAR (designated uPAR-CHO) have been described (17). The α5-deficient CHO cells (B2 variant) expressing human α5 (wild type or mutant) have been described (21).

**Methods**

**Generation of Wild-type uPA and uPA Fragments**—cDNA encoding wild-type single-chain uPA (scuPA) was generated and subcloned into pHIT/BpPV5 (Invitrogen) as described previously (22). cDNA encoding the amino-terminal fragment (ATF, amino acids 1–143), kringle (amino acids 47–143), ΔGFd-scuPA (amino acids 47–411), and FLAG-LMW-uPA (amino acids 136–411) were generated by PCR with full-length UK/pUN121 (23) as a template. The PCR products were digested with BglII and subcloned into pHIT/BpPV5 at the EcoRI and Xhol sites. Recombinant proteins were expressed using the Drosophila expression system (Invitrogen) in Schneider S2 cells according to the manufacturer's recommendations. Wild-type scuPA, ΔGFd-scuPA, and FLAG-LMW-uPA were purified from S2 medium by affinity chromatography using anti-LMW uPA mAb immobilized onto Sepharose (IKTEK Ltd.). ATF 1–143 and kringle were purified from S2 medium by affinity chromatography using an anti-kringle uPA mAb immobilized onto Sepharose (IKTEK Ltd.). Synthesis of soluble uPA (D2D3 form) has been described (17).

**Adhesion Assays**—Adhesion assays were performed as previously described (24). Briefly, wells in 96-well Immulon-2 microtiter plates (Dyneact Laboratories, Chantilly, VA) were coated with 100 μl of phosphate-buffered saline (10 mM phosphate buffer, 0.15 M NaCl, pH 7.4) containing uPA binding substrates at a concentration of 50–1000 nM and were incubated 1 h at 37°C. Remaining protein binding sites were blocked by incubating with 0.2% BSA (Calbiochem) for 1 h at room temperature. Cells (105 cells/well) in 100 μl of Hepes-Tyrode buffer (10 μM HEPES, 150 mM NaCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, 2.5 mM KCl, 0.1% glucose, 0.02% BSA) supplemented with 2 mM MgCl2 were added to the wells and incubated at 37°C for 1 h unless stated otherwise. After non-bound cells were removed by rinsing the wells with the same buffer, bound cells were quantified by measuring endogenous phosphatase activity (25). Antibodies were used at a 250-fold dilution for ascites (KH72 and 135-13C) and at 10 μg/ml for purified antibodies or IgG. Data are shown as means ± S.D. of three independent experiments. We confirmed that equivalent amounts of the fragments and mutants of uPA were coated on the plate by enzyme-linked immunosorbent assay (data not shown).

**Mitogen-activated Protein Kinases (MAPK) Activation Assay**—uPAR-CHO cells were plated into 6-well tissue culture plates at 2 × 104 cells/ml in Dulbecco’s modified Eagle’s medium supplemented with 0.5% fetal calf serum, 1% penicillin-streptomycin-glutamine solution and incubated for 4 days at 37°C in 5% CO2 humidified atmosphere. The cell culture media was removed, and the cells were washed once with prewarmed serum-free Dulbecco’s modified Eagle’s medium. The cells were incubated for 3 h with serum-free Dulbecco’s modified Eagle’s medium with or without the MEK inhibitor PD98059 (50 μM) at 37°C in a 5% CO2 humidified atmosphere. The cells were stimulated with different concentrations of soluble scuPA for 5 min at 37°C. The reaction was terminated by removing the stimulation media and washing the cells with 1 ml of ice-cold phosphate-buffered saline containing 1 mM NaPO4, followed by incubation with 100 μl of ice-cold radioimmunune precipitation assay buffer for 20 min on ice. The whole cell lysate was collected, and the nuclear material was pelleted by centrifugation at 14,000 × g for 10 min. The supernatant from each treatment was retained and stored at −20°C until required. Whole cell lysates (40 μg of protein) were fractionated using 4–20% SDS-polyacrylamide gel electrophoresis and electrophotographed and electrophotographed transferred to nitrocellulose for Western blotting. The membranes were blocked for 1 h at room temperature with blocking buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5, supplemented with 0.1% Tween 20 and 5% BLOTTO; Biorad, Hercules, CA). To determine the phosphorylation changes in MAPKs, the blots were washed twice for 5 min with wash buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5, supplemented with 0.1% Tween 20) and incubated overnight at 4°C with a 1:1000 dilution of rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-204) antibody (Cell Signaling Technology, Beverly, MA) in blocking buffer. The blots were washed 3 times for 5 min with wash buffer and probed with a 1:2000 dilution of anti-rabbit horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology) in blocking buffer at room temperature for 1 h. The blots were washed 3 times for 5 min and developed using the Immun-Star horseradish peroxidase chemiluminescence substrate kit (Bio-Rad). The blots were stripped by incubating with stripping buffer (0.1 M glycine, pH 2.6, and 2% SDS) for 30 min at 50°C. The blots were washed 3 times with Tris-HCl, pH 7.5, removed from the blotter, and subjected to 1 h of temperature with blocking buffer, washed 3 times for 5 min, incubated with a 1:10000 dilution of rabbit anti-phospho-p44/42 MAPK (Thr-202/Tyr-204) antibody (Cell Signaling Technology) overnight at 4°C in blocking buffer, and processed as above to determine the total MAPK levels of each lane.

**Migration Assays**—Cell migration was analyzed using tissue culture-treated 24-well Transwell plates (Costar, Cambridge, MA).
carbonate 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 a serum-free migration buffer (Dulbecco’s modified Eagle’s medium supplemented with 10 m M Hepes, 0.5% bovine serum albumin, and 1/1000 penicillin-streptomycin), and cells (100,000/ml) suspended in the same buffer (8 × 10^5 cells/ml) were added to the upper chamber. The cells were incubated at 37 °C in 5% CO_2 for 20 h. Cells in the upper chamber were removed by wiping, and those 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 in each well is the mean cell number of 4 randomly selected high magnification microscopic fields from triplicate experiments. In some experiments, anti-integrin antibodies (10 μg/ml) were incubated with cells for 15 min before to the assay. 

Co-precipitation of uPAR and Integrin α_5β_1—Soluble uPAR was radioiodinated with Na- 125I using Iodo beads (specific activity 14,500 cpm/ng). Purified human α_5β_1 integrin was obtained from Chemicon International. Purified α_5β_1 (6 μg/ml), mAb HA5 (9 μg/ml), 125I-labeled soluble uPAR (6 μg/ml), and uPA (12 μg/ml) were incubated with protein G-agarose beads either in the presence or absence of RGD peptide (150 μg/ml) in serum-free RPMI 1640 medium supplemented with 10 mM HEPES, pH 7.4, 0.02% bovine serum albumin at 4 °C for 4 h. As a control, experiments were performed in the absence of uPA. Beads were washed 3 times in RPMI supplemented with 10 mM HEPES, 0.02% bovine serum albumin. Bound materials were extracted into reducing SDS-PAGE sample buffer and analyzed by SDS-PAGE and autoradiography.

RESULTS

uPAR-dependent Cell Adhesion to uPA—It has been reported that CHO cells express endogenous hamster uPAR (17). We detected low level endogenous hamster uPAR on the mock-transfected CHO cells with polyclonal anti-human uPAR antibodies (Fig 1a). Consistent with this finding, mock-transfected CHO cells adhered to immobilized uPA in a dose-dependent manner (Fig. 1c). We transfected CHO cells with cDNA encoding human uPAR and cloned stable cell lines expressing high levels of receptor (designated uPAR-CHO). uPAR-CHO cells expressed uPAR at a much higher level (Fig. 1b) and showed greater adhesion to uPA than mock-CHO cells (Fig. 1d). Anti-uPAR polyclonal rabbit IgG completely blocked adhesion of uPAR- and mock-transfected CHO cells to uPA (Fig. 1d). To further test whether uPAR is involved in this process, we treated uPAR-CHO cells with phosphatidylinositol-specific phospholipase C to remove the glycosylphosphatidylinositol-anchored uPAR. The phosphatidylinositol-specific phospholipase C treatment removed more than 90% of uPAR on the cell surface, as determined by flow cytometry with anti-uPAR (Fig.
Role of Integrins in uPAR-mediated Adhesion to uPA—It has been proposed that integrins may be critically involved in the uPA-uPAR signaling (2). To examine this hypothesis we first determined whether integrins contribute to cell adhesion to uPA. To do so, we tested the effects of anti-integrin mAbs on adhesion of uPAR-CHO cells to uPA. CHO cells have endogenous hamster integrins αβ1, αβ2, and αβ2 (data not shown). We found that RGD peptide (100 μM) blocked adhesion of uPAR-CHO cells to uPA, whereas control RGE peptide did not (Fig. 3a). Consistent with the findings that RGD-dependent integrin(s) is involved in this process, anti-α5 mAb (KH72) completely blocked adhesion of uPAR-CHO cells to uPA, whereas anti-α5 mAb (P1F6) or control ascites did not (Fig. 3a). These results suggest that adhesion of uPAR-CHO cells to immobilized uPA is α5β1-dependent. Then we tested whether the level of α5β1 expression affects cell adhesion to uPA using the B2 variant of CHO cells, which expresses ~2% of wild type α5β1 compared with parental CHO cells (28). The B2 cells adhered to uPA at a level lower than CHO cells (Fig 3b). This adhesion was completely blocked by anti-uPAR polyclonal antibodies and anti-integrin α5 (data not shown). These results suggest that cell adhesion to uPA is dependent on the level of α5β1, but that a small amount of α5β1 on the B2 cells still supports the adhesion to uPA to some extent. Another possibility is that integrin α5β1 may also be involved in this process, although we were not able to test this hypothesis since function-blocking anti-hamster α5 mAb is not currently available. To test the specific contribution of α5β1 to uPA-uPAR-dependent cell adhesion, we used immobilized anti-uPAR mAb as a uPAR ligand. We found that uPAR-CHO cells adhered to anti-uPAR and that this adhesion was not inhibited by anti-integrin α5 mAb (Fig. 3c), suggesting that α5β1 was not required for this process. Taken together, adhesion of CHO cells to uPA requires uPAR and α5β1, and α5β1 is specifically involved in this process only when uPA is used as a ligand.

coating concentration of uPA was 500 nM. Cells were incubated with immobilized uPA in the presence of control IgG, P1F6 (anti-integrin α5β1), KH72 (anti-integrin α5), RGD, or RGE peptide. P1F6 and KH72 cross-react with endogenous hamster α5β1, and α5β2, respectively. The results suggest that integrin α5β1 (RGD-dependent) is critical for cell adhesion to uPA: cont., control; b, effect of α5 expression levels on cell adhesion to uPA. The B2 variant of CHO cells (28) express low level α5β1 (about 2% of wild type). B2 cells were tested for their ability to adhere to immobilized uPA as a function of the coating concentration of uPA. The results suggest that B2 cells adhered to uPA at a significantly lower level than CHO cells. c, effect of anti-α5 mAb on adhesion to uPA as a ligand. Anti-uPAR mAb (anti-D2D3, 20 μg/ml) was used instead of uPA as substrate. uPAR-CHO cells were incubated with immobilized anti-uPAR mAb in the presence of KH72 or control mouse IgG. The results suggest that integrin α5β1 is not critical for cell adhesion to anti-uPAR mAb.
The Domains of uPA Required for Cell Adhesion to uPA—To identify which uPA domains are involved in uPAR-dependent cell adhesion to uPA, we used several uPA fragments including the ATF, the kringle domain, the LMW-uPA, ΔGFD-uPA, which lacks GFD, and ΔKringle-uPA, which lacks the kringle domain (Fig 4a). We found that ATF and ΔKringle-uPA supported cell adhesion at levels comparable with that of wild-type uPA. Kringle, LMW-uPA, or ΔGFD-uPA did not support the adhesion at all (Fig 4b). These results suggest that GFD is primarily involved in cell adhesion to uPA, but other domains are not.

As a second approach, we tested whether mAbs to different domains of uPA block cell adhesion to uPA. We found that anti-GFD (AD3471) and anti-kringle mAb (Ab963) blocked adhesion of uPAR-CHO cells to uPA, whereas anti-LMW (UNG-5) did not (Fig 4c). Ab963 has been observed to inhibit the binding of uPA to whole cells despite the fact that its epitope has been mapped to the kringle domain. These results are consistent with the results with uPA fragments (Fig. 4b) with the exception of the effect of the anti-kringle antibody. GFD is required for uPA to bind to uPAR (10). Taken together these studies suggest that uPAR-mediated cell adhesion to uPA is also critically dependent on the interaction with GFD.

Mutations in Integrin α5 Affect Cell Adhesion to uPA—We have recently reported that uPAR is a ligand for several integrins (17). Specifically, soluble uPAR supports integrin-mediated cell adhesion, and glycosylphosphatidylinositol-linked uPAR binds to integrins in apposing cells in trans and supports cell-cell interaction. We have reported that mutations in the ligand binding region of integrin α5 subunit blocked adhesion of α5β1-transfected CHO cells to immobilized soluble uPAR (17), suggesting that uPAR binds to α5β1 as a ligand. These critical residues are located within the ligand binding site in integrins based on the crystal structure of integrin α5β1 (29, 30). We suspected that cell adhesion to uPA in the present study involves interaction between uPAR and α5β1 on the same cell surface (in cis). We have reported that similar mutations (the Tyr-186 to Ala (Y186A), F187A, and W188A) in the ligand binding site of α5β1 blocked fibronectin binding to α5β1 (21). We therefore studied whether uPAR binds to α5β1 as a ligand during adhesion to uPA using these α5 mutants.

We first tested the effects of these function-blocking mutations of α5 (21) on adhesion to soluble uPAR. We used B2 cells expressing wild type and Y186A, F187A, and W188A mutants of integrin α5 (designated α5β2, α5Y186A-B2, α5F187A-B2, and α5W188A-B2, respectively). Expression levels of integrin α5β1 among those transfectants were comparable as measured by flow cytometry with non-function blocking anti-human α5 mAb (VC-5) (21). CHO cells, but not B2 cells, bind to coated soluble uPAR upon Mn²⁺ activation (17), indicating that α5β1 requires activation to bind to uPAR. We found that α5β2 cells adhered to soluble uPAR if activated with Mn²⁺ (Fig 5a) and that the Y186A and W188A mutations completely, and the F187A mutation partially, blocked the adhesion to soluble uPAR.

We then tested the effect of these α5 mutations on cell adhesion to uPA. In this experiment we used ATF instead of wild-type uPA to exclude the possible contribution of the serine coating concentration of uPA fragments is 500 nM. Adhesion of uPAR-CHO cells to immobilized uPA fragments was determined. The data suggest that only ATF supported significant adhesion, c, effect of anti-uPA mAbs on adhesion to uPA. The coating concentration of uPA is 500 nM. uPAR-CHO cells were incubated with uPA in the presence of mAbs to uPA. The data suggest that anti-kringle and anti-GFD effectively blocked the adhesion.
protease domain because we found that both B2 and α5-B2 cells bind weakly to LMW-uPA when α5 is activated with Mn2+. We found that α5-B2 cells adhered to ATF more than parental B2 cells, but that α5/Y86A-B2 and α5/W188A-B2 cells adhered at the level comparable with or lower than that of parental B2 cells (Fig 5b). The adhesion of parent B2 cells to ATF may be due to endogenous low levels of α5 on B2 cells or due to endogenous α5, as noted above. These results suggest that Tyr-186 and Trp-188 of α5 are critical for cell adhesion to both soluble uPAR and ATF.

Taken together these results suggest that uPAR-mediated cell adhesion to uPA requires the intact ligand binding function of α5. This is consistent with the observation that RGD peptide blocked adhesion of uPAR-CHO cells to uPA in the present study (Fig. 3a) and that the anti-α5 mAb we used in the present study (KH72) binds to the ligand binding site of α5. We propose that uPAR binds to α5β1 in cis as a ligand upon cell adhesion to uPA.

2 T. Tarui and Y. Takada, unpublished data.
3 T. Tarui and Y. Takada, unpublished observation.

uPA-induced Cell Migration Also Depends on Both uPAR and Integrin α5β1—It has been reported that physiological concentrations of uPA stimulate a chemotactic response in human monocytic THP-1 through binding to uPAR (11). We tested whether uPAR and α5β1 are involved in uPA-induced cell migration. We found that wild-type uPA was chemotactic for uPAR-CHO cells, whereas ΔGFD-uPA or kringle was not (Fig 6a), indicating that GFD is critical for this process. We found that anti-human uPAR polyclonal antibodies, anti-uPAR mAb (anti-D2D3), and anti-α5 mAb (KH72) effectively blocked uPA-induced migration (Fig 6b). Neither control rabbit IgG nor anti-integrin α5 mAb blocked uPA-induced migration. Taken together these results suggest that GFD, uPAR, and integrin α5β1 are critically involved in uPA-induced cell migration.

uPA-induced Binding of uPAR to Integrin α5β1—The results in the present study predict that binding of uPA to uPAR...
through GFD induces uPAR binding to $\alpha_5\beta_1$. We tested whether uPA directly induces binding of uPAR and $\alpha_5\beta_1$ in a cell-free system. We incubated labeled soluble uPAR with isolated $\alpha_5\beta_1$ in the presence and absence of uPA. We isolated $\alpha_5\beta_1$-uPAR complex with anti-$\alpha_5$ mAb HA5 (non-function blocking). We found that uPAR co-precipitated with anti-$\alpha_5\beta_1$. Immunoprecipitated proteins were fractionated by SDS-PAGE, and gels were exposed to film. Autoradiographs were analyzed by densitometry and are presented as percentage of relative density units normalized to uPA alone.

**DISCUSSION**

In the present study we demonstrate that immobilized uPA induces cell adhesion and migration in an uPAR-dependent manner. Interestingly, signaling from immobilized uPA is similar to that from soluble uPA. Cell adhesion to uPA required signaling because this process was sensitive to temperature and to a MEK-1 inhibitor. We have shown that uPA-induced cell adhesion and migration required uPA binding to uPAR through GFD of uPA, since 1) antibodies against uPAR and GFD effectively blocked this interaction and 2) depletion of uPAR reduced, and overexpression of uPAR enhanced, cell adhesion to uPA. Also, we have shown that the catalytic domain of uPA was not critical for cell adhesion to uPA.

A major finding of the present study is that cell adhesion and migration to uPA required the ligand binding function of $\alpha_5\beta_1$. We have previously reported that uPAR binds (as a ligand) to integrins in trans and that trans interaction between uPAR and integrins supported cell adhesion and cell-cell interaction (17). In the present study we have shown that function-blocking anti-$\alpha_5$ mAb blocked uPA-induced cell adhesion and migration and that RGD peptide and the $\alpha_5$ mutations that block ligand binding effectively blocked cell adhesion to uPA. The crystal structure of integrin $\alpha_5\beta_1$ has only a single RGD binding site between $\alpha_5$ and $\beta_1$ subunits (30) but did not show the position or existence of allosteric RGD binding sites. The integrin mutations we used in the present study (Y186A, F187A, and W188A in $\alpha_5$) are very close to the RGD peptide in the $\alpha_5\beta_1$-RGD complex (30) (the distances between their backbones are within 10 Å), suggesting that these mutations directly block ligand binding to $\alpha_5\beta_1$. Thus, it is highly likely that RGD peptide and the integrin mutations directly blocked uPAR binding to $\alpha_5\beta_1$ and thereby blocked cell adhesion to uPA. We do not, however, preclude the possibility that RGD peptide binds to allosteric RGD binding sites and affects uPAR binding to $\alpha_5\beta_1$.

Because anti-uPAR antibodies effectively blocked adhesion of uPAR-CHO cells to uPA, $\alpha_5\beta_1$, alone cannot directly support adhesion to uPA. Consistent with this observation, no $\alpha_5\beta_1$, ligand other than uPAR was required for uPAR-uPA signaling in the present study. It is highly likely that uPAR is a primary ligand for $\alpha_5\beta_1$ upon uPA-induced cell adhesion and migration in the present study and that uPAR binds to $\alpha_5\beta_1$ as a ligand even when uPAR and $\alpha_5\beta_1$ interact in cis. Thus uPA-induced cell adhesion and migration involves the following sequence. 1) uPA binds to uPAR, 2) uPAR then binds to $\alpha_5\beta_1$, and 3) signal transduction is mediated through $\alpha_5\beta_1$ (Fig. 8). CHO and B2 cells express $\alpha_5\beta_1$ and $\alpha_6\beta_1$, and we have shown that anti-$\alpha_5\beta_1$ (P1B6) does not block adhesion to uPA. However, we do not rule out the possibility that $\alpha_5\beta_1$ is involved in uPAR binding in cis. We are not able to test whether $\alpha_5\beta_1$ is involved because anti-$\alpha_5$ antibody that cross-reacts with hamster $\alpha_5$ is not available.

It has previously been reported that the avidity of $\alpha_5\beta_1$, in uPAR-rich human carcinoma Hep3 cells for fibronectin was higher than that of uPAR-poor dormant Hep3 cells (8). The levels of MAPK activation by fibronectin were much higher in uPAR-rich cells than uPAR-poor cells. It is unclear, however, how fibronectin, uPAR, and $\alpha_5\beta_1$ are involved because both...
fibronectin and uPAR are ligands for $\alpha_5\beta_1$. We suspect that 1) the initial uPAR binding to a limited number of $\alpha_5\beta_1$ in cis induces the increased signaling through $\alpha_5\beta_1$ and activated MAPK, 2) $\alpha_5\beta_1$ is activated by inside-out signaling, and 3) fibronectin binds to activated unoccupied $\alpha_5\beta_1$, leading to more outside-in signaling in uPAR-rich cells. In uPAR-poor cells, in contrast, the initial uPAR-$\alpha_5\beta_1$ binding may not be enough to induce $\alpha_5\beta_1$ signaling. This would be a possible reason that reduction in the level of uPAR induced a protracted state of dormancy in tumor cells. We were not able to detect stress-fiber formation upon adhesion of uPAR-CHO or mock-transfected CHO cells to immobilized uPA in the absence of fibronectin (Fig. 2d). It is unclear whether signal transduction through $\alpha_5\beta_1$ on adhesion to uPA stabilized the interaction between uPAR and immobilized uPA and/or induced re-organization of cytoskeletal proteins in the present study.

Several recent papers suggest that uPAR binds to unique “non-ligand binding sites” in repeat 4 of the $\alpha$ subunits in several integrins (31, 32). Several integrin peptides from the predicted 2–3 loop in repeat 4 (e.g., PRHRHMGAVFLSSEQG, residues 240–257 of $\alpha_5$) have been reported to block uPAR-integrin interaction. Several laboratories including ours have previously identified many amino acid residues within repeats 2–4 of the integrin $\alpha$ subunits that are critical for binding to integrin ligands (21, 33–38). A recent crystal structure of integrin $\beta_3$ subunits that are critical for binding to uPAR and immobilized uPA and/or induced re-organization of cytoskeletal proteins in the present study.

We recently reported that the angiostatin, a plasminogen derived peptides described above might block the binding of uPA to integrins using co-immunoprecipitation (8, 32, 40, 41) although to date the mechanism underlying this phenomenon has remained unclear. Consistent with these reports, we demon-strated that uPA induces RGD-dependent binding of soluble uPAR to isolated $\alpha_5\beta_1$ in solution in the present study (Fig. 7). These results predict that uPA binding to uPAR induces uPAR binding to $\alpha_5\beta_1$ in cis on the cell surface. What is the mechanism of uPA-induced uPAR binding to integrins? It has been reported that soluble uPAR forms dimers and oligomers (42). Interestingly, the addition of an equimolar concentration of uPA leads to the dissociation of these dimers and oligomers. It is tempting to speculate that the ability of uPAR to binding to integrins is related to dissociation of uPAR, i.e. that GPD binding to uPAR may induce dissociation of uPAR dimers and oligomers, exposing the integrin binding sites within uPAR. Additional studies to examine the role of uPA and uPAR in $\alpha_5\beta_1$ activation are currently under way.

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REFERENCES

1. Preissner, K. T., Kanse, S. M., and May, A. E. (2000) Curr. Opin. Cell Biol. 12, 621–628

2. Osowski, L., and Aguirre-Ghiso, J. A. (2000) Curr. Opin. Cell Biol. 12, 613–620

3. Waltz, D. A., Sailor, L. Z., and Chapman, H. A. (1993) J. Clin. Invest. 91, 1541–1552

4. Gyetko, M. R., Todd, R. F., III, Wilkinson, C. C., and Sitrin, R. G. (1994) J. Clin. Invest. 93, 1388–1387

5. Basset, N., Mazur, S. K., Legrand, D., Waxman, S., and Osowski, L. (1994) J. Cell Biol. 126, 259–270

6. Odekon, L. E., Sato, Y., and Rikit, D. B. (1992) J. Cell. Physiol. 150, 258–263

7. Rabin, S. A., Mazur, A. P., Bernier, S. M., Hig, M., Bolivar, L., Henkin, J., and Goltzman, D. (1992) J. Biol. Chem. 267, 14151–14156

8. Aguirre Ghiso, J. A., Kovalski, J., and Osowski, L. (1999) J. Cell Biol. 147, 89–104

9. Fischer, K., Lutz, V., Wilhelim, O., Schmitt, M., Graeff, H., Heiss, P., Nishiguchi, T., Harbeck, N., Kessler, H., Luther, T., Magdolen, V., and Reuning, U. (1998) FEBS Lett. 438, 101–105

10. Appella, E., and Blasi, P. (1997) Ann. N. Y. Acad. Sci. 511, 192–195

11. Resmaiti, M., Gutterman, M., Valacomonica, S., Sidenius, N., Blasi, F., and Fazio, F. (1990) EMBO J. 15, 1572–1582

12. Hynes, R. O. (1994) Cell 69, 11–25

13. Hynes, R. O., and Lander, A. D. (1992) Cell 68, 303–322

14. Xue, W., Kindzelskii, A. L., Todd, R. F., III, and Petty, H. R. (1994) J. Immunol. 152, 4650–4660

15. Xue, W., Minukami, I., Todd, R. F., III, and Petty, H. R. (1997) Cancer Res. 57, 1682–1689

16. Wei, Y., Lukashev, M., Simon, D. I., Bodary, S. C., Rosenberg, S., Doyle, M. V., and Chapman, H. A. (1994) Science 267, 1551–1553

17. Tarui, T., Mazur, A. P., Cines, D. B., and Takada, Y. (2001) J. Biol. Chem. 276, 3983–3990

18. Fokine, P., Racchi, A., Rosau, J., and Kennel, S. (1988) Cancer Res. 48, 816–821

19. Min, H. Y., Semmanni, R., Minukami, I. F., Wast, K., Todd, R. F. D., and Liu, D. Y. (1992) J. Immunol. 148, 3656–3662

20. Gom, R., Juare, J., Allsagey, H., Mazur, A., Wang, Y., and Boyd, D. (1998) Oncogene 17, 213–225

21. Irie, A., Kamata, T., Puzon-McLaughlin, W., and Takada, Y. (1995) EMBO J. 14, 5542–5549

22. Bilde, K., Kuo, A., Mazur, A., Sachais, B. S., Xian, W., Gawlak, S., Harris, S., Higashi, A. A., and Cines, D. B. (2000) J. Biol. Chem. 275, 28532–28538

23. Axelrod, J. H., Reich, E., and Miskin, R. (1989) Mol. Cell. Biol. 9, 2135–2141

24. Zhang, W. P., Kamata, T., Tanie, K., Puzon-McLaughlin, W., and Takada, Y. (1998) J. Biol. Chem. 273, 7345–7350

25. Prater, C. A., Plotkin, J., Jaye, D., and Frasier, W. A. (1991) J. Cell Biol. 112, 1031–1040

26. Takada, Y., and Puzon, W. (1993) J. Biol. Chem. 268, 17759–17760

27. Nguyen, D. H., Hussain, I. M., and Gonia, S. L. (1998) J. Biol. Chem. 273, 8502–8507

28. Bauer, J. S., Schreiner, C. L., Giancotti, F. G., Ruoslahti, E., and Juliano, R. L. (1992) J. Biol. Chem. 267, 10234–10240

29. Xiong, J.-P., Stelhe, T., Diefenbach, B., Zhang, R., Dunker, R., Scott, D. L., Andrej, J., Goodman, S. L., and Arnaout, M. A. (2001) Science 294, 339–345

30. Xiong, J.-P., Stelhe, T., Zhang, R., Joachimiak, A., Frech, M., Goodman, S. L., and Arnaout, M. A. (2002) Science 296, 151–155

31. Simon, D. I., Wei, Y., Zhang, L., Rao, N. K., Xu, H., Chen, Z., Liu, Q., Rosenberg, S., and Chapman, H. A. (2000) J. Biol. Chem. 275, 10228–10234

32. Wei, Y., Eble, J. A., Wang, Z., Kreidberg, J. A., and Chapman, H. A. (2001) Mol. Biol. Cell 12, 2975–2986

33. Kamata, T., Tieu, K. K., Irie, A., Springer, T. A., and Takada, Y. (2001) J. Biol. Chem. 276, 44275–44283

4. G. Parry and A. Mazar, unpublished observations.
34. Kamata, T., Irie, A., and Takada, Y. (1996) J. Biol. Chem. 271, 18610–18615
35. Irie, A., Kamata, T., and Takada, Y. (1997) Proc. Natl. Acad. Sci. U.S.A. 94, 7198–7203
36. Puzon-McLaughlin, W., Kamata, T., and Takada, Y. (2000) J. Biol. Chem. 275, 7795–7802
37. Mould, A., Burrows, L., and Humphries, M. (1998) J. Biol. Chem. 273, 25664–25672
38. Humphries, M. J. (2000) Biochem. Soc. Trans. 28, 311–339
39. Tarui, T., Miles, L. A., and Takada, Y. (2001) J. Biol. Chem. 276, 39562–39568
40. Yehra, M., Goretzki, L., Pinzler, M., and Mueller, B. M. (1999) Exp. Cell Res. 250, 231–240
41. Czekay, R. P., Aertgeerts, K., Curriden, S. A., and Loskutoff, D. J. (2003) J. Cell Biol. 160, 781–791
42. Shliom, O., Huang, M., Sachais, B., Kuo, A., Weisel, J. W., Nagaswami, C., Nassar, T., Bayir, K., Hiss, E., Gawlak, S., Harris, S., Mazar, A., and Higazi, A. A. (2000) J. Biol. Chem. 275, 24304–24312
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