Urokinase-type Plasminogen Activator Receptor (CD87) Is a Ligand for Integrins and Mediates Cell-Cell Interaction*

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The receptor for urokinase-type plasminogen activator (uPA),1 uPAR/CD87, is a glycoprotein (M, 35,000–65,000) composed of 283 amino acid residues. It is anchored to the plasma membrane by a glycosyl phosphatidylinositol (GPI) linkage (reviewed in Refs. 1 and 2). uPAR is the cellular receptor for urokinase, a serine protease that is constitutively or inducibly secreted by most uPAR-expressing cells. Receptor-bound uPA can convert plasminogen to plasmin, which mediates pericellular proteolysis of extracellular matrix proteins in the path of cellular invasion. uPAR is expressed by activated leukocytes, endothelial cells, fibroblasts, and different types of cancer cells (reviewed in Refs. 1 and 2 and references therein). Expression of uPAR has been shown to correlate with the prognosis of many human cancers (2). In murine tumor models, expression or administration of uPAR antagonists has a marked inhibitory effect on the metastatic ability of cancer cells (4) and on the growth of the primary tumor (5), and the down-regulation of uPAR leads to dormancy of carcinoma cells in vivo (6, 7). Thus, uPAR expression has been implicated in cancer progression. In addition, it appears that uPAR is up-regulated on cells that are in motion. Migratory/chemotaxis-inducing stimuli (e.g. vascular endothelial growth factor, fibroblast growth factor, platelet-derived growth factor, and interleukins) up-regulate uPAR in endothelial cells, smooth muscle cells, and leukocytes in vitro, whereas unstimulated cells do not have detectable expression of uPAR (reviewed in Refs. 1 and 2 and references therein). Thus, uPAR may play a role in leukocyte recruitment, angiogenesis, and tumor metastasis.

uPAR has been reported to associate with many signaling molecules and to mediate signal transduction (8–10). In recent reports the binding of uPA to uPAR in tumor or endothelial cells has been shown to activate the mitogen-activated protein kinases, extracellular regulated kinase 1 and 2 (11–13). However, a major question is how uPAR mediates cellular signaling, because the molecule has no transmembrane structure. The existence of one or more hypothetical “transmembrane adapter molecules” that connects uPAR and signaling molecules inside cells has been proposed (14).

It has been shown that the β1, β2, and β3 integrin receptor families interact with uPAR using immunoprecipitation, immunocolocalization, and resonance energy transfer approaches (15–17). The uPAR-integrin interaction may be significant, because many integrin receptors activate intracellular signals coupled to the pathways used by both receptor and nonreceptor tyrosine kinases (18–20). Integrin- and receptor tyrosine kinase-mediated signals may complement each other to fully activate cell survival and proliferation pathways (21, 22). It has been proposed that uPAR forms cis-interactions with integrins on the same cell surface as an integrin-associated protein (reviewed in Ref. 1). However, it has not been established that this is the dominant mode of interaction responsible for signal transduction events.

In the present study, we designed experiments to examine the uPAR-integrin interaction in detail using isolated domains derived from recombinant soluble uPAR (suPAR) and cells expressing recombinant β1 or β3 integrins. These studies establish that uPAR binds to integrins in a manner that is very...
similar to that of known integrin ligands (e.g. vascular cellular adhesion molecule-1 (VCAM-1)). Additionally, we have found that uPAR interacts with integrins on apposing cells (trans-interaction). These unexpected findings may help to clarify the complex role of uPAR in signal transduction, inflammation, and cancer.

**EXPERIMENTAL PROCEDURES**

**Materials**

TS2/16, an activating anti-β1 mAb (23), was obtained from American Type Culture Collection (Manassas, VA). AIIB2, a function-blocking anti-β1 mAb (24), was obtained from Developmental Studies Hybridoma Bank (Iowa City). IA. P1B5 (anti-α3) (25) was a gift from E. Wayner and W. G. Carter (University of Washington, Seattle, WA). SG73 (anti-α4) (26) and KH72 (anti-α5) were provided by K. Miyake (Sagai Medical School, Saga, Japan). 135-13C (anti-β3) was obtained from S. J. Kennel (Oak Ridge National Laboratory, Oak Ridge, TN), and Y9A2 (anti-α9) (28) was obtained from D. Sheppard (University of California, San Francisco, CA). 7E3 (anti-β3) (29) was provided by B. S. Coller (Mount Sinai Hospital, New York, NY). 8C8 (anti-α2) and 15 (anti-β3) were provided by M. H. Ginsberg (The Scripps Research Institute). Anti-uPAR monoclonal antibody (3B10) (30) was provided by R. F. Todd III (University of Michigan Medical Center, Ann Arbor, MI).

Recombinant VCAM-1-mouse Ck chain fusion protein was provided by Novartis (Basel, Switzerland). Recombinant domain 2 and domain 3 forms and a combination of the two forms generated in Chinese hamster ovary cells were prepared as described previously (31). GRGDs and GRGES peptides were purchased from Advanced ChemTech (Louisville, KY). α-Bungarotoxin and rabbit IgG were obtained from Sigma. Human fibrinogen was obtained from Enzyme Research Laboratories (South Bend, IN).

CHO cells and human erythroleukemia K562 cells were obtained from the American Type Culture Collection. Integrin αβ5-deficient B2 variant CHO cells (32) were provided by R.L. Juliano (University of North Carolina, Chapel Hill, NC), and Jurkat human T-cell leukemic cells were provided by M. H. Ginsberg (The Scripps Research Institute). CHO cells expressing human α9 (designated α9-CHO) (33) were provided by D. Sheppard (University of California, San Francisco, CA). CHO cells expressing other human integrin α and/or β subunits (with type and mutant) have been described (34). B2 cells expressing human α2, α3, and α4 (designated α2-B2, α3-B2, and α4-B2 cells, respectively) were prepared as described for CHO cells expressing these integrins (34). These cells express human α/hamster β1 or hamster α/human β3 hybrid. K562 cells expressing recombinant human α4 have been described (35). K562 cells expressing recombinant human α9 (α9-K562) have been described (36). K562 cells expressing recombinant human αβ3 (αβ3/K562) (37) are a gift from E. J. Brown (Washington University, St Louis, MO).

CHO cells expressing the three domain forms of human uPAR (designated HuPAR-CHO) were prepared by transfecting full-length human uPAR cDNA (provided by L. A. Miles, The Scripps Research Institute) into a pcDNA3 vector (Invitrogen) together with a plasmid containing a neomycin-resistant gene. Cells were selected with G-418 (0.7 mg/ml medium). Approximately 50% of cells stably expressed uPAR after selection with mAb 3B10. CHO cells stably expressing uPAR were sorted by FACSter (Becton-Dickinson) to obtain cells homogenously expressing uPAR at a high level.

**Methods**

**Production of suPAR in Drosophila S2 Cells—cDNAs encoding soluble wild-type uPAR (amino acids 1–277) and soluble domains 2 and 3 (amino acids 88–277) were generated by polymerase chain reaction using pTracer-full-length uPAR as a template. The fragments were digested with BgIII and XhoI and sub-cloned into the expression vector (pMT/BIP/V5, Invitrogen). Soluble suPAR domains were expressed in Drosophila Schneider S2 cells (DES system, Invitrogen) as described by the manufacturer.**

**Production of suPAR Expressed in S2 Cells—Small scale preparations of suPAR and variants were purified from the medium using a polyclonal anti-uPAR antibody affinity column. For large scale preparations, S2 cell culture supernatants were filtered, and the medium were loaded onto a 40-g hydroxyapatite column equilibrated with 10 mM K2HPO4, pH 7.0. The column was then eluted with a gradient of 10–200 mM K2HPO4, pH 7.0, and the suPAR-containing fractions were identified by Western blotting. Fractions containing monomeric suPAR eluted between 50 and 120 mM K2HPO4. Fractions containing monomeric suPAR were pooled, concentrated, and further purified using C8 reverse-phase HPLC. Crude suPAR (20 mg of total protein) was loaded in a volume of 2 ml onto a semi-preparative (10 × 250 mm) C8 column and eluted at a flow rate of 4 ml/min with a linear gradient of 0–60% solvent B. Solvent A was 100 mM acetonitrile, 0.1% trifluoroacetic acid, and solvent B was 100% acetonitrile, 0.1% trifluoroacetic acid. suPAR eluted as a single broad peak under these conditions with a retention time of ~27 min. SDS-polyacrylamide gel electrophoresis analysis of suPAR purified in this manner demonstrated a single major peak at 35 kDa under nonreducing conditions. Also observed was a slight lagging effect due to the four minor higher molecular weight species. These were determined to be SDS-stable aggregates of suPAR, which disappeared when SDS-polyacrylamide gel electrophoresis was performed under reducing conditions. Matrix-assisted laser desorption ionization-time of flight mass spectrometry revealed a single broad peak with an average mass of 34,797 Da. The predicted mass based on the amino acid sequence is 30,672 Da, indicating the presence of about 4 kDa in glycosylation. Expression levels of suPAR were typically 30 mg/liter determined by enzyme-linked immunosorbant assay. Purification yielded about 10–12 mg of pure suPAR protein per liter of culture supernatant.

**Enzymatic Digestion of Wild-type suPAR—**suPAR was digested with chymotrypsin to generate the soluble D1 and D2D3 fragments. Chymotrypsin was added to suPAR (1 mg/ml in phosphate-buffered saline) at a final molar ratio of suPAR/chymotrypsin of 1000:1, and the digest was incubated for 1 h at room temperature. The digest was quenched by addition of Pefablock (100 μM final concentration). The D1 and D2D3 fragments were separated using C8 reverse phase-HPLC. The D1 and D2D3 fragments eluted with a retention time of ~27 and 25 min, respectively. The purified D1 fragment was sequenced, and a single N terminus was observed beginning with RS (representing the two extra amino acids present in this construct) followed by LR, amino acids 1 and 2 in the mature suPAR sequence. Sequencing of the D2D3 fragment revealed a single N terminus as well, beginning with amino acids SRS, corresponding to amino acids 88–90 in the mature suPAR sequence. Matrix-assisted laser desorption ionization-time of flight mass spectral analysis revealed a single peak for the D1 fragment (mass = 11,041 Da) and several peaks for the D2D3 fragment (mass = 23,846; 23,691; 22,936; 22,800). These were presumed to be glycosylation isoforms, although additional digestion of the D2D3 fragment from the C terminus could not be excluded. However, no consensus chymotrypsin cleavage site that could result in the observed mass differences is present at the C terminus of the D2D3 fragment.

**Affinity-purified Anti-suPAR—**A polyclonal antibody against suPAR was generated as described (38). Briefly, recombinant human uPAR was expressed in SP2/0 cells and purified using a single-chain uPA-Sepharose column. Serum was collected from rabbits immunized with purified suPAR, and the IgG was obtained by a 50% ammonium sulfate precipitation. This material was dialyzed (10,000 ×) against phosphate-buffered saline and further purified using a suPAR-Sepharose column to generate affinity-purified anti-suPAR IgG.

**Cell Adhesion Assays—**Adhesion assays were performed as described previously (34). Briefly, wells in 96-well Immulon-2 microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated with 100 μl of phosphate-buffered saline (10 mM phosphate buffer, 0.15 mM NaCl, pH 7.4) containing substrates at a concentration of 0.25–5 μg/ml and were incubated overnight at 4 °C. Remaining protein-binding sites were blocked by incubation with 0.2% bovine serum albumin (Calbiochem) for 1 h at room temperature. Cells (105 cells/well) in 100 μl of Hepes-Tyrode buffer (10 mM HEPES, 150 mM NaCl, 12 mM NaHCO3, 0.4 mM NaH2PO4, 2.5 mM KCl, 0.1% glucose, 0.02% bovine serum albumin) supplemented with 2 mM MgCl2 were added to the wells and incubated at 37 °C for 1 h, unless stated otherwise. After nonbound cells were removed by rinsing the wells with the same buffer, bound cells were quantified by measuring endogenous phosphatase activity (39). Antibodies were used at 250 × dilution of ascites (TS2/16, 8C8, P1B5, SG73, KH72, and 135–13C), at 4 μg/ml (AIIB2, 7E3, and Y9A2), and at 3.4 μg/ml (anti-uPAR and rabbit IgG). Data are shown as means ± S.D. of three independent experiments.

**Cell-Binding Assay—**Integrin-transfected, mock-transfected (vector only), or parental K562 cells were cultured in 7.7 × 102 ml of a 56-carboxyfluorescein diacetate (CFDA-AM) (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions. The labeled cells (104 cells/well) were added to the monolayer of parent, mock-transfected, or HuPAR-CHO cells and incubated for 30 min at 37 °C. After the wells were rinsed with medium to remove unbound cells, bound cells were quantified by assaying fluorescence (excitation 485 nm, emission 530 nm) using an FL500 microplate fluorescence reader.
Antibodies on adhesion to suPAR D2D3 was studied. The concentration of suPAR D2D3 used for coating was 5 µg/ml. Bovine serum albumin, closed columns; suPAR D2D3, open columns; and suPAR D2D3 plus function-blocking antibodies, shaded columns. The antibodies used were SG73 (anti-a) for a4-CHO cells, KH72 (anti-a5) for CHO cells, 135–13C (anti-a6) for a6-CHO cells, Y9A2 (anti-a9) for a9-CHO cells, and 7E3 (anti-b3) for b3-CHO cells. E, the effect of affinity-purified anti-uPAR antibody on avb3-suPAR D2D3 interaction was studied. Polyclonal anti-uPAR or control rabbit IgG was included in the assay medium at 3.4 µg/ml. SuPAR D2D3 was used at a coating concentration of 5 µg/ml. F, the capability of α-bungarotoxin, a protein structurally similar to uPAR, to support adhesion of CHO cells was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E). We then characterized the uPAR-integrin interaction throughout this study. The rationale of using D2D3 is that removal of D1 leaves uPAR lacking endogenous α5β1, and avb3-suPAR D2D3 interaction was studied. Specific antibodies against uPAR. We found that antibodies against uPAR effectively blocked suPAR binding to avb3 (Fig. 1E).
tested whether α-bungarotoxin, which has a “three-finger protein” structure similar to the uPAR structure, binds to αvβ3. The toxin showed only weak, if any, affinity to αvβ3 (Fig. 1F). These results suggest that uPAR binding to integrins is specific to the uPAR sequence and structure.

Effect of Cations and β1 Integrin Activation and Inactivation on uPAR Binding—Ligand binding to integrins is tightly regulated by activation/inactivation of integrins through inside-out signal transduction (45). β3-CHO cells significantly adhere to suPAR D2D3 in the presence of Mg2+ but not in the presence of Ca2+ (Fig. 2A). CHO cells do not adhere to suPAR in either condition. These results suggest that αvβ3 requires Mg2+ for binding to suPAR D2D3 under the conditions employed. Although α2-CHO, α3-CHO, and CHO cells do not strongly adhere to suPAR D2D3 under the assay conditions used, it is still possible that binding of α2β1, α3β1, and α5β1 may require more activation. Therefore, we studied whether Mn2+ (0.1 mM), which universally activates integrins, facilitates uPAR binding to these integrins (Fig. 2A). We found that α5β1-deficient B2 variant CHO cells did not significantly adhere to suPAR D2D3 in the presence of Mn2+. α2-B2 cells did not adhere to suPAR D2D3 under any conditions tested. Ca2+ did not significantly support adhesion of these cells. These results suggest that α3β1, α4β1, and α5β1 but not α2β1 bind to suPAR D2D3 in an activation-dependent manner.

We then asked whether the activation status of β1 integrins that is required for uPAR binding to natural integrins is similar to the requirements for the recombinant integrins that we studied. To answer this question, we studied uPAR binding to nonrecombinant integrins on Jurkat human T-cell leukemia cells (α4β1+, α5β1+). We can stimulate or suppress β1 integrin-ligand interaction from outside cells using anti-β1 antibodies (e.g., TS2/16, activating; AIIB2, inhibiting) (reviewed in Ref. 46). We found that adhesion of Jurkat cells to suPAR D2D3 is stimulated by TS2/16 and blocked completely by AIIB2 and SG73 (anti-α4 mAb) and partially by KH72 (anti-α5 mAb) (Fig. 2B). These results suggest that nonrecombinant α4β1 and α5β1 in Jurkat cells may interact with suPAR D2D3 in an activation-dependent manner.

Does uPAR Share Common Binding Sites in Integrins with Known Integrin Ligands?—We then asked whether uPAR competes with VCAM-1, a known α4β1 ligand, for binding to α4β1. To address this question, we examined the adhesion of α4-B2 cells to suPAR D2D3 in the presence of soluble VCAM-1. We used B2 cells to completely eliminate the contribution of α5β1 in uPAR binding. We found that adhesion of α4-B2 cells to suPAR D2D3 was blocked by VCAM-1 in a dose-dependent manner (Fig. 3A) but not by irrelevant ligand fibrogenin. These results suggest that the inhibitory effect of VCAM-1 is specific to α4β1-suPAR D2D3 interaction, and thus uPAR and VCAM-1 compete for binding to α4β1. We also studied the effect of the GRGDS peptide, a widely distributed integrin-binding motif, on uPAR-αvβ3 interaction (Fig. 3B). We found that GRGDS peptide completely blocked binding of αβ3-CHO cells to suPAR D2D3, but control GRGES peptide did not, suggesting that uPAR and the ligand-derived RGD motif compete for binding to αvβ3. Thus it is highly likely that the uPAR-binding sites in these integrins may overlap with those of known integrin ligands.

We next studied whether uPAR binding is inhibited by mutations in these integrins that block binding of known ligands. We have previously reported that the mutation to Ala of several amino acid residues, Tyr-187, Trp-188, and Gly-190 in α4 (designated Y187A, W188A, and G190A mutations, respectively), blocks VCAM-1 and fibronectin connecting segment-1 (CS-1) peptide binding to α4β1 (47). These residues are located within the putative ligand-binding site in α4, and the corresponding residues in αIIb or α5 have also been reported to be critical for ligand binding to αIIbβ3 or α5β1 (47, 48). We studied whether these α4 mutations block adhesion of α4β1 to suPAR D2D3. We found that these mutations blocked uPAR-α4β1 interaction, but several other mutations did not (Fig. 4). The overall effect of these mutations on uPAR binding to α4β1 is similar to their effect on VCAM-1 or CS-1 binding to α4β1. These results suggest that the uPAR-binding site in α4 overlaps with those for VCAM-1 and CS-1.

The Asp-130 residue of β1 (42, 49) and the corresponding Asp residues in β2 (50), β3 (Asp-119) (51), and β6 (52) have been reported to be critical for ligand binding. We tested whether mutation of Asp-119 to Ala in β3 affected suPAR D2D3 binding to αvβ3. The wild type or Asp-119 to Ala (D119A) dominant-negative mutant of human β3 was transiently expressed in CHO cells, and the ability of cells to adhere to suPAR D2D3 was determined. Wild-type or mutant β3 is expressed as hamster αv/human β3 hybrid. We found that cells transiently expressing the αvβ3(D119A) mutant did not adhere to suPAR...
binding to mutant shows a dominant-negative effect on VCAM-1 and CS-1 (Fig. 5A). The residual adhesion of B cells express human α4β1/human α4 complex. This results suggest that Asp-130 of β1 is critical for uPAR binding to αβ1 and that the conserved Asp residues in β1 and β3, which are required for known integrin ligand binding, are critical for uPAR binding as well. Taken together, these results strongly suggest that uPAR is an integrin ligand.

uPAR-Integrin Trans-interaction Supports Cell-Cell Adhesion—uPAR has been proposed to interact with integrins on the same cells (cis-interaction) (reviewed in Ref. 1). Because the present results suggest that uPAR may interact with integrins as a ligand, it is possible that uPAR may interact with integrins on apposing cells (trans-interaction), analogous to the interaction between VCAM-1 and αβ1. To determine whether uPAR interacts in-trans with integrins, we tested whether K562 erythroleukemic cells expressing recombinant αβ1 (designated α4-K562 cells) interact with unsorted CHO cells expressing human uPAR (the three-domain form, designated HuPAR-CHO cells) (Fig. 6A). We found that α4-K562 cells showed significantly greater adhesion to HuPAR-CHO cells than to parental CHO cells and that this adhesion was blocked by SG73 (anti-α4). Mock-transfected and parental K562 or CHO cells generated essentially the same results, and the data with parental K562 or CHO cells are shown. Endogenous αβ1 in K562 cells and endogenous hamster uPAR in CHO cells (53) may explain the background binding of K562 cells to CHO cells. We obtained essentially the same results with K562 cells expressing recombinant αβ1 and αβ3 (designated α9- and αβ3-K562 cells, respectively) and cloned HuPAR-CHO cells (Fig. 6, B and C). Y9A2 and 7E3 blocked binding of α9- and αβ3-K562 cells to cloned HuPAR-CHO cells, respectively, suggesting that these interactions are specific to the respective integrins. These results suggest that GPI-anchored uPAR specifically interacts with several integrins on the apposing cells and supports cell-cell interaction.

**DISCUSSION**

uPAR as a Ligand for Several Non-1 Domain Integrins—The present study for the first time establishes that uPAR is a
ligand for several β1 and β3 integrins. We demonstrated that 1) these integrins adhere to suPAR D2D3 in a dose-dependent and cation-dependent manner and 2) specific antibodies against these integrins or uPAR block these interactions. Another structurally related protein, α-bungarotoxin, showed only weak integrin binding relative to suPAR D2D3, suggesting that binding to integrins is a specific property of uPAR. We also demonstrated that the uPAR-binding sites in α4β1 and αvβ3 overlap with the previously reported putative ligand-binding site in these integrins. This is based on the observations that 3) soluble ligands for α4β1 and αvβ3 compete with suPAR D2D3 for binding to these integrins and 4) the known integrin mutations that block ligand binding to α4β1 and αvβ3 block binding of suPAR D2D3 to these integrins as well. These results suggest that uPAR may directly compete with other ligands for binding to these integrins, rather than only operating indirectly by regulating the binding affinity of integrins to other ligands as an integrin-associated protein. These unexpected results are consistent with the observation that soluble uPAR blocks fibronectin binding to α5β1 (17) but do not necessarily agree with the current view that uPAR interacts with integrins exclusively as an associated protein rather than as a ligand for several β1 and β3 integrins.

Fig. 5. Conserved Asp residues in the β subunits are critical for uPAR binding. A, the wild-type or Asp-119 to Ala (D119A) dominant-negative mutant of human β3 was transiently expressed in CHO cells. Expression vector (pB3-1) was transiently transfected in CHO cells as control. Transfected wild-type or mutant β3 is expressed as hamster αv/human β3 hybrid. Forty-eight h after transfection, the ability of cells to adhere to suPAR D2D3 was determined. The level of β3 expression is 35.8% in cells expressing wild-type β3, 43.8% in cells expressing mutant β3, and 8.8% (background) in mock-transfected cells with anti-human β3 antibody (mAb 15). The concentration of suPAR D2D3 used for coating was 5 μg/ml. B, the wild type or Asp-130 to Ala (D130A) dominant-negative mutant of human β1 was expressed in clonal CHO cells together with human α4. The resulting α4β1- and α4β1(D130A)-CHO cells were tested for their capacity to adhere to suPAR D2D3. The expression levels of human α4 and β1 are comparable. α4β1(D130A)-CHO cells show lower adhesion to suPAR D2D3 than do α4β1- or α4-CHO cells (dominant-negative effect). It should be noted that the remaining binding in α4β1(D130A)-CHO cells to suPAR D2D3 is due to endogenous intact hamster β1.

Fig. 6. uPAR-mediated cell-cell interaction. A, fluorescence-labeled parent K562 cells or K562 cells expressing recombinant human α4 (α4-K562) were incubated with a monolayer of CHO cells expressing human uPAR (HuPAR-CHO) or CHO cells in RPMI 1640 medium. Labeled α4-K562 cells were incubated with HuPAR-CHO cells in the presence of mAbs against integrin α4 (SG73). The HuPAR cells used were not clonal (~50% of the population was positive in human uPAR expression). B and C, fluorescence-labeled parent K562 cells or K562 cells expressing recombinant human α9 (α9-K562) (B) or αvβ3 (αvβ3-K562) (C) were incubated with a monolayer of HuPAR-CHO or CHO cells in Hepes-Tyrode buffer supplemented with 2 mM Mg++. Labeled α9- or αvβ3-K562 cells were incubated with HuPAR-CHO cells in the presence of mAbs (Y9A2 to α9, or 7E3 to β3), respectively. HuPAR-CHO cells used in B and C are clonal. Very similar results were obtained with parent or mock-transfected CHO or K562 cells in A, B, and C.

(reviewed in Ref. 1). Integrin specificity experiments were performed using suPAR D2D3. It is possible that if we had used suPAR D1 or full-length suPAR (D1D2D3), differences in integrin specificity or avidity would have become apparent. Although all of the recombinant suPAR fragments used supported the α4β1- and αvβ3-mediated cell adhesion to the same extent, it is not yet conclusive whether each of the domains
The trans-interaction model (Fig. 7B) predicts that tyrosine phosphorylation of proteins and the resulting induction of gene expression occur in the cells that have integrin receptors but not in the cells in which uPAR alone is expressed. In uPAR-integrin-mediated cell-cell interaction, both of the apposing cells may express uPAR and integrins, and uPAR-integrin interaction may occur reciprocally; thus tyrosine phosphorylation might occur on both sides.

Implication of uPAR as an Integrin Ligand in Pathological Situations—It has been reported that down-regulation of uPAR makes a human epidermoid carcinoma Hep3 dormant in vivo (6, 7) as a result of a reduced proliferation rate rather than an increased apoptotic rate (7). It has been proposed that reduction of uPAR expression makes cells either incapable of responding to a signal, or unable to generate a sufficient signal, to propel them through G0/G1 in vivo (10). However, cells expressing less than 50% of the normal level of uPAR grow indistinguishably from parental cells in culture (10). It is unclear why the altered uPAR expression level affects the proliferation of cancer cells in vivo but not in culture. We suspect that uPAR-integrin trans-interaction, which occurs during three-dimensional growth in tumor mass in vivo but may not occur in culture, may contribute to this discrepancy. Trans-interactions between uPAR and integrins as described in this study have the potential to transduce proliferative signals within tumor masses through integrin-dependent pathways. It is possible that uPAR may also function as a ligand for integrin through cis-interactions (Fig. 7C), thereby providing autocrine-type proliferative signals. If this is the case, cancer cells that express high levels of uPAR may have the potential to grow faster than cells that express uPAR at lower levels.

Soluble uPAR is present in ascites of ovarian cancer patients (55). Soluble uPAR levels in plasma increase in patients with rheumatoid arthritis (56), ovarian cancer (57), and leukemia (58). Soluble uPAR levels correlate with resistance to chemotherapy in leukemia (58) and with tumor volume in animal models (59). It has also been shown that soluble uPAR induces extracellular regulated kinase activation when added to uPAR-low Hep3 cells (10). The present study suggests that soluble uPAR might also interact as a ligand with integrins on leukemic cells, solid tumor cells, or inflammatory leukocytes and transduce proliferative signals (Fig. 7D). Thus binding of GPI-anchored uPAR (trans-interaction) or soluble uPAR to integrins as a ligand is a potential therapeutic target in cancer, inflammation, or other pathological situations.

It has been reported that leukocyte rolling and adhesion dramatically increase in mesenteric postcapillary vessels in adjuvant-induced chronic vasculitis in rats (60). An integrin α4 antibody significantly blocks this leukocyte rolling and adhesion. Interestingly, this α4-dependent interaction is not dependent on VCAM-1 or the CS-1 region of fibronectin. It has also been reported that platelet and endothelial uPAR is involved in the survival of platelets in the circulation in mice (61). Injection of tumor necrosis factor increases the number of platelets in the lung alveolar capillaries in wild-type mice, but platelet trapping is insignificant in mice deficient in uPAR. uPAR may be a candidate integrin ligand in these cases. It is possible that uPAR-integrin trans-interaction may be involved in these leukocyte-endothelium and platelet-endothelium interactions during inflammation.

In summary, we have shown that uPAR is a ligand for several integrins and mediates cell-cell adhesion through trans-interactions with these integrins. These findings also help to clarify how uPAR binding to integrins as a ligand transduces signals through integrin pathways. In addition, the present findings predict that interaction of uPAR with integrin...
as a ligand (cis or trans) may be involved in transduction of proliferative or activating signals in cancer and inflammatory cells. Additional studies will be required to clarify the role of uPAR-integrin interactions in these processes.

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