Domain 2 of the Urokinase Receptor Contains an Integrin-interacting Epitope with Intrinsic Signaling Activity

GENERATION OF A NEW INTEGRIN INHIBITOR*

We investigated the interaction between the urokinase receptor (uPAR) and the integrin αvβ3. Vitronectin (VN) induces cell migration by binding to αvβ3, but expression of the uPAR boosts its efficacy. Thus, uPAR may regulate VN-induced cell migration by interacting laterally with αvβ3. In contrast, cells expressing a uPAR mutant lacking domain 2 do not migrate in response to VN. This effect is overcome by D2A, a synthetic peptide derived from the sequence of domain 2. In addition, D2A has chemotactic activity that requires αvβ3 and activates αvβ3-dependent signaling pathways such as the Janus kinase/Stat pathway. Moreover, D2A disrupts uPAR-αvβ3 and uPAR-α5β1 co-immunoprecipitation, indicating that it can bind both of these integrins. We also identify the chemoktaecically active epitope harbored by peptide D2A. Mutating two glutamic acids into two alanines generates peptide D2A-Ala, which lacks chemotactic activity but inhibits VN-, FN-, and collagen-dependent cell migration. In fact, the GEEG peptide has potent chemotactic activity, and the GAAG sequence has inhibitory capacities. In summary, we have identified an integrin-interacting sequence located in domain 2 of uPAR, which is also a new chemotactic epitope that can activate αvβ3-dependent signaling pathways and stimulate cell migration. This sequence thus plays a pivotal role in the regulation of uPAR-integrin interactions. Moreover, we describe a novel, very potent inhibitor of integrin-dependent cell migration.

The urokinase receptor (uPAR) regulates pericellular proteolysis and fibrinolysis by localizing urokinase (uPA) and plasminogen activation on the cell surface. However, uPAR−/− mice do not show thrombotic disorders (1, 2), and it has now become clear that uPAR function is in the regulation of cell migration (3, 4). By binding uPA, uPAR induces migration of both adherent and nonadherent cells in culture. In uPAR−/− mice, neutrophil recruitment in response to pulmonary Pseudomonas aeruginosa infection is weaker than in wild type, and leukocyte recruitment to sites of acute inflammation is dramatically reduced (5–8). Thus, uPAR is involved in physiological and pathological processes requiring cell migration such as angiogenesis, tumor invasion, and inflammation. Furthermore, uPAR promotes cell adhesion directly by binding vitronectin (VN), a serum and extracellular matrix molecule (9, 10). Finally, uPAR affects cell adhesion through lateral interactions with integrins (11–13). uPAR has no cytoplasmic domain and is bound to the plasma membrane by a glycosylphosphatidylinositol anchor. This receptor consists of three homologous domains, and its N terminus, the domain 1, is the primary binding site for uPA. However, domain 3 is also involved, and the presence of domains 2 and 3 enhances the affinity of domain 1 for uPA (14). uPAR affinity for VN is also enhanced by the binding of uPA (10). The linker region of uPAR, located between domains 1 and 2, contains a strong chemotactic epitope (15–17). uPA binding to uPAR induces a conformational change in the receptor that exposes this previously masked SRSRY epitope (16), switching uPAR into a ligand for FPRL1, a seven-membrane-spanning domains G protein-coupled receptor, which in turn stimulates cell migration (15, 16, 18, 19). Therefore, uPAR might be considered as a membrane-anchored chemokine (4, 20). Despite the lack of a cytoplasmic domain, uPAR is capable of complex signaling. Hck, c-Src, focal adhesion kinases (FAK), and protein kinases A and C have been shown to regulate uPAR-dependent signaling pathways (21). uPA binding to uPAR activates downstream signaling pathways including mitogen-activated protein kinases (17, 21). uPAR also controls small G proteins, like Rac (22), that are involved in the regulation of the cell cytoskeleton (23) and cell morphology. uPAR interacts with numerous molecules in the plasma membrane, including FPRL1 (15), the epidermal growth factor receptor (24), gp130 (25), and integrins (26), activating or modulating signaling pathways (3, 4, 13). We have previously shown that uPA- and VN-induced chemotaxis, cytoskeleton reorganization, and cell shape changes required the formation of a uPAR-αvβ3 signaling complex (19, 21). Several studies

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† The abbreviations used are: uPAR, urokinase receptor; ATF, amino-terminal fragment; FCS, fetal calf serum; VN, vitronectin; IBMX, isobutylymethanxilantane; LN, laminin; uPA, urokinase; RSMC, rat smooth muscle cells; SMC, smooth muscle cells; VN, vitronectin; DME, Dulbecco’s modified Eagle’s medium; FITC, fluorescein isothiocyanate; DAPI, 4’,6-diamidino-2-phenylindole; PBS, phosphate-buffered saline; Chaps, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; suPAR, soluble uPAR; Jak, Janus kinase; TRITC, tetramethylrhodamine isothiocyanate.
An Integrin-interacting, uPAR Chemotactic Epitope

have identified amino acid sequences in the α subunit of integrins that bind uPAR and interfere with uPAR-integrin interactions (26–28). Similar information is not available regarding sequences in uPAR for integrin binding. In this study, we have identified a sequence in domain 2 of uPAR that plays a structural and functional role in this interaction. A synthetic peptide, D2A, generated on this basis has chemotactic activity, inhibits soluble uPAR (suPAR)-integrin interaction in vitro, and signals through the VN integrin receptor. A mutant of this peptide (D2A-Ala) inhibits integrin-dependent cell migration.

EXPERIMENTAL PROCEDURES

Materials, Cell Culture, and Adenoviral Infection—Mouse LB6 and NIH 3T3 parental and transfected cells and human embryonic kidney cells (HEK-293 and transfected HEK-293-uPAR) were cultured in DMEM plus 10% FCS. LB6 and NIH 3T3 cells express a low level of murine uPAR (29–32). The constructs used to transfect the cells with human wild-type or mutated uPAR have been described (29, 31). LB6 clone 19 and NIH 3T3-uPAR cells express about 500,000 and 300,000 human receptors/cell, respectively (29). LB6-D1H3D and LB6-D1D2 clones express about 500,000 and 90,000 uPAR molecules/cell on their surface, respectively (29). Despite the lack of one domain, the dissociation constants of D1H3D (2 nM) and D1D2 (8 nM) clones for uPA were not very different from wild type (1.5 nM) (29).

Rat smooth muscle cells (RSMC) were cultured in DMEM plus 10% FCS. For adenoviral infection, RSMC were grown up to 80–80% confluence in 100-mm Petri dishes and infected for 3 h with recombinant adenovirus at a multiplicity of infection of 500 plaque-forming units/cell. The recombinant adenoviruses encoding wild-type Tyk2 and a dominant-negative deletion mutant of Tyk2 have been described (33, 34). RSMC were infected for 2 days after the second infection. Human smooth muscle cells (AoSMC, CASMC) from the aortic and coronary arteries, respectively, were cultured according to the supplier (Clonetics, Charlotte, NC). Human smooth muscle cells (RSMC) were harvested from Molecular Jack Henkin (Abbott Park, IL). Soluble human uPAR was generously supplied by Dr. Douglas Cines (University of Pennsylvania, Philadelphia, PA) (35). Human active two-chain uPA was purchased from American Diagnostica. Mouse monoclonal anti-Stat1 antibody was from BD Biosciences, Transduction Laboratories (Lexington, KY). Mouse monoclonal anti-αvβ3 (LM 609) and anti-α5β1 antibodies, anti-mouse Ig and horseradish peroxidase-conjugated secondary antibodies, and FITC-phalloidin were from Molecular Probes (Eugene, OR). Human fibronectin (10 μg/ml) and gelatin (100 μg/ml) were from BD Biosciences, Transduction Laboratories (Lexington, KY). Human active two-chain uPA was purchased from American Diagnostica. 293 cells (HEK-293 and transfected HEK-293-uPAR) were cultured in DMEM plus 10% FCS, washed with phosphate-buffered saline (PBS), and cultured for another 2 days after the second infection. 

Cell Adhesion Assays—Binding of recombinant uPA to NIH 3T3-uPAR and NIH 3T3 parental cells was determined by incubating the cells on ice for 90 min in binding buffer (DMEM containing 0.1% (w/v) BSA and 10 mM Hepes, pH 7.4) plus 125I-suPAR. The radioactivity was counted. Cell number was determined in parallel wells by direct counting, and the results were expressed as cpm/106 cells.

Pull-down Assay—suPAR was radiodinated (as for VN; see above) at a specific activity of 1.5 × 106 cpm/μg. A mix composed of 2 μg of either purified αvβ3 or α5β1 integrin and 2 μg of 125I-suPAR was incubated for 14 h at 4 °C in the absence (control) or in the presence of 4 μg of uPA with or without 5 μg of either D2A or D2A-Ala peptide in binding buffer (RPMI plus 0.02% BSA, 10 mM HEPES, pH 7.4; total volume 300 μl). Complexes were immunoprecipitated with 3 μg of anti-αvβ3 (LM609) or anti-α5β1 monoclonal antibody (HA5) and 60 μl of protein G-agarose beads (Amersham Biosciences). Beads were washed three times with ice-cold binding buffer and once with ice-cold PBS and lysed in a 1% SDS, 1% Triton X-100 solution. Lysates were analyzed by autoradiography and densitometry, and the results are presented as percentage of relative density units normalized to the band obtained with the addition of uPA alone (100%).

Cell Adhesion Assays—The cell adhesion assay was performed using uPAR-overexpressing HEK-293 cells (both clone 14 and clone 19). Vitronectin (1 μg/ml)-coated 96-well tissue culture plates were preincubated for 2 h at 37 °C, followed by a 1-h incubation at 37 °C with 2% BSA in PBS. After two washes with PBS, 3 × 105/ml cells suspended in 100 μl of DMEM plus 0.1% BSA were seeded in duplicate on protein-coated 96-well plates and incubated for 1 h at 37 °C. After three washes with PBS, attached cells were fixed and stained with Diff-Quick (Dade Diagnostics). The data were quantified by measuring absorbance at a wavelength of 570 nm. The peptides c325, scrambled a325, D2A, and D2A-Ala (100 μM) were added to the cell suspension immediately before seeding cells in the wells.

Statistical Analysis—Statistical analysis was performed with the Prism software using Student’s t test for pairwise comparisons of treatments or an analysis of variance model for the evaluation of treatments with increasing doses of a reagent.

RESULTS

uPAR Expression Effects VN-induced Migration—We have examined the chemotactic effect of VN on control mouse uPAR and on the same cells transfected with human uPAR cDNA. NIH 3T3 and LB6 murine cells express only low levels of murine uPAR, whereas NIH 3T3-uPAR and LB6 clone 19 cells

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express 300,000 and 500,000 human receptors/cell, respectively. VN has chemotactic activity in both transfected and untransfected cells (Table I); however, an increased migratory response to VN was observed in the cells transfected with uPAR. At 5 μg/ml, VN-directed cell migration of LB6 clone 19 cells was significantly enhanced (p values < 0.001) 3.3-fold, with respect to untransfected LB6 cells (Table I). Similar results were observed with NIH 3T3 and human HEK-293 cells, the latter being naturally devoid of uPAR (Table I). Thus, uPAR expression affects the migratory response to VN, in agreement with a direct role of uPAR in VN-induced chemotaxis (21).

**TABLE I**

| Cells                  | Expression of human uPAR | No addition | VN |
|------------------------|--------------------------|-------------|----|
| LB6                    | Wild-type uPAR           | 100 ± 2.7   | 274 ± 3.8 |
| LB6 clone 19           | Wild-type uPAR           | 165 ± 4.7   | 906 ± 22.8 |
| NIH 3T3                | Wild-type uPAR           | 100 ± 2.4   | 222.7 ± 4.8 |
| NIH 3T3-uPAR           | Wild-type uPAR           | 134.2 ± 4.0 | 397 ± 11.1 |
| HEK-293                | Wild-type uPAR           | 100 ± 3.8   | 199.1 ± 12.8 |
| HEK-293-uPAR           | Mutant D1D2-uPAR         | 87.1 ± 16.6 | 393.9 ± 7.8 |
| LB6-D1HD3              | Mutant D1HD3-uPAR        | 100 ± 2.0   | 102 ± 5.1 |
| LB6-D1D2               | Mutant D1D2-uPAR         | 100 ± 3.5   | 280 ± 6.0 |
| NIH 3T3-D1HD3          | Mutant D1HD3-uPAR        | 100 ± 3.0   | 100 ± 4.0 |

However, since anti-uPAR antibodies also inhibited VN-dependent chemotaxis (21), uPAR must be also directly involved. Therefore, uPAR involvement in the migration-promoting effect of VN might be due to a lateral interaction with the integrins (26, 42). Since human wild-type uPAR and D1D2-uPAR enhanced the effect of VN, whereas a mutant lacking domain 2 prevented VN-induced cell migration (Fig. 1B), we hypothesized that domain 2 of uPAR might be directly involved in an interaction with an integrin. A region of domain 2, rich in charged amino acid residues and hence likely to be exposed at the surface of uPAR, attracted our attention (peptide D2A).

Other sequences within the same domain were not tested.

**D2A, a Peptide Derived from the Sequence of Domain 2 of Human uPAR, Has Chemotactic Activity**—We have explored the sequence of domain 2 of uPAR and have focused on peptide 130IQEGEEGRPKDDR142 (D2A) as its sequence suggested that it might be present on the surface of the protein. Peptide D2A per se was able to stimulate migration of LB6 and LB6-D1HD3 cells with a maximal 2-fold increase at 1 μM for the former and 10 μM for the latter (Fig. 2, A and B). Importantly, D2A restored the response to VN of the LB6-D1HD3 cells, counteracting the dominant negative effect of D1HD3 (Fig. 2B).

We also tested the effect of peptide D2A in a well-characterized cell system, RSMC (19, 21, 41, 42), which express uPAR and αvβ3 and migrate in response to both uPA and VN challenge. Fig. 2C shows that D2A dose-dependently stimulated the migration of RSMC with a maximum at 1 μM. The chemotactic effects of D2A and VN were not additive, since the combination of optimal doses of peptide D2A (1 μM) and VN (1 μg/ml) did not further increase cell migration (Fig. 1D). Similar results were obtained with a combination of VN-(40–459) and D2A (Fig. 1D).

**Identification of Essential Chemotactic Residues of Peptide D2A**—We next compared the chemotactic activity of peptides D2A and D2B (see “Experimental Procedures”) that share the same amino acids composition but with a reversed sequence. Both peptides were equally active in stimulating migration of RSMC (Fig. 3A), suggesting the presence of a common epitope in both D2A and D2B. Examination of the two sequences revealed a common GEEG sequence, which might explain why the reverse D2A sequence, D2B, was still chemotactic. We substituted the two glutamic acids in D2A for two alanines, giving a GAAG sequence. When this new peptide, D2A-Ala, was compared with D2A and D2B in chemotaxis, it failed to stimulate cell migration (Fig. 3A). These data suggested that the GEEG sequence of both D2A and D2B peptides was responsible for their chemotactic activity. This conclusion was further supported by the use of smaller synthetic GEEG and GAAG peptides. The GEEG peptide dose-dependently stimulated cell migration with a maximum at 1 μM (Fig. 3B). Thus, this GEEG peptide acted in a way similar to the longer D2A peptide (Fig. 1C).
In addition, a scrambled version of peptide D2A has no effect on cell migration (Fig. 3B). However, the mutated GAAG peptide used at the same concentrations did not affect cell migration (Fig. 3B). Therefore, both GAAG and D2A-Ala have no chemotactic activity.

**D2A Peptide Has αβ3-dependent, Not uPAR-dependent, Chemotactic Activity.**—We previously showed that uPA and VN synergized in RSMC chemotaxis because they activated different signaling pathways (21). We reported, that unlike uPA-induced cell migration, VN-induced cell migration required down-regulation of PK-A and was not extracellular signal-regulated kinase-dependent (21). On the other hand, uPA-promoted cell migration was independent of PK-A and required extracellular signal-regulated kinase activation (21). Therefore, we pharmacologically investigated the pathways involved in D2A chemotaxis. The effects of both VN and D2A were blocked by increasing the intracellular cAMP concentration with forskolin and IBMX (Fig. 4A), a combination that does not inhibit uPA-induced cell migration (21). In contrast, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, PD98059, which completely blocks uPA-induced cell migration, actin cytoskeleton reorganization, and extracellular signal-regulated kinase translocation into RSMC nucleus (21), had no effect on VN- and D2A-induced cell migration (Fig. 4B). In contrast, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, PD98059, which completely blocks uPA-induced cell migration, actin cytoskeleton reorganization, and extracellular signal-regulated kinase translocation into RSMC nucleus (21), had no effect on VN- and D2A-induced cell migration (Fig. 4B). These data suggest that VN and D2A stimulate analogous signaling pathways. In addition, AG-490, a specific inhibitor of the Janus kinases (Jaks), inhibited both VN and D2A chemotaxis in RSMC (Fig. 4C). Once again, the fact that AG-490 acted in a similar way and dose-dependently inhibited cell migration induced by either D2A or GEEG peptide, the shorter form of D2A, reinforces the idea that the GEEG sequence is the chemotactically active epitope of D2A (Fig. 4D). Random cell migration was not affected by the same concentrations of AG-490 (Fig. 4D). These results suggest that both VN and D2A (or the GEEG peptide) can activate the Jak-dependent signaling pathway. We have not yet identified the Jak(s) involved. However, we have excluded the Janus kinase Tyk2, which has been shown to play an important role in regulating uPA-induced SMC migration (25, 33, 34). We investigated whether Tyk2 would be involved in mediating the migratory signal induced by D2A, GEEG, and VN. RSMC were adenovirally infected to express wild-type Tyk2 (RSMC/Tyk2)
or a dominant-negative mutant of Tyk2 (RSMC/K\textsubscript{H9004}L). When challenged by D2A, GEEG, or VN, RSMC/Tyk2 showed a 2-fold increase in chemotaxis compared with RSMC/Tyk2 migrating toward medium alone (Fig. 4E). However, both RSMC/K\textsubscript{H9004}L and uninfected RSMC exhibited a similar increase (Fig. 4E). In contrast, RSMC/K\textsubscript{H9004}L did not migrate in response to PAI-1 (38). Thus, these data show that Tyk2 is not required for D2A-, GEEG-, and VN-induced cell migration. These data also suggest that another Jak kinase(s) is involved in mediating D2A and VN chemotaxis and that in good agreement with a previous study VN (or D2A) and uPA induce cell migration through different signaling pathways (21). Moreover, both VN- and D2A-induced cell migration required \(\alpha_v\beta_3\) integrin, since they were inhibited by LM 609, a monoclonal antibody against \(\alpha_v\beta_3\) (Fig. 4F). Therefore, D2A and VN appear to act through the same signaling pathways.

Chemoattractants induce the reorganization of actin cytoskeleton concomitantly with cell motility (45). Thus, as a chemoattractant, D2A should affect cell shape and actin cytoskeleton organization. In addition, since members of the Jak kinase family are involved in mediating the chemotactic signal

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**FIG. 2.** Peptide D2A has a permissive effect for VN and a chemotactic activity. A, peptide D2A stimulates chemotaxis of untransfected parental LB6 cells. Effect of increasing doses of peptide D2A in the presence or absence of VN (1 \(\mu\)g/ml). The single asterisk (\(p < 0.01\)) and the double asterisk (\(p < 0.05\)) indicate statistical significance compared with control. B, peptide D2A reverts the block in VN-induced migration in LB6-D1HD3 cells (see Fig. 1b). Effect of increasing doses of peptide D2A in the presence or absence of VN (1 \(\mu\)g/ml). *, statistical significance (\(p < 0.001\)) compared with control.

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**FIG. 3.** Identification of the chemotactically active sequence of peptide D2A. A, comparison of the effects of peptides D2A, D2B, and D2A-Ala on migration of RSMC, *, statistical significance (\(p < 0.0001\)). B, D2A and its shorter form, the GEEG peptide, are equally chemotactic. Both D2A and GEEG peptide stimulate migration of RSMC in a dose-dependent manner. In all cases, in the presence of D2A or GEEG peptide, the results are statistically significant (\(p < 0.05\)) compared with control. GAAG peptide, the smaller version of D2A-Ala, has no chemotactic activity. Migration of RSMC toward medium alone (control) is considered to be 100% migration.
FIG. 4. Effect of signaling inhibitors on RSMC chemotaxis. A, both D2A and VN chemotactic effects on RSMC are completely inhibited by the addition of a combination of forskolin plus IBMX. The dilution buffer used to solubilize the mix of forskolin and IBMX has no effect. *, statistical significance ($p < 0.0001$) compared with control lacking VN. B, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor, PD98059, fails to block D2A- and VN-induced RSMC migration. C, AG-490, a specific inhibitor of the Jak kinases, blocks both D2A- and VN-dependent chemotaxis of RSMC. *, statistical significance ($p < 0.0001$) compared with control lacking VN. D, the inhibitor, AG-490, dose-dependently inhibits RSMC chemotaxis induced by either D2A or GEEG peptide, the shorter version of D2A. E, Tyk2 is not involved in the mediation of the migratory signal induced by either D2A, GEEG peptide, or VN. Noninfected RSMC or infected RSMC expressing wild-type Tyk2 (RSMC/Tyk2) or a dominant negative mutant (RSMC/KΔL) were subjected to chemotaxis assay and migrated toward D2A, GEEG peptide, or VN. Control is represented by noninfected cells migrating in the absence of chemoattractant. In the presence of PAI-1, the migration of RSMC/KΔL is significantly different ($p < 0.001$) from that of RSMC/Tyk2. In the other conditions tested, no significant differences were observed between noninfected RSMC and infected RSMC. F, a monoclonal antibody against αvβ3 (LM 609) inhibits both D2A- and VN-induced RSMC migration. ***, a difference highly significant compared with control lacking antibody ($p < 0.0001$); *, significant difference ($p < 0.05$). The 100% value represents the number of cells migrating in the absence of attractant.
induced by D2A and VN (Fig. 4, C–E), we also explored the state of activation of Stat1, a downstream effector of some of the Jaks (see Ref. 46). Subconfluent cultures of serum-starved RSMC were stimulated with 1 pM D2A peptide or 1 μg/ml VN for 30 min at 37 °C and analyzed for actin cytoskeleton organization and Stat1 distribution. Cells were triple-labeled with FITC-phalloidin, DAPI, and primary anti-Stat1 antibodies, followed by TRITC-secondary antibodies to visualize the actin cytoskeleton, the nucleus, and Stat1, respectively. Unstimulated cells kept at 37 °C for 30 min represented the control conditions. Most RSMC under control conditions exhibit numerous stress fibers and a nonpolarized cell shape (Fig. 5). After 30 min of stimulation with D2A, the RSMC acquired an elongated, polarized morphology, semiring actin structures, and membrane ruffling at the leading part of the cell. Actin filaments were also observed flanking the nucleus and in the dragging trail. As expected (21), VN induced similar changes. As far as Stat1, it was mainly cytoplasmic (inactive) in unstimulated RSMC, whereas both VN and D2A induced its translocation to the nucleus (Fig. 5).

These results show that D2A and GEEG peptides stimulate cell migration through signaling pathways that are common to the αvβ3-dependent and different from the uPA-uPAR-FPRL1-dependent signaling pathways (18, 21, 47), supporting the idea that the GEEG epitope harbored by peptide D2A may be involved in the uPAR-αvβ3 interaction.

**Peptide D2A Interferes with the Formation of suPAR-Integrin Complexes in Vitro**—Since uPAR and αvβ3 form complexes on the cell surface and in vitro (48–50), we investigated the effects of peptide D2A in a cell-free, uPA-dependent, suPAR-integrin co-immunoprecipitation assay (see “Experimental Procedures”). Fig. 6A shows that the addition of uPA is required for 125I-suPAR to be immunoprecipitated by αvβ3 antibodies (lane 1 versus lane 2). D2A (lane 3) reduced the amount of coimmunoprecipitated suPAR by about 50%. D2A also inhibited suPAR-α5β1 co-immunoprecipitation by about 55% (Fig. 6B), indicating that D2A can interact with multiple integrins.

**D2A-Ala Inhibits VN-induced Cell Migration**—We further tested D2A-Ala by investigating its effects on VN-induced migration. Surprisingly, unlike D2A, D2A-Ala completely abrogated the chemotactic effect of VN on RSMC (Fig. 7A) as well as primary cultures of human smooth muscle cells from the coronary artery (CASMC) and from the aorta (AoSMC) (Fig. 7, B and C). Thus, D2A-Ala behaves as an inhibitor of VN-induced cell migration. Since the only difference between peptide D2A and D2A-Ala is the presence of a GAAG sequence in the latter, we investigated the inhibitory effect of this GAAG tetrapeptide on VN-induced chemotaxis. GAAG peptide completely inhibited VN-induced RSMC migration (Fig. 7A), showing that the GAAG sequence is responsible for the inhibitory properties of the D2A-Ala peptide.

To determine the IC50 of D2A-Ala inhibitor, we tested increasing concentrations of D2A-Ala on VN-stimulated RSMC. Fig. 7D shows that D2A-Ala inhibited VN-induced cell migration in a dose-dependent manner. A complete inhibition was
obtained at 1 pm with an IC50 of 10–20 fm. Thus, D2A-Ala inhibition is extremely potent. We also tested the effect of D2A-Ala on uPA-dependent suPAR-v3 co-immunoprecipitation in vitro. D2A-Ala also prevented uPAR-v3 co-immunoprecipitation (Fig. 6A), indicating that it can still interact with v3. However, D2A-Ala did not promote the change of morphology and reorganization of actin cytoskeleton (Fig. 5) or Stat1 translocation to the nucleus (Figs. 5 and 8A). These data indicate that D2A-Ala interacts with v3 in an antagonistic way, preventing the transduction of a chemotactic signal. The exact molecular mechanism is presently unknown but will be the focus of future experiments.

D2A-Ala Is a General Inhibitor of Integrin-dependent Cell Migration—Since v3 mediates the chemotactic effect of VN, and since uPAR interacts with several integrins, D2A-Ala might inhibit migration induced by other matrix proteins such as FN and LN. Indeed, FN and LN bind to and act through different integrins, including α5β1. D2A-Ala inhibited both FN- and LN-induced migration (Fig. 8B). Peptide GAAG, the shorter form of D2A-Ala also inhibited FN-induced cell migration (Fig. 8C). In conclusion, D2A-Ala (or GAAG peptide) is a powerful and general inhibitor of matrix-induced, integrin-dependent, cell migration.

The Migration-promoting Effect of Peptide D2A Does Require the Presence of uPAR on the Cell Surface—LB6 and NIH 3T3 cells express low levels of murine uPAR. Therefore, to test whether the effects of peptides D2A and D2A-Ala required the expression of uPAR on the cell surface, we used HEK-293 cells that do not express uPAR (18). D2A elicited a strong chemotactic effect on HEK-293-uPAR cells but failed to stimulate the migration of HEK-293 cells (Table II), indicating that uPAR expression is a prerequisite for the D2A chemotactic effect. D2A-Ala did not induce migration in either HEK-293 or HEK-293-uPAR cells (Table II). VN induced chemotaxis in HEK-293 cells, and this effect was increased in the HEK-293-uPAR cells (Table II). The addition of D2A did not modify the response to VN in either of the cell types. However, D2A-Ala did not induce migration in either HEK-293 or HEK-293-uPAR cells (Table II). VN induced chemotaxis in HEK-293 cells, and this effect was increased in the HEK-293-uPAR cells (Table II). The addition of D2A did not modify the response to VN in either of the cell types. However, D2A-Ala blocked VN-dependent chemotaxis also in the cells that do not express uPAR (Table II). Thus, whereas D2A needs uPAR expression to stimulate migration, the inhibitor D2A-Ala can inhibit VN-
Effects of uPAR expression on the agonistic and antagonistic effects of peptides D2A and D2A-Ala, respectively, on cell migration

Chemotaxis assay was performed as described in the “Experimental Procedures” section. Data are expressed as percentage of control ± S.D. The value 100% (control) represents the number of HEK-293 cells migrating in the absence of attractant.

| Attractant  | HEK-293 | HEK-293-uPAR |
|-------------|---------|--------------|
| No addition | 100.00 ± 24.52 | 92.28 ± 33.80 |
| D2A (1 pM)  | 106.98 ± 11.78 | 325.48 ± 14.42 |
| D2A-Ala (1 pM) | 110.19 ± 26.63 | 108.72 ± 17.37 |
| VN (1 μg/ml) | 210.84 ± 26.95 | 417.25 ± 4.64 |
| VN (1 μg/ml) + D2A (1 pM) | 190.23 ± 35.24 | 400.58 ± 17.41 |
| VN (1 μg/ml) + D2A-Ala (1 pM) | 94.50 ± 19.59 | 108.18 ± 13.90 |

Induced chemotaxis even in the absence of uPAR.

In conclusion, these data show that the agonistic effect of D2A and the antagonistic effect of D2A-Ala use two slightly different mechanisms, which require the presence of uPAR on the cell surface for the former but not for the latter. Therefore, the sequence of peptide D2A contains both “interaction” and “signaling” information, and the two can be dissociated.

D2A/D2A-Ala and α325 Have Different Effects on Cell Adhesion onto VN—D2A/D2A-Ala both inhibit uPAR-integrin association (see Fig. 6). This is the same effect exerted by the α325 integrin peptide (27). It is possible, therefore, that the D2A peptide represents the binding site for α325. To test this possibility, we have performed cell adhesion assays onto VN using two different clones of HEK-293 uPAR cells and verified the effect of these peptides. As shown in Table III, α325 totally blocked cell adhesion on VN in both clones. However, neither D2A nor D2A-Ala inhibited adhesion nor had any effect on the inhibitory effect of α325. We conclude, therefore, that although all of these peptides inhibit uPAR-integrin co-immunoprecipitation, the D2A sequence does not represent the binding site for α325 on uPAR and that the two peptides act through a different mechanism.

**DISCUSSION**

Although the glycosylphosphatidylinositol membrane-bound uPAR was first thought to be a key regulator of plasminogen activation, it is now recognized that it is also (perhaps mostly) a signaling and adhesion receptor. The fact that uPAR does not have a cytoplasmic domain suggests that its effects must be mediated through interactions with other receptors on the cell surface. A wide diversity of transmembrane receptors have been reported to interact with uPAR, including for instance LDL-receptor-related protein and other internalization receptors, the epidermal growth factor receptor and the G protein-coupled receptor FPRL1 (for reviews, see Refs. 3, 11, 13, and 51). In the case of FPRL1, uPA binding to uPAR induces a conformational change that exposes the chemotactic sequence located in the linker region between domains 1 and 2 of uPAR. This conformational change turns uPAR into a ligand for FPRL1, which mediates at last the chemotactic signal of uPA (15, 16, 18, 19).

Integrins are another important family of receptors that can interact with uPAR (8, 26, 35, 50, 52, 53), in particular with αβ2 (Mac-1), αβ1, αβ3, and ανβ3 (26–28, 35). Integrins are...
well known for their role in the regulation of cell adhesion and migration, but unlike uPAR, they possess a cytoplasmic domain connected to downstream signaling molecules. Furthermore, integrins are capable of bidirectional signaling, conveying outside-in and inside-out signals. Perhaps for this reason, integrins interact with numerous membrane proteins such as integrin-associated protein, tetraspans, and uPAR (for a review, see Ref. 54). The role of uPAR in these interactions has been examined, and it has been suggested that uPAR behaves as a modulator of integrin function (26, 27, 42, 49, 55).

The role of uPAR in cell migration is not confined to mediating the uPA signal. In fact, it is also involved in other signals, like that of formylmethionylleucylphenylalanine and VN (21, 40, 56). In this study, we have investigated the role of uPAR in VN-mediated cell migration and have explored the consequences of uPAR-integrin interactions on cell signaling, cytoskeleton organization, cell morphology, and cell migration. We confirm here that uPAR is an important regulator of integrin function. Although it is not absolutely required (see the effect of VN on HEK-293 cells, which do not produce uPAR), uPAR overexpression considerably enhances VN-induced cell migration. In untransfected mouse cells (LB6 and NIH 3T3 cells), VN also stimulates migration (Table I); hence we speculate that mouse uPAR can interact with mouse integrins, although we have not directly addressed this point. Moreover, we also assume that human uPAR can interact with mouse integrins. It is known that the uPA-uPAR interaction is species-specific (57, 58), but there is no evidence that the human uPAR cannot interact with mouse integrins. Although it is known that integrin engagement induces proteases including uPA (59), the effect of endogenous uPA on the uPAR-dependent increase of VN chemotactic activity in transfected cells can probably be ignored because of the species specificity of the uPA-uPAR interaction (57, 58).

The impact of human uPAR on mouse integrin αvβ3 is further demonstrated by the influence of two mutants of uPAR, D1D2-uPAR and D1H3-uPAR. Whereas the first only slightly alters the response to VN, the second prevents it altogether (Table I and Fig. 1B). The effects of these mutants rule out the possibility that uPAR mediates VN chemotaxis by a direct binding mechanism, since neither of the two mutants can bind VN (Fig. 1) (39). Furthermore, VN-(40–459), the truncated form of VN that lacks the somatomedin B domain harboring the binding site for uPAR but still conserving the RGD site (52), promoted cell migration as well as full-length VN (Fig. 1D). Moreover, VN can stimulate migration of HEK-293 cells that are devoid of uPAR. Therefore, the antibody inhibition data of Fig. 3D confirm that VN stimulates cell migration by binding to its own integrin receptors, particularly αvβ3 as previously reported (21, 40, 60), exploiting the lateral interactions between integrins and uPAR (10).

This hypothesis is plainly supported by the identification of peptide D2A, which is located in domain 2 (IQEGEGRPK-DDR) of uPAR. This peptide on the one hand abolished the inhibitory effect of the expression of D1H3-uPAR and, on the other hand, showed direct signaling properties identical to VN. Peptide D2A blocks the formation of uPAR-αvβ3 and uPAR-α5β1 complexes, indicating that it can interact directly with at least two integrins. D2A binding also appears to be functionally relevant, since it stimulated migration of cells expressing uPAR and, even more importantly, overcame the inhibitory effect of D1H3-uPAR expressing LB6 cells (Fig. 2). Like VN-, D2A-induced cell migration was completely blocked by LM609, a monoclonal antibody against αvβ3. Thus, D2A not only binds to the integrin but also generates a signal through it. The investigation of the downstream signaling pathways activated by peptide D2A fully agrees with this idea. Using previously identified inhibitors that discriminate between uPAR- and VN-dependent signaling (21), we found that D2A stimulated migration via VN-dependent, uPAR-independent signaling pathways. Indeed, on the one hand, increasing intracellular cAMP using forskolin and IBMX, which has no effect on uPA-directed cell migration (21), totally inhibited both D2A- and VN-induced chemotaxis. On the other hand, PD98059, the mitogen-activated protein kinase/extracellular signal-regulated kinase kinase inhibitor that prevents mitogen-activated protein kinase activation and blocks uPA-induced cell migration (21, 61), failed to inhibit either D2A or VN-dependent chemotaxis (Fig. 3). Finally, both D2A and VN activated the Jak/Stat signaling pathway as observed for numerous other chemottractants such as chemokines (46). In fact, both D2A- and VN-promoted chemotaxis were blocked by AG-490, an inhibitor of the Janus family of kinases, suggesting that D2A can activate at least one Jak. Moreover, both D2A and VN induced Stat1 relocalization to the nucleus of RSMC (Fig. 5). Stats are the downstream effectors of Jak, and once activated, these latent cytoplasmic transcription factors translocate into the nucleus (46). Importantly, D2A (or GEEG peptide) and VN did not induce cell migration through activation of the Tyk2 member of Jak kinases, which was reported to be the major Jak mediating uPA-induced SMC migration (33, 34). In fact, the dominant negative Tyk2 mutant did not affect VN-, D2A-, or GEEG-induced cell migration (Fig. 4E). The Jak family member responsible for the Stat-1 activation remains to be identified, as well as the mechanism involved, since Stat-1 nuclear localization depends on a balance of Stat-1 import and export from the nucleus (62). In addition, D2A promotes the appearance of the elongated morphology typical of motile cells (sometimes also called a hand mirror shape) and the reorganization of actin cytoskeleton that plainly reflects this motile morphology (Fig. 5). These results suggest that besides the Jak/Stat pathway, D2A can activate other downstream signaling molecules such as small GTP-binding proteins that are known to regulate the organization of actin cytoskeleton (23). This conclusion would be in keeping with previous observations in fibroblasts (19, 22). Taken together, these observations show that D2A has signaling capacities, acting through αvβ3-dependent and not uPAR-controlled pathways, and that the Jak/Stat pathway is directly involved in the regulation of D2A-induced cell migration.

Based on these considerations, we propose that D2A contains two types of information, including sequences that mediate its binding to integrins αvβ3 or α5β1, and sequences for the activation of integrin-dependent signaling pathways involved in the regulation of cell migration. The dissociation between these two sets of properties was observed when we introduced muta-
tions into the sequence of D2A. Our attention on a particular epitope (i.e. GEEG) resulted from the observation that although peptide D2B had a sequence that was the reverse of D2A, it was equally active in chemotaxis, and both peptides contain the same GEEG sequence. Thus, we modified GEEG into a GAAG epitope and tested the chemotactically active activity of the new peptide named D2A-Ala. This peptide was identical to D2A except for the substitution of two alanines for the two glutamic acid residues. Peptide D2A-Ala was not chemotactic and had no signaling activity (Figs. 4, 6, and 7), demonstrating that the GEEG sequence is the chemotactically active epitope harbored by peptides D2A and D2B. Despite this, D2A-Ala could still interact with αvβ3 and α5β1, as indicated by its inhibition of the suPAR-integrin co-precipitation (Fig. 6). Strikingly, the introduction of the GAAG epitope converted D2A into a powerful inhibitor of VN-induced cell migration with an extremely low IC50, about 10–20 nM (Fig. 7). In addition, D2A-Ala also inhibited Stat1 activation as well as the appearance of the motile cell morphology and actin cytoskeleton reorganization promoted by VN. Moreover, D2A-Ala inhibited migration induced by other extracellular matrix proteins such as FN and LN, suggesting that it can block other integrins. Therefore, the inhibitory ability of D2A-Ala might reside in its ability to block the formation of and/or disrupt uPAR-integrin complexes such as uPAR-αvβ3 and uPAR-α5β1. Thus, the mutation introduced in the GEEG epitope of D2A destroyed its signaling information without affecting its ability to interact with αvβ3 integrin.

When investigating the effects of D2A and D2A-Ala on cells that do not express uPAR but express αvβ3 and α5β1 (27), we found that peptide D2A failed to stimulate migration of HEK-293 cells, whereas D2A-Ala succeeded in inhibiting VN-induced chemotaxis in the same cells. Thus, the induction of cell migration by D2A requires the presence of uPAR on the cell surface, whereas the inhibition of VN chemotaxis just requires the binding of D2A-Ala to the integrin. These data reveal subtle differences in the agonistic and antagonistic mechanisms of peptides D2A and D2A-Ala and suggest that binding of uPAR can modify the affinity and/or the avidity of integrins.

When considered altogether, the agonistic effects of D2A and the dominant negative behavior of D1HD3-uPAR suggest that uPAR can exert both a positive and a negative regulation on integrins. However, it seems likely that besides the D2A epitope, other sites of interaction between uPAR and integrins exist. These sites might be located in domain 3 because of the apparent dominant negative activity of the D1HD3 variant and because the D1D2 cells were less sensitive to low doses of VN (Fig. 1). Moreover, D2A and D2A-Ala do not share with the α325 peptide the property of inhibiting uPAR-mediated cell adhesion on VN (Table III). Therefore, D2A does not represent the binding site of the α integrin subunit onto uPAR. This is a further argument for suggesting that uPAR and the integrins interact through at least two sites.

A model based on our data might explain the activating effects of uPAR on integrins; uPAR can first “anchor” (or contact) integrins through a still undefined epitope, which would allow the subsequent interaction of the D2A sequence with the integrin, reinforcing the interaction and allowing signaling. This is a real possibility now that the uPAR x-ray structure has been elucidated. Indeed, the D2A region lies outside of the site of interaction with uPA and is thus accessible to interaction with other molecules (63). Integrin ligands, such as VN, could bind to the “activated” integrin and induce signaling, although it is possible that VN binding to integrin may be required for the uPAR-integrin interaction. In our model, the D2A region has two functions, integrin binding and signaling, and these functions can be dissociated, since in the context of the entire D2A peptide the GEEG sequence is required for signaling but not for binding (also D2A-Ala can inhibit suPAR-integrin co-immunoprecipitation) (Fig. 6).

Under certain conditions, uPAR may negatively regulate the integrin, as in the case of the D1HD3-uPAR variant. When uPAR contacts the integrin only through binding site(s) located within domain 1 or 3 of uPAR (mutant D1HD3), the integrin appears to be locked in a signaling-inactive state. This block is bypassed by the addition of the D2A peptide.

In summary, in this study we have identified a new chemotactic sequence located in domain 2 of uPAR. Peptide D2A, the synthetic peptide bearing that sequence, binds to integrins and acts through these receptors activating integrin-dependent signaling pathways. The two glutamic acid residues in the GEEG epitope are crucial for signaling, and the GEEG sequence itself has full chemotactic activity. In addition, we have also identified a form of uPAR, D1HD3, that behaves as a dominant negative mutant. These data suggest that uPAR may induce positive or negative regulation of integrin function by inducing different conformations through different multiple interactions. The positive regulation would be achieved by binding to multiple sites, including D2A located on domain 2, and by extrapolation from the D1HD3 results, on domain 3 of uPAR. The negative regulation would rather be obtained by interactions involving site(s) located only on domain 3 (and/or domain 1). Finally, by introducing mutations into the D2A sequence, we have generated a powerful integrin inhibitor, D2A-Ala. This inhibitor is extremely interesting and might be effective against the many physiological and pathological processes in which uPAR and integrins are involved, such as cancer and inflammatory diseases. Further studies will have to validate these points.

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Domain 2 of the Urokinase Receptor Contains an Integrin-interacting Epitope with Intrinsic Signaling Activity: GENERATION OF A NEW INTEGRIN INHIBITOR
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