Activation of urokinase receptor by a novel interaction between the connecting peptide region of urokinase and αvβ5 integrin

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Summary

The serine protease urokinase (uPA) binds to the urokinase receptor (uPAR) through its growth-factor domain (GFD, residues 1-49), affecting cell migration, adhesion and growth. Here, we show that uPA can promote cytoskeletal rearrangements and directional cell migration in a GFD-independent manner, through a new and specific interaction between an internal uPA domain coined 'connecting peptide' (residues 132-158) and cell-surface integrin αvβ5. Remarkably, a peptide corresponding to this region (CPp, residues 135-158) retains the ability to bind to αvβ5, eliciting cytoskeletal rearrangements and directing cell migration at a concentration as low as 1-10 pM. These effects are lost in cells not expressing uPAR, indicating that the uPAR is required for CPp-dependent signaling. Furthermore, the CPp–αvβ5-integrin interaction enhances F-actin-enriched protrusions and cell migration induced by the well-established interaction between the uPAR-binding peptide (GFDp, residues 12-32) of uPA and uPAR. These results provide new insight into the function of uPA, which – through individual domains – can engage two different surface receptors (uPAR and αvβ5 integrin), thus initiating and potentiating intracellular signaling and migration.

Key words: uPA, uPAR signaling, Cytoskeleton

Introduction

Cell migration is a spatially and temporally coordinated process requiring a biochemically based cyclic generation of driving forces. Migrating cells respond to a motogen gradient through the acquisition of a polarized morphology and the extension of adhesive protrusions, which serve as traction sites for forward locomotion (Ridley et al., 2003). This complex process requires the coupling of extracellular signals with the internal signaling machinery that controls cytoskeleton dynamics and cell adhesion. In vivo, 3D migration is facilitated by controlled enzymatic cleavage of the extracellular matrix (ECM) by metalloproteinases and plasminogen activators (Chang and Werb, 2001).

Urokinase (uPA), a serine protease originally identified for its plasmin-generating capacity and its, thus indirect, fibrin and ECM-degrading function (Dano et al., 1985), has also been shown to posses motogen activity. Its dual function in enhancing migration while lowering the physical resistance of ECM underlies the rate-limiting nature of this factor in tumor invasion and metastasis (Andreasen et al., 1997). This multidomain protease consists of two disulfide-bridge-linked polypeptide chains: the N-terminal polypeptide containing a uPA growth-factor-like domain (GFD; residues 1-49), a kringle domain (residues 50-131) and a linker or 'connecting peptide' region (CP; residues 132-158) extending to the Lys158-Ile159 pro-enzyme activation site and a large C-terminal, serine protease polypeptide (residues 159-411). Urokinase has the catalytically independent ability to elicit a dynamic reorganization of the actin cytoskeleton and adhesion to the extracellular matrix, through the Rho family small guanosine triphosphate (GTP)-binding proteins and downstream mediators, such as ERK1/2 serine kinases (Jo et al., 2002). Urokinase-dependent signaling leading to MDA-MB 231 breast cancer cell migration involves disruption of the interaction between β1 integrins and N-WASP, which subsequently translocates to the actin cytoskeleton (Sturge et al., 2002). In monocyte-like cells, expression of the Src family kinase p56/59^{hck}, in the constitutively active or kinase-inactive forms, prevents urokinase-dependent induction of adhesion or motility, indicating that a specific activation state of p56/59^{hck} is required for each cell response (Chiaradonna et al., 1999). The phosphorylation state of uPA has been shown to be important in the regulation of cell motility, because phosphorylated uPA is unable to convey a stimulatory signal for cell migration (Franco et al., 1997).

The majority of the uPA-dependent signaling effects are mediated by uPA binding, through the growth-factor domain (GFD), to the glycosyl-phosphatidylinositol (GPI)-anchored cell-surface urokinase receptor (uPAR) (Vassalli et al., 1985; Stoppelli et al., 1986; Appella et al., 1987; Geiger et al., 2001). The uPAR belongs to the Ly-6 protein family and contains three domains (D1 to D3) that are homologous to CD59, Ly6E

and α-neurotoxins (Blasi and Carmeliet, 2002). Increasing evidence shows that uPAR signals by forming associations with transmembrane receptors like the G-protein-coupled receptor FPRL1 (formyl peptide receptor 1), integrins such as α5β1, αvβ3, αvβ5, αMβ2, CD11b/CD18 and the rafts-associated caveolin (Ossowski and Aguirre Ghiso, 2000). The uPAR ability to modify integrin activity as well as integrin-dependent signaling implies that it is a cis-acting integrin regulator (Wei et al., 1996; Aguirre Ghiso et al., 1999; Carriero et al., 1999). Also, the uPAR has been reported to be a true integrin ligand (Tarui et al., 2001). A direct uPAR-Mac-1 (CD11b/CD18) interaction was shown by the use of a 25 amino-acid-long peptide, which disrupts physical association and signaling (Simon et al., 2000). The D1 domain of uPAR is required for the association of uPAR with integrin, and signaling (Liu et al., 2002; Montuori et al., 2002). At least in one case, the uPAR-integrin interaction site has been mapped within the α5 propeller (residues 242-246) of α5β1 integrin (Wei et al., 2005; Simon et al., 2000) and within domain III of uPAR (Chaurasia et al., 2006).

Although, the precise molecular interactions leading to complex formation between uPAR and integrins, and to the initiation of signaling are still unclear, there is strong evidence that supports the existence of such interactions. Of interest to the current study is the association of uPAR with αvβ5 integrin, which is increased by the amino-terminal fragment (ATF; residues 1-135 of the human uPA sequence) binding to uPAR (Carriero et al., 1999). Moreover, αvβ5 protein levels are upregulated by uPAR engagement (Silvestri et al., 2002), suggesting some interdependence of these two receptors.

We previously showed that a proportion of uPA can become phosphorylated on two serine residues, one of which (Ser138) resides in the CP region of uPA, and that this modification abolishes its ability to induce cell migration and cytoskeletal rearrangements (Franco et al., 1997; Carriero et al., 2002). Furthermore, the fact that phosphorylation or substitution of Ser138 with glutamic acid does not change the binding affinity, yet inhibits migration, of uPA to uPAR through the classic GFD, suggests that the CP region, centered around Ser138, plays an important regulatory function. In this article, we map the uPA region involved in a new functional cooperation with the uPAR-binding GFD and identify the integrin that participates in this interaction. Taken together, the data highlight the simultaneous concurrent interaction of individual uPA domains with two distinct surface receptors, leading to a full uPAR-dependent cell migration.

Results

We previously demonstrated that a phosphomimetic form of uPA, in which Ser138 was substituted with glutamic acid, exhibited an impaired ability to induce migration, suggesting the chemotactic relevance of the region surrounding Ser138 (Franco et al., 1997; Franco et al., 1998). We have now focused on the role of the CP region (residues 132-158) in the uPA-dependent signaling.

GFD-independent effects of uPA on cell migration

In an effort to characterize the role of the CP region and its functional relationship with the growth-factor-domain (GFD), several uPA variants were generated. Histidine-tagged wild-type human uPA (His-uPA) and a ΔGFa variant carrying the deletion of the uPAR-binding region (amino acids 9-45) were obtained as histidine-tagged pro-enzymes (Fig. 1A), and characterized by their ability to activate plasminogen and interact with uPAR. Once activated, both products retained enzymatic activity (data not shown) and, as expected, only His-uPA retained the ability to bind to uPAR. Competition-binding assays of ^{125}I-His-uPA to U937 cells – which express uPAR (Stoppelli et al., 1985) – show that His-uPA competes efficiently for binding to uPAR, whereas the ΔGFa variant, which lacks residues 9-45, does not (Fig. 1B). It is noteworthy that 90-95% of His-uPA and ΔGFa are still in the single-chain form, even at the end of incubation (data not shown).

To test whether ΔGFa was able to induce migration and whether uPAR expression was required, parental human embryonic HEK-293 cells that do not express detectable uPAR (Montuori et al., 2002) were stably transfected with pcDNA3-uPAR, and two clones with approximately 3×10^3 uPAR/cell (HEK-293/uPAR-12) and 3.5×10^5 uPAR/cell (HEK-293/uPAR-25) were isolated. The relative uPAR expression level determined by western blot analysis is shown in Fig. 1C. In agreement with existing literature, His-uPA induced migration only in uPAR-bearing HEK-293 cells (Fig. 1D). Importantly, in agreement with our previous data that suggested a chemotactic function for the CP region, the ΔGFa was able to induce HEK-293/uPAR-12, HEK-293/uPAR-25 and monocyte-like U937 cell migration (Fig. 1D). It is noteworthy that in uPAR-lacking cells, ΔGFa and His-uPA were slightly inhibitory rather than eliciting migration, (Fig. 1D).

To further analyse the effect of uPAR engagement on chemotaxis, we employed a peptide that corresponds to residues 12-32 of human uPA (GFDp, Fig. 1A) and had been shown to be the minimal requirement for receptor binding. This peptide has a K_d value of 40 nM for uPAR (Appella et al., 1987). To directly compare the extent of migration induced by GFDp and ΔGFa, HEK-293/uPAR-25 cells were exposed to increasing concentrations of the two effectors. As expected, binding of GFDp to uPAR was followed by a dose-dependent enhancement of cell migration, with optimal effect being observed at ~100 pM (Fig. 1E). Surprisingly, the dose-dependence of ΔGFa was slightly less, with peak activity reached at 10 pM (Fig. 1E). The equimolar mixture of GFDp and ΔGFa produced a somewhat stronger motogenic effect than the individual proteins and the effect extended over a wide concentration range, suggesting cooperation between GFD and a domain in the ΔGFa protein.

Specific uPAR-independent binding of uPA to αvβ5 integrin through the CPp region

The observation that ΔGFa was unable to compete for His-uPA binding to uPAR, even though it stimulated HEK-293/uPAR-25 cell migration, prompted us to consider that it might specifically associate with the cell surface, independently of uPAR. To test this possibility, the binding of ΔGFa was assessed in a ^{125}I-ΔGFa-competition assay with HEK-293 cells. As shown in Fig. 2A, ~10 pM of ΔGFa was sufficient to reduce surface-bound ^{125}I-ΔGFa by 50%, indicating the occurrence of a specific interaction. Unlabeled uPA 1-158 (corresponding to the first 158 uPA residues) and the peptide corresponding to residues 135-158 of human uPA (CPp) (Fig. 1A) display a similar relative affinity, because 50% competition was achieved at ~10 pM. By contrast, GFDp was

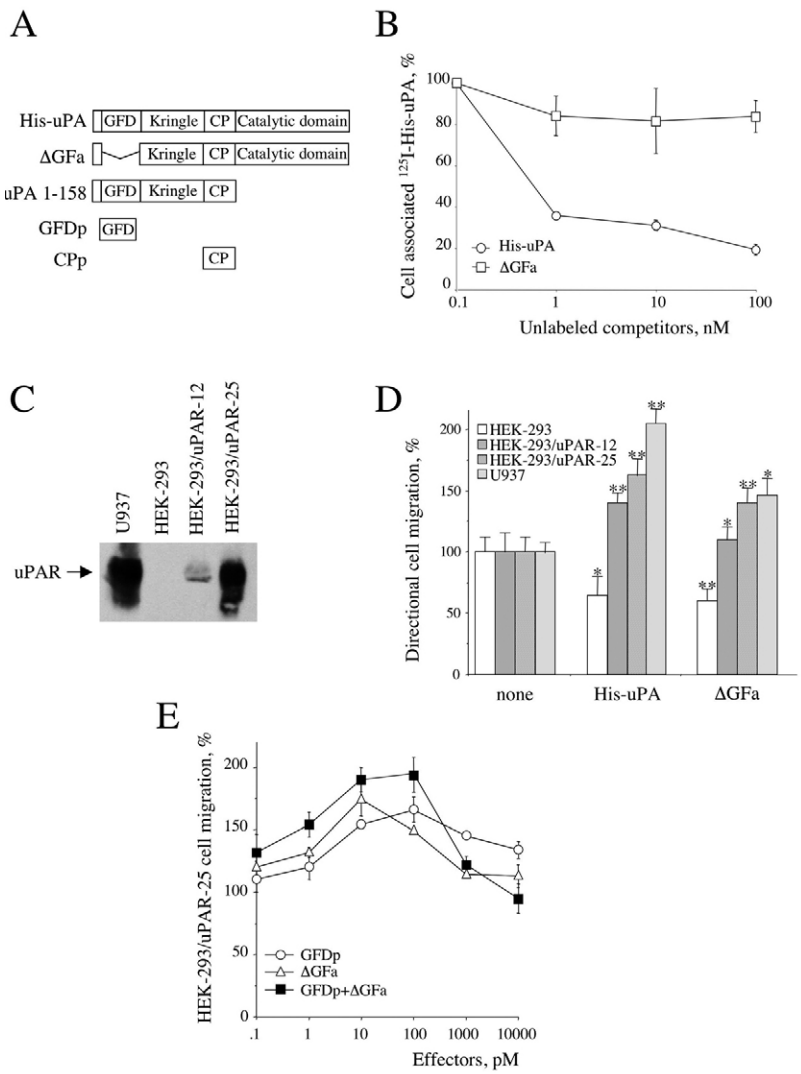

Fig. 1. GFD-independence of uPA chemotactic ability. (A) Schematic representation of the human urokinase structure, showing the N-terminal growth-factor-like domain (GFD, residues 1-49), the kringle domain (residues 50-131), the CP region (residues 132-158) and the catalytic domain (residues 159-411). Histidine-tagged wild-type human uPA (His-uPA), the histidine-tagged variant lacking amino acid residues 9-45 (ΔGFa) and the untagged N-terminal 158 aminoacids (uPA 1-158) were obtained as secreted products in the *P. pastoris* expression system. The two peptides (CPp, residues 135-158 and GFDp, residues 12-32) employed in this analysis are also indicated. (B) Radioreceptor competition assay of ^{125}I-His-uPA (10^5 cpm/sample) and the indicated nanomolar concentrations of unlabeled His-uPA or ΔGFa to U937 cells. The results are the mean of two independent experiments performed in duplicate, error bars indicate ±s.d. (C) 50 μg of membrane extracts of U937, HEK-293, HEK-293/uPAR-12 or 2.5 μg of HEK-293/uPAR-25 were resolved on a 10% SDS-PAGE followed by western blotting with R2 anti-uPAR monoclonal antibody. (D) HEK-293, HEK-293/uPAR-12 and HEK-293/uPAR-25 cell lines were subjected to a directional migration assay in Boyden chambers. 10^5 cells/sample were allowed to migrate for 3 hours towards 0.1 nM His-uPA or ΔGFa or diluents. Migration in the absence of effectors or random migration was taken as 100%. Data are presented as the mean ± s.d. of three separate experiments performed in duplicate. Statistical analysis was with Student's *t*-test. **P*<0.01; ***P*<0.0001 when compared with the untreated relative control cells (none). (E) HEK-293/uPAR-25 cells were assayed for directional migration towards the indicated effectors over the specified concentration range as specified above. In the combinations, a molar ratio of 1:1 was employed. The results are expressed as mean ± s.d. of three independent experiments performed in triplicate.

ineffective, indicating that the uPAR-binding region is not involved in this interaction. This experiment suggests the presence of a specific, high-affinity site on the surface of HEK-293 cells that interacts with ΔGFa and uPA 1-158 through the CPp region, encompassing residues 135 to 158. This notion is directly supported by the finding that His-uPA, ΔGFa, uPA 1-158 and CPp were able to compete for the binding of ^{125}I-CPp peptide to the cell surface, whereas GFDp was not (Fig. 2B). Since ^{125}I-CPp bound to HEK-293 and to HEK-293/uPAR-12 with a similar K_d, the uPAR seems to be dispensable for uPA binding to the cell surface through the CP region. However, to rule out the possibility that uPA was binding directly to uPAR through the CP region, a competition experiment was set up. Purified uPA 1-158 was incubated with cell culture medium of LB6 cells, expressing the soluble uPAR (suPAR) (Masucci et al., 1991) in the presence of excess GFDp or CPp. The resulting products were cross-linked and loaded onto an SDS-PAGE followed by western blotting with anti-uPAR antibody. The association of uPA 1-158 with suPAR yielded a ~55 kDa band which was abrogated by 5 μM GFDp. On the contrary, CPp did not reduce the extent of uPA 1-158/suPAR complex up to a concentration of 50 μM, showing that it is unable to bind with high affinity to the suPAR (Fig. 2C). These data, taken together, indicate that uPA binds to a non-uPAR component on cell surface through the CP region.

In an attempt to identify the binding partner for ^{125}I-CPp on cell surface, we explored integrins as possible candidates. HEK-293 cells were tested for integrin expression by FACS analysis. The following integrin subunits were found: α1, α2, α3, α4, α5, αv, β1, β3 and β5. Although some published reports suggest that αvβ5 integrin is not expressed (Li et al., 2001), our analysis indicates that it is present but not at a high level (data not shown). Before testing the effect of integrin-blocking antibodies on CPp binding, the antibody function was tested in an adhesion-blocking assay using HEK-293 cells and collagen, laminin, vitronectin and fibronectin as matrix proteins. As shown in Table 1, all antibodies tested were effective in blocking adhesion to the respective, specific matrix protein. To examine whether any of the integrins expressed on HEK-293 cells were responsible for ^{125}I-CPp binding, the cells were exposed to the indicated antibody prior to incubation with ^{125}I-CPp at 4°C. Results shown in Fig. 3A indicate that anti-αv integrin, anti-β5 integrin and anti-αvβ5 integrin antibodies caused a 50-60% reduction in the radioactivity specifically associated with the cell surface. Antibodies against α2 integrin, α3 integrin, α4 integrin, β1 integrin and β3 integrin were

ineffective in blocking the binding of ^{125}I-CPp, and anti-α5 antibody produced a small (20%), but statistically significant, inhibition. We do not know whether the reason for the antibody blockade of CPp binding to αvβ5 integrin is the result of an overlap between the CPp- and the vitronectin-binding site or an allosteric effect that masks the CPp-binding site. However, the fact that the blocking antibodies against αv integrin, β5 integrin and αvβ5 integrin all prevent CPp binding suggests some overlap between the native ligand binding domain and CPp-binding domains. Importantly, two additional experimental results support the specific role of αvβ5 integrin. First, cloned HEK-293 cells stably overexpressing αv integrin exhibited increased specific association of ^{125}I-CPp to cell surface (Fig. 3A). Second, whereas preincubation of purified αvβ5 integrin with CPp reduced the binding of ^{125}I-CPp to cells by 80-90%, no reduction was observed following preincubation with α1β1 integrin (Fig. 3A). Further evidence of ΔGFa binding to αvβ5 integrin was obtained from an experiment in which the adherence of HEK-293 cells to ΔGFa-coated dishes was examined. After 1 hour at 37°C, approximately 40% of untreated HEK-293 cells adhered to the ΔGFa-coated plates, but this number was reduced by 60-70% when HEK-293 cells were preincubated with anti-αv, anti-β5 or anti-αvβ5 antibodies (Fig. 3B). In control experiments, about 90% of the total HEK-293 cells were found to adhere to vitronectin-coated plates, whereas only 3.9% adhered to BSA-coated plates. Finally, direct interaction of αvβ5 integrin and ΔGFa was tested in vitro by preloading anti-uPA polyclonal antibody conjugated to protein A-Sepharose with ΔGFa or diluents, and further incubating it with purified αvβ5 integrin. Examination of bead-associated and supernatant αvβ5 integrin by western blotting revealed that, although both supernatants contained αvβ5 integrin, only the beads that were incubated with ΔGFa had bound αv integrin (Fig. 3C). When purified α1β1 integrin was added to the anti-uPA-ΔGFa-Sepharose beads, no integrin bound to Sepharose (data not shown).

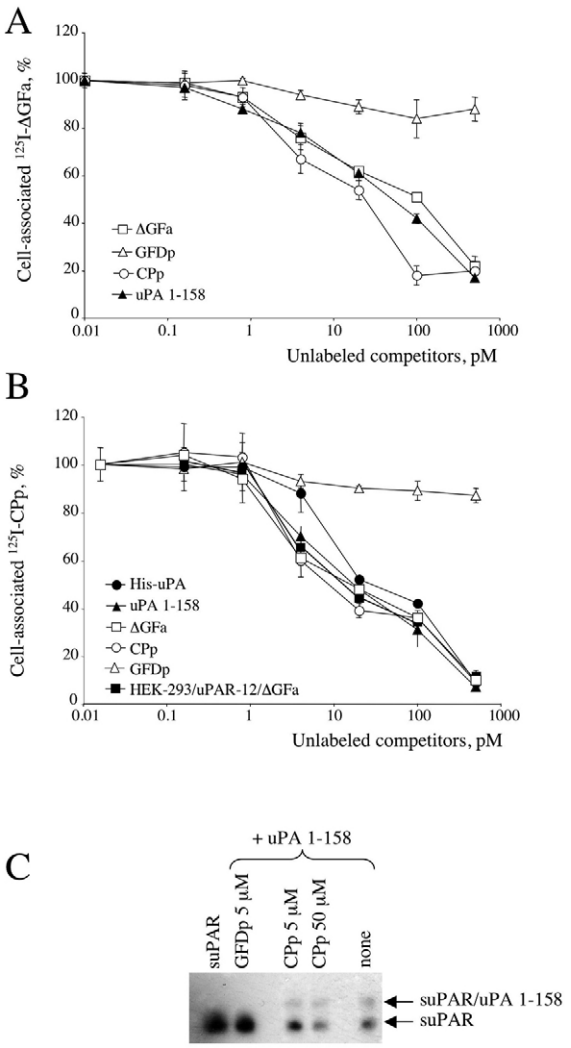

Fig. 2. High-affinity specific binding of ΔGFa and CPp to HEK-293 cells. (A) ^{125}I-ΔGFa (150,000 cpm/sample) was incubated with HEK-293 cells with increasing concentrations of unlabeled effectors for 3 hours at 4°C. After extensive washing the cell-surface-associated radioactivity was determined. The ^{125}I-ΔGFa-specific binding to cells in the absence of competitor was 1300 cpm. Results are presented as the mean of specific binding ± s.d. in percent. Experiments were carried out in duplicate and data shown here are representative of three independent experiments. (B) ^{125}I-CPp (150,000 cpm/sample) was incubated with HEK-293 or HEK-293/uPAR-12 cells with increasing concentrations of the indicated unlabeled effectors for 3 hours at 4°C as described above. The ^{125}I-CPp-specific binding to cells in the absence of competitor was 2270 cpm. Results are presented as the mean of specific binding ± s.d. in percent. Experiments were carried out in duplicate and data shown are representative of three independent experiments. (C) 50 nM uPA 1-158 was incubated with 5 μl of conditioned medium from LB6 cells expressing soluble uPAR (suPAR) for 1 hour at 37°C with or without the indicated concentrations of CPp or GFDp. Samples were crosslinked with 1 mM DSS for 15 minutes on ice and loaded onto a 12.5% SDS-PAGE. Arrows indicate the suPAR and the complex between suPAR and uPA 1-158 (suPAR/uPA 1-158).

Pattern of F-actin rearrangements in response to uPA and uPA-derived peptides

Reorganization of the actin cytoskeleton, which generates the driving forces supporting migration, is an early event in the stimulation of cell migration. Here, we analyse the relationship of uPA-induced cell migration and cytoskeletal rearrangements, using a novel technique employing a Dunn-type chamber that allows the observation of directional migration towards a preformed chemotactic gradient under a

Table 1. HEK-293 cell-adhesion-blocking activity of anti-integrin antibodies to different matrix proteins

Antibodies	Collagen (%)	Laminin (%)	Vitronectin (%)	Fibronectin (%)
None	100	100	100	100
Anti-α1+ anti-β1	23±3	–	–	–
Anti-αv+ anti-β5	94±8	111±11	21±4	–
Anti-α3+ anti-β1	–	27±6	–	–
Anti-α4+ anti-β1	–	–	88±10	67±8
Anti-α5+ anti-β1	–	–	–	36±4

Sub-confluent 1×10^5 HEK-293 cells were pre-incubated with the indicated anti-integrin antibodies or diluents for 1 hour and seeded in wells of a 24-wells plate coated with 50 μg/ml of collagen, 20 μg/ml laminin, 25 μg/ml vitronectin, 20 μg/ml fibronectin or 1 mg/ml BSA and kept at 37°C for 1 hour. Adherent cells were extensively washed in 1× PBS with 1 mg/ml BSA and counted. This value was taken as 100% and the values obtained in the presence of antibodies are expressed relative to that. The percentage of cells adherent to BSA-coated dishes was 3.9±0.4%.

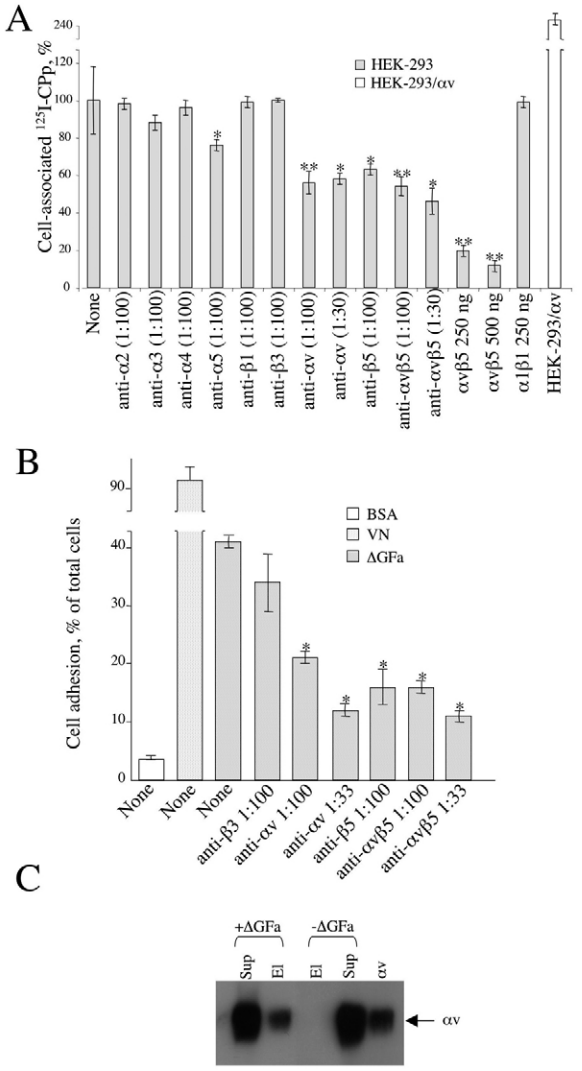

Fig. 3. Specific binding of ΔGFa and CPp to αvβ5 integrin. (A) ^{125}I-CPp was incubated with aliquots of HEK-293 cells (as described in the legend to Fig. 2B) that had been preincubated with the indicated antibody dilution for 1 hour at 4°C. When indicated, ^{125}I-CPp was preincubated either with purified αvβ5 integrin (250 or 500 ng) or α1β1 integrin (250 ng) in a total volume of 0.2 ml for 1 hour prior to radioreceptor-binding assay. The white bar (furthest right) refers to the radioactivity specifically bound to HEK-293-αv cells, which had been exposed to ^{125}I-CPp for 3 hours at 4°C. Non-specific binding was assessed by including additional duplicate samples containing unlabeled 10 nM CPp. Specific binding to HEK-293 cells was taken as 100% and the extent of inhibition by anti-integrin antibodies or by αvβ5 integrin or α1β1 integrin was calculated relative to this value. Values represent the mean ± s.d. of two independent experiments performed in duplicate. *P<0.005; **P<0.0006. (B) 24-well cell culture dishes were coated with 1 mg/ml BSA or 25 μg/ml vitronectin or 100 pM ΔGFa overnight at 4°C and extensively washed with 1× PBS. For each sample, 10^5 HEK-293 cells were preincubated with the indicated dilution of antibodies or non-treated (none) in a final volume of 0.3 ml for 1 hour and allowed to adhere in an 1-hour-assay at 37°C. The number of adherent cells was counted and is given as the percentage of the total cell population, representing the average of three different experiments performed in duplicate. *P<0.0001 compared with ΔGFa. (C) 6% SDS-PAGE followed by western blotting with anti-αv integrin polyclonal antibody. 15 μl of protein A–anti-uPA antibody Sepharose with (+ ΔGFa) or without (– ΔGFa) 5 μg of ΔGFa was incubated for 2 hours at 25°C in a total volume of 60 μl, washed and further incubated with 500 ng of αvβ5 integrin. Sepharose-bound proteins were isolated by centrifugation and the supernatants (Sup) and the eluates (El) were separated by 7.5% SDS-PAGE. 100 ng of purified αvβ5 integrin protein was loaded as a control.

microscope. Owing to the design of the Dunn chamber, it is possible to distinguish between migrated and stationary cells. We counted the number of migrating cells displaying cytoskeletal rearrangements in response to an uPA 1-158 gradient and compared it with migrating cells exhibiting F-actin protrusions in the absence of chemoattractant. The results showed that, in the presence of uPA 1-158 gradient, 66±4% of cells had F-actin-enriched protrusions (Fig. 4B), whereas only 19±1% of control cells showed this characteristic (Fig. 4A). Therefore, uPA specifically promotes cytoskeletal rearrangements in almost half (47%) of the migrating cells.

To examine whether F-actin reorganization is affected by the GFD-uPAR interaction and whether the CP region participates to some extent, HEK-293/uPAR-25 cells were treated for 1 hour with different uPA-related effectors and stained with Rhodamine-phalloidin. To reduce F-actin background staining due to adhesion, cells were kept in suspension throughout the treatment with the selected effectors according to a published procedure (Carriero et al., 1999; Gargiulo et al., 2005). Cell exposure to GFDp caused the appearance of a single protruding region in which F-actin and uPAR were co-localized (Fig. 5A). A similar pattern was observed following HEK-293/uPAR-25 cell incubation with CPp (data not shown). A quantitative analysis was performed by examining 200 cells/sample. Cells exhibiting phalloidin-positive protrusions were counted and expressed as the percentage of total cell number. The percentage of untreated cells exhibiting single phalloidin-positive protrusions (5%-8%) was subtracted to obtain the net effector-dependent values. The analysis revealed a statistically significant net increase in the percentage of F-actin-enriched protrusions following exposure to His-uPA, GFDp or CPp (between 30% and 45%). In all cases, this effect was prevented by preincubation of cells with anti-uPAR polyclonal antibody, blocking uPAR engagement by GFD, by anti-αvβ5 integrin, anti-αv integrin, anti-β5 integrin antibodies and by the RGD peptide, blocking the integrins (Fig. 5B). Treatment of cells with anti-α2 integrin antibody did not significantly reduce the percent of cells with phalloidin-positive protrusions, supporting the specific role of αvβ5 integrin. A dose-dependence analysis of cytoskeleton rearrangements revealed that GFDp is active at concentrations exceeding 10 pM, whereas the effect of CPp begins to increase at 0.1 pM and reaches a peak between 1 and 10 pM (Fig. 5C). Interestingly, a 1:1 molar ratio of CPp and GFDp resulted in an enhancement of the response with a peak at 1 pM (Fig. 5C). These data show that the GFD-uPAR interaction does promote cytoskeletal rearrangements and further support the possibility that the CP region cooperates to generate a full response.

Cooperation between CP and GF domains in the stimulation of chemotaxis and uPAR-integrin association

Unless uPAR is present on cell surface, the incubation of HEK-293 cells with uPA or uPA-related molecules does not result in

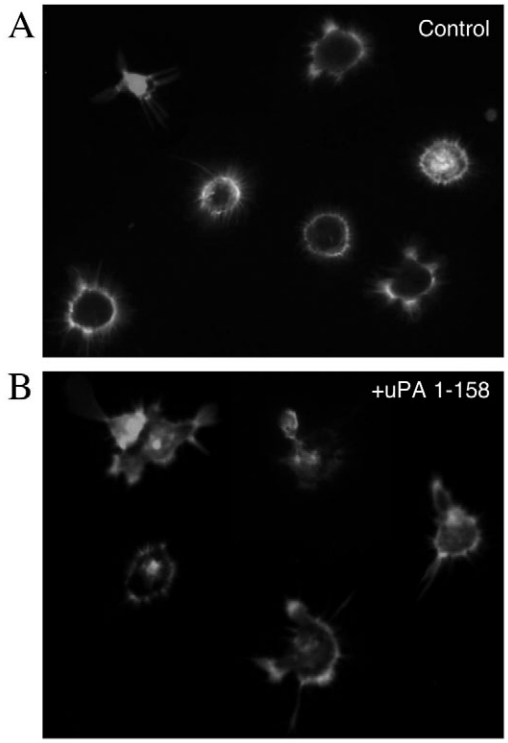

Fig. 4. Cytoskeletal rearrangements in migrating HEK-293/uPAR-25 cells. (A,B) HEK-293/uPAR-25 cells were seeded onto a glass slide and allowed to migrate towards 1 nM uPA 1-158 or diluents (Control) for 4 hours in a Dunn chamber (Allen et al., 1998). At the end of the incubation, cells were stained with Rhodamine-phalloidin, observed under an inverted fluorescence microscope and analysed as described in Materials and Methods. Original magnification, ×400.

increased motility. When uPAR is expressed, both ΔGFa and CPp become chemotactic. Since uPA is a multi-domain complex molecule with individual domains displaying a degree of functional independence, it was important to assess the integrated functional impact of the individual domains on migration, by testing equimolar combinations of GFDp and CPp. Thus, following exposure to different concentrations of GFDp and/or CPp, the HEK-293/uPAR-25 cell migration was analysed and quantified (Fig. 6A). Individually, both GFDp and CPp were chemotactic for HEK-293/uPAR-25 in a dose-dependent manner, their optimum being at ~100-1000 pM and 1-10 pM, respectively (Fig. 6A). The equimolar combination of GFDp with CPp (between 0.1 pM and 10,000 pM) produced a stronger motogenic effect than the individual proteins. Importantly, a time course of chemotaxis towards the same concentration (10 pM) of CPp or GFDp suggested that CPp was a faster acting motogen for HEK-293/uPAR-25 (Fig. 6B). The combination of the two peptides was more effective than the individual peptides, and the complete N-terminal uPA region (residues 1-158) was the most effective. It is interesting to note that the chemotactic response to CPp is not restricted to HEK-293/uPAR-25 cells: freshly isolated monocytes from human blood and U937 monocyte-like cells, exhibit an increased motility towards 10 pM CPp (approximately 190%±10 and 172%±8, respectively). Vice-versa, HEK-293 cells do not respond to CPp (data not shown).

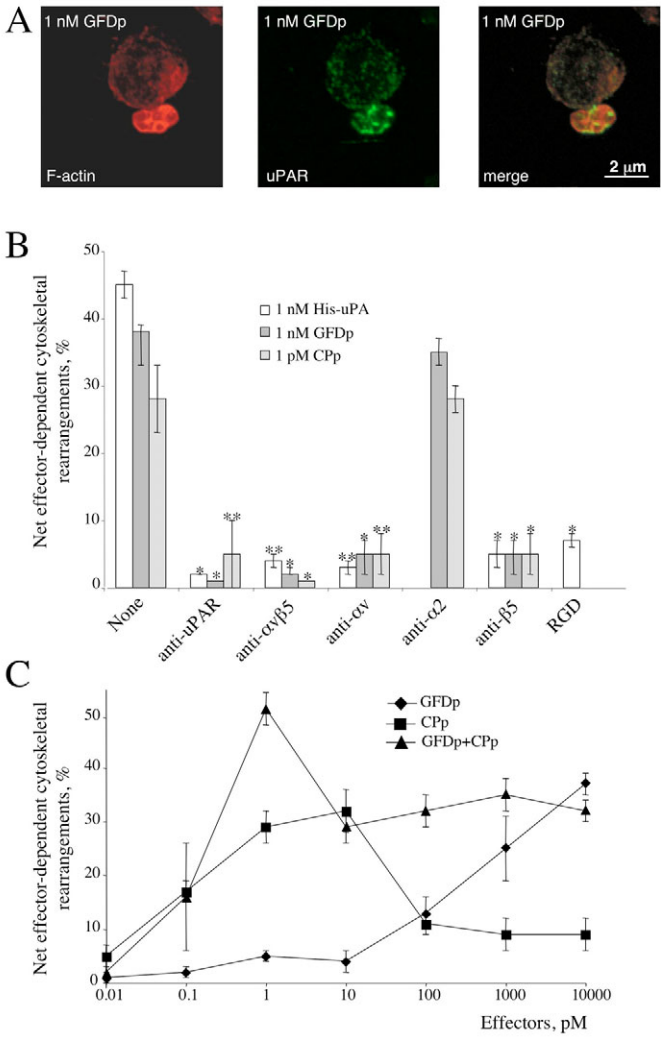

Fig. 5. Cytoskeletal rearrangements in HEK-293/uPAR-25 cells exposed to GFDp and/or CPp. Cells were collected by a mild tryspinization, incubated in suspension with diluents or His-uPA or GFDp or CPp at the indicated concentrations for 1 hour at 23°C. In the combinations, a molar ratio of 1:1 CPp:GFDp was employed. When specified, cells were incubated with diluents (none), or preloaded with 50 μg/ml RGD peptide, 5 μg/ml anti-uPAR 399 polyclonal or with the specific anti-integrin antibodies at a dilution of 1:30 for 1 hour. F-actin was detected with Rhodamine-phalloidin; uPAR was detected with anti-uPAR 399 polyclonal antibody followed by a secondary FITC-conjugated anti-rabbit antibody, as specified in Materials and Methods. (A) A representative confocal image of GFDp-treated HEK-293/uPAR-25 cells double-stained with Rhodamine-phalloidin and anti-uPAR antibodies. Original magnification, ×630. (B,C) Values reported on the *y*-axis correspond to the net percentage of cells exhibiting F-actin-enriched protrusions upon exposure to the indicated effectors. Data represent the mean of three independent experiments ± s.d. (error bars), performed in triplicate and evaluated by two independent observers. **P*<0.005; ***P*<0.0001.

To explore the mechanism through which the newly identified uPA chemotactic CP region induces migration, we examined the association of CPp with αvβ5 integrin and its effect on the interaction of integrin with uPAR. We found that the motogenic effect of CPp preincubated with purified αvβ5

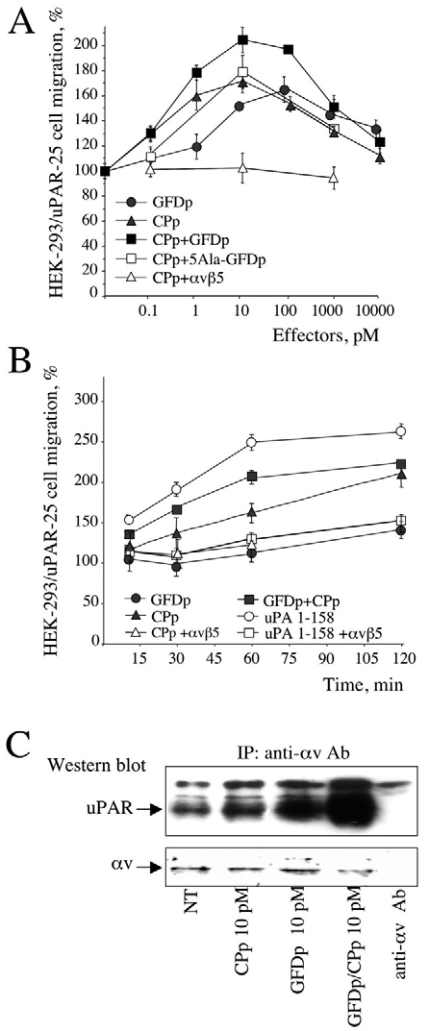

Fig. 6. Combined effects of individual uPA domains leading to enhancement of cell migration and association of uPAR with αvβ5 integrin. (A,B) HEK-293/uPAR-25 cells were allowed to migrate towards (A) increasing concentrations of the indicated effectors for 3 hours or (B) 0.1 nM of the effectors for 15, 30, 60 and 120 minutes (B). In the combinations, a molar ratio of 1:1 was employed. When indicated, CPp or uPA 1-158 were preincubated with 100 ng of purified αvβ5 integrin in 0.2 ml of 0.1% BSA in DMEM for 1 hour at 37°C. In all cases, cells were assayed for directional migration in Boyden chambers as specified in the legend to Fig. 1. The results are expressed as mean ± s.d. of three independent experiments performed in triplicate. (C) Lysates (400 μg/sample) of HEK-293/uPAR-25 cells exposed for 60 minutes to the indicated peptides or diluents (NT), were immunoprecipitated with 5 μg/ml VNR147 anti-αv integrin monoclonal antibody. 1 μg of anti-αv integrin antibody was loaded as a control. The resulting proteins were analysed by western blotting using R4 anti-uPAR or polyclonal anti-αv integrin antibodies.

integrin was strongly reduced, indicating that CPp binds to the integrin in solution (Fig. 6A,B). In control experiments, preincubation of CPp with the non-uPAR-binding peptide 5Ala-GFDp was ineffective. Interestingly, preincubation of uPA 1-158 with purified integrin brought chemotaxis down to the level induced by GFDp, suggesting that αvβ5 integrin binds and blocks one of the two chemotactically active regions of uPA 1-158. Taken together, our results suggest that two distinct sequences of uPA cooperate to induce cell migration: the GFD (binding directly to uPAR) and the CP (binding to αvβ5 integrin), which also requires uPAR for its motogenic activity. We reasoned that uPA affects signaling in a CP-dependent manner, by increasing the physical association of αvβ5 integrin with uPAR. To test this possibility, HEK-293/uPAR-25 cells were incubated with CPp, lysed and proteins immunoprecipitated with anti-αv integrin antibodies. In control samples, cells were exposed to GFDp or to an equimolar combination of GFDp and CPp. As shown in Fig. 6C, the amount of uPAR that co-immunoprecipitated with αv integrin increased in cells incubated with CPp and was greater following cell exposure to the CPp-GFDp mixture. This indicates that CPp, although unable to bind to the uPAR, stimulates the physical association of uPAR with αvβ5 integrin.

Table 2. Effect of cell signaling inhibitors on HEK-293/uPAR-25 directional migration

Inhibitor	100 pM GFDp	10 pM CPp
None	165±4***	173±16**
Anti-uPAR (10 μg/ml)	67±6***	132±8***
Anti-αvβ5 (1:100)	140±25*	61±4***
Worthmannin (1 μM)	125±3**	91±14***
LY 294002 (10 μM)	129±7**	75±5***
PD 98059 (25 μM)	82±6***	79±4***
SB 203580 (20 μM)	84±4***	73±4***
Y 27632 (10 μM)	93±6***	79±19**
PP2 (10 μM)	91±6***	64±18***

Subconfluent HEK-293/uPAR-25 cells were detached by mild trypsinization and pre-incubated with the indicated antibodies, signaling inhibitors or diluents (none) for 30 minutes at the specified concentrations in DMEM with 0.1 mg/ml BSA, and then allowed to migrate towards GFDp or CPp in Boyden chambers. Random migration was taken as 100%. The results are presented as the mean ± s.d. of two experiments performed in triplicate. *P<0.002; 0.0002>**P>0.00001; 0.00001>***P>0.0000001.

All our experimental results indicate that induction of migration by uPA involves uPAR and αvβ5 integrin, and that binding of GFDp and CPp to the integrin potentiates the uPAR-integrin interaction and enhances migration. Therefore, we reasoned that one of the partners (uPAR or integrin) affects the response to both uPA-derived peptides. To test this possibility, HEK-293/uPAR-25 cells were pretreated with anti-uPAR or anti-αvβ5 integrin antibodies (to block uPAR or integrin) and allowed to migrate towards either GFDp or CPp. As shown in Table 2, each of the antibodies prevented CPp- and GFDp-dependent directional migration, suggesting the involvement of αvβ5 integrin and uPAR in both cases. In particular, polyclonal anti-uPAR antibody was the most effective inhibitor of GFDp, whereas the monoclonal anti-αvβ5 integrin antibody reduced CPp-dependent migration more effectively. This result might reflect the ability of anti-uPAR and anti-αvβ5 integrin antibody to directly inhibit binding of GFDp (not shown) and CPp (Fig. 3A), respectively. Finally, cells were pre-exposed to a number of signaling inhibitors. With the exception of the PI 3-kinase inhibitors worthmannin and LY294002, which seem to prevent CPp-dependent signaling more effectively than GFDp-dependent signaling, all compounds were able to reduce peptide-dependent signaling to a similar extent. The latter observation suggests that CPp-dependent signaling shares, at least some, downstream mediators with the GFDp-uPAR-dependent pathway.

These findings indicate that uPAR can be either engaged directly through the GFD and/or, indirectly, through the specific interaction between the CP region in uPA and the αvβ5 integrin receptor.

Discussion

The results presented here reveal a new and unsuspected function for the CP domain of uPA (residues 132-158) in the regulation of uPA-uPAR-dependent cell migration that involves αvβ5 integrin. Our working model proposes that the multi-domain serine protease urokinase plasminogen activator, in addition to its well established interaction with uPAR through GFD (residues 1-49), interacts simultaneously with αvβ5 integrin through the CP region, thus, eliciting a full chemotactic response.

We have based our model on the findings that both CPp, a peptide corresponding to most of the human CP region (Fig. 1A), and a truncated uPA without the uPAR-binding domain (ΔGFa) bound to αvβ5 integrin. In the presence of uPAR, this interaction leads to an increased integrin-uPAR association, F-actin-enriched protrusions and, ultimately, cell migration. Although ΔGFa and CPp bind to the surface of HEK-293 cells, these effects are not detected in cells that lack uPAR, showing that uPAR is required for CPp-dependent signaling (data not shown). Importantly, we have shown that CPp does not bind to uPAR at less than 50 μM (Fig. 2C), suggesting that the functional effects of CPp – which are occurring at picomolar concentrations – cannot depend on a direct interaction of CPp with uPAR.

In the presence of uPAR, simultaneous exposure to CPp and GFDp results in enhanced cytoskeletal rearrangements and cell migration, suggesting a functional cooperation between the two regions in the intact protein. The latter possibility is further supported by the partial inhibition of chemotaxis towards uPA 1-158 preincubated with αvβ5 integrin (Fig. 6B). The relevance of the GFD-uPAR interaction is highlighted by a report showing that, in Chinese hamster ovary (CHO) cells, uPAR adhesion to uPA is abolished by the deletion of uPA residues 1-46, indicating that direct binding to uPAR is required (Tarui et al., 2003; Tarui et al., 2006). However, consistent with our data, other reports show an interaction of uPA with the cell surface that is independent of uPAR and GFD. For example, a weak interaction between a recombinant uPA kringle domain (residues 43-156) and an unidentified cell-surface target, elicited cell migration in the absence of uPAR (Mukhina et al., 2000). Remarkably, uPA has been described to directly interact with αMβ2 integrin through the kringle-domain (residues 47-135) and the proteolytic domain (residues 136-411) on the surface of leukocytes (Pluskota et al., 2003). Our results clearly identify αvβ5 integrin as the integrin involved in HEK-293-cell migration, displaying a clear-cut GFD-independent ability to associate with the CP of uPA (residues 132-158). Consistently, the physiological relevance of the CP region is supported by the finding that a peptide corresponding to residues 136-143 of uPA has anti-invasive and anti-angiogenic properties (Guo et al., 2002) and is thought to act by competing with a secondary, weaker interaction between uPA and uPAR. However, in our system the binding of CPp (and ΔGFa) to the cell surface was completely uPAR-independent but αvβ5-integrin-dependent. Also, binding of CPp and ΔGFa to the cell surface was blocked by preincubation of these proteins with purified αvβ5 integrin and by anti-αvβ5 integrin antibodies, indicating that this was their primary binding site. Moreover, the existence of a GFD-independent interaction between uPA and αv integrin is further supported by the finding that HEK-293-cell clones that had been stably transfected to overexpress the αv integrin subunit, exhibit a proportionally increased specific association of ^{125}I-ΔGFa and ^{125}I-CPp to their surface. An interesting possibility is that, activation of pro-uPA, through enzymatic cleavage of the Lys158-Ile159 bond, might change the local protein conformation and, therefore, the binding properties of the CP region. This, as well as the integrin-binding characteristics of pro-uPA phosphorylated on Ser138, will be the object of further investigation.

The crystal structure of a soluble form of uPAR has been recently solved, revealing a central cavity where the GFD binds and a large external surface accessible to interactions with other partners, such as integrins (Llinas et al., 2005). These findings do not exclude the possibility that uPA simultaneously associates with uPAR and with a different membrane partner through a region not involved in uPAR-binding, such as the CP. In support of this idea, formation of a ternary complex for the signaling mechanism of GPI-linked receptors has been suggested in the glial-derived neurotropic factor (GDNF) receptor that associates with the transmembrane tyrosine kinase receptor c-RET, favoring the interaction of the complex with GDNF (Cik et al., 2000). Our previous work with another integrin, α5β1, has shown that its interaction with uPAR induced signal transduction that was further enhanced by the presence of uPA (Aguirre Ghiso et al., 2001).

Some authors (Wei et al., 1996; Carriero et al., 1999) have proposed that uPAR/integrin complexes pre-exist in cells not previously exposed to uPA and/or can be formed in vitro in the absence of uPA. That appears to contradict the proposed model. However, our co-immunoprecipitation experiments (Fig. 6C) with anti-αvβ5 integrin antibodies to pull down uPAR, indicate that a modest amount of the complex exists in absence of exogenously added uPA. Whether this is due to small amount of uPA being produced or due to uPA-independent complex formation has not been established. However, treatment of cells with either CPp or GFDp substantially increased the amount of uPAR associated with the integrin, and the mixture of the two at picomolar concentrations produced a further tenfold increase in αvβ5-integrin-associated uPAR. The data clearly indicate that the association of CPp with αvβ5 integrin is insufficient to mobilize cells for migration unless uPAR is present. On the contrary, in the absence of uPAR, His-uPA and ΔGFa downregulate basal migration of HEK-293 cells (Fig. 1D). Whether this effect is due to the interaction of CP with αvβ5 integrin remains to be investigated.

This last finding further supports the role of uPAR as a positive regulator of cell migration and agrees well with our previous observations that the physical and functional association of uPAR with αvβ5 integrin alters the signaling specificity of αvβ5 (Carriero et al., 1999). It is possible that, once the ternary complex (uPAR-uPA-integrin) is formed, new mediators are then recruited: this possibility deserves further investigation by analyzing the partners of CPp–αvβ5-integrin complex in the presence and in the absence of uPAR. Remarkably, in the presence of uPAR, the chemotactic activity of CPp and GFDp is prevented by the same set of signaling

inhibitors, indicating that they share, at least several, downstream effectors (Table 2). This evidence further supports the finding that CPp acts through uPAR to stimulate cell migration. It will be interesting to assess whether the impact of the CP region on cell physiology is limited to motility or extends to the proliferative and anti-apoptotic effects of uPA (Alfano et al., 2005; Alfano et al., 2006). In conclusion, the data presented in here indicate that the intact uPA protein simultaneously binds to αvβ5 integrin and to uPAR, perhaps forming a bridge between the two receptors that initiates and potentiates uPAR-dependent signaling and migration.

Materials and Methods

Reagents

Anti-uPAR R2 and R4 antibodies were a gift of G. Hoyer-Hansen, Finsen Institute, Copenhagen, Denmark. Anti-uPA polyclonal antibody was provided by P. A. Andreasen, Aarhus, Denmark. 5B4 Agarose was a gift of M. L. Nolli (Areta Intl., Gerenzano, Italy). β1 integrin polyclonal kit, purified αvβ5 and α1β1 integrin, VNR147 anti-αv integrin and P1F6 anti-αvβ5 integrin monoclonal antibodies were from Chemicon Int. Inc. (Temecula, CA). N-19 goat anti-αv integrin polyclonal antibody was from Santa Cruz (Santa Cruz, CA). 399 rabbit anti-uPAR polyclonal antibody was from American Diagnostica (Greenwich, CT). Rhodamine-conjugated phalloidin and FITC-conjugated antibodies were from Sigma (Milan, Italy). $Na^{125}I$ (17.4 mCi/μg) and the enhanced chemiluminescence detection system (ECL) were from Amersham (Milan, Italy). All cell culture reagents were purchased from Gibco (Gaithersburg, MD). Complete™ protease inhibitor cocktail was from Roche (Penzberg, Germany). The pPIC9 vector and Pichia strain GS115 were obtained from Invitrogen Corp. (San Diego, CA). Collagen type IV, laminin, fibronectin, vitronectin, the MAPK inhibitors PD 98059 and SB 203580, the PI 3-kinase inhibitors worthmannin and LY 294002, the ROCK inhibitor Y 27632 and the PKC inhibitor PP2 were from Sigma.

The C-terminal histidine-tagged uPA human variants shown in Fig. 1A (His-uPA and ΔGFa) and the untagged uPA 1-158 (corresponding to the first 158 amino acids of human uPA) have been expressed as secreted products in the methylotrophic yeast *P. pastoris*. Tagged proteins were purified by Ni^{2+}-NTA chromatography, as previously described (Franco et al., 1997). 95% of the purified His-uPA and ΔGFa are in the single-chain pro-urokinase form. Untagged uPA 1-158 was purified by 5B4-agarose chromatography, as described (Stoppelli et al., 1985).

Plasmids

pPIC9-His-uPA encoding His-uPA was obtained by ligation of a double-strand oligonucleotide (5′-AATTCAGCAATGAACTTCATCAAGTTCCAT-3′ and 5′-CGATGGAACTTGATGAAGTTCATTGCTG-3′) to the 370 bp *Taq*I-*Fsp*I fragment excised from pcDNAneo-His-uPA plasmid and the *Nco*I-*Xba*I fragment from pcDNAneo-His-uPA (Franco et al., 1997). To obtain pPIC9 coding for histidine-tagged ΔGFa, the region encoding amino acid residues 66-411 of uPA, included in the 3099 bp *Stu*I-*Nco*I fragment, was excised from pPIC9-His-uPA, ligated into the *Stu*I-*Not*I sites of pPIC9 multicloning site, together with a double-strand oligonucleotide coding for the first eight residues of uPA and for amino acid residues 46-65 (5′-GGCCGCGAGCAATGAACTTCATCAAGTTCCAAAGTCAAAAAC-CTGCTATGAGGGGAATGGTCACTTTTACCGAGGAAAGGCCAGCACTGAC-AC-3′ and 5′-CATGGTGTCAGTGCTGGCCTTTCCTCGGTAAAAGTGACCAT-TCCCCTCATAGCAGGTTTTTGACTTTGGAACTTGATGAAGTTCATTGCTC-GC-3′), thus introducing the 9-45 deletion. The uPAR expression vector pcDNA3-uPAR was constructed by inserting the 1027 bp *Eco*RI-*Eco*RI fragment from the pBluescript II SK vector, containing the whole uPAR-cDNA coding sequence in the pcDNA3 vector. The αv-pcDNA3 vector was kindly provided by D. Cheresh, UCSD, La Jolla, CA.

Peptide synthesis and purification

The peptides employed in this analysis correspond to the human uPA sequence, amino acid residues 12-32 (GFDp, DCLNGGTAVSNKYFSNIHWCN), its non-binding version carrying the substitution of the crucial residues with five Ala residues (5Ala-GFDp, DCLNGGTAVSAAAAANIHWCN), or to the uPA sequence, amino acid residues 135 to 158 (CPp, KKPSSPPEELKFQCGQKTLRPRFK). Briefly, peptides were synthesized using the solid phase approach with standard Fmoc methodology in a manual reaction vessel (Stewart, 1997). Purification was achieved with semi-preparative RP-HPLC C 18 bonded silica column (Vydac 218TP1010). The purified peptide was 99% pure as determined by analytical RP-HPLC. The correct molecular weight of the peptide was confirmed by mass spectrometry and amino acid analysis.

Cell culture and generation of stable transfectants

Human embryonic kidney (HEK)-293 cells, LB6 mouse cells expressing soluble uPAR (suPAR) (Masucci et al., 1991) and the stably transfected cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). U937 histiocytic lymphoma cells were cultured in RPMI 1640 medium, containing 10% heat-inactivated FBS. All cell lines were grown in the presence of 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C, under 5% CO_2 atmosphere.

Stable HEK-293 transfectants were obtained by electroporating 10^7 subconfluent HEK-293 cells with 80 μg of plasmid DNA, in 0.9 ml of culture medium. The expression of uPAR by HEK-293/uPAR clones was quantitated by western blotting with anti-uPAR R2 antibody in total cell lysates and by radioreceptor-binding assay with ^{125}I-His-uPA (Stoppelli et al., 1985). Expression of the αv integrin subunit by HEK-293/αv clones was quantitated by western blotting with anti-αv integrin antibody in total cell lysates. Conditioned medium from LB6 cells expressing suPAR was obtained as described previously (Carriero et al., 1994).

^{125}I-labeling and radioreceptor-binding assay

200 ng of ΔGFa or His-uPA were labeled with 1 mCi of $Na^{125}I$ (Amersham) using IODO-BEADS from Pierce (Rockford, IL) in 0.1 M sodium phosphate, 0.15 M NaCl pH 7.2 for 10 minutes at 25°C in a final volume of 100 μl. Specific activity was 12 μCi/μg for ΔGFa and 1 μCi/μg for His-uPA. In both cases the preparations retained 70% enzymatic activity as determined by an indirect chromogenic assay according to Gardell et al. (Gardell et al., 1989). The connecting peptide (CPp) was iodinated with 1 mCi of a mixture of monoiodinated and di-iodinated Bolton-Hunter reagent (MP Biomedicals) according to Bolton and Hunter (Bolton and Hunter, 1973). Briefly, 10 μg of CPp was incubated with the ^{125}I–Bolton-Hunter reagent in 0.1 M sodium-borate buffer pH 8.5 for 1 hour at 0°C and then eluted through a D-salt polyacrylamide 1800 desalting column (Pierce) with a 0.05 M phosphate buffer containing 0.1% gelatin. Specific activity was 0.7 μCi/μg.

For binding studies, 2×10^6 HEK-293 or U937 cells were harvested and incubated for 3 hours at 4°C with the indicated amounts of the ^{125}I-labeled proteins or the ^{125}I-labeled peptide in DMEM (HEK-293) or RPMI (U937) containing 1 mg/ml BSA and 10 mM Hepes pH 7.4 (binding buffer). At the end of incubation, cells were washed three times with binding buffer and the surface-associated proteins were recovered by treating cells with an acidic wash (50 mM glycine-HCl buffer pH 3.0, containing 0.1 M NaCl) for 2 minutes at room temperature and quantitated by measuring γ-radiation (Stoppelli et al., 1986). Each experiment was carried out in duplicate and the results were plotted as mean cpm ± standard deviation (s.d.).

Chemotaxis assay

Chemotaxis assays were performed in Boyden chambers with 8-μm-pore-size PVPF-free filters (insert growth area 0.33 cm^2, coated with collagen type IV) according to Carriero et al. (Carriero et al., 1999) with minor modifications. Briefly, 10^5 cells were detached by mild trypsinization, incubated in DMEM with 10% FBS for 1 hour, treated with acidic wash and inoculated into the upper compartment. Chemoattractants were diluted in DMEM with 0.1% BSA and added to the lower compartment. After 3 hours of incubation at 37°C, the cells on the upper side of the membrane were removed by scraping and cells on the lower side of the filters were counted under an inverted microscope. Cell migration in the absence of chemoattractant or random migration was referred to as 100%.

Dunn-chamber assay

HEK-293/uPAR cells were seeded on 20×20 mm coverslips for 24 hours and then treated with an acidic wash. Before inverting the cover slip on top of a double-concentric chamber, cells on the cover slip covering the outer chamber were carefully scraped away (Allen et al., 1998). A gradient of a chemoattractant was created by placing serum-free medium in the inner chamber and 1 nM uPA 1-158 in the outer chamber. The ring separating the inner and outer chambers permits slow diffusion between the chambers. For control experiments both wells were filled with serum-free medium. After 4 hours, the coverslip was removed from the chamber and the cytoskeleton was visualized by staining with Rhodamine-conjugated phalloidin. A total of 100 cells/sample that translocated to the area corresponding to the outer well was examined with a fluorescence-inverted microscope and images were taken with a videocamera. Quantitative analysis of the images was performed by counting the number of cells exhibiting oriented, F-actin-containing filamentous structures and were reported as percentage of total migrating cell number. Data represent the results of two experiments.

Binding of ΔGFa to purified αvβ5

100 μg of anti-uPA antibodies were incubated with 200 μl of protein A-Sepharose in 0.2 M sodium borate buffer pH 9.0 for 1 hour at room temperature. After extensive washing, bound proteins were crosslinked with 20 mM dimethylpimelimidate for 30 minutes at room temperature and the reaction was finally stopped with 0.2 M ethanolamine pH 8.0. Then, 5 μg of ΔGFa were incubated with 15 μl of protein-A–anti-uPA antibody Sepharose in 60 μl of 50 mM potassium phosphate buffer, 0.5 M NaCl, 0.1% Triton X-100 for 2 hours at 4°C. The resin was further incubated with 500 ng of αvβ5 integrin or α1β1 integrin in the presence of 1 mM $MgCl_2$ for 2 hours at 4°C and washed twice. Bound proteins

were eluted with 0.1 M glycine-HCl, 0.1% Triton X-100, 0.5 M NaCl pH 2.8, diluted with loading buffer and analysed on a 7.5% SDS-PAGE under non-reducing conditions, followed by western blotting with 1 μg/ml of goat polyclonal anti-αv integrin antibody.

Analysis of cytoskeleton and uPAR distribution

Subconfluent cells were harvested by a mild trypsinization and incubated with DMEM with 10% FBS for 1 hour at 37°C and acid-treated to strip any membrane-bound growth factors (Carriero et al., 1997). The cells were then incubated in suspension in DMEM with the indicated effectors for 1 hour at 23°C and/or preloaded with diluents (without antibody) or 5 μg/ml of anti-uPAR 399 polyclonal or 50 μg/ml RGD peptide or the specific anti-integrin antibodies at 1:30 dilution for 1 hour at 23°C. Then, cells were washed, centrifuged and kept in suspension throughout the procedure. To analyse uPAR distribution, cell pellets were incubated with anti-uPAR 399 antibody followed by a secondary FITC-conjugated anti-rabbit IgG antibody. To study F-actin distribution, cells were fixed with 2.5% formaldehyde, permeabilized with 0.1% Triton X-100 for 10 minutes at 4°C and incubated with 0.1 μg/ml Rhodamine-phalloidin for 40 minutes as previously described (Carriero et al., 1999; Gargiulo et al., 2005). In all cases, after extensive washing with PBS, cells were placed on a clean glass slide and examined either with an inverted or a confocal microscope (Leica Microsystems, Milan, Italy). To generate quantitative data, a total of 200 cells/sample was examined and the percentage of cells exhibiting a rearranged cytoskeleton was assessed. The percentage of cells exhibiting F-actin rearrangements in the absence of treatment was subtracted to obtain the net effector-dependent values, as described (Gargiulo et al., 2005). Data represent the mean of three independent experiments performed in triplicate and evaluated by two independent observers with error bars indicate the s.d.

Co-immunoprecipitation assay

Detached HEK-293/uPAR-25 cells were treated with acidic wash, exposed for 60 minutes to different effectors and then lysed in RIPA buffer (140 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Triton X-100, 1 mM Na_2VO_4) and protease inhibitor mixture. Four hundred μg/sample was immunoprecipitated overnight at 4°C with 5 μg/ml VNR147 anti-αv integrin monoclonal antibody. The G-Sepharose-absorbed proteins were separated by 10% SDS-PAGE under non-reducing conditions, followed by western blotting with 2 μg/ml of R4 anti-uPAR monoclonal or with anti-αv integrin polyclonal antibodies for 2 hours at 4°C, according to Carriero et al. (Carriero et al., 1999).

Statistics

The results were analysed using the Student's *t*-test. A value of $P<0.05$ was considered to be significant. Data are presented as the mean ± s.d. and the number of experiments performed is indicated in the figure legends.

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