

Interaction between Kringle and Growth-Factor-Like Domains in the Urokinase Molecule: Possible Role in Stimulation of Chemotaxis

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Abstract—The results presented in this paper suggest the presence of an interaction between the kringle- and the growth-factor-like urokinase domains. This interaction regulates chemotactic properties of urokinase. We also show that interaction of urokinase with its “classical” receptor (uPAR) has a “permissive” effect on the interactions between the kringle domain and other targets on the cell surface. On the basis of our data we can suggest that uPAR serves as an “adaptor” for urokinase, and the binding of urokinase kringle domain to its receptor causes immediate activation of intracellular signaling and induction of cell migration.

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Urokinase-type plasminogen activator (urokinase, uPA) is a multifunctional protein directly involved in inflammation, tissue remodeling, angiogenesis, tumor growth, and metastasis [1, 2]. This multifunctionality is due to the existence of three domains in its structure: growth-factor-like domain (GFD), kringle domain (KD), and proteolytic domain (PD) (Fig. 1). The GFD, structurally homologous to epidermal growth factor, provides binding of the uPA molecule to urokinase receptor

(uPAR). The PD performs a proteolytic cleavage of a peptide bond in the plasminogen molecule and converts it to plasmin. The kringle domain is involved in regulation of cell migration by uPA [3]. It was also shown that the KD stabilizes the urokinase–uPAR complex [4].

Urokinase receptor consists of three domains having a high degree of homology (Fig. 2). First domain provides binding with urokinase [5], while two others increase affinity of the receptor to a ligand [6-8]. uPAR is anchored in the membrane through glycosyl phosphatidylinositol (GPI) [9].

A number of groups have shown that urokinase binding to uPAR on the plasma membrane of cell leads to activation of intracellular signaling pathways which in turn regulate migration [10-13], adhesion [14, 15], proliferation, and differentiation of cells [16, 17]. Since uPAR structure does not contain transmembrane and cytoplasmic domains, complex formation between uPAR and transmembrane proteins such as integrins [18], caveolin [19], vitronectin [20, 21], and L-selectin [22] is necessary for signal transduction from uPAR to intracellular signaling systems. It was also shown that urokinase can simultaneously bind two receptors on the cell surface—uPAR through the growth-factor-like (“growth”) domain and integrin $\alpha_5\beta_1$

Abbreviations: ATF) N-terminal fragment of urokinase; BSA) bovine serum albumin; CHO) Chinese hamster ovary; GFD) growth-factor-like domain; GPI) glycosyl phosphatidylinositol; HEK 293) transformed human embryonic kidney cell line; HGF) hepatocyte growth factor; KBP) kringle-binding protein; KD) kringle domain; LRP) low density lipoprotein receptor related protein; PBS) phosphate-buffered saline; PD) proteolytic domain; PVDF) polyvinylidene fluoride; RAP) receptor-associated protein; SMC) smooth muscle cells; uPA (urokinase)) urokinase-type plasminogen activator; uPA–GFD) urokinase without growth-factor-like domain; uPAmut) modified urokinase; uPAwt) wild type urokinase; uPAR) urokinase receptor; U937) human leukemic monocytic lymphoma cell line.

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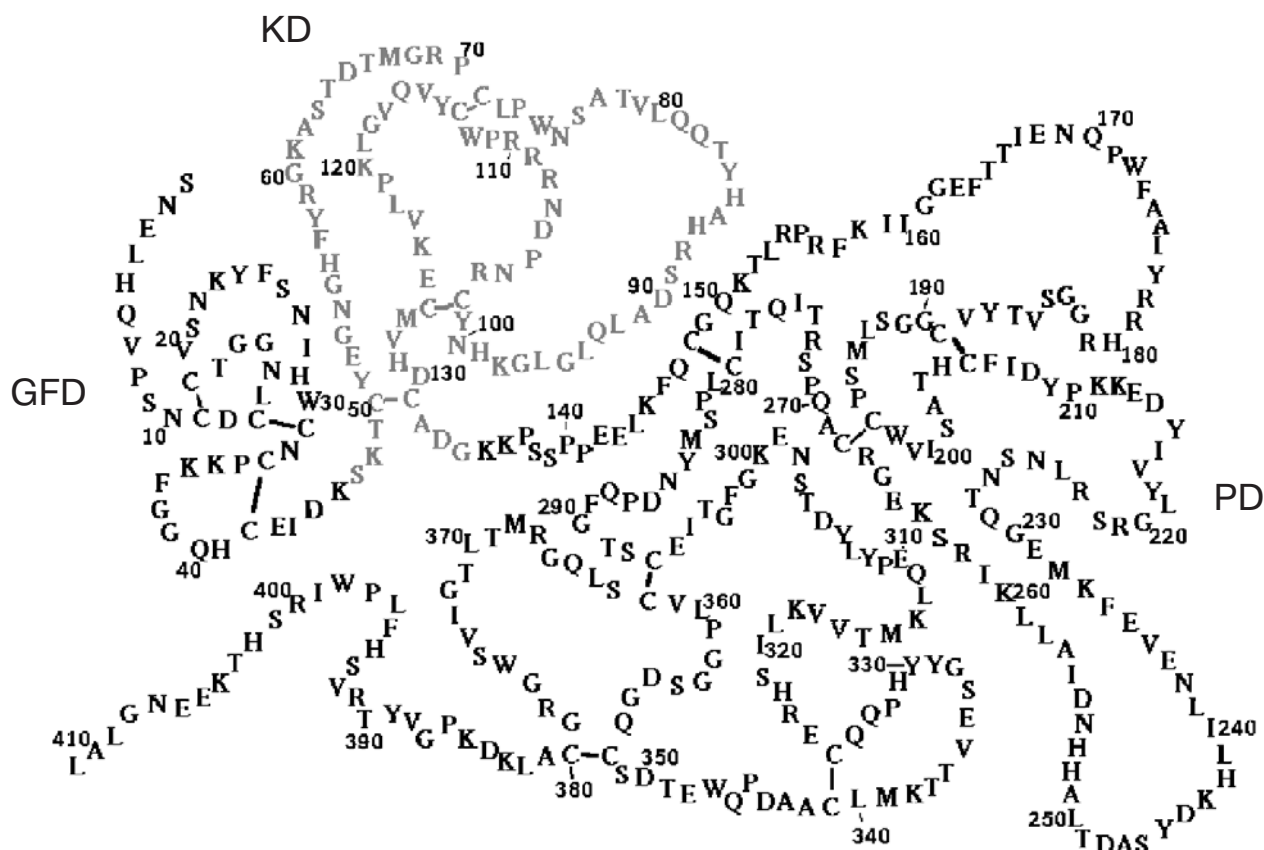


Fig. 1. uPA structure. uPA consists of growth-factor-like domain (GFD) (1-46 a.a.), kringle domain (KD) (47-135 a.a.), and proteolytic domain (PD) (136-411 a.a.).

through the kringle and proteolytic domains [23]. Although urokinase can bind integrins in the absence of uPAR, the maximal effect of uPA is observed during simultaneous expression of integrins and uPAR on the cell surface.

Data obtained by our group indicate that both the kringle domain of urokinase and urokinase without GFD, which does not bind classic uPAR, are able to activate p38 and p42/44 MAPKs and cause cell migration. We demonstrated that the kringle domain has bound protein different from uPAR and integrins on the surface of smooth muscle cells (SMC) [3]. These data indicate that in this case activation of migration by KD can take place even without uPAR. Moreover, we showed [3] that uPA without KD does not have chemotactic properties despite its ability to bind uPAR. Therefore, binding of “growth” domain to uPAR is not sufficient for the activation of migration. Nevertheless, it was necessary for both “growth” and kringle domains to bind their receptors—uPAR and kringle-binding protein (KBP), respectively—for the activation of migration by full length urokinase containing both domains. In our previous works, we also used modified urokinase (uPAmut) containing altered GFD that could not bind uPAR, while the kringle domain structure was preserved. According to our data, uPAmut

did not activate migration despite the presence of intact KD [24]. To explain this apparently paradoxical phenomenon we suggested that there were intermolecular interactions between kringle and “growth” domains causing “shielding” of KD effector region by “growth” domain. In other words, GFD might hinder KD interaction with its receptor. We also assumed that this effect of GFD disappears upon its interaction with uPAR accompanied by exposure of KD “active” site.

In the present work, we report possible interaction between kringle and “growth” domains of urokinase. We found that kringle domain both isolated or in uPA might compete with uPAR for binding to GFD of urokinase. The interaction of urokinase with uPAR leads to exposure of KD sites able to bind with yet unknown cell surface receptors activating migration. We suggest that simultaneous binding of urokinase to uPAR and kringle-binding target is a leading factor triggering cell migration by uPA.

MATERIALS AND METHODS

Materials. Analytical grade reagents were purchased from Sigma (USA), Serva (Germany), Fluka (Switzer-

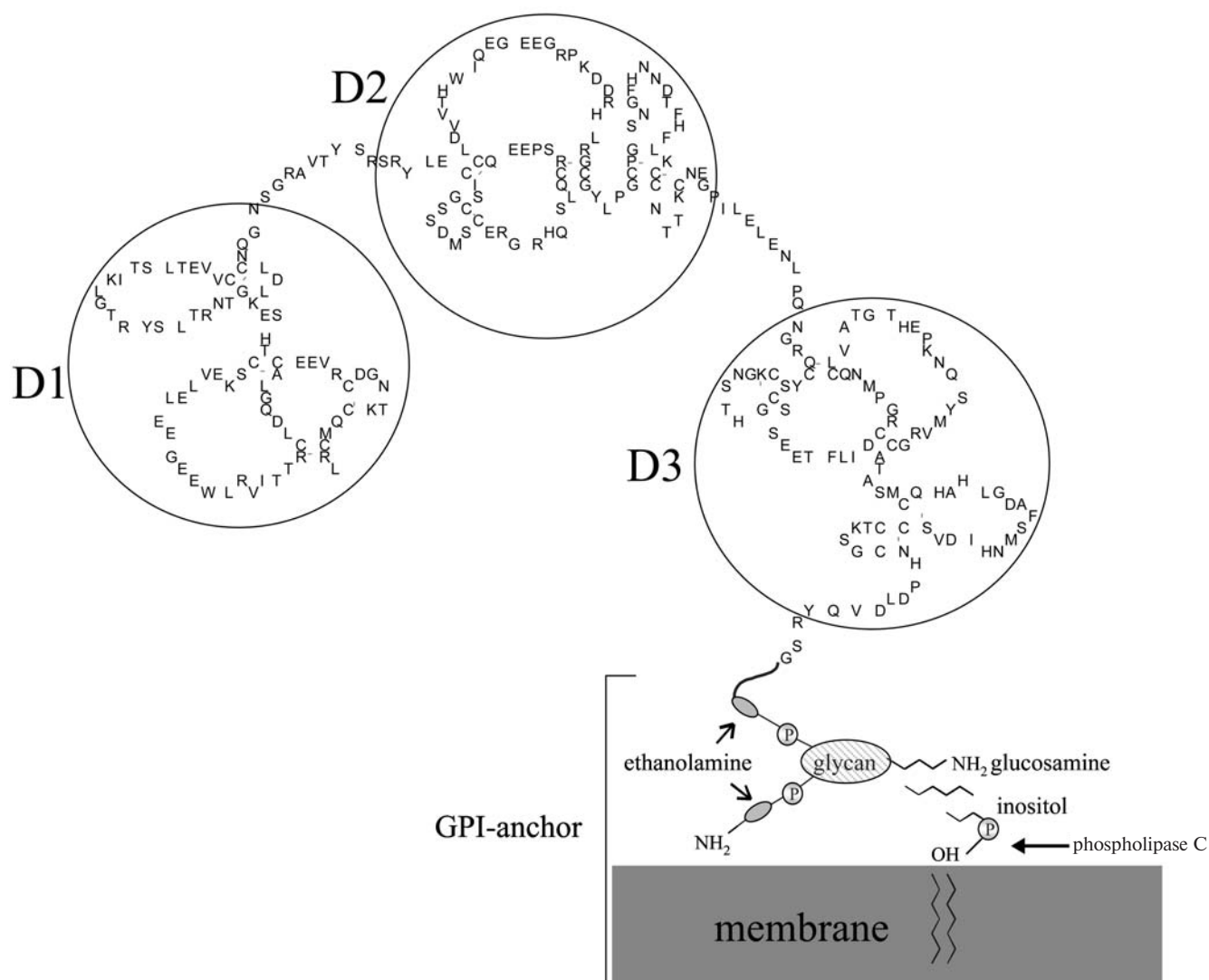


Fig. 2. uPAR structure. The receptor consists of three domains: D1 (1-77 a.a.), D2 (93-177 a.a.), and D3 (193-272 a.a.).

land), and Reakhim (Russia). Growth medium and reagents from Gibco (USA) and Invitrogen Corp. (USA) and fetal calf serum from HyClone (USA) were used.

Urokinases. The following recombinant urokinases expressed in *Escherichia coli* were used in the present work: 1) wild type urokinase (uPAwt); 2) urokinase without “growth” domain (uPA–GFD); 3) N-terminal fragment of urokinase (ATF) containing “growth” and kringle domains; 4) kringle domain of urokinase (KD), and 5) modified urokinase with replacement of 24 N-terminal amino acid residues for 13 random amino acid residues (uPAmut) (Fig. 3) [24]. ATF and KD contain eight histidine residues at the C-terminus allowing their purification by metal-chelating chromatography using Ni^{2+} -support.

Purification of recombinant urokinases. *Escherichia coli* cells were grown in LB broth medium (Sigma).

Proteins were isolated and purified according to a previously described protocol [3].

Protein biotinylation in solution. Protein solution (1–2 mg/ml) in PBS (phosphate-buffered saline) was added to freshly prepared aqueous solution of sulfo-NHS-LC-biotin (Pierce, USA) (2 mg/ml) at 1 : 10 protein/biotin molar ratio. The reaction mixture was incubated at 25°C for 30 min. Excess unreacted substances were removed by gel filtration of the biotinylated proteins on PD-10 columns (Amersham Biosciences, Great Britain). The quality of the resulting preparations was tested by SDS-PAGE followed by electrotransfer onto PVDF (polyvinylidene fluoride) membrane. Biotinylated proteins were stained with neutravidin conjugated to polyperoxidase (Pierce).

Cell cultures. U937 cells were grown in RPMI-1640 medium containing antibiotics and 10% fetal bovine

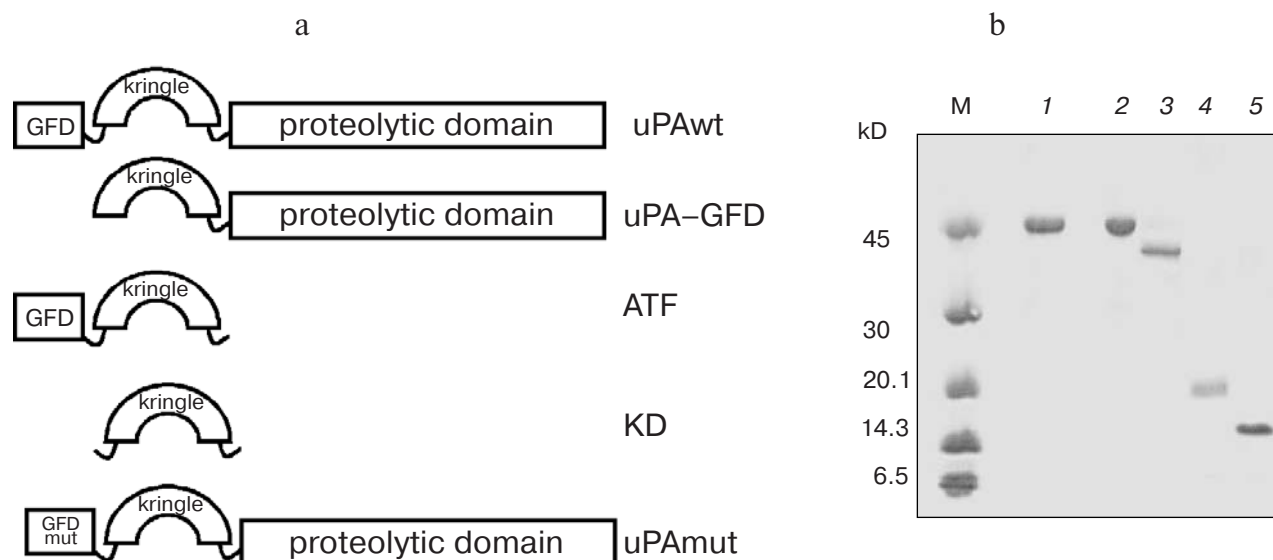


Fig. 3. Recombinant uPA proteins used in the present work: a) uPAwt, full length urokinase; uPA-GFD, urokinase without growth-factor-like domain; ATF, N-terminal fragment of urokinase without proteolytic domain; KD, kringle domain; uPAmut, urokinase with amino acid substitution in "growth" domain (1-24 a.a., SNELHQVPSNCDCLNGGTCVSNKY) to 13 "foreign" amino acid residues (ITPSL-HACRSTLD); b) 13% SDS-PAGE. Protein separation was performed in non-reducing conditions. Lanes: 1) uPAwt; 2) uPA-GFD; 3) uPAmut; 4) ATF; 5) KD. Five micrograms of protein were loaded on each lane; proteins were stained with Coomassie Blue.

serum. Human SMC, CHO-LRP⁻, and HEK 293 cells were grown in DMEM medium containing antibiotics and 10% fetal bovine serum.

Preparation of cell lysates. All steps were performed at 0°C. Cells were chilled on ice and lysed in buffer I (100 mM Tris-HCl, pH 8.1, 1% Triton X-114, 5 mM Chaps, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml pepstatin A, 100 U/ml aprotinin) (0.1-0.2 ml per 10⁶ cells) during 15 min. The resulting lysates were centrifuged at 30,000g for 30 min to remove insoluble components. Fresh cell lysates were used in the experiments.

Urokinase immobilization on cyanogen bromide-activated Sepharose. BrCN-activated Sepharose 4 Fast Flow (Amersham Biosciences) was used for immobilization of recombinant proteins.

Determination of concentration of protein immobilized on Sepharose. Immobilized protein concentration was determined using modified Bradford assay [25] in the sample of the following content: 0.95 ml reagent (Coomassie Brilliant Blue G-250) and 0.05 ml suspension of Sepharose with immobilized protein. To obtain a standard curve, 1-8 µg urokinase and 0.05 ml of control Sepharose were added into the sample (final volume 1 ml). Sepharose activated in the absence of the protein as described above was used as a control. Immobilized protein concentration was calculated for 1 ml of precipitated Sepharose. In the experiments, it was usually 0.4-0.8 mg/ml.

Affinity chromatography. For competitive binding of uPA to the receptor, we used BrCN-activated Sepharose

sorbents with immobilized different urokinases (see above). Freshly prepared cell lysates were incubated with affinity sorbents for 2 h at room temperature with stirring. Then the sorbents were washed with 20 volumes of solution A (PBS, 2 mM Chaps), 40 volumes of solution A containing 0.85 M NaCl, 20 volumes of solution A, and then with five volumes of 50 mM Tris-HCl, pH 6.8, containing 2 mM Chaps. Proteins bound to the sorbent were eluted with 1% SDS, 10 mM Tris-HCl, pH 7.0. Proteins bound to the affinity sorbents were analyzed by SDS-PAGE and ligand- and immunoblotting.

Immunoblotting. Proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane [26]. The membrane was incubated in blocking buffer I (PBS containing 5% dry fat-free milk and 0.05% Tween-20). Then the membrane was incubated with primary antibodies. Primary antibodies against human uPAR (Monozyme ApS, Denmark) (dilution 1 : 1000) or mouse monoclonal antibodies against human uPA (concentration 0.2 µg/ml) (UNG, Russia) were used. After washing, the membrane was incubated with goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (AffiniPure (H+L); Jackson ImmunoResearch, USA) (dilution 1 : 2000). Protein was detected with chemiluminescent substrate ECLTM (Western Blotting Detection Reagents; Amersham Biosciences). The resulting film was scanned with an Epson or Kodak digital camera (Kodak, USA).

Ligand blotting. Proteins were separated by SDS-PAGE and electrotransferred to PVDF membrane as for

the immunoblotting. After transfer, the membrane was incubated in blocking buffer II (PBS containing 0.5% gelatin and 0.05% Tween-20) for 1 h at 25°C. Then the membrane was incubated for 1 h with neutravidin conjugated to horseradish peroxidase (Pierce) (dilution 1 : 25,000) diluted in blocking buffer II. Protein was detected with chemiluminescent substrate as described above.

Binding of iodinated human recombinant urokinases to cells. Cells grown in 48-well plates until confluence were washed twice with PBS, pH 7.4, containing 0.1% BSA (bovine serum albumin). After preincubation with 0.5 ml of binding medium (DMEM containing 0.1% BSA and 100 KIU/ml aprotinin) for 1 h at room temperature and subsequent aspiration of the medium, 100 µl of iodinated urokinase at 3 nM concentration and 100 µl buffer or non-labeled competitor in different concentrations were added to the cells. The cells were incubated with iodinated urokinases for 2 h at 4°C. Then the medium was aspirated and the cells were washed 3 times with PBS containing 0.1% BSA and lysed by addition of 500 µl of 1% SDS in 0.5 M NaOH. Radioactivity in cell lysates was determined using a CompuGamma γ -counter (LKB Wallac, Finland).

Cell migration. Cell migration was assayed according to a previously described method [24] using a Boyden chamber (Neuroprobe Inc., USA). Confluent cells were cultured in serum-free medium, DMEM/0.1% BSA, for 24 h before experiments. Different human urokinases placed in lower wells of the Boyden chamber were used as chemoattractants. The chemoattractants and cells were divided by a polycarbonate pore filter with pore diameter of 5 µm (Nucleopore Corp., USA) coated with collagen type I (100 mg/ml) (Vitrogen 100; Celtrix Pharmaceuticals Inc., USA). The upper well of the chamber was loaded with 50 µl of cell suspension in serum-free medium (DMEM/0.1% BSA) obtained during the treatment of cell monolayer with trypsin/EDTA (0.05%/0.02%) solution. The number of cells added per well was varied with cell type and was 35,000–130,000 cells per well. Cells were incubated in a CO₂-incubator at 37°C for 2.5–12 h depending on cell culture. Non-migrated cells were removed from the upper side of the filter, and cells on the lower side were fixed with methanol and stained with Dif Quick (Baxter, USA). The membrane with stained cells was scanned on a ScanJet II CX scanner using Deskscan and NIH Image software. Migration intensity was assessed using peak areas obtained by scanning of the field of stained cells. Data were represented as a ratio of peak area to control value obtained for cells passed through the filter in the absence of chemoattractant.

Statistical analysis of results. Mean $\pm$ standard error from three independent experiments each in triplicates are presented if not indicated otherwise. At the 0.05 level of Student's *t*-test, the two means are significantly different.

RESULTS AND DISCUSSION

Analyzing structure of urokinase and its separate domains by NMR, it was proposed that GFD and KD of uPA are structurally independent [27, 28]. However, stabilization of “growth” domain interactions with uPAR by KD [4] and our data on the influence of cell migration by different urokinases [3] suggest that the KD and GFD can interact to each other and mutually affect their binding with cell receptors.

It was demonstrated for a number of proteins that homopolymeric structures were formed by so-called “mutual exchange” of domains interacting in the protein monomer [29]. Therefore, we proposed that under some conditions the KD and GFD of uPA might interact when being in different molecules. To investigate the ability of isolated kringle domain or KD in N-terminal fragment of uPA (ATF) to bind the “growth” domain in full-length urokinase, recombinant uPA was immobilized on BrCN-Sepharose. Sepharose with immobilized BSA was used as a control sorbent. Recombinant kringle domain or ATF (0–1.7 nmol/400 µl) was incubated with affinity and control sorbents as described in “Materials and Methods”. As Fig. 4 shows, both the kringle domain and ATF can bind full-length uPA immobilized on Sepharose. Lack of binding with the control sorbent indicates the specificity of uPA interaction with isolated KD or kringle domain in ATF. It should be noted that 20% of the protein in recombinant ATF preparations are dimers (Fig. 5b, see color insert). Dimer formation in preparations of HGF (hepatocyte growth factor) fragment containing the kringle domain was described previously in the literature [30]. Dimer is likely to form as a result of intermolecular domain exchange [29]. Figure 5a shows one of the possible dimeric structures explaining the presence of dimers and oligomers in ATF preparations.

It was mentioned above that the “growth” domain of uPA binds uPAR with high affinity. Since we proposed that the “growth” domain can interact with kringle domain, we considered the possibility of competition between uPAR and kringle domain for binding the “growth” domain of urokinase. This could support the hypothesis that kringle domain interacts with “growth” domain being located in different molecules and possibly inside the same molecule. We proposed that if isolated soluble KD might interact with GFD of uPA immobilized on sorbent it could displace uPAR bound to the uPA sorbent. First of all it was necessary to confirm that uPAR binds uPA through the “growth” domain only, and that the kringle domain could not bind uPAR.

We immobilized uPAwt, kringle domain, and uPA–GFD on BrCN-Sepharose to obtain affinity matrices. These matrices then were incubated with lysates from U937 cells serving as a source of uPAR. Figure 6 shows that only uPAwt containing “growth” domain binds

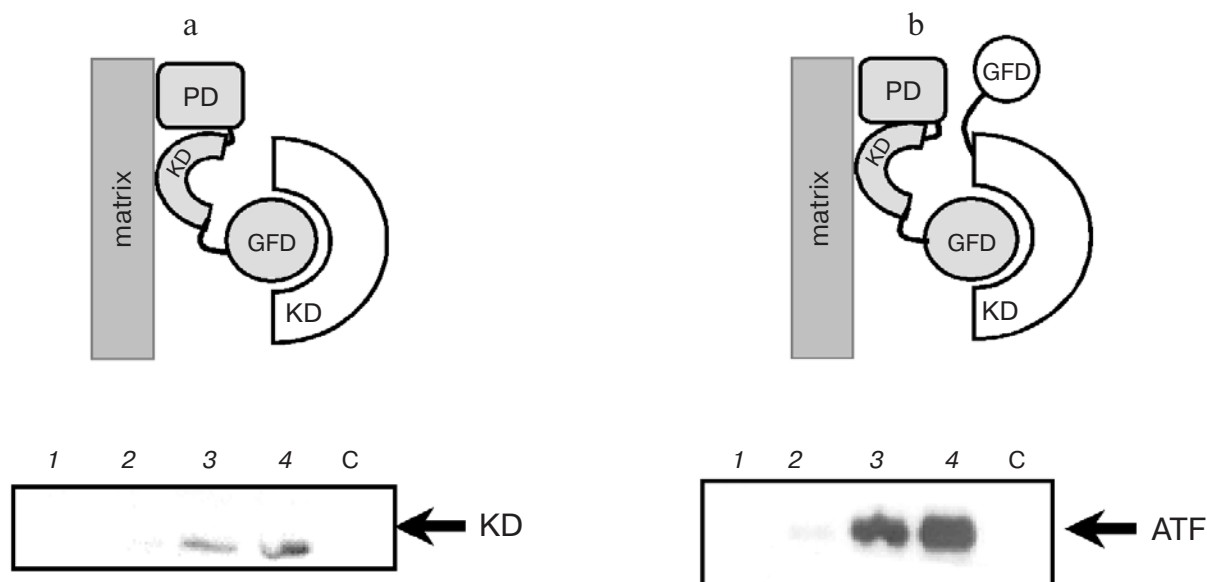


Fig. 4. Kringle domain (a) and ATF (b) binding to uPA-Sepharose. uPAwt was immobilized on BrCN-Sepharose 4B. U937 cell lysate containing uPAR was incubated with affinity matrix in absence and in presence of increasing concentrations of kringle domain (a) or ATF (b). Lanes: 1) 0; 2) 0.17; 3) 0.89; 4) 1.7 nmol. C, control sorbent incubated with 1.7 nmol of kringle domain or ATF. Bound proteins were eluted from the matrix, separated by SDS-PAGE, transferred to PVDF membrane, and detected using monoclonal antibodies against kringle domain of uPA.

uPAR, while neither isolated kringle domain nor uPA-GFD are able to bind the receptor.

Therefore, the model system described might help to elucidate the ability of kringle domain to compete with uPAR for binding with "growth" domain of uPA immobilized on the sorbent. uPAR was binding affinity sorbent during incubation of uPAwt matrix with lysates of U937 cells. So it seemed possible to add increasing quantities of kringle domain, uPA-GFD, or uPAwt to lysate samples in the presence of affinity uPA sorbent, and to estimate the efficacy of uPAR binding with the matrix.

Aliquots of uPAwt-Sepharose (15 μ l) were incubated with 250 μ l of U937 lysates (1.5 mg of total protein) in the absence and in the presence of soluble preparations of uPAwt, KD, and uPA-GFD (Fig. 7, a-c, respectively). As expected, uPAwt added to lysate bound uPAR from lysate, thus impeding receptor association with immobilized uPAwt. Figure 7a shows dose-dependent decrease of uPAR binding to affinity sorbent upon increase of uPAwt concentration in solution.

Although the kringle domain does not bind uPAR, applying increasing quantities of KD (Fig. 7b) or uPA-GFD (Fig. 7c) also led to decrease in uPAR binding to immobilized uPAwt. These data indicate that kringle domain is able to compete with uPAR for binding with uPAwt sorbent, namely, with its "growth" domain leading to "elution" of sorbent-bound uPAR.

In our previous publications, we used recombinant urokinase containing modified GFD unable to bind uPAR (uPAmut). We demonstrated earlier that this pro-

tein, in contrast to full-length urokinase, did not cause cell migration [24]. Since uPAmut contains intact kringle domain we suggested that addition of uPAmut excess in lysate solution in the presence of uPAwt sorbent also lead to uPAR elution from affinity matrix due to uPAmut binding with immobilized uPAwt. However, uPAR displacement was not observed upon addition of uPAmut (Fig. 7d). uPAmut is unable to interact with uPAR in solution. So the absence of competition might be explained in a way that kringle domain in uPAmut inter-

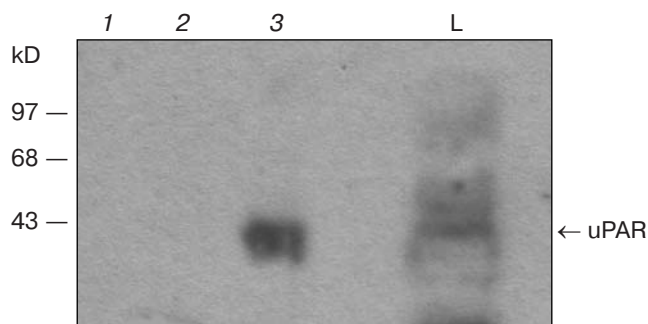


Fig. 6. uPAR from U937 lysate binding to immobilized uPA. Different uPA constructs were immobilized on BrCN-Sepharose 4B. U937 cell lysate containing uPAR was incubated with affinity matrix. Bound proteins were eluted from the matrix, separated by SDS-PAGE, transferred to PVDF membrane, and detected with monoclonal anti-uPAR antibodies (Monozyme). Lanes: 1) uPA-GFD; 2) KD; 3) uPAwt; L) U937 cell lysate.

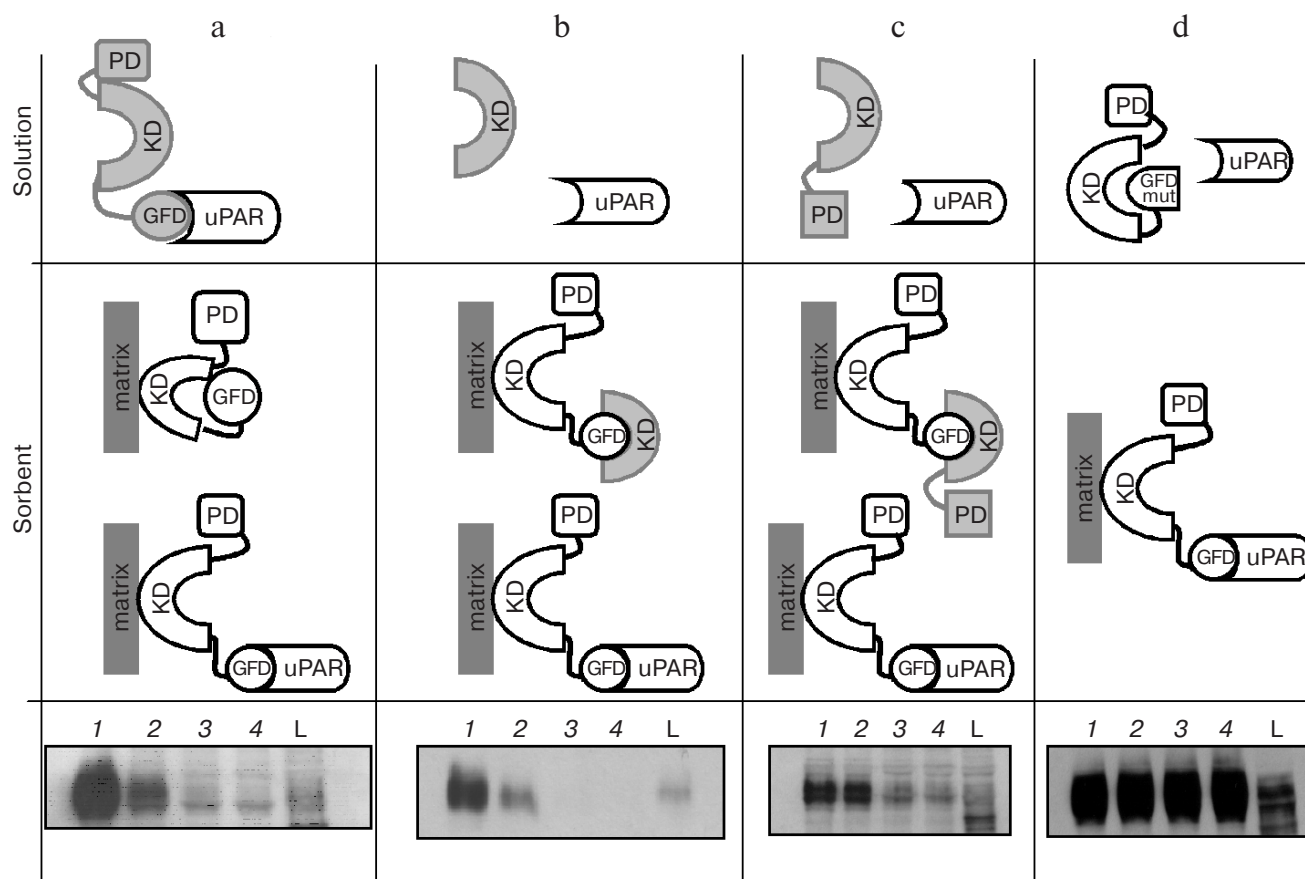


Fig. 7. uPAR binding with uPA-Sepharose in the presence of different uPA forms. uPAwt was immobilized on BrCN-Sepharose 4B. U937 cell lysate containing uPAR was incubated with affinity matrix in the absence and in the presence of increasing concentrations of uPAwt (a), KD (b), uPA-GFD (c), and uPAmut (d). Lanes: 1) 0; 2) 0.17; 3) 0.89; 4) 1.7 nmol of protein; L) U937 lysate. Bound proteins were eluted from matrix, separated by SDS-PAGE, transferred to PVDF membrane, and detected with monoclonal anti-uPAR antibodies.

acts with modified “growth” domain inside the same protein molecule, thus being prevented from binding with GFD of immobilized uPAwt.

Therefore, the data suggest that the “growth” and kringle domains can interact, being part of both the same and different molecules in the case intramolecular interactions are disrupted due to binding with other proteins. It also can be proposed that “growth” domain shields KD in urokinase and hinders its binding with the receptor until GFD would bind uPAR.

It was demonstrated earlier by our group that besides uPAR, urokinase is able to bind other sites on the surface of a number of cells such as SMC, HEK 293 [3], and CHO. This additional site is different from uPAR and integrins [31, 32] since it binds kringle domain of uPA [3], thus causing kringle-dependent activation of cell migration [3, 33, 34]. We proposed that the “growth” domain can shield an “active” epitope on the kringle domain and prevent its binding with the receptor in the case when uPA is not bound to uPAR. So we decided to elucidate the contribution of different domains of uPA in binding with cell surface of a CHO cell line that does not

express LRP (low density lipoprotein receptor related protein) (CHO-LRP^{-/-}). These cells were chosen to exclude binding of uPA with LRP [35] (Fig. 8). As expected, excess of uPAwt inhibited specific binding of ¹²⁵I-labeled uPAwt. Receptor-associated protein (RAP), the universal inhibitor of uPA binding with LRP/ α_2 -MR receptor, did not inhibit specific binding, thus supporting the absence of urokinase binding with LRP in this cell type [35]. uPA-GFD, which does not bind uPAR [33], inhibited ¹²⁵I-labeled uPAwt binding to cell surface only by 40%. This indicates that ¹²⁵I-labeled uPAwt binds to cell surface through both the “growth” and kringle domains.

uPAmut, which is not able to bind uPAR but has intact kringle domain, did not compete for binding to ¹²⁵I-labeled uPAwt. These data suggest that “growth” domain of uPAmut continues to shield KD due to its inability to bind uPAR, and thus prevents interaction of kringle domain with its receptor on the cell surface.

Comparing chemotactic properties of different urokinases, we found that uPAwt and uPA-GFD showed chemotactic activity being bound to both uPAR and KD-

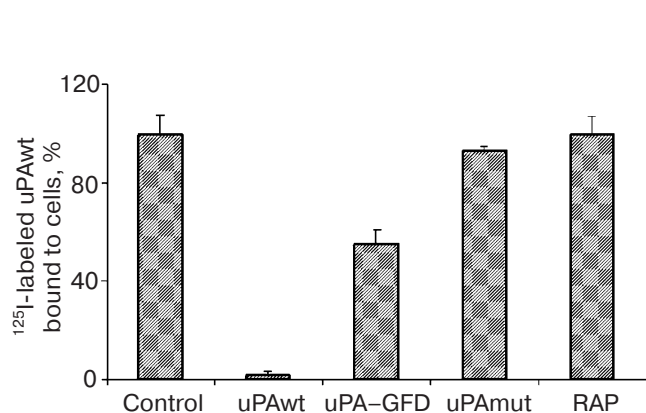


Fig. 8. Different urokinases compete for ^{125}I -labeled uPAwt. CHO-LRP⁻ cells were incubated with ^{125}I -labeled uPAwt (3 nM) and non-labeled uPAwt, uPA-GFD, uPAmut, and RAP (300 nM). Data presented in the graph are mean values from nine experiments.

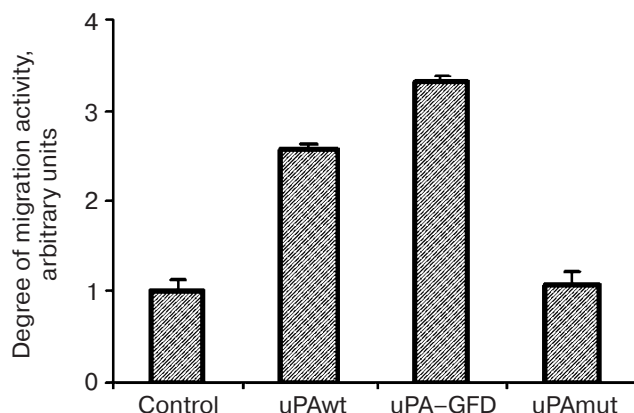


Fig. 9. Chemotactic action of different uPAwt on migration of human SMC. Different urokinases were added to cells: uPAwt, uPA-GFD, and uPAmut in concentration of 10 nM. Data presented in the graph are mean values from nine experiments.

binding targets (KBP) or to only KBP. uPAmut neither bound to cells (Fig. 8) nor activated migration (Fig. 9) [24]. These data suggest the possibility of intramolecular interactions between kringle and “growth” domains of urokinase affecting its chemotactic properties.

In a series of papers, it was shown that signal functions of uPA did not require its proteolytic activity and might be mediated by urokinases with no proteolytic activity. Moreover, it was demonstrated that uPA can trigger intercellular signaling without the participation of

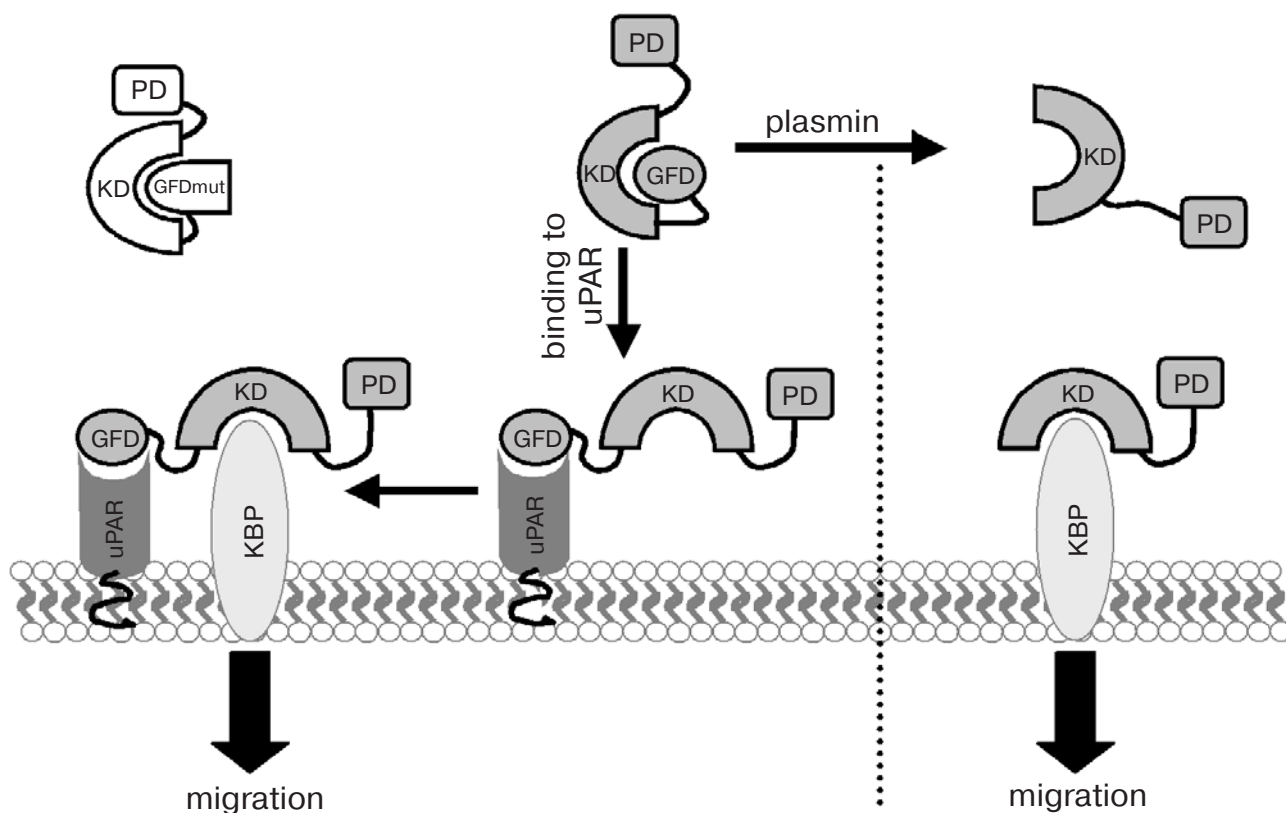


Fig. 10. Model of uPAwt molecule. KD is “closed” as a result of interaction with GFD and “opened” after cleavage of GFD with plasmin upon interaction of GFD with uPAR. “Opening” of KD leads to its recognition by several integrins and kringle-binding protein (KBP) with subsequent triggering of cell migration.

uPAR [3, 34]. Based on these data, we suggest that some uPAR adaptor proteins can interact with binding site located outside the urokinase “growth” domain. Even more, we and our colleagues have shown that kringle domain mediated at least some chemotactic and pro-adhesive properties of uPA [3, 23]. We also demonstrated that both full-length uPA and uPA–GFD caused similar chemotactic response in smooth muscle cells of the human respiratory system.

In the case of uPAwt, not only the kringle domain is important to triggering intracellular signaling, but also binding to uPAR, while for uPA–GFD the presence of KD is the only important factor [3]. Nevertheless, it is still unclear how uPAR interaction with “growth” domain of uPA affects the interaction of KD with its receptor.

Our studies partially explain these apparently conflicting data. We found that GFD reversibly affected chemotactic properties of KD. Based on our data, it can be suggested that the “growth” domain of uPAwt, which is not bound to uPAR, shields KD epitopes responsible for binding with its receptor(s) on the cell surface (KBP). Exposure of KD active sites is likely to take place upon interaction of GFD with uPAR, thus allowing the kringle domain to bind its receptor.

Based on these and previously obtained data, we suggest the uPAwt model where KD is “closed” as a result of interaction with GFD. The kringle domain becomes “opened” due to plasmin cleavage of GFD or due to the interaction of uPA with uPAR (Fig. 10). KD “opening” leads to its recognition by several integrins or other kringle-binding receptors for triggering migration. These data make clearer the still not understood function of KD and can provide support for suggesting a structure of one of the kringle-binding proteins.

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