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The Chemotactic Action of Urokinase on Smooth Muscle Cells Is Dependent on Its Kringle Domain

CHARACTERIZATION OF INTERACTIONS AND CONTRIBUTION TO CHEMOTAXIS*

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Urokinase plasminogen activator (uPA) is thought to exert its effects on cell growth, adhesion, and migration by mechanisms involving proteolysis and interaction with its cell surface receptor (uPAR). The functional properties of uPA and the significance of its various domains for chemotactic activity were analyzed using human airway smooth muscle cells (hAWSMC). The wild-type uPA (r-uPAwt), inactive urokinase with single mutation (His^{204} to Gln) (r-$uPA^{H/Q}$), urokinase with mutation of His^{204} to Gln together with a deletion of growth factor-like domain (r-$uPA^{H/Q}$-GFD), the catalytic domain of urokinase (r-uPA^{LMW}), and its kringle domain (r-KD) were expressed in *Escherichia coli*. We demonstrate that glycosylated uPA, r-uPAwt, r-$uPA^{H/Q}$, and r-$uPA^{H/Q}$-GFD elicited similar chemotactic effects. Half-maximal chemotaxis (EC_{50}) were apparent at approximately 2 nM with all the uPA variants. The kringle domain induced cell migration with an EC_{50} of about 6 nM, whereas the denaturated r-KD and r-uPA^{LMW} were without effect. R-uPAwt-induced chemotaxis was dependent on an association with uPAR and a uPA-kringle domain-binding site, determined using a monoclonal uPAR antibody to prevent the uPA-uPAR interaction, and a monoclonal antibody to the uPA-kringle domain. The binding of iodinated r-uPAwt with hAWSMC was due to interaction with a high affinity binding site on the uPAR, and a lower affinity binding site on an unidentified cell surface target, which was mediated exclusively through the kringle domain of urokinase. Specific binding of r-$uPA^{H/Q}$-GFD to hAWSMC involved an interaction with a single site whose characteristics were similar to those of the low affinity site of r-uPAwt binding to hAWSMC. uPAR-deficient HEK 293 cells specifically bound r-uPAwt and r-$uPA^{H/Q}$-GFD via a single, similar type of binding site. These cells migrated when stimulated by r-$uPA^{H/Q}$-GFD and uPAwt, but not r-uPA^{LMW}. HEK 293 cells transfected with the uPAR cDNA expressed two classes of sites that bound r-uPAwt; however, only a single site was responsible for the binding of r-$uPA^{H/Q}$-GFD. Together, these findings indicate that uPA-induced chemotaxis is dependent on the binding of the uPA-kringle to the membrane surface of cells and the association of uPA with uPAR.

Plasminogen activators are directly involved in inflammation, angiogenesis, tissue remodeling, and tumor growth and invasion (for review, see Refs. 1–4). Of the two types of plasminogen activators expressed by tissues, urokinase-type plasminogen activator (uPA[1] or urokinase) and tissue-type plasminogen activator (tPA), uPA rather than tPA promotes cell migration (5–8) and proliferation (9–11) via interactions with high affinity cell surface receptors (uPAR/CD87). In addition to interacting with uPA, the significance of uPAR in chemotaxis also appears dependent upon interaction with integrins (12), and/or exposure of a chemotactic epitope within uPAR following ligand binding or proteolytic cleavage (13). The urokinase/uPAR system also participates in the chemotaxis initiated by growth factors which activate tyrosine kinase receptors, as well as several chemoattractant molecules such as formylmethionylleucylphenylalanine (14–17).

Urokinase is secreted by many cell types, as a single chain polypeptide that possesses little or no proteolytic activity (18). It is composed of three structurally independent domains, a proteolytic domain (PD), a kringle domain (KD), and a growth factor-like domain (GFD). The active serine protease is generated from a single cleavage between Lys^{158} and Ile^{159} (18), and further plasminolysis generates the two-chain low molecular weight form uPA^{LMW}, commencing at Lys^{136} and consisting of mainly PD (19), and amino-terminal fragment (ATF) (20).

Urokinase PA and the ATF have been shown to bind with similar high affinity to uPAR/CD87 via the GFD (9, 21). This interaction induces cell migration and the activation of a number of signal transduction pathways within the cell cytoplasm and transcriptional apparatus; events which are independent of proteolysis (6–8, 22–28). Several studies indicate that the ability of uPA to initiate a chemotactic response is dependent

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[1] The abbreviations used are: uPA, urokinase-type plasminogen activator; GFD, growth factor-like domain of uPA; KD, kringle domain of uPA; PD, proteolytic domain of uPA; r-uPAwt, recombinant single-chain urokinase with wild type structure; r-$uPA^{H/Q}$, proteolytically inactive urokinase with substitution of His^{204} in active center for Gln; r-$uPA^{H/Q}$-GFD, proteolytically inactive urokinase with substitution of His^{204} in active center for Gln, lacking GFD; uPA^{LMW}, low molecular weight form of uPA, containing mainly PD; uPA-KD, uPA deletion mutant lacking kringle domain; uPAR, uPA receptor; tPA, tissue-type plasminogen activator; hAWSMC, human airway smooth muscle cells; HEK cells, human embryonic kidney cells; mAb, monoclonal antibody; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; PAGE, polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

only on its proteolytic activity (29, 30), implicating a direct or plasmin-mediated activation, or release of the mitogenic growth factors-basic fibroblast growth factor, hepatocyte growth factor/scatter factor, and VEGF− from the extracellular matrix (31–33). Others have demonstrated that uPA-induced cell migration is dependent on its proteolytic activity, interaction with uPAR, as well as interactions with low density lipoprotein receptor-related protein/α2-macroglobulin receptor (LRP/α2-MR) or very low density lipoprotein receptor (34, 35). Together these studies indicate that signal transduction pathways utilized by uPA in initiating chemotaxis exhibit marked diversity depending upon cell type. The importance of uPA in cell migration and tissue remodeling was recently demonstrated in knockout mice (36), where there was marked reduction in the size of the neointima which forms after intra-vessel injury in the uPA-deficient mice, when compared with tPA-deficient and normal animals. In contrast, when mice deficient in uPAR were similarly injured neointima size was unaffected (37). Together these observations suggest that mechanisms, distinct or additional to uPA/uPAR interaction might mediate processes of the uPA-dependent tissue remodeling.

Since uPAR is attached to the cell membrane via glycosylphosphatidylinositol anchor and lacks transmembrane and cytoplasmic regions, alone it is not capable of initiating a chemotactic signal. An obligatory partner of yet unknown nature is probably required for uPAR to act as a signaling receptor (13, 22), that can be activated either by a uPAR fragment (38) or following binding of uPA. Such an adaptor might be integrins, which can interact directly with uPAR and promote cell adhesiveness and chemotaxis (39–44).

Most investigating uPA-dependent chemotaxis has utilized full-length uPA, DFP-inactivated uPA, or its ATF. However, the ATF includes the growth factor-like and kringle domains, which behave as independently folded domains (45). Urokinase PA-kringle is highly homologous to the kringle structure-containing fragments of blood plasma proteins, which affect cell motility and/or proliferation (46, 47). Despite these observations, the precise mechanism mediating the uPA-dependent effects on cell migration and the impact of the different domains of uPA on cell migration remain unclear.

To elucidate the role of uPA structural domains in cell migration, we have constructed and produced recombinant uPA forms deficient in the GFD, as well as the individual domains of urokinase. Recently we reported that a form of uPA lacking proteolytic activity and the growth factor-like domain is capable of inducing cell migration to a similar extent as uPA. It exhibited the atypical binding of urokinase, which was unrelated to any interaction with uPAR via GFD (48). Here we report that besides the growth factor-like domain, which interacts with uPAR, the kringle domain of urokinase can also be involved in the induction of cell chemotaxis by uPA.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and Lipofectin™ were purchased from Life Technologies, Inc.. Chromogenic substrate S-2444 was obtained from Chromogenix, Mölndal, Sweden. Glycosylated high molecular weight urokinase purified from urine (glycosylated uPA) was from Medac, Hamburg, Germany, and tPA (Actilyse) was from Roche Molecular Biochemicals. A mouse monoclonal antibody clone 3 (R-3-01) which reacts with domain I of human uPAR was from Monozyme, Denmark. The goat anti-mouse IgG conjugated with horseradish peroxidase and chemiluminescent substrate "Super Signal Substrate" were from Pierce. Plasminogen, a mouse IgG, and two different murine monoclonal antibodies of the IgG_1 subtype (UIG-1 and UNG-5) raised against human urinary uPA (49) were kindly provided by Dr. S. P. Domogatsky. An anti-uPA mAb UIG-1 detected high and low molecular forms of uPA by Western blots. An anti-uPA mAb UNG-5 detected urokinase forms containing the kringle domain, (specifically, glycosylated uPA, r-uPAwt, r-uPA$^{H/Q}$, r-uPA$^{H/Q}$-GFD, and r-KD); it does not interact with r-uPA^{LMW}, lacking the kringle domain, tPA, and plasminogen. UNG-5 did not interact with denaturated r-KD, uPAwt, and r-uPA$^{H/Q}$-GFD.

Recombinant Urokinase Derivatives—Recombinant uPA with wild-type structure (r-uPAwt), proteolytically inactive urokinase with substitution of His^{204} in active center for Gln (r-uPA$^{H/Q}$), and proteolytically inactive urokinase with deletion of NH_2-terminal 1–43 amino acids called "growth factor-like domains" (r-uPA$^{H/Q}$-GFD) were expressed in *Escherichia coli* and purified as described previously (14, 48).

Two-chain low molecular weight urokinase with the catalytic domain r-uPA^{LMW} was prepared by proteolytic cleavage of r-uPAwt by plasmin, followed by purification with affinity chromatography, using monoclonal antibody against urokinase catalytic domain coupled to CNBr-Sepharose 4B (Amersham Pharmacia Biotech).

The recombinant kringle domain (r-KD) was made in *E. coli* as follows. The region of uPA cDNA corresponding to amino acids 42–210 (50, 51) was amplified using primers: M3, 5′-CTGTGATCTAGATA-AGTCAAAAACCTGCTATGAGGG-3′, and M7, 5′-AGCACTGTGTGG-CGCTGATCACCCAGCAAGGGCTG-3′.

Primer M3 was designed to introduce an *Xba*I site (underlined) between GFD and KD of uPA without changing the amino acid sequence. The PCR product was digested with *Xba*I and *Eco*RI enzymes, generating a fragment coding urokinase amino acids 42–166. The expression vector was constructed by modifying a pTZ19 plasmid between *Hin*dIII and *Xba*I sites. This modification was done using a synthetic fragment containing a new translation initiation region and coding for the first 5 amino acids (MKSTL). This plasmid allowed the cloning of coding inserts between the *Xba*I and *Bam*HI sites. The *Xba*I-*Eco*RI fragment of uPA and the *Eco*RI and *Bam*HI ending synthetic duplex: 5′-AATTCACCACCCTGCACCATCACCATCACCATTAATAG-3′; 3′-G-TGGTGGGACGTGGTAGTGGTAGTGGTAATTATCCTAG-5′ were ligated between the *Xba*I and *Bam*HI sites; the duplex sequence contains a hexahistidine tag and stop codon after the *Eco*RI site. The structure of the resulting construct was confirmed by sequencing. The cloning procedures outlined above resulted in a pKR-his6 plasmid coding for a polypeptide identical to r-uPAwt amino acids 43–166 as shown: MKSTLEIDK-SKTCYEGNGHFYRGKASTDTMGRPCLPWNSATVLQQTYHAHRSDA-LQLGLGKHNYCRNPDNRRRPWCYVQVGLKPLVKECMVHDCADGK-KPSSPPEELKFQCGQKTLRP**RF**KIIGGEFTTLHHHHHH. The non-urokinase amino acids acquired from the vector are underlined. *E. coli* strain JM109 was transformed by the resulting pKR-his6 plasmid. Purification of r-KD was carried out as follows: inclusion bodies were isolated and the proteins denatured in buffer containing 6 M guanidine chloride. Subsequent reconstitution was carried out by gradually removing the denaturing agents while maintaining appropriate redox conditions with a glutathione-containing buffer (52). The primary structure of r-KD allowed us to use nickel-chelate affinity chromatography for its initial purification. Subsequent purification to homogeneity was achieved by affinity chromatography on Sepharose coupled with the monoclonal antibody UNG-5. Then the kringle polypeptide was treated with thrombin, to cleave the peptide bond (Arg^{156}-Phe^{157}) in urokinase (53) (shown in bold); affinity chromatography using anti-kringle mAb removed the hexahistidine tag, any uncleaved peptides and thrombin. The resulting purified r-KD corresponded to urokinase amino acids Glu^{43}-Arg^{156}.

The homogeneity of recombinant urokinase forms was confirmed by SDS-PAGE and Western blotting using monoclonal antibodies directed against the urokinase catalytic and kringle domains. The amidolytic activities of the glycosylated uPA and recombinant uPA forms were determined using the chromogenic urokinase substrate S-2444, as described previously (54). The proteolytic activities of glycosylated uPA, r-uPAwt, and r-uPA^{LMW} were ~5000 units/nmol; the other urokinase forms with substitution of His^{204} in active center for Gln (r-uPA$^{H/Q}$ and r-uPA$^{H/Q}$-GFD) as well as r-KD were proteolytically inactive. R-uPAwt and r-uPA$^{H/Q}$ coupled to CNBr-Sepharose 4B (Amersham Pharmacia Biotech) specifically precipitated uPAR from cell lysates with similar efficiency, while r-uPA$^{H/Q}$-GFD and r-uPA^{LMW} did not.

Reduction and Alkylation of the Recombinant Kringle Domain—This was performed as described previously (46, 47). Briefly, r-KD (110 μg, 1 ml) was reduced by dithiothreitol (0.5 M, 15 min at room temperature). The reduced r-KD was alkylated by the addition of 0.25 M iodoacetamide for 1 h at 4 °C and then dialyzed against PBS. Both the denatured r-KD and the intact r-KD were analyzed by SDS-PAGE and Western blotting using anti-kringle mAb UNG-5.

Cell Lines and Culture—Human airway smooth muscle cells (hAW-SMC) were isolated from trachea and characterized as previously described (55). Human embryonic kidney cells HEK 293 were from Cardiology Research Center Cell Collection, Moscow. The cell lines were cultured in DMEM supplemented with 10% fetal bovine serum, 10 mM

HEPES, 5 mM glutamine, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C and 5% CO_2.

uPAR Receptor Expression Plasmids and Transfection of HEK 293 Cells—The *Xba*I-*Eco*RI fragment from pB SK-(uPAR) plasmid containing human uPAR-cDNA (GenBank accession number X51675), was ligated into pUC19 (New England Biolabs) producing an intermediate plasmid pUC-uPAR. Then the *Hin*dIII-*Eco*RI fragment from pUC-uPAR was inserted into the pcDNA3 expression vector (Invitrogen). The resultant plasmid pcDNA-uPAR transfected into HEK 293 cells using Lipofectin, according to the manufacturers protocol. Two days later the cells were plated onto 96-well plates for clone selection. The transfected cells were cultured in DMEM with 10% fetal bovine serum containing 1.5 mg/ml G418 (Life Technologies, Inc.) for 25 days and G418-resistant clones were analyzed. Recombinant-uPAR expressed by these HEK 293 cells specifically bound recombinant uPAwt, assessed using an affinity uPAR precipitation method from cell lysates and immobilized r-uPAwt. Cells HEK 293 transfected by plasmid pcDNA3-anti-βGAL bearing a 3-kilobase fragment of *E. coli* β-galactosydase gene inserted in the antisense orientation (HEK-control cells) were used as controls.

Western Blotting and uPAR Analysis—Cells were scraped into 10 mM HEPES buffer (pH 7.2), containing 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM phenylmethylsulfonyl fluoride, and centrifuged at 1500 × *g* for 3 min at 4 °C. The pellet was resuspended in 10 mM Tris-HCl buffer (pH 8.0), containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride. After incubation for 20 min at 4 °C, the suspension was centrifuged at 5,000 × *g* for 15 min at 4 °C. The proteins in the supernatant were mixed with SDS sample buffer and subjected to 10% SDS-PAGE/Western blotting onto polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane was immersed overnight at 4 °C in PBS containing 1% casein, 0.05% Tween 20, and 1 μg/ml anti-uPAR mAb (Monozyme). After multiple washings the membranes were incubated with a goat anti-mouse IgG conjugated to horseradish peroxidase and visualized using the chemiluminescent substrate "Super Signal Substrate."

Chemotaxis Assays—Migration of hAWSMC was determined as described previously, using a micro-Boyden chamber (14). Briefly, to determine the role of uPAR in uPA-induced cell migration, a hAWSMC cell suspension in DMEM supplemented with 0.1% BSA was preincubated with the anti-uPAR mAb (50 μg/ml) or a mouse Ig (50 μg/ml) for 1 h prior to seeding the cells into the upper wells of the Boyden chamber. When the role of the urokinase-kringle domain in uPA-induced cell migration was to be assessed, r-uPAwt, r-uPA$^{H/Q}$-GFD, or r-KD and the anti-kringle mAb UNG-5 (20 μg/ml) or with total mouse Ig (20 μg/ml) were preincubated for 1 h prior to placement into the lower wells. HEK cell suspension was prepared in DMEM supplemented with 1% BSA. The extent of cell migration was evaluated after incubation for 3 h at 37 °C in the CO_2 incubator. Data are presented as peak area on scanned fields with stained cells and expressed as percentage of cells that migrate across a filter compared with control chemotaxis. Comparisons between cell migration under the different conditions were analyzed using Student's *t* test. A $p < 0.05$ value was considered statistically significant. All results are expressed as mean ± S.E.

Binding Assay—Urokinase constructs (60 μg) were iodinated with 1 mCi of $Na^{125}I$ and 0.1 mg of IODO-GEN (Pierce) for 8 min at room temperature and the reaction was terminated with excess L-tyrosine. Purification of iodinated peptides was carried out by chromatography on PD-10 columns (Amersham Pharmacia Biotech) equilibrated with PBS. Iodinated peptides could be precipitated (95–98%) by trichloroacetic acid and had specific activities from 3.0 to 4.0 × 10^5 cpm/pmol. Binding studies with the iodinated peptides were carried out on confluent cells in 48-well dishes, washed (2 times) with PBS containing 0.1% BSA, and preincubated for 1 h with 500 μl of binding medium (DMEM supplemented with 20 mM HEPES, 0.1% BSA, 100 KIU/ml aprotinin). After aspirating the medium the increasing amounts of radiolabeled urokinase in 100 μl of binding medium were added to the confluent hAWSMC together with either 100 μl of unlabeled r-uPA (4 μM in DMEM) or 100 μl of DMEM and incubated at 4 °C for 2 h. In the competitive binding studies the cells were incubated with 200 μl of radiolabeled urokinase alone or in the presence of different concentrations of unlabeled competitors (see later). To determine the contribution of uPAR to uPA binding to hAWSMC, the cells were preincubated with anti-uPAR mAb (50 μg/ml) for 1 h. The role of the kringle domain in uPA binding to the cells was assessed by preincubating the ^{125}I-uPAwt with the anti-kringle mAb (40 μg/ml) or mouse IgG (40 μg/ml) for 1 h, prior to addition to cell monolayers. Incubations were terminated by aspirating the medium, washing cell monolayers with PBS/BSA, and solubilizing the cells with 1% SDS, 0.5 M NaOH. Cell associated radioactivity was determined in a γ-counter and the results analyzed using the program "Ligand" for estimating ligand binding parameters for one- or two-site model system (56). All results except those depicted in the Scatchard plots are expressed as mean ± S.E.

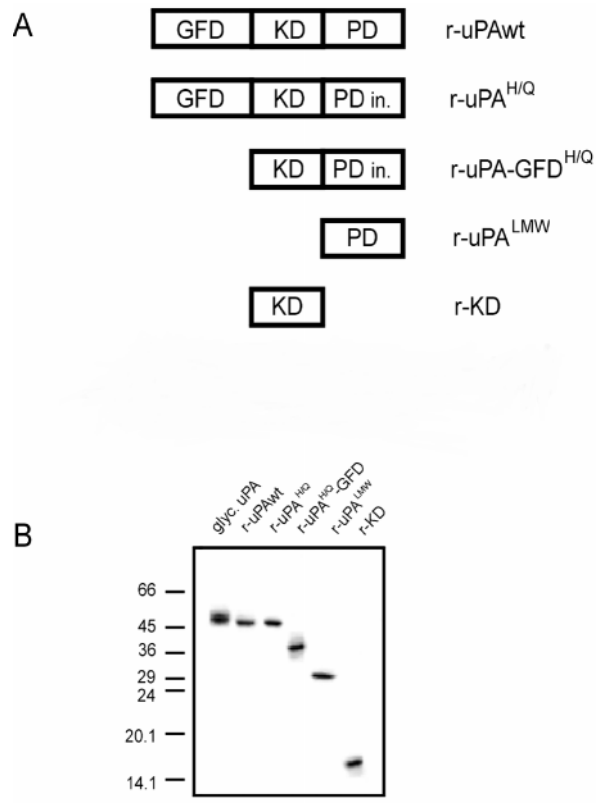


FIG. 1. **Urokinase peptide variants produced in *E. coli* and used in the cell migration and ligand binding studies.** *Panel A,* schematic representation of the different urokinase peptide variants used in the study. An *open box* represents the various domains of uPA. *PD in,* the inactive protease domain. Recombinant (r)-uPAwt represents recombinant wild type urokinase, r-uPA$^{H/Q}$, the proteolytically inactive urokinase with a substitution His^{204} for Gln in the active center; r-uPA$^{H/Q}$-GFD represents the proteolytically inactive urokinase with a His^{204} substitution for Gln in the active center, and also lacking the GFD. Urokinase (u)-PA^{LMW} represents the low molecular weight uPA, containing mainly PD, r-KD, the recombinant kringle domain of uPA. *Panel B,* SDS-PAGE analysis of the purified uPA variants. One μg of each purified peptide was loaded per lane and subjected to SDS-PAGE (12% gel), under nonreducing conditions; the peptides were stained with Commassie Blue.

RESULTS

Effects of Different Urokinase Mutants on hAWSMC Migration—To determine the roles of proteolysis, uPA/uPAR interaction, and the involvement of putatively key domains in uPA-induced cell migration**,** we expressed the following mutant recombinant urokinase peptides in *E. coli*: the single-chain wild type urokinase (r-uPAwt), a proteolytically inactive urokinase with substitution of His^{204} for Gln within the active center (r-uPA$^{H/Q}$), and a proteolytically inactive urokinase lacking the growth factor like domain (r-uPA$^{H/Q}$-GFD) (Fig. 1).

Both the native glycosylated uPA and the recombinant single-chain urokinase with the wild type structure (r-uPAwt) initiated dose-dependent chemotaxis in the Boyden chamber migration assay. Cell migration with r-uPAwt was stimulated over the concentration range, 0.5 to 40 nM (EC_{50} values ~2 nM). The proteolytically inactive form, r-uPA$^{H/Q}$, which interacts with uPAR/CD87 via the GFD, induced cell migration over a similar concentration range, with half-maximal stimulation at 2 nM (Fig. 2).

Recombinant-uPA$^{H/Q}$-GFD, which lacks the uPAR-binding

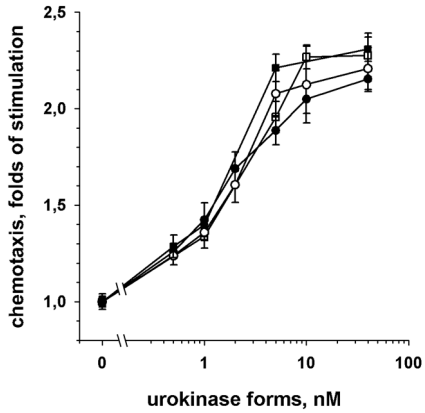


FIG. 2. **Concentration-dependent chemotactic effects of urokinase peptide variants on hAWSMC.** hAWSMC were exposed to different concentration of r-uPAwt (●), glycosylated uPA (■), r-uPA$^{H/Q}$ (□), and r-uPA$^{H/Q}$-GFD (○), and cell migration was measured as described under "Experimental Procedures." Results are expressed as fold stimulation relative to hAWSMC migration in the absence of the uPA variants. Each point is the mean of two experiments performed in triplicate; the *error bars* represent the S.E.

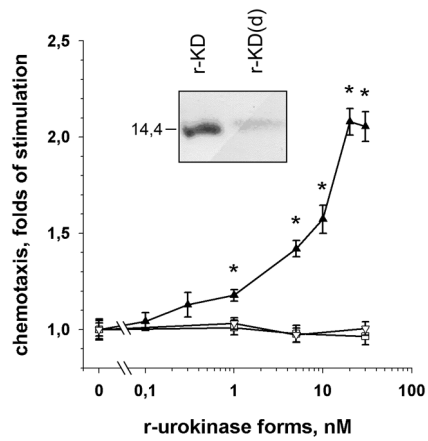


FIG. 3. **Dose-dependent effects of individual urokinase domains on hAWSMC migration.** hAWSMC migration studies were performed in the Boyden Chamber as described under "Experimental Procedures" using r-KD (▲), r-uPA^{LMW} (□), denatured r-KD (▽). Results are expressed as fold stimulation relative to hAWSMC migration in the absence of these agents. Each point is the mean of two experiments performed in triplicate; *error bars* represent the S.E.; *, $p < 0.01$ compared with controls. *Inset,* Western blot analysis of the intact and reduced r-KD using an anti-kringle mAb. r-KD was reduced with dithiothreitol, then alkylated with iodoacetamide, as described under "Experimental Procedures." One μg of r-KD and denatured r-KD (r-KD(d)) was loaded per lane, subjected to SDS-PAGE, electroblotted, and detected with the anti-kringle mAb.

domain (the GFD), induced hAWSMC chemotaxis similarly to r-uPAwt and r-uPA$^{H/Q}$ over the concentration range, 5–40 nM, also with an $EC_{50} \sim 2$ nM (Fig. 2). Together these findings suggest a mechanism of uPA-induced cell migration that does not necessitate the uPA-GFD binding to uPAR.

Urokinase-induced Cell Migration Is Dependent on uPA-Kringle Domain—To determine which domain within r-uPA$^{H/Q}$-GFD is responsible for its chemotactic property, we produced both the kringle domain polypeptide (r-KD) and the proteolytic domain (r-uPA^{LMW}) peptide (as described under "Experimental Procedures") (Fig. 1). As shown in Fig. 3, r-KD also stimulated cell migration in a dose-dependent fashion over the concentration range 1–30 nM, with maximal stimulation at 20 nM and an $EC_{50} \sim 6$ nM. In contrast, r-uPA^{LMW} was without effect.

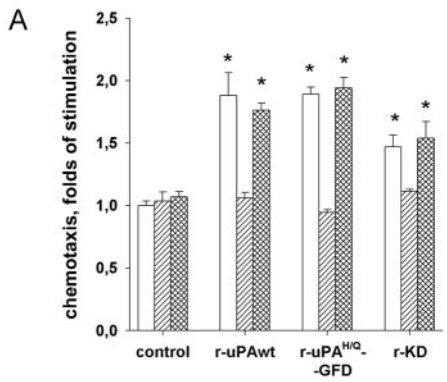
To determine the requirement of intact structure in the kringle polypeptide (r-KD) for chemotaxis, its disulfide bounds were reduced by dithiothreitol and alkylated by iodoacetamide to prevent subsequent refolding. The reduced r-KD was analyzed by SDS-PAGE and Western blotting using monoclonal antibody UNG-5 (see "Experimental Procedures"). This monoclonal antibody which specifically interacts with the kringle domain of uPA, and recognizes the intact recombinant kringle polypeptide, does not recognize reduced r-KD (Fig. 3, *inset*). This reduced r-KD did not induce cell chemotaxis (Fig. 3).

To confirm that uPA-induced chemotaxis requires the kringle domain, we examine the effect of the monoclonal antibody interacting with the urokinase kringle domain (UNG-5) on cell migration. This antibody inhibited hAWSMC migration induced by r-uPAwt, r-uPA$^{H/Q}$-GFD, and r-KD; a non-immune mouse Ig had no effect (Fig. 4*A*).

Thus uPA-induced cell migration is critically dependent on its kringle domain, since: (i) the "neutralization" of the kringle domain by a specific antibody results in the abolition of the chemotactic properties of r-uPAwt and r-uPA$^{H/Q}$-GFD; (ii) the kringle domain polypeptide stimulates hAWSMC migration in a dose-dependent manner.

Involvement of uPAR/CD87 in Urokinase-induced hAWSMC Migration—To define whether urokinase receptor binding is necessary for the r-uPAwt-induced cell migration, a monoclonal anti-uPAR antibody that prevents uPA interaction with uPAR was used in the chemotaxis assays. The anti-uPAR mAb significantly attenuated the chemotactic effect of r-uPAwt, but had no effect on r-KD-induced chemotaxis (Fig. 4*B*). This suggests that the chemotactic effect of full-length urokinase is dependent both on an association with uPAR and the binding of the urokinase kringle domain to the cell surface. However, the removal of GFD from the uPA structure, as in the case of r-uPA$^{H/Q}$-GFD or r-KD, excludes the requirement of uPAR binding for chemotaxis.

Binding of Urokinase Variants to hAWSMC—Analysis of ^{125}I-r-uPAwt specific binding to hAWSMC by Scatchard plots indicated the presence of two different classes of binding sites (Fig. 5*A*). The high affinity binding sites were exhibited a $K_d \sim$ of 1.4 nM and $B_{max} \sim 120{,}000$ sites/cell; the K_d and B_{max} of the lower affinity binding site was 20.3 nM and 780,000 sites/cell. Analysis of ^{125}I-r-uPA$^{H/Q}$-GFD binding to hAWSMC indicated binding to only one site (Fig. 5*B*) with a $K_d \sim 16.8$ nM ($B_{max} \sim$ 770,000 sites/cell), a site similar/identical to the low affinity binding of ^{125}I-r-uPAwt. The average from three experiments indicated the following: (i) for ^{125}I-r-uPAwt specific binding, $K_{d,1} = 1.51 \pm 0.06$ nM and $B_{max,1}$ 140,000 ± 11,000 sites/cell, and $K_{d,2} = 20.27 \pm 1.01$ nM and $B_{max,2}$ 815,000 ± 133 000 sites/cell; (ii) for ^{125}I-r-uPA$^{H/Q}$-GFD specific binding, $K_d = 20.29 \pm 2.22$ nM and B_{max} 1,028,000 ± 129,000 sites/cell. The parameters defining the high affinity binding of r-uPAwt are similar to those previously reported for uPAR (23). To confirm that high affinity binding sites represent binding to uPAR, we also carried out binding studies in the presence of the anti-uPAR mAb, to prevent any interaction between uPA and uPAR. Scatchard analysis of the specific binding under these conditions revealed only one site with a low affinity (Fig. 6*A versus* Fig. 5*A*). Thus, the high affinity site not seen in the presence of the anti-uPAR mAb most likely represents binding to uPAR. To address the question of whether the binding of uPA to the low affinity site is mediated by kringle domain, ^{125}I-r-uPAwt was preincubated with anti-kringle monoclonal antibody UNG-5 before addition to cells. UNG-5 (up to 100 μg/ml) prevented the specific binding of ^{125}I-r-uPAwt, in a dose-dependent manner, by up to 70%. The non-immune mouse Ig had no effect (data not shown). After preincubation of ^{125}I-r-uPAwt with UNG-5 antibody, Scatchard analysis of the specific binding indicated interaction with a single class of binding site with characteristics close to those for the high affinity binding sites (Fig. 6*B versus*



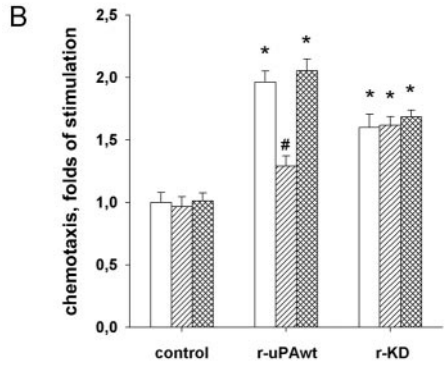
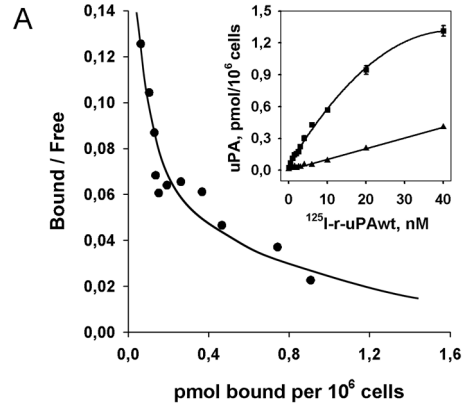


FIG. 4. **Urokinase KD and uPAR in hAWSMC migration.** *Panel A,* the effects of the anti-kringle mAb on hAWSMC chemotaxis induced by the following r-uPA variants, r-uPAwt (10 nM), r-uPA$^{H/Q}$-GFD (10 nM), and r-KD (10 nM), alone (*open bars*), following preincubation with either the anti-kringle mAb UNG-5 (*hatched bars*), or non-immune mouse Ig (*double hatched bars*) for 1 h prior to addition into the lower wells of Boyden chamber. Controls were the anti-kringle mAb UNG-5 and non-immune mouse Ig in the absence of any uPA variants. Results are expressed as fold stimulation relative to hAWSMC migration in the absence of added agents. Each point is the mean of two experiments performed in triplicate; *error bars* represent the S.E.; *, $p < 0.001$ compared with controls. *Panel B,* the effects of the anti-uPAR mAb on cell migration induced by r-uPA-variants. Before initiating cell migration, the hAWSMC were preincubated for 1 h with DMEM/BSA (*open bars*), with the anti-uPAR mAb (50 μg/ml), to prevent binding of uPA to uPAR (*hatched bars*), or with a non-immune mouse Ig (50 μg/ml) (*double hatched bars*). Then the cells were added to the upper wells of the Boyden chamber and r-uPAwt (10 nM), r-KD (10 nM), or DMEM/BSA placed in its lower wells. Results are expressed as fold stimulation relative to hAWSMC migration in the absence of these agents and are the means of two experiments performed in triplicate; the *error bars* represent S.E.; *, $p < 0.001$ and #, $p < 0.05$ compared with controls.



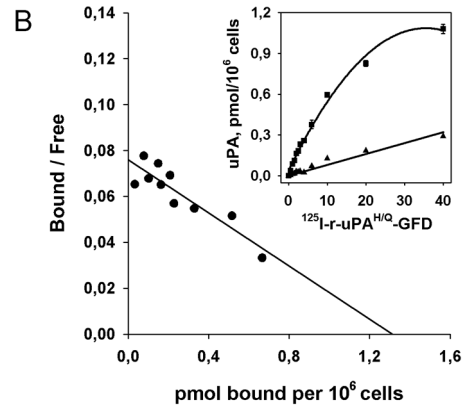


FIG. 5. **Scatchard plots of specific binding of ^{125}I-r-uPAwt and ^{125}I-uPA$^{H/Q}$-GFD to hAWSMC.** ^{125}I-r-uPAwt (*A*) or ^{125}I-r-uPA$^{H/Q}$-GFD (*B*) (0.5 to 40 nM) were added to hAWSMC and incubated for 2 h at 4 °C. Nonspecific binding was determined by adding a 50-fold excess of the same but unlabeled agent. The *insets* show the binding curve curves obtained in each instance. Nonspecific binding is represented by ▲, and total binding by ■. The mean ± S.E. of triplicate determinations from such an experiment is shown for each urokinase variant.

Fig. 5*A*).

In competitive binding studies where ^{125}I-r-uPAwt (0.5 nM) was added to the cell monolayers with r-uPA$^{H/Q}$-GFD indicated that it was unable to compete for binding; in contrast, half-maximal inhibition of ^{125}I-r-uPAwt was achieved with about 2 nM unlabeled r-uPAwt (Fig. 7*A*). Urokinase-PAwt, r-uPA$^{H/Q}$-GFD, r-KD, r-uPA^{LMW}, and denatured r-KD were also examined in competition studies with ^{125}I-r-uPAwt and ^{125}I-r-uPA$^{H/Q}$-GFD to further characterize the low affinity binding sites. The competitive isotherm generated, using ^{125}I-r-uPAwt (3 nM) and r-uPAwt and r-uPA$^{H/Q}$-GFD were very similar (Fig. 7*B*). Also, r-uPAwt and r-uPA$^{H/Q}$-GFD competed similarly with ^{125}I-r-uPA$^{H/Q}$-GFD (Fig. 7*C*). Recombinant-KD also competed for binding with ^{125}I-r-uPAwt and ^{125}I-r-uPA$^{H/Q}$-GFD, but was weaker, compared with either r-uPAwt or r-uPA$^{H/Q}$-GFD (Fig. 7, *B* and *C*). Neither reduced r-KD nor r-uPA^{LMW} were capable of displacing the ^{125}I-r-uPAwt or ^{125}I-r-uPA$^{H/Q}$-GFD from hAWSMC (Fig. 7, *B* and *C*). Thus, hAWSMC bind uPA via interaction with at least two different sites: the uPAR/CD87 that associates with its GFD, and a site on the membrane surface that interacts with uPA via its kringle domain. The binding of r-uPAwt and r-uPA$^{H/Q}$-GFD to the low affinity sites was specific only for uPA kringle, since other substances containing multiple kringle domain structures, tPA and plasminogen, in concentrations up to 1 μM did not compete for binding (Fig. 7*C*).

Binding of Kringle-containing Urokinase Variants to HEK 293 Cells and r-uPAR-HEK Cells Results in Promotion of Cellular Motility—The curvy nature of the Scatchard plot can reflect either two classes of binding sites or negative cooperativity within a single class of binding site. To demonstrate that the low affinity binding site represents a site distinct from uPAR/CD87, we next examined the binding of ^{125}I-r-uPAwt and ^{125}I-r-uPA$^{H/Q}$-GFD to human embryonic kidney cells (HEK 293 cells) which do not express uPAR. Both uPA variants bound to the HEK 293 cells with similar characteristics, ($K_d \sim$ of 12.5 nM and $B_{max} \sim$ 50,600 sites/cell for ^{125}I-r-uPAwt and $K_d \sim$ of 11.2 nM and $B_{max} \sim$ 45,800 sites/cell for ^{125}I-r-uPA$^{H/Q}$-

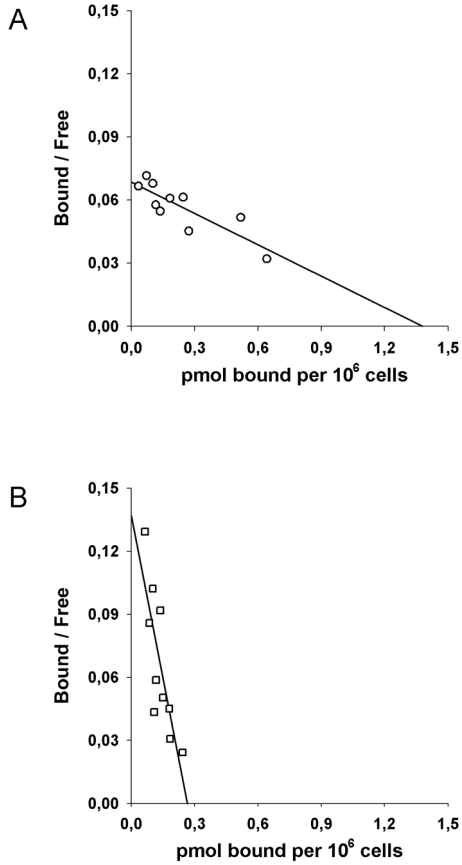


FIG. 6. **Effect of an anti-uPAR mAb and an anti-kringle mAb on ^{125}I-r-uPAwt specific binding to hAWSMC.** *Panel A,* ^{125}I-r-uPAwt was added to hAWSMC which had been preincubated for 1 h with anti-uPAR mAb (50 μg/ml) and its specific binding determined and analyzed according to Scatchard as described in the legend of Fig. 5. *Panel B,* ^{125}I-r-uPAwt was preincubated for 1 h with the anti-kringle mAb (40 μg/ml) then added to the hAWSMC which were preincubated for 1 h in culture medium (□). The Scatchard plot shows specific binding to a single site. Specific binding was defined as the difference between total radioligand bound and that bound in the presence of a 50-fold excess of (unlabeled) r-uPAwt.

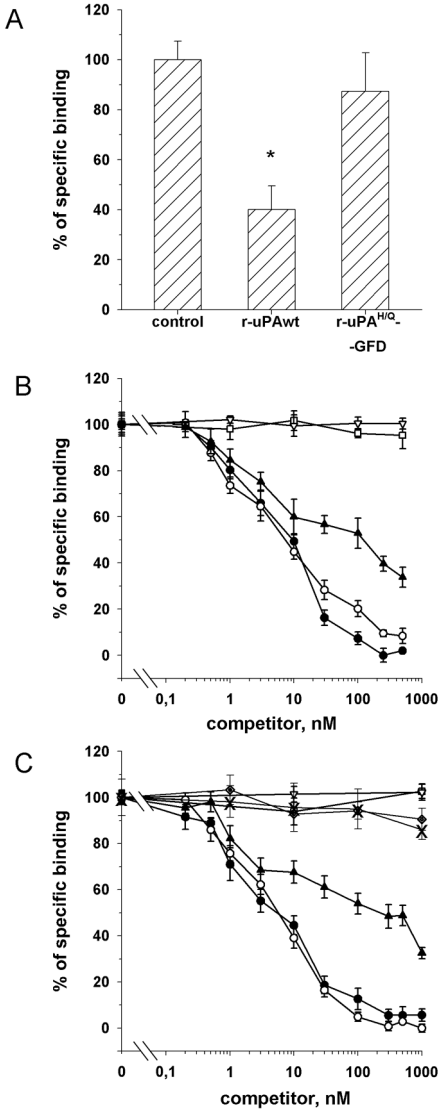
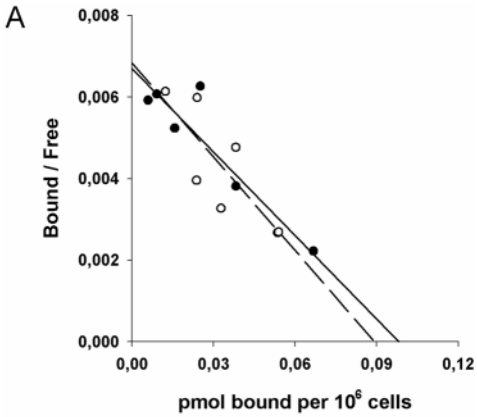


FIG. 7. **Competitive binding interactions between recombinant urokinase variants in hAWSMC.** *Panel A,* competitive binding isotherms generated by incubation of hAWSMC with ^{125}I-r-uPAwt (0.5 nM) and unlabeled r-uPAwt (2 nM) and r-uPA$^{H/Q}$-GFD (2 nM). Nonspecific binding was defined as ^{125}I-r-uPAwt bound in the presence of r-uPAwt (50 nM). *Panels B* and *C,* competitive binding isotherms between 3 nM ^{125}I-r-uPAwt (*B*) or 3 nM ^{125}I-r-uPA$^{H/Q}$-GFD (*C*) and unlabeled r-uPAwt (●), r-uPA$^{H/Q}$-GFD (○), r-uPA^{LMW} (□), r-KD (▲), reduced r-KD (▽), tPA (◇), and Plg (×). The hAWSMC were incubated for 2 h with ^{125}I-r-uPAwt or ^{125}I-r-uPA$^{H/Q}$-GFD in the absence or presence of the indicated urokinase variants. The results are expressed as percent of the specific binding, determined in the absence of the urokinase variants and shown as mean ± S.E. from six experiments.

GFD) (Fig. 8*A*). When HEK 293 cells expressing r-uPAR were used (Fig. 8*B, inset*), ^{125}I-r-uPAwt bound to two distinct sites ($K_{d,1}$~ of 2.0 nM and $B_{max,1}$ ~ 14,500 sites/cell, and $K_{d,2}$ ~ of 14.1 nM and $B_{max,2}$ ~ 49,400 sites/cell). The characteristics of ^{125}I-r-uPA$^{H/Q}$-GFD binding to the r-uPAR-HEK cells remained unaltered when compared with untransfected HEK cells (K_d ~ of 15.2 nM and B_{max} ~ 46,400 sites/cell) (Fig. 8*B*). Thus r-uPAwt and r-uPA$^{H/Q}$-GFD can associate with the cell surface of the uPAR-deficient HEK 293 cells in the similar way, but after transfection with the uPAR cDNA a high affinity binding site is observed for the full-length uPA, but not for uPA$^{H/Q}$-GFD. Because both r-uPAwt and r-uPA$^{H/Q}$-GFD are capable of binding to HEK293 cells, we asked whether the two uPA variants could induce cellular motility. Both r-uPAwt and r-uPA$^{H/Q}$-GFD stimulated HEK 293 cell migration across the filter in the Boyden chamber, in contrast to the uPA^{LMW} variant which was ineffective (Fig. 9). Thus, the uPA-kringle-binding target on HEK 293 cells is sufficient for the promotion of cell migration by the kringle-containing uPA variants, in the absence of uPAR.

DISCUSSION

Our experimental data indicate that human airway smooth muscle cell migration, induced by urokinase, is critically dependent on its kringle domain. Our finding that recombinant forms of uPA lacking both proteolytic activity and the uPAR-binding growth factor-like domain, as well as recombinant uPA-kringle polypeptides are able to induce cell migration, is the first available evidence for functional activity of urokinase kringle domain. We demonstrate an interdependence between the uPA-kringle domain-dependent interaction, and the association of uPA with uPAR in initiating the chemotactic action of the full-length uPA form. The binding of the monoclonal antibody UNG-5 to the kringle region and preventing the interaction between uPA and uPAR with the monoclonal antibody R3, inhibited uPA-induced hAWSMC migration. This latter observation is in agreement with previous reports which indicate that the induction of cell migration by uPA, DFP-inactivated uPA, and ATF are dependent on their interacting with the uPAR (6, 8, 22, 23, 39). These uPA variants possess the uPAR-



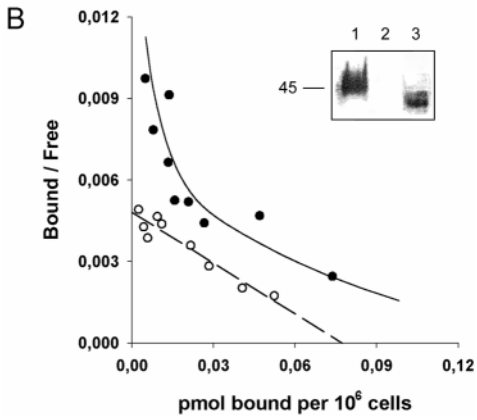


FIG. 8. **Scatchard plots of ^{125}I-r-uPAwt- and ^{125}I-uPA$^{H/Q}$-GFD-specific binding to HEK-control cells and r-uPAR-HEK cells.** 125I-r-uPAwt (●) and ^{125}I-r-uPA$^{H/Q}$-GFD (○) were added to control-transfected HEK 293 (*A*) or HEK 293 transfected with the uPAR cDNA (*B*), in concentrations ranging from 0.5 to 30 nM, then incubated for 2 h at 4 °C. Scatchard plots represent specific binding of the radioligands calculated by subtracting total radioligand binding from the nonspecific binding, determined in the presence of a 50-fold molar excess of unlabeled r-uPAwt or r-uPA$^{H/Q}$-GFD. *Inset,* Western blot of uPAR expressed by the hAWSMC (*lane 1*), control transfected HEK 293 cells (*lane 2*), and the HEK 293 transfected with the uPAR cDNA (*lane 3*). The cells were solubilized in 10 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride, then subjected to 10% SDS-PAGE, electroblotted onto nitrocellulose membrane, and probed with anti-uPAR mAb.

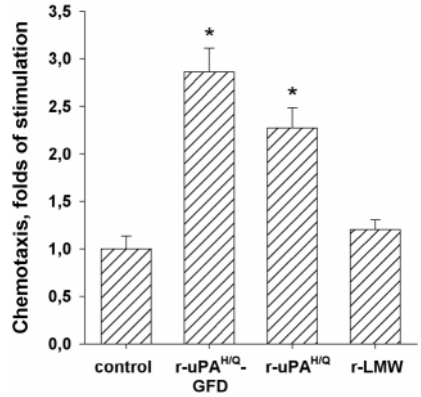


FIG. 9. **Chemotactic actions of urokinase variants on HEK 293 cells.** HAWSMC migration in the Boyden chamber, initiated by r-uPAwt, r-uPA$^{H/Q}$-GFD, and r-uPA^{LMW} (10 nM for all uPA-variants), and measured as described under "Experimental Procedures." Results are expressed as fold stimulation relative to HEK 293 cell migration in the absence of the uPA variants. Each point is the mean of two experiments performed in triplicate; *error bars* represent the S.E.

binding growth factor-like domain and the kringle domain. The present data demonstrates that deletion of the growth factor-like domain from uPA, and as a consequence, abolishing its normal ability to bind to uPAR with high affinity, does not abolish its chemotactic properties, demonstrated by the functional properties of the r-uPA$^{H/Q}$-GFD. Indeed, the concentrations at which half-maximal chemotactic effect were observed with the full-length r-uPAwt and r-uPA$^{H/Q}$-GFD were nearly identical. Thus, it is likely that the major mechanism responsible for uPA-induced cell motility involves signaling dependent on the kringle domain.

Kringle-like structures with homology to that found in urokinase are also present in a variety of other proteins, tPA (57), plasminogen (58), prothrombin (59), hepatocyte growth factor/scatter factor (60), as well as in apolipoprotein *a* (61). The kringle domains in these proteins are involved in interactions with other cell surface target proteins, annexin II (62, 63), cell surface actin (64), and the 45-kDa endothelial cell surface protein (65). In contrast to kringle domains of these proteins, there is limited information on the functional significance of uPA kringle domain, except the fact that the kringle domain is capable of binding to heparin (66). However, this is a very lower affinity interaction (K_a ~57 mM^{-1}), distinct from the kringle binding described in our study. It has been demonstrated that kringle-containing polypeptides, obtained by limited proteolysis of plasminogen, hepatocyte growth factor/scatter factor, and prothrombin can influence both cell migration and mitogenesis (46, 47, 67–71). It would appear that proteolytic processing of a kringle-containing proteins changes their ability to interact, a consequence of multiple fragments being generated, in comparison with the original molecule or the exposure of new epitopes on separate domains which can interact with other effector systems.

Such studies suggest the presence of sites in addition to uPAR on the hAWSMC as being important for uPA biological activities, in particular, the kringle domain. Binding experiments with r-uPAwt indicate the presence of two binding sites on the surface of the hAWSMC. The site exhibiting the higher affinity (K_d ~1.51 nM) appears to present the uPAR/CD87, since the interactions were prevented with the monoclonal uPAR antibody. Our results indicate that uPA binding to the sites exhibiting the lower affinity (K_d ~20.27 nM) was dependent on the presence of the kringle domain, since the binding of uPA to this site was abolished by the monoclonal antibody which interacts with the kringle region. These data combined with the fact that cell migration stimulated by uPA was attenuated in the presence of anti-kringle antibodies suggest that the binding of uPA via the kringle domain to the cell surface is a necessary prerequisite for uPA-induced cell migration. This interaction seems to be a unique feature of the kringle structure of uPA, since tPA and plasminogen which also contain kringle structures were unable to compete with either r-uPA$^{H/Q}$-GFD or r-uPAwt for binding to the membrane surface of hAWSMC.

To date the high affinity binding sites on SMC surface that interact with uPA kringle have not been characterized. They could represent a membrane protein, which specifically binds the urokinase kringle domain. It is also possible that the kringle-binding site is on the uPAR molecule. This possibility was explored by examining the binding of ^{125}I-r-uPAwt and ^{125}I-r-uPA$^{H/Q}$-GFD HEK 293 cells, which are devoid of uPAR, and comparing the binding with r-uPAR-HEK cells. The HEK 293

cells were able to bind r-uPAwt and r-uPA$^{H/Q}$-GFD in a similar fashion, via a single type of binding site. Transfection of HEK 293 cells with the uPAR cDNA resulted in appearance of an additional high affinity site for r-uPAwt. Furthermore, the binding characteristics of ^{125}I-uPA$^{H/Q}$-GFD to the r-uPAR-HEK 293 cells remained unaffected when compared with those from the untransfected cells or the cells transfected with the another unrelated (control) plasmid. Thus, a membrane target distinct from uPAR/CD87 is most likely responsible for the binding of r-uPAwt or r-uPA$^{H/Q}$-GFD via the kringle domain. Our findings indicate that HEK cells can respond chemotactically to either r-uPAwt or r-uPA$^{H/Q}$-GFD even in the absence of uPAR. Indeed, both kringle-containing uPA variants clearly promoted migration of HEK cells, while the catalytic portion of uPA (r-uPA^{LMW}) could not induce chemotaxis.

Finally, the question still remains as to whether the uPA-induced chemotaxis is entirely dependent upon the kringle domain. The kringle domain-containing uPA variants are chemotactic even for uPAR-deficient cells; and the uPA form lacking the GFD is chemotactic for uPAR-expressing cells. On the other hand, ability of the full-length uPA to induce chemotaxis in uPAR-expressing cells requires both the kringle- and uPAR-binding sites. Our findings that uPA might regulate cell motility via its kringle, apparently independently of the uPAR, implies an intrinsic duality of uPA actions on which cells express and do not express the uPAR. Clearly, the binding of uPA to uPAR-deficient cells as well as the signaling mechanisms that are responsible for cell migration requires further studies. The presence of uPAR on the cell surface and its ability to function either as an adhesion molecule (reviewed in Ref. 12) or a cell surface chemokine (reviewed in Refs. 72 and 73) may possibly provide some explanation into the mechanisms responsible for the interplay and interdependence of the uPAR and the uPA-kringle binding target in stimulating cell migration. One possibility is that although uPAR binds the uPA with high affinity, nevertheless, due to the lack of a transmembrane and a cytoplasmic domain, uPAR requires an additional partner to transduce the uPA chemotactic signal. One might suppose that uPA kringle-binding target, characterized in this study, could be such a partner for the uPAR-related signaling complex responsible for the uPA-mediated chemotactic stimulus. The binding of some growth factors to a signaling receptor through the agency of glycosylphosphatidylinositol-anchored molecule is not unusual. As was shown recently, neurotrophic factors (ciliary neurotrophic factor, neurturin, and glial cell-derived neurotrophic factor) bind to tandem "signaling" receptors and glycosylphosphatidylinositol-anchored receptor(s), a requirement for their activity (74–78). In uPAR-expressing cells such an interaction of uPA with uPAR and the kringle-binding target could result in exposure of an effector site on uPA molecule, responsible for initiating the chemotactic stimulus. It should not be over looked that the r-uPA$^{H/Q}$-GFD mimics the "conformationally activated" uPA. It is also possible that the uPA bound to the surface of uPAR-deficient cells undergoes some proteolytic modification, cleaving the GFD, thereby forming an "activated" uPA. Clearly further studies on the involvement of the urokinase kringle domain in uPA-induced chemotaxis has to be studied in conjunction with a greater understanding of the nature of the kringle-binding target and how uPA interacts with the surface of cells deficient in uPAR.

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The Chemotactic Action of Urokinase on Smooth Muscle Cells Is Dependent on Its Kringle Domain: CHARACTERIZATION OF INTERACTIONS AND CONTRIBUTION TO CHEMOTAXIS

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