Binding of High Molecular Weight Kininogen to Human Endothelial Cells Is Mediated via a Site within Domains 2 and 3 of the Urokinase Receptor

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Abstract

The urokinase receptor (uPAR) binds urokinase-type plasminogen activator (u-PA) through specific interactions with uPAR domain 1, and vitronectin through interactions with a site within uPAR domains 2 and 3. These interactions promote the expression of cell surface plasminogen activator activity and cellular adhesion to vitronectin, respectively. High molecular weight kininogen (HK) also stimulates the expression of cell surface plasminogen activator activity through its ability to serve as an acquired receptor for prekallikrein, which, after its activation, may directly activate prourokinase. Here, we report that binding of the cleaved form of HK (HKa) to human umbilical vein endothelial cells (HUVEC) is mediated through zinc-dependent interactions with uPAR. These occur through a site within uPAR domains 2 and 3, since the binding of ^{125}I-HKa to HUVEC is inhibited by vitronectin, anti-uPAR domain 2 and 3 antibodies and soluble, recombinant uPAR (suPAR), but not by antibody 7E3, which recognizes the β chain of the endothelial cell vitronectin receptor (integrin $\alpha_v\beta_3$), or fibrinogen, another $\alpha_v\beta_3$ ligand. We also demonstrate the formation of a zinc-dependent complex between suPAR and HKa. Interactions of HKa with endothelial cell uPAR may underlie its ability to promote kallikrein-dependent cell surface plasmin generation, and also explain, in part, its antiadhesive properties. (*J. Clin. Invest.* 1997. 100:1481–1487.)

Key words: plasminogen • vitronectin • prourokinase • prekallikrein • integrin $\alpha_v\beta_3$

Introduction

In the presence of zinc ions, high molecular weight kininogen (HK)[1] binds to endothelial cells specifically, saturably, and reversibly, with a K_d of 28–35 nM and B_{max} of 0.9–3.2 × 10^6 sites per endothelial cell (1–4). Cell-associated HK, or its cleaved, two-chain form, HKa, can serve as an acquired endothelial cell receptor for plasma prekallikrein or Factor XI (5–7), the latter of which is converted to Factor XIa by Factor XIIa, allowing the assembly and expression of an intrinsic Factor IX activator complex on endothelial cells (5). Prekallikrein bound to HK is converted to kallikrein on the endothelial surface (8), subsequently liberating bradykinin from HK and resulting in the generation of HKa (9). Bradykinin binds to endothelial B2 receptors (10), and may stimulate endothelial cell hyperpolarization (11), as well as the synthesis and release of prostacyclin (12), superoxide anion (13), tissue plasminogen activator (14), and nitric oxide (15). In comparison to HK, relatively little attention has been devoted to characterization of the binding of HKa to cells, though data from one report suggested that only ~ 25% as much HKa as HK specifically bound to endothelial cells (16). Whether these differences result from different affinities of these species for a single receptor, or binding to different receptors, has not been determined.

Structural determinants on HK which mediate its binding to endothelial cells have been described. HK is composed of six defined domains, denoted D1–D6 (17). The release of bradykinin (D4) results in the generation of HKa, which consists of an NH_2-terminal 65-kD heavy chain composed of three cystatin-like domains (D1, D2, and D3), and a COOH-terminal 56-kD light chain containing a histidine-glycine-lysine–rich domain (D5) and a prekallikrein and Factor XI binding domain (D6). Endothelial cell binding sites reside on both the heavy and light chains (18). One site on D3 (19), near the carboxy terminus of the heavy chain, has been localized to a 13–amino acid sequence containing a disulfide loop (20), while a second high-affinity binding site, located on D5 of the light chain (21), is essentially identical to one of the two sites which mediate the binding of HK to anionic artificial surfaces (22, 23). Additionally, a low-affinity site on D4 overlaps with the bradykinin sequence (16). The absence of this region in HKa has been suggested as a potential explanation for the differences in binding of HK and HKa to endothelial cells (16).

Through its ability to bind urokinase and focus the expression of cell surface plasminogen activator activity to discrete sites on the cell surface, the urokinase-type plasminogen activator receptor (uPAR) facilitates the generation of cell surface plasminogen activator activity (24, 25). This receptor plays a central role in diverse processes involving cellular migration, including angiogenesis (26, 27), formation of the placental vasculature after embryo implantation (28), tumor metastasis (29), and the migration of leukocytes into inflammatory foci.

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1. *Abbreviations used in this paper:* HK, high molecular weight kininogen; HKa, cleaved, bradykinin-free HK; HUVEC, human umbilical vein endothelial cells; GPI, glycosylphosphatidylinositol; PA, plasminogen activator; PI-PLC, phosphatidylinositol-specific phospholipase C; suPAR, a soluble recombinant form of uPAR; uPAR, urokinase-type plasminogen activator receptor.

The uPAR is a glycosylphosphatidylinositol (GPI)-linked protein consisting of three domains (30, 31). The NH_2-terminal domain, D1, at least when contained within the intact receptor, mediates high-affinity binding of pro- or two-chain urokinase (32–34), while domains 2 and 3 contain a binding site for, and mediate cellular adhesion to, vitronectin (35, 36).

In this report we demonstrate that the urokinase receptor mediates the binding of HKa to cultured human umbilical vein endothelial cells (HUVEC). HKa binds to a site within domains 2 and 3 of uPAR which is identical to or nearby that which mediates the binding of vitronectin. We also demonstrate that endothelial cell $\alpha_v\beta_3$ is not a receptor for HKa. These observations provide a potential explanation for the different binding properties of HKa and HK to endothelial cells (16), as well as the ability of HK to inhibit endothelial cell adhesion to vitronectin (37) and promote the expression of plasminogen activator activity on the endothelial cell surface (8).

Methods

Human fibrinogen, HK, and HKa were purchased from Enzyme Research Laboratories (South Bend, IN). Fibrinogen was homogeneous as judged by SDS-PAGE, and was plasminogen-free and > 95% clottable. HKa and prourokinase were radiolabeled with ^{125}I-Na (ICN Pharmaceuticals, Irvine, CA) using Iodogen or Iodobeads, respectively (Pierce Chemical Co., Rockford, IL) (38). The specific radioactivity of HKa, which retained > 95% of its procoagulant activity, varied from 0.5 to 1.5 mCi/μg. Vitronectin was obtained from GIBCO BRL (Gaithersburg, MD). A monoclonal antibody to the integrin β_3 chain, which inhibits vitronectin binding to $\alpha_v\beta_3$, the primary endothelial cell vitronectin receptor, as well as the binding of fibrinogen to its receptor on platelets ($\alpha_{IIb}\beta_3$), was kindly supplied as purified IgG by Dr. Barry Coller (Mt. Sinai Medical School, New York).

Expression of soluble recombinant uPAR (suPAR). Human suPAR cDNA (encoding aa 1–281 with the signal peptide) was prepared from U937 cells by RT-PCR and cloned into pDEMp, an expression vector for murine hybridoma cells. This vector was transfected into the murine hybridoma cell line Sp2/0 and the clones were selected by growing the cells in DMEM containing 10% FBS and increasing concentrations of methotrexate (150 nM to 1 μM). The concentration of suPAR secreted into the medium was determined by ELISA (American Diagnostica, Greenwich, CT). Large scale expression was accomplished by growing the stably transfected cells in spinner flasks containing serum-free medium (Gibco Laboratories, Grand Island, NY). Media from large scale cultures (100 liters, 20–40 mg suPAR/liter) were obtained on day 8 after seeding, stabilized with protease inhibitors (5 mM EDTA, 100 μM PMSF, 5 μM aprotinin, 5 μM leupeptin), concentrated, and stored at −70°C until purified.

To isolate suPAR, pro-uPA (500 mg) was immobilized on CNBr-agarose, and equilibrated with PBS containing 10 μM EDTA. Concentrated medium (200 ml) was applied to the column, which was then sequentially washed with PBS, PBS containing 1 M KCl, and PBS alone until the A_{280} of the flow-through returned to 0. suPAR was then eluted using 0.2 M glycine, pH 3.0. The eluted protein was gel-filtered on Sephacryl S-300 (Pharmacia, Piscataway, NJ), and migrated as a single band between 35 and 45 kD on SDS-PAGE.

Production of uPAR domain 2 and 3–specific polyclonal antibodies. suPAR was cleaved using chymotrypsin (5,000:1 molar ratio) for 24 h at 4°C, as previously described (32). The uPAR domain 1 was separated from domains 2 and 3 (aa 88–281) using Sephadex-G50 equilibrated with PBS. Further purification of each domain was achieved using C8 reverse-phase chromatography, with a linear gradient of 0–70% acetonitrile/0.1% TFA over 60 min. Purified uPAR domains 1, 2, and 3 were then used to generate domain-specific polyclonal antibodies in rabbits. Immunoglobulins were isolated from the serum of immunized rabbits by ammonium sulfate precipitation, followed by affinity chromatography on a suPAR-Sepharose column.

Isolation and culture of endothelial cells. HUVEC were isolated as described (39, 40), and cultured in medium 199 containing 10% FBS, 100 μg/ml endothelial cell growth factor [purified as described by Maciag (41)], 2 mM glutamine, and penicillin-streptomycin (Mediatech, Herndon, VA). All studies were performed using HUVEC of passage 3 or less. Before binding studies, cells were released from culture dishes using trypsin-EDTA (Mediatech), then replated and cultured until confluent in fibronectin-coated 96-well Immulon II plates (Dynatech, McLean, VA), which caused less nonspecific binding of ^{125}I-HKa than standard 96-well polystyrene tissue culture plates. At confluence, each well of the 96-well plate contained a mean of 35,000±5,000 cells per well. In selected experiments, HUVEC were preincubated at 37°C with 1 U/ml phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma, St. Louis, MO) which cleaves the glycophosphatidylinositol linkage of uPAR to the cell membrane, for 2 h before binding experiments. The efficacy of uPAR release after PI-PLC treatment was confirmed by measuring the specific binding of ^{125}I-prourokinase to control and PI-PLC–treated cells.

Binding of ^{125}I-HKa and ^{125}I-prourokinase to endothelial cells. After achieving confluence, HUVEC were chilled to 4°C. Conditioned medium was then removed, and the cells were preincubated for 20 min in cold Hepes buffer (10 mM Hepes, 137 mM NaCl, 4 mM KCl, 11 mM glucose, 0.5 mg/ml fatty acid–free BSA, and 1 mM $CaCl_2$, pH 7.35) containing (for total binding) or lacking (for nonspecific binding) 50 μM $ZnCl_2$. The binding of ^{125}I-HKa, diluted in 100 μl of Hepes buffer (in the absence or presence of 50 μM $ZnCl_2$), to individual wells of HUVEC was then determined. After a 60-min incubation period, the concentration of "free" ligand in each well was determined by measuring the radioactivity in a 10-μl aliquot of the binding solution, and the cell-associated radioactivity by solubilizing the cells in 150 μl of 1% SDS after washing twice with Hepes buffer. Specific binding was defined as the difference between total and nonspecific (no added $ZnCl_2$) binding. Data obtained in preliminary studies agreed with those of previous reports (1–4), in which it was observed that the binding of ^{125}I-HKa in the absence of $ZnCl_2$ and in the presence of a 50-fold molar excess of HKa, was equivalent, and hence representative of nonspecific binding. In selected studies, the effects of specific antibodies or other putative inhibitors of ^{125}I-HKa binding to cells were assessed by preincubating cells for 60 min with the test reagent before addition of ^{125}I-HKa. Specific inhibitors which were tested included fibrinogen (0–3,500 nM), vitronectin (0–180 nM), mAb 7E3 (0–2,000 nM), prourokinase (0–500 nM), and soluble urokinase receptor (0–600 nM). Total or specific binding for each experiment is expressed as picomoles of HKa bound, normalized per 10^6 cells. For studies in which the effects of competitors on the binding of a fixed concentration of ^{125}I-HKa were assessed, the data are expressed as percentage of the original ligand bound in the absence of competitor.

The binding of ^{125}I-prourokinase to endothelial cells was determined in a similar manner, though these studies were performed in PBS containing 1% BSA, with nonspecific binding determined in the presence of a 100-fold molar excess of unlabeled prourokinase, as previously described (42).

Demonstration of a ^{125}I-HKa–suPAR complex by gel filtration. G-200 Superfine Sepharose (Pharmacia) was packed in a 0.9 × 60 cm polystyrene column. The gel was equilibrated in a buffer consisting of 0.01 M Hepes, 0.137 M NaCl, 0.004 M KCl, 0.011 M glucose, 0.001 M $CaCl_2$, and 50 μM $ZnCl_2$, pH 7.35 (equilibration buffer). Mixtures of ^{125}I-labeled HKa or HK (65–75 nM) and unlabeled suPAR or suPAR domains 2 and 3 (1 μM) were incubated for 60 min at room temperature in the equilibration buffer containing 500 μg/ml BSA, used as a carrier protein. To determine whether complex formation between test proteins was zinc dependent, control experiments were performed in an identical manner, but with $ZnCl_2$ omitted from the equilibration buffer. To confirm that complex formation was not due to the association of ^{125}I-HKa with BSA, control experiments were

performed in which prourokinase (550 μg/ml) was substituted for BSA as a carrier protein. After the 1-h incubation, chromatography was performed at room temperature with a stable upward flow rate of 4 ml/h, maintained using a P-1 peristaltic pump (Pharmacia). 0.5-ml fractions were collected in an LKB 2112 Redirack fraction collector and analyzed for radioactivity after their collection.

The elution volume of ^{125}I-labeled HKa or HK is expressed as the Kav, which represents the fraction of the stationary gel volume available for diffusion of a given solute species. As defined by Ackers (43), $Kav = Ve - Vo/Vt - Vo$, where Ve is the elution volume of the sample, Vt is the total volume of the packed bed, and Vo is the void volume of the column, determined by measuring the elution volume of ^{125}I-labeled fibrinogen in independent experiments. The significance of differences in the Kav for ^{125}I-HKa or ^{125}I-HK under differing experimental conditions was determined using the Student's *t* test for paired samples.

Results

Evaluation of the endothelial cell vitronectin/fibrinogen receptor ($\alpha_v\beta_3$) as an HKa binding site. Under our experimental conditions, the binding of HKa was linear over a concentration range of 10–80 nM, with nonspecific binding (determined in the absence of $ZnCl_2$) constituting < 20% of total binding. Therefore, we selected an HKa concentration of 70 nM to use in competitive binding studies in order to detect concentration-dependent inhibition of binding. Since HK had been shown previously to bind to $\alpha_M\beta_2$ (Mac-1), a neutrophil integrin receptor which binds fibrinogen (5), we initially postulated that a related receptor on endothelial cells, $\alpha_v\beta_3$ (44), might also function as a receptor for HKa. To address this hypothesis, we assessed the ability of vitronectin to compete with ^{125}I-HKa for binding to HUVEC. We observed that over a concentration range of 20–175 nM, vitronectin competitively inhibited the binding of 70 nM ^{125}I-HKa (Fig. 1), with complete inhibition obtained at a concentration of 150 nM. These results were consistent with inhibition of binding of ^{125}I-HKa to $\alpha_v\beta_3$ by vitronectin. To further investigate this hypothesis, we determined whether fibrinogen, another $\alpha_v\beta_3$ ligand, also inhibited the binding of HKa to HUVEC. Despite the fact that ^{125}I-fibrinogen bound to endothelial cells saturably over a range of 50–400 nM (not shown), unlabeled fibrinogen did not inhibit the binding of 70 nM ^{125}I-HKa to HUVEC, even at concentrations up to 3,500 nM, a 50-fold molar excess relative to the concentration of ^{125}I-HKa (Table I).

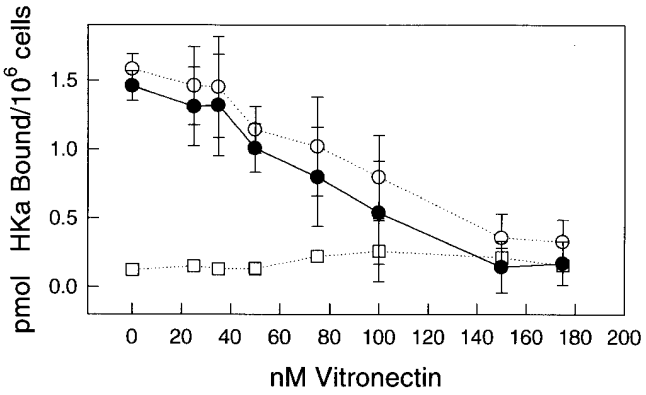

Figure 1. ^{125}I-HKa binding to HUVEC in the presence of increasing concentrations of vitronectin. Mixtures of ^{125}I-HKa (70 nM) and various concentrations of vitronectin (0–175 nM) were incubated with HUVEC for 60 min at 4°C and binding was measured as described in Methods. Total (*open circles*), nonspecific (*open squares*), and specific (*filled circles*) binding are depicted. Each data point represents the mean±SD of triplicate determinations. This figure is representative of three experiments performed.

Table I. Effect of Fibrinogen, mAb 7E3, and Prourokinase on Binding of ^{125}I-HKa to HUVEC

Fibrinogen		mAb 7E3 (A) and control mouse IgG (B)			Prourokinase	
Conc (nM)	% control	Conc (nM)	% control (A)	% control (B)	Conc (nM)	% control
0	100±17	0	100±19	100±25	0	100±20
140	84±15	133	106±17	104±27	50	136±13
420	90±16	533	101±21	107±25	100	104±14
560	91±9	933	98±21	117±35	200	119±6
910	88±6	1333	116±25	91±25	300	123±11
1400	96±11	1600	119±20	85±24	500	88±9
3500	107±30	2000	60±10	97±12		

Data represent the mean±SEM of triplicate points of at least two experiments, and are depicted as the percent binding relative to that in the absence of the test protein. *Conc*, concentration.

Since these results did not support the hypothesis that ^{125}I-HKa bound to $\alpha_v\beta_3$, we further investigated the potential interactions of HKa with this receptor by determining whether mAb 7E3, which is directed to the β_3 integrin chain and inhibits the binding of fibrinogen and vitronectin to $\alpha_v\beta_3$, inhibited the binding of ^{125}I-HKa to HUVEC. We observed that 7E3 did not significantly inhibit the binding of ^{125}I-HKa to HUVEC, even at concentrations up to 2,000 nM (a 29-fold molar excess) (Table I). Thus, the results of these latter experiments suggested that the observed inhibition of ^{125}I-HK binding to HUVEC was mediated through interactions with a receptor distinct from $\alpha_v\beta_3$.

Binding of HKa to the endothelial cell urokinase receptor. Previous studies have demonstrated that in addition to $\alpha_v\beta_3$, vitronectin binds to the urokinase receptor at a site contained within domains 2 and/or 3 of uPAR (35, 36). This site is functionally distinct from uPAR domain 1, which binds pro- and two-chain urokinase (32, 33). Since uPAR are expressed by HUVEC (45–47), we hypothesized that the observed inhibition of HKa binding to these cells might involve interactions with this alternative vitronectin binding site. To address this issue, we first determined whether prourokinase affected the binding of ^{125}I-HKa to endothelial cells. Consistent with our hypothesis that HKa did not interact with domain 1 of uPAR, we observed no inhibition of its binding to endothelial cells by prourokinase, even at prourokinase concentrations of up to 500 nM (Table I). Next, we determined whether the binding of ^{125}I-HKa to HUVEC was inhibited by affinity-purified polyclonal antibodies raised against domains 2 and 3 of uPAR. These antibodies blocked the binding of 70 nM ^{125}I-HKa to HUVEC in a concentration-dependent manner (IC_{50} = 180 nM), with essentially complete inhibition achieved at an antibody concentration of 700 nM (~ 100 μg/ml) (Fig. 2). No inhibition of ^{125}I-HKa binding to HUVEC was caused by similar concentrations of nonimmune rabbit IgG. These observations

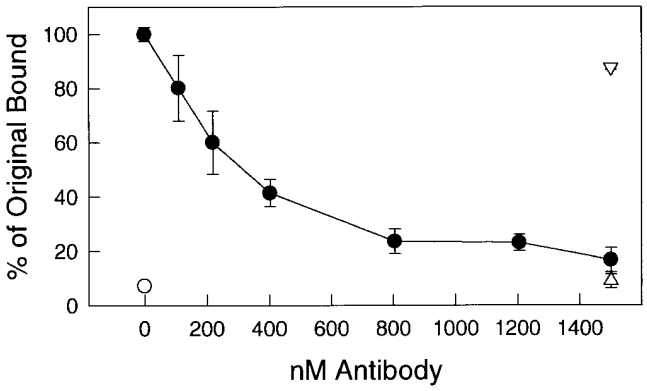

Figure 2. ^{125}I-HKa binding to HUVEC in the presence of increasing concentrations of rabbit polyclonal antibody specific to domains 2 and 3 of uPAR. HUVEC were incubated with various concentrations of anti-D2+D3 antibody (0–1,500 nM) or control rabbit IgG for 30 min at 4°C before the addition of 70 nM ^{125}I-HKa. Incubation was continued for an additional 60 min, and binding measured as described in Methods. Total binding in the presence of 50 μM $ZnCl_2$ (*filled circles*); nonspecific binding in the absence of $ZnCl_2$ (*open circle*); total binding in the presence of control antibody and 50 μM $ZnCl_2$ (*triangle pointing down*); nonspecific binding in the presence of control antibody, in the absence of $ZnCl_2$ (*triangle pointing up*). The data represent the mean±SD of triplicate points from a single experiment representative of two performed.

suggested that HKa bound to a site within domains 2 and 3 of uPAR which was identical or closely apposed to the site which mediates the binding of vitronectin (35, 36).

To further address this possibility, we determined whether suPAR, containing amino acids 1–281 of the native receptor (truncated 2 amino acids NH_2-terminal to the GPI linkage site), inhibited the binding of ^{125}I-HKa to HUVEC. We observed that preincubation of 70 nM ^{125}I-HKa with suPAR, in the presence of 50 μM $ZnCl_2$, reduced its subsequent binding to HUVEC by 85% (Fig. 3). The IC_{50} for this interaction was 80 nM. These observations further support the hypothesis that HKa interacts directly with uPAR, probably with 1:1 stoichiometry.

Effect of preincubation of endothelial cells with PI-PLC on binding of ^{125}I-HKa. The urokinase receptor is a GPI-linked protein, and previous studies have demonstrated that this receptor can be released from cell surfaces by treatment with PI-PLC (31). Therefore, to further assess the role of uPAR in binding of ^{125}I-HKa, we measured binding after preincubation of HUVEC with PI-PLC. The specific binding of ^{125}I-prourokinase was measured in parallel to determine the efficiency of uPAR release.

Pretreatment of HUVEC with PI-PLC caused an 87% reduction in the specific binding of ^{125}I-prourokinase, as expected. A parallel reduction was observed in the specific binding of ^{125}I-HKa to the treated cells, although its magnitude was slightly less than that observed for ^{125}I-prourokinase binding (Fig. 4).

Demonstration of complex formation between HKa and uPAR domains 2 and 3. To this point, our studies suggested that HKa bound directly to a site within domains 2 and 3 of endothelial cell uPAR. To further confirm this hypothesis, we determined whether HKa and suPAR interacted directly in a cell-free system. Gel-sieving chromatography, a method previously used to demonstrate the complexation of HK with kallikrein (1478), was used to illustrate the formation of complexes between HKa and suPAR. Incubation of 65 nM ^{125}I-HKa ($M_r \sim 120{,}000$) with 1 μM suPAR ($M_r \sim 55{,}000$), in the presence of 50 μM $ZnCl_2$, led to the formation of a high molecular weight complex which eluted near the void volume of the column (Table II). The Kav for this complex was 0.167±0.008, and was significantly different from that of HKa alone, whether measured in the absence (0.218±0.013) or presence (0.232±0.006) of $ZnCl_2$. The zinc dependence of this complex was confirmed by the observation that the formation of a high molecular weight complex between ^{125}I-HKa and suPAR did not occur in the absence of $ZnCl_2$ (Kav 0.218±0.013). Furthermore, formation of the complex was not due to nonspecific as-

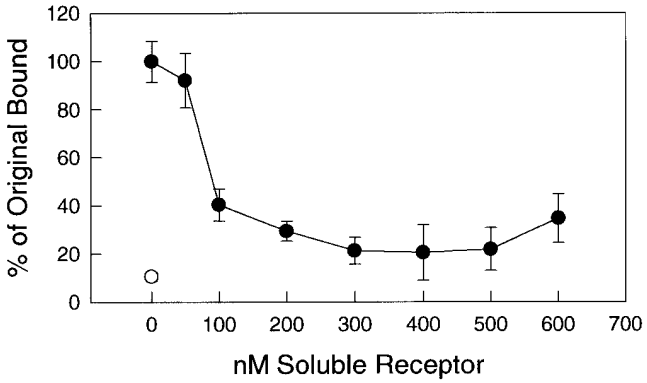

Figure 3. ^{125}I-HKa binding to HUVEC in the presence of increasing concentrations of suPAR. Mixtures of ^{125}I-HKa (70 nM) and various concentrations of suPAR (0–600 nM) were preincubated at room temperature for 60 min, then chilled to 4°C. The mixtures were then added to HUVEC and incubated for 60 min at 4°C. Binding was measured as described in Methods. Total binding in the presence of 50 μM $ZnCl_2$ (*filled circles*); nonspecific binding in the absence of $ZnCl_2$ (*open circle*).

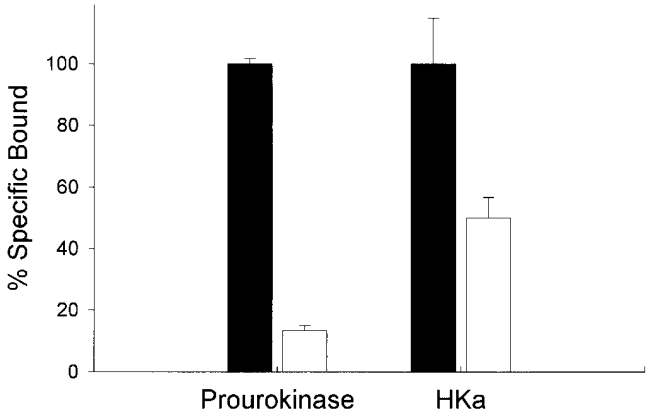

Figure 4. Effect of PI-PLC treatment on the binding of ^{125}I-HKa and ^{125}I-proUK to HUVEC. Cells were preincubated with 1 U/ml of PI-PLC as described in Methods. The total and nonspecific binding of each of the radiolabeled ligands to control and PI-PLC–treated cells was then determined, and the specific binding, displayed in the figure, was calculated. These data represent the mean±SEM of quadruplicate points from a single experiment representative of three performed.

Table II. Analysis of HK(a)–uPAR Complex Formation by Gel Filtration

	Condition	Kav*	*n*	*P*‡
1	HKa + Zn^{2+}	0.232±0.006	5	
2	HKa + Zn^{2+} + suPAR	0.167±0.008	6	< 0.001 (vs. 1)
3	HKa − Zn^{2+}	0.218±0.013	4	
4	HKa − Zn^{2+} − suPAR	0.212±0.001	3	0.695 (vs. 3)
				0.007 (vs. 2)
5	HKa + Zn^{2+} (proUK)§	0.223	1	
6	HKa + Zn^{2+} + suPAR (proUK)	0.170	2	0.829 (vs. 2)
7	HK + Zn^{2+}	0.266	1	
8	HK + Zn^{2+} + suPAR	0.271	2	
9	HK − Zn^{2+}	0.234	2	
10	HK − Zn^{2+} + suPAR	0.223±0.006	3	

*Kav (defined in Methods) is depicted as the mean±SEM of *n* experiments. §ProUK depicts experiments in which prourokinase (550 μg/ml) was substituted for BSA as a carrier protein. ‡*P* values were derived using the Student's *t* test for paired samples.

sociation of HKa with the BSA carrier protein, since complex formation was not observed in the absence of suPAR and $ZnCl_2$, regardless of whether BSA or prourokinase was used as the carrier protein. Complex formation also did not occur when uncleaved ^{125}I-HK was substituted for ^{125}I-HKa, further demonstrating the specificity of the interaction between suPAR and HKa (Table II). Finally, a high molecular weight complex with a Kav similar to that of the HKa–suPAR complex was observed after incubation of ^{125}I-HKa with isolated uPAR domains 2 and 3, demonstrating that the structural information necessary for HKa binding is contained within this region of the receptor (Fig. 5).

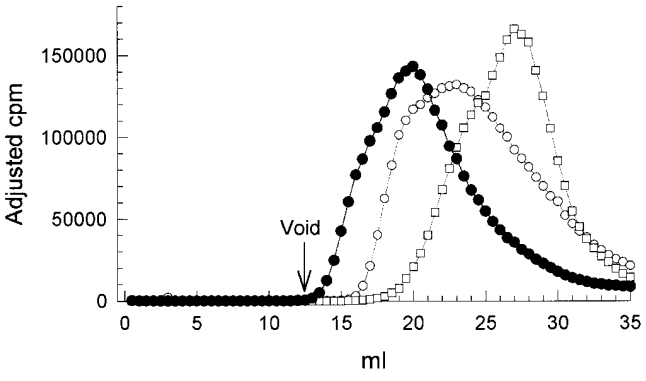

Figure 5. Gel filtration chromatography of HKa and suPAR domains 2 and 3. G-200 Sepharose Superfine was packed into a 0.9 × 60 cm polystyrene column and equilibrated with a buffer containing 50 μM $ZnCl_2$ (see Methods). A stable flow rate of 4 ml/h was maintained in an upward direction using a P-1 peristaltic pump (Pharmacia). A 150-μl volume of each sample was injected, and 0.5-ml fractions were collected from the time of injection. Symbols are as follows: *open squares*, 340 nM ^{125}I-suPAR; *open circles*, 18 nM ^{125}I-HKa; and *filled circles*, 18 nM ^{125}I-HKa + 7.2 μM suPAR domains 2 and 3. The void volume was determined using ^{125}I-labeled fibrinogen.

Discussion

The binding of HK and HKa to endothelial cells has been appreciated for many years, although the cellular binding sites which mediate these interactions have not been identified. The studies described above suggest that at least one such binding site for the latter is the urokinase receptor. This conclusion is based on several observations. First, the binding of ^{125}I-HKa to endothelial cells was inhibited by vitronectin, suggesting that HKa and vitronectin compete for the same binding site on the endothelial cell surface. One site to which both of these ligands might bind is the vitronectin receptor $\alpha_v\beta_3$. However, the binding of HKa to endothelial cells was not inhibited by either a known $\alpha_v\beta_3$ ligand, fibrinogen, or a specific anti-β_3 integrin monoclonal antibody known to inhibit vitronectin binding to $\alpha_v\beta_3$, suggesting that the vitronectin receptor does not mediate the binding of HKa to endothelial cells. In contrast, the binding of ^{125}I-HKa to endothelial cells was inhibited by both polyclonal antibodies raised against domains 2 and 3 of the urokinase receptor, as well as the recombinant, soluble receptor itself. Furthermore, the binding of HKa to HUVEC was reduced after pretreatment of cells with PI-PLC, which releases the GPI-linked uPAR from the cell surface. These observations suggest a direct interaction of HKa with a binding site within domains 2 and 3 of uPAR, which is likely identical to that previously shown to mediate the binding of vitronectin (35, 36). This hypothesis is strengthened by the demonstration of a zinc-dependent interaction between ^{125}I-HKa and both suPAR and uPAR domains 2 and 3 in a cell-free system, as evidenced by an increase in molecular size (decreased Kav) on gel filtration (Table II). Of interest is the fact that we were unable to demonstrate the formation of a complex between ^{125}I-labeled HK and suPAR. This observation suggests that uncleaved HK does not bind to uPAR, or does so less avidly than HKa, with the lower affinity complex dissociating during gel filtration. Consistent with the results of previous studies that demonstrated, using circular dichroism (48) and electron microscopy (49), that HK undergoes extensive conformational change upon its activation to HKa, it is notable that the apparent molecular size of HK, as assessed by gel filtration, differed from that of HKa (Table II). Such considerations make it plausible that the primary endothelial receptor for HK may be a protein other than uPAR. After the activation of cell bound HK to HKa, the latter might then bind with greater affinity to the urokinase receptor.

Recently, Herwald et al. (50) isolated a protein from EAHY cells, identified as the gC1q receptor, which bound to immobilized HK (51). Joseph et al. (52) have also demonstrated that this protein serves as a binding site for HK on HUVEC. The latter, but not the former, group reported that the binding of HK to the gC1q-R was zinc dependent (52), and also that the binding of ^{125}I-labeled HK to HUVEC could be inhibited by selected antibodies raised against this protein, although C1q itself did not compete for binding. The relationship between this binding site and uPAR is uncertain. HK appears to have different receptors on different cell types; for example, it binds in a zinc-dependent manner to Mac-1 (CD11b/CD18) on neutrophils (53) and glycoprotein Ib on unactivated platelets (54). On activated platelets, HK binds to cell-associated thrombospondin, released from α-granules after platelet activation, although zinc dependence of this interaction has not been demonstrated (55).

The inability of pretreatment of HUVEC with PI-PLC to reduce the binding of HKa to the same extent as prourokinase suggests that an additional endothelial binding site for HKa, in addition to uPAR, may exist. However, the essentially complete inhibition of specific HKa binding by antibodies directed against uPAR domains 2 and 3 or soluble recombinant uPAR suggests that such a site might, under normal conditions, either exist in a complex, or at least in close spatial proximity to uPAR, and therefore be sterically hindered by reagents which bind to uPAR domains 2 and 3. Such a site might thus appear more prominent after PI-PLC treatment of cells. A precedent for such an association is the Mac-1–uPAR complex on monocytes (56), and direct associations of uPAR with other cellular integrins have also been demonstrated (57). Regardless, our studies expand knowledge concerning cellular kininogen binding sites by suggesting that different species of HK, specifically the uncleaved and cleaved forms, might bind to different primary sites on the same cell.

The potential physiological implications of interactions between HKa and uPAR are numerous. One implication concerns the capacity of the contact activation system to facilitate the expression of cell surface plasminogen activator activity. A major role of uPAR is to focus the expression of cell surface plasminogen activator activity to discrete areas on the cell surface (25, 29, 58). Ellis et al. have shown that the u-PA–mediated activation of plasminogen is greatly facilitated in the presence of uPAR-expressing U937 cells, the effect of which is primarily to increase the catalytic efficiency (k_{cat}/K_m) of the reciprocal reaction involving plasmin-mediated activation of prourokinase (59). However, receptor-bound prourokinase may also be directly activated by kallikrein, which binds with high affinity to endothelial cells via interactions with cell-bound HK or HKa. We have shown recently that preincubation of HUVEC with HK and kallikrein enhances the ability of these cells to promote the generation of cell surface plasmin, and that this enhancement may be blocked by peptides which inhibit the binding of kallikrein to cell-bound HK(a) (60). Based on these results, as well as the studies presented in this report, we hypothesize that promotion of cell surface plasminogen activator activity by HK and kallikrein results from the formation of a multiprotein complex which includes these two proteins, as well as uPAR and proUK. The formation of this complex may be initiated by binding of HKa, perhaps generated from cell-bound HK by the actions of either an endothelial-derived cysteine protease (8), or Factor XIIa (61), to uPAR domains 2 and 3. HKa may then function as an acquired receptor for kallikrein, which, as a consequence of its favorable spatial localization relative to uPAR domain 1, may directly activate uPAR-bound prourokinase.

Yet another process which may be influenced by the binding of HK to uPAR is that of cell adhesion. In addition to its role in facilitating the expression of cell surface plasminogen activator activity, the urokinase receptor may promote cellular adhesion by mediating the binding of cells to vitronectin (35, 36). Asakura et al. (37) have demonstrated the ability of HKa, but not uncleaved HK, to inhibit the adhesion and spreading of endothelial, osteosarcoma or melanoma cells on vitronectin, as well as other substrates. However, the mechanisms accountable for these effects were not defined, and we propose that the ability of HKa to compete for vitronectin binding sites on uPAR domains 2 and 3 may be involved in inhibition of cellular adhesion to vitronectin. This hypothesis, if confirmed, would suggest that through its ability to promote kallikrein-mediated prourokinase activation and inhibit cellular adhesion to matrix-associated vitronectin, HK may play a prominent role in processes involving cellular migration and/or invasion, such as angiogenesis, tumor metastasis, placentation, and the migration of leukocytes into inflammatory foci. Our model also suggests that the antiadhesive effects of HKa and plasminogen activator inhibitor type 1 (PAI-1), which has been shown recently to inhibit uPAR-dependent cellular adhesion by competing with uPAR for binding to the same region of the vitronectin somatomedin B domain (62), may be mediated through a similar pathway.

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