MAPPING THE INTERACTION BETWEEN HIGH MOLECULAR WEIGHT KININOGEN AND THE UROKINASE PLASMINOGEN ACTIVATOR RECEPTOR

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Running Title: HK Binding Site(s) on uPAR

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SUMMARY

The urokinase plasminogen activator receptor (uPAR) is a multifunctional, GPI-linked receptor that modulates cell adhesion/migration and fibrinolysis. We mapped the interaction sites between soluble uPAR (suPAR) and high molecular weight kininogen (HK). Binding of biotin-HK to suPAR was inhibited by HK, 56HKa, and 46HKa with an IC$_{50}$ of 60, 110, and 8 nM, respectively. We identified two suPAR binding sites, a higher affinity site in the light chain of HK and 46HKa (H$^{477}$-G$^{496}$) and a lower affinity site within the heavy chain (C$^{333}$-K$^{345}$). HK predominantly bound to suPAR fragments containing domains 2 and 3 (S-D2D3). Binding of HK to domain 1 (S-D1) was also detected and addition of S-D1 to S-D2D3 completely inhibited biotin-HK or -46HKa binding to suPAR. Using sequential and overlapping 20 amino acid peptides prepared from suPAR, two regions for HK binding were identified. One on the carboxyterminal end of D2 (L$^{166}$-T$^{195}$) blocked HK binding to suPAR and to human umbilical vein endothelial cells (HUVEC). This site overlapped with the urokinase binding region and urokinase inhibited the binding of HK to suPAR. A second region on the aminoterminal portion of D3 (Q$^{215}$-N$^{255}$) also blocked HK binding to HUVEC. Peptides that blocked HK binding to uPAR also inhibited PK activation on HUVEC. Therefore, HK interacts with suPAR at several sites. HK binds to uPAR as part of its interaction with its multiprotein receptor complex on HUVEC and the biological functions that depend upon this binding are modulated by urokinase.
INTRODUCTION

Recent investigations indicate that high molecular weight kininogen (HK\textsuperscript{1}) binds to endothelial cell membranes through an interaction with a multiprotein receptor complex comprising at least cytokeratin 1 (CK1), gC1qR, and the urokinase plasminogen activator receptor (uPAR) (1-5). The three proteins co-localize on the endothelial cell membrane (6). The same three proteins form a receptor complex for factor XII (7), but binding of factor XII \textit{in vivo} is likely limited both by the low plasma concentration of free Zn\textsuperscript{2+}, which is below the requirement for FXII binding, and by the much higher plasma concentration of HK (7). Binding of HK to this multiprotein receptor complex predominates, localizing prekallikrein (PK) to the cell surface. The plasma concentration of PK and the ambient free Zn\textsuperscript{2+} concentration in plasma also prevent FXI from binding to HK under conditions where platelets or other cells are not activated (8). PK bound to HK on endothelial cells is proteolyzed by membrane-expressed prolylcarboxypeptidase to form plasma kallikrein (9,10). This multiprotein receptor complex thereby regulates the assembly and activation of the plasma kallikrein/kinin system.

The requirements for HK binding to each component of this receptor complex and the effect of other biologically relevant ligands, e.g. urokinase, on this binding, has not been well delineated. It is known that both the heavy and light chains of HK interact with a region of CK1 coded by exon 1 (2). Antibody to this region completely inhibits HK binding to CK1 as well as to the receptor complex (6). Likewise, some antibodies to gC1qR and uPAR completely block HK binding to the proteins individually as well as when they are part of the complex expressed on endothelial cells (6,8). However, it is unclear whether each component of the cellular complex recognizes discrete or overlapping portions of HK and whether each molecule of HK binds to more than one component of the complex at the same time. To begin to address these issues, we sought to identify the regions in HK and uPAR required for binding. The results
indicate the existence of multiple potential sites of interaction between this ligand and receptor and provide insight into the assembly of HK on its multireceptor complex and the influence of urokinase on this interaction.
EXPERIMENTAL PROCEDURES

**Proteins and materials:** Single-chain and two-chain HK (specific activity of 13-17 U/mg and 17 U/ml, respectively) in 4 mM sodium acetate-HCl and 0.15 M NaCl, pH 5.3, plasma kallikrein, and prekallikrein (PK) (specific activity of 22-27 U/mg) in 4 mM sodium acetate-HCl and 0.15 M NaCl, pH 5.3 were purchased from Enzyme Research Laboratories, Inc., South Bend, IN. Carboxymethylated papain was prepared as previously reported (11). 56 kDa (56HKa) and 46 kDa (46HKa) kallikrein-cleaved HK was prepared by dialyzing 1 mg of single-chain HK into HEPES carbonate buffer (137 mM NaCl, 3 mM KCl, 12 mM NaHCO₃, 14.7 mM HEPES, 5.5 mM dextrose containing 2 mM CaCl₂ and 1 mM MgCl₂) pH 7.4. After dialysis, plasma kallikrein in a molar ratio of 1:200 to HK was added (12). The mixture was incubated at 37°C for various amounts of time, and the reaction was stopped by adding sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Aliquots from each time point were analyzed by SDS-PAGE. Once the optimal time of incubation to generate 56 kDa and 46 kDa HK was determined, larger amounts were incubated at the appropriate molar ratios, and the reactions were stopped by adding 1 mM DFP. The cleaved HK then was dialyzed against HEPES carbonate buffer, pH 7.4 to remove the DFP. The integrity of the cleaved HK was analyzed on a 8% SDS-PAGE and the protein concentration was estimated using the Bio-Rad protein assay. A biotinylation kit and immunoPure streptavidin horseradish peroxidase dihydrochloride (turbo-TMP) were supplied by Pierce Chemical Co. (Rockford, IL). Prestained low molecular weight standard, nitrocellulose and polyacrylamide were purchased from Bio-Rad (Richmond, CA). SDS gel electrophoresis samples were stained with Coomassie Brilliant Blue R-250 (BioRad). cDNA encoding full-length uPAR was generously provided by Dr. F. Blasi, Copenhagen, Denmark.

**Peptides and Antibodies:** Peptides corresponding to domains 3, 4 and 5 of HK (See Table I) were synthesized at the Protein and Carbohydrate Structure Facility, University of Michigan, Ann Arbor MI as previously reported (13-15). Sequential and overlapping peptides
corresponding to each domain of suPAR were synthesized at Multiple Peptide Systems (San Diego, CA) (See Table II). All peptides from suPAR are numbered based upon the full-length sequence including the 22 amino acid signal peptide of uPAR. The peptides used were colorless, odorless, and >95% pure as determined by reverse phase HPLC and mass spectrometry. A monoclonal antibody against uPAR (3B10FC) was generously provided by Dr. Robert F. Todd III from the University of Michigan, Ann Arbor, MI (16). Monoclonal anti-uPAR antibodies E180 and E33 were the kind gifts of Dr. A. Mazar (Attenuon, San Diego, CA).

**Preparation of wild type and mutant suPAR:** Soluble urokinase plasminogen activator receptor (suPAR), isolated domains of suPAR (1,2,3), a fragment containing recombinant soluble suPAR domains 1 and 2 and 3 (S-D1, S-D2D3) and scuPA were prepared and expressed using the Drosophila Expression System (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, as described previously (17,18). Isolated domains 1,2, and 3 of suPAR were prepared from wild type protein by sequential digestion with chymotrypsin and pepsin as previously reported (19). Mutagenesis of suPAR in pMT/Bip/V5 (Invitrogen) was performed with the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) (17). The suPAR domain 2 and 3 mutants are shown in Table III. Wild-type and variant suPARs were purified from the media using a monoclonal (E180 or E33) anti-uPAR antibody affinity column. Wild-type scuPA was purified from the media using a monoclonal anti-uPA antibody column and were analyzed by SDS-PAGE and western blot (17). All suPAR mutants are numbered based upon the full-length sequence of suPAR including the signal peptide.

**Gel Electrophoresis and Immunoblot Analysis:** Proteins were separated using 8-15% acrylamide SDS-PAGE and then transferred to nitrocellulose membranes at 100 volts for 1 h. The electroblots then were incubated in blocking buffer [5% (wt/v) dry milk, 0.05% Tween 20, 0.15 M NaCl, and 20 mM Tris-HCl, pH 7.4] for 1 h (20). The membranes were then incubated with monoclonal antibody to uPAR diluted at 1:100 for 1 h. The membranes were washed and incubated with horseradish peroxidase conjugated sheep anti-mouse (1:2000) for 1 h and
antibody binding was detected with the ECL system from Amersham (Arlington Height, IL). All steps were carried out at room temperature.

**Endothelial Cell Culture:** Human umbilical vein endothelial cells (HUVEC), endothelial cell growth medium (EGM), trypsin-EDTA and trypsin neutralizing solutions were purchased from Clonetics (San Diego, CA). The cells were cultured according to the manufacturer’s recommendations. Cells between the 1st-5th passages were subcultured onto fibronectin-coated, 96-well plates 24 h prior to the start of the experiment (14). Cell viability was determined using trypan blue exclusion. Cell numbers were determined by direct counting on a hemocytometer.

**Biotinylation and iodination:** Five mg of HK in 200 μl was dialyzed against 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 (14). A 5-fold molar excess of sulfo-NHS-LC-Biotin was added. After incubation for 2 h on ice, the sample was loaded onto 10 ml Econo-Pac 10 DG column (Bio-Rad). Biotinylated-HK (biotin-HK) was monitored by absorbance at 280 nm using an extinction coefficient of 7.0 for HK and a protein assay (Bio-Rad). Biotin-HK had a specific activity of 13 U/mg. 46HKa was biotinylated in the same way. Peptides PGS20, HKH20, CNA13, QCY21, and DCR20 were synthesized with biotinylated labels at Multiple Peptide Systems. SuPAR and HK were radiolabeled with Na125I using Iodogen pre-coated tubes (Pierce, Rockford, IL) at a ratio of 100 μCi of 125I/100 μg protein to avoid oxidative injury (17). Free 125I was removed by gel filtration using Sephadex G25 (PD-10, Amersham Pharmacia Biotech, Piscataway, NJ).

**Biotin-protein or -peptide binding studies:** Biotinylated proteins or peptides in the absence or presence of competing proteins or peptides in HEPES carbonate buffer, pH 7.4 (8), were added to microtiter plates coated with monolayers of HUVEC or with other proteins or peptides (1 μg/ml) coated by an overnight incubation at 4°C in 0.1 M sodium carbonate, pH 9.6. After blocking the wells with 1% gelatin, the cuvettes were incubated with biotinylated-HK, -46HKa, or -peptides for 1 h at 37°C. The wells were washed 3 times with HEPES carbonate buffer, pH
7.4. The bound biotinylated proteins or peptides were measured using ImmunoPure streptavidin horseradish peroxidase conjugate (Pierce) and peroxidase-specific fast reacting substrate, 3,3', 5,5' tetramethylbenzidine dihydrochloride (turbo-TMP, Pierce) by measuring the absorbance of the reaction mixture at 450 nm using a microplate autoreader EL 311 (Bio-Tek Instrument, Winooski, VT), as previously described (14,21).

**Binding of iodinated HK to suPAR.** suPAR was immobilized onto Immulon 4HB plates (Thermo Labs Systems, Franklin, MA) in 0.01 M sodium phosphate, 0.15 M NaCl, pH 7.4 overnight at 4°C at a concentration of 1 μg/ml. Binding of 125I-HK to suPAR was measured in the presence of varying concentrations of unlabeled HK in HEPES carbonate buffer. 125I-suPAR binding to CHO cells expressing scuPA was performed as previously reported (17).

**Prekallikrein activation on endothelial cells.** The ability of peptides from suPAR to block PK activation on endothelial cells was determined. HUVEC were grown as monolayers in microtiter plate wells. HK and PK (20 nM each) were then added to the wells in the absence or presence of 100 μM peptide DLC20, LRG20, YLP20, PGS20, FHN20, TKC19, QCY21, or DCR20 from domains 1 to 3 of suPAR (See Table II) in HEPES carbonate buffer, pH 7.4 for 1 h at 37°C. After washing 3 times with HEPES carbonate buffer, pH 7.4, 0.4 mM H-D-Pro-Phe-Arg-pNA was added and hydrolysis of the substrate was measured over the next 1 hr at 405 nm (22).
RESULTS

**Characterization of HK species:** One μg of HK, 46HKa, and 56HKa was analyzed using 8% SDS-PAGE under reduced and non-reduced conditions. Under reduced conditions, HK, 56HKa, and 46HKa migrated predominantly at 120 kDa, 64 and 56 kDa, and 64 and 46 kDa, respectively (Figure 1A). These results indicate that 56HKa and 46HKa retained their heavy and their respective light chains. Under non-reduced conditions, HK, 56HKa and 46HKa migrated as single bands at 120 kDa, 110 kDa, and 108 kDa, respectively (Figure 1B), consistent with previous reports of a change in the molecular mass of plasma HK when it is activated by plasma kallikrein (20).

**Binding of biotin-HK to HUVEC and suPAR.** We then determined if these forms of HK differed in their affinity for HUVEC or for suPAR. Initial investigations were with HUVEC. Native HK, 56HKa, and 46HKa blocked the binding of biotinylated-HK to HUVEC with an IC₅₀ of 115 nM, 115 nM, and 50 nM, respectively (Figure 2A). Native HK, 56HKa, and 46HKa blocked biotin-HK binding to suPAR with an IC₅₀ of 60 nM, 110 nM, and 8 nM, respectively (Figure 2B). These data indicate that 46HKa is a more potent inhibitor of the binding of native HK to HUVEC and suPAR than are the less activated forms of HK. This outcome was consistent with previously reported information that kallikrein-cleaved HK bound more avidly to uPAR than did intact HK (5). The data also demonstrated that native HK also bound to suPAR, albeit with lower affinity than its cleaved counterparts. This information suggested that cleavage of HK enhanced its binding to suPAR. This increase in affinity of binding for 46HKa was less pronounced on HUVEC which express additional binding sites for the kininogens.

**Binding sites for suPAR within domain 5 of HK.** It has been shown in previous studies that HK bound to cells through determinants within both its light and heavy chains (13,15). Therefore, we next sought to identify the region(s) in domain 5 of HK that bound to suPAR and inhibited the binding of native HK and 46HKa (13-15). Binding of biotin-HK to suPAR was blocked by
peptides GKE19, HNL21, GHG19, HKH20, and HVL24 with an IC$_{50}$ of 300 µM, 20 µM, 30 µM, 2 µM, and 0.7 µM, respectively (Table I). HKH20 was a weaker inhibitor of biotin-46HKa binding (Table I). Peptides HKH20, HNL21, and HVL24 inhibited biotin-46HKa binding to suPAR with an IC$_{50}$ of 20 µM, 10 µM and 0.5 µM, respectively (Table I). These data indicate that the HVL24 region, corresponding to amino acids 471-494 of domain 5 of HK, has the highest affinity for suPAR, although other portions of D5 also had the capacity to bind.

**Binding sites for suPAR within domain 3 of HK.** A similar investigation was performed with peptides within the cell-binding region in domain 3 of HK (13) (Table I). Peptides NAT26, LDC27, and CNA13 inhibited biotin-HK binding to suPAR with an IC$_{50}$ of 20 µM, 20 µM, and 15 µM, respectively (Table I), whereas peptide KIC11 from the amino terminal end of this domain was inactive (Table I). The protein CM-papain, a domain 3 binding protein, inhibited the binding of biotin-HK to suPAR with an IC$_{50}$ of 1.5 µM (data not shown). NAT12, LDC27, and CNA13 inhibited biotin-46HKa binding to suPAR with an IC$_{50}$ of 20 µM, 30 µM, and 10 µM, respectively (Table I). CM-papain was an equipotent inhibitor of biotin-46HKa and biotin-HK binding (IC$_{50}$=1.5 µM) (data not shown). Taken together, these data suggested that HK bound to suPAR through regions found on both its heavy and light chains, although the binding of suPAR to domain 5 peptides was at least 10-fold more avid (compare IC$_{50}$ of HVL24 with CNA13). This conclusion was supported by the finding that biotin-HKH20 and biotin-CNA13 bound specifically to suPAR (Figure 3).

**Binding sites for HK in suPAR.** We next investigated the binding sites in suPAR for HK. As a first step, the epitope recognized by a monoclonal anti-uPAR antibody (3B10FC) that blocked HK binding to cultured endothelial cells (6,16) was partially mapped. On immunoblot, 3B10FC bound to suPAR domain 2 and to fragments containing domains 2 + 3 (D2D3, S-D2D3); 3B10FC bound weakly to D3, but not to D1, under the same experimental conditions (Figure 4). These data are consistent with previous studies in which it was reported that a polyclonal antibody to domains 2 and 3 of uPAR blocked HK binding to HUVEC (5,6). Additional
investigations were then performed to determine if HK bound directly to domains 2 and 3 of suPAR (5). Ten μM purified S-D2D3 inhibited the binding of biotin-HK and -46HKa to full-length suPAR by ~55-60% (Figures 5A and 5B). At the same concentration, isolated domain 1 of suPAR blocked binding 20-25% (Figure 5A and 5B). When combined, isolated S-D1 and S-D2D3 blocked biotin-HK and -46HKa binding to an even greater extent than that seen with intact suPAR. Based on these findings, we focused our attention on identifying the HK binding regions with domains 2 and 3 of suPAR.

**Binding sites for HK within domains 2 and 3 of suPAR.** To map the region(s) within this fragment of suPAR that recognize HK, a series of 20 amino acid peptides were synthesized starting at the carboxyterminus of domain 1 and spanning domains 2 and 3 (Table II). Peptide PGS20, located in the carboxyterminus of domain 2, inhibited binding of biotin-HK with an IC$_{50}$ of 1.8 μM (Table II). Additional overlapping peptides that incorporated or flanked the PGS20 region, LRG20, YLP20, and FHN20, inhibited biotin-HK binding to suPAR with an IC$_{50}$ of 0.9 μM, 20 μM, and 1 μM, respectively (Table II). These data suggested that full-length suPAR contains a binding site for HK within amino acids 166-195 of domain 2. This finding is biologically important because these peptides also inhibited biotin-HK binding to HUVEC (Table II). Binding of biotin-HK to HUVEC also was inhibited by PGS20, YLP20, LRG20, and FHN20 with an IC$_{50}$ of 40 μM, 25 μM, 7 μM, and 4 μM, respectively (Table II).

A similar approach was employed to begin to map HK binding site(s) within suPAR domain 3 (Table II). Two peptides from the amino terminal portion of domain 3 of uPAR, QCY21 and DCR20, inhibited biotin-HK binding to HUVEC with an IC$_{50}$ of 12 and 22 μM, respectively (Table II). These data suggested that an HK binding site also was contained within amino acids 215-255 of domain 3 of uPAR.

**Binding of a uPAR-domains 2+3 peptide to HK.** Based on its capacity to inhibit HK binding to suPAR, we next determined if biotinylated PGS20 from suPAR bound directly to HK. Biotin-
PGS20 bound to HK linked to microtiter plate cuvette wells in a specific, concentration-dependent and saturable manner (Figure 6A). Binding of biotin-PGS20 to immobilized HK was blocked by HK and PGS20. Biotin-QCY21 or -DCR20 also bound specifically to HK (Figure 6B) and 100-fold molar excess unlabeled QCY21 or 50-fold molar excess unlabeled DCR20 blocked the binding of their biotinylated forms to HK (Figure 6B). These data suggest that regions on domains 2 and 3 combined to form an HK binding site.

**Mapping the HK binding site in suPAR using site-directed mutagenesis.** As a second, independent approach to identify the binding sites for HK in domains 2 and 3 of suPAR, site-directed mutagenesis was performed on the charged amino acids in those regions that had been implicated in binding based on peptide inhibition. The suPAR mutant H\textsuperscript{182}A, N\textsuperscript{184}A lost its ability to inhibit biotin-HK binding to suPAR (Figure 7A, Table III). In contrast, suPAR mutant H\textsuperscript{182}A, D\textsuperscript{185}A inhibited biotin-HK binding with an IC\textsubscript{50} of 10 \(\mu\)M, a value identical to wild type suPAR (Figure 7A, Table III). These results indicated that position H\textsuperscript{182} contributed little to HK binding to suPAR. To examine this interpretation, N184 was then mutated to the more conservative residue glutamine rather than to alanine. SuPAR N\textsuperscript{184}Q at 10 \(\mu\)M inhibited biotin-HK binding unlike suPAR H\textsuperscript{182}A, N\textsuperscript{184}A, but less so than the wild type protein (Figure 7A, Table III). These data indicated that N184 was important for the suPAR-HK interaction. These data were also consistent with the peptide inhibition data in Table II and indicate the C-terminal region of domain 2 from amino acids L\textsuperscript{166}-T\textsuperscript{195} contributed to the HK binding region on suPAR. Consistent with this result, suPAR variants R\textsuperscript{159}K\textsuperscript{161}R\textsuperscript{164}H\textsuperscript{165}R\textsuperscript{167}A that had been shown previously to bind single chain urokinase with markedly reduced affinity (\(K_d=33\) and 55 nM, respectively, vs. 0.33 nM for wild-type suPAR) (17) inhibited the binding of biotin-HK to suPAR with an IC\textsubscript{50}=1 \(\mu\)M and 7 \(\mu\)M, respectively, a potency comparable to the inhibition observed with full-length suPAR (IC\textsubscript{50}=7 \(\mu\)M) (Figure 7A.)

Although previous investigations suggested that urokinase did not block HK binding to suPAR (5), more recent studies implicated the region C\textsuperscript{175} to C\textsuperscript{192} as a urokinase binding site.
(23). Consistent with these recent studies, single chain urokinase (scuPA) inhibited the binding of biotin-HK to suPAR with an IC$_{50}$ of 53 nM (Figure 8). Similar data were seen with $^{125}$I-HK binding to suPAR (data not shown). Moreover, suPAR mutants H$^{182}$A, N$^{184}$A and N$^{184}$Q were weak competitors of $^{125}$I-suPAR binding to CHO cells expressing cell-associated scuPA (data not shown.). suPAR-N$^{184}$Q also bound urokinase with a reduced affinity (data not shown). These results indicated that the site in domain 2 of suPAR involved in the binding of domain 5 of HK overlapped with the binding site for urokinase. The affinity of scuPA for suPAR is 113-fold greater than that of HK.

Studies were also performed to map the binding site for HK in domain 3 of suPAR. The variant suPAR mutant N$^{222}$Q did not inhibit biotin-HK binding to suPAR (Figure 7B, Table III). In contrast, point mutations in neighboring region (suPAR mutant E$^{230}$A, E$^{231}$A) inhibited biotin-HK binding with an IC$_{50}$=6 μM, similar to wild type suPAR. These data pointed to the involvement of amino acid 222 in domain 3 for binding of HK, an amino acid localization consistent with the results of the peptide mapping experiments shown in Figure 9.

**Effect of suPAR peptides on prekallikrein activation.** We then asked whether peptides from suPAR that bound HK interfered with PK activation on cultured endothelial cells (21). Peptide FHN20 inhibited PK activation on HUVEC by 78% (Table II) (Figure 9), consistent with its capacity to inhibit HK binding to these cells (Table II). Likewise, peptides LRG20, YLP20, and PGS20 that blocked HK binding to suPAR with an IC$_{50}$ of 7, 30, and 70 μM, respectively, also inhibited PK activation on HK by 62%, 50%, and 38%, respectively (Figure 9). Thus, interference of HK binding by peptides to the HK binding region on suPAR blocked PK activation on cells, supporting the relevance of the data obtained using suPAR and indicating an obligatory role for uPAR in PK activation.
DISCUSSION

These investigations address the mechanism by which the HK and PK complex assembles on the uPAR-containing multiprotein HK receptor complex on endothelial cells. Our studies indicate that the binding of HK to uPAR is mediated through regions on both its domain 3 heavy chain and domain 5 light chain. These results are consistent with our previous work, which showed that domains 3 and 5 of HK are involved in cell binding (13,14). It is of interest that the domain 5 region of HK, which contains its anti-angiogenic and anti-proliferative activity, binds to suPAR with a 10-fold higher affinity than does the domain 3 region, suggesting that uPAR may contribute to these biological functions.

It has been reported previously that native HK does not bind to suPAR in contrast to cleaved HK (HKa) (5). These results are at odds with our finding that biotin-HK bound to uPAR on cultured endothelial cells (6). This difference may be explained by findings in the present study which show that intact HK or 56 kDa kallikrein-cleaved HK (56HKa) binds suPAR with 7.5- to 14-fold lower affinity than does 46 kDa kallikrein-cleaved HK (46HKa). Thus, proteolysis of HK liberating the bradykinin moiety and an 11 kDa amino terminal portion from the light chain of HK promotes its binding to uPAR, rather than necessarily invoking the involvement of a novel receptor (24). These findings have implications for the anti-angiogenic and anti-proliferative activity of HKa. Intact HK, which under most physiologic circumstance is in molar excess to HKa, is likely to inhibit HKa binding to uPAR under physiological conditions (24-26). Bradykinin liberated from HK expresses pro-angiogenic activity (26,27). Thus it is likely that HKa inhibits angiogenesis only in situations where sufficiently large concentrations of these proteolytic fragments have been generated to overcome the competing influence of native plasma HK. Thus, the local ratio of HK to HKa may modulate its pro- and anti-angiogenic activities (24-27).
The HK binding region within domain 2 of uPAR overlaps with a region implicated in the binding of uPA (23) (Figure 10). In contrast to a previous study (5), we found that scuPA inhibited the binding of HK to suPAR. We attribute this difference to the source of reagents as we found that several commercial sources of uPA showed little or no inhibition as well. The plasma concentration of HK is 700 nM and that of scuPA 1-2 nM, implying that HK may limit the binding of uPA to uPAR \textit{in vivo}. On the other hand, we, and others, have found that plasma kallikrein is a potent activator of scuPA and that endothelial cell membranes provide a kinetically favorable environment for this process (22,28). This apparent paradox may be explained by the fact that scuPA has a 113-fold tighter affinity for uPAR than does HK. Thus, at physiological plasma concentrations, approximately one-quarter of the uPA receptors would be predicted to be occupied by scuPA, the remainder by HK. Higher occupancy rates of uPAR by uPA may occur when cells have been stimulated to migrate and synthesize uPA locally. Formation of mixed homodimers (29) on endothelial cells might permit HK-uPAR to approximate scuPA-uPAR. Alternatively, or additionally, binding of HK to the other components of this multiprotein receptor complex may facilitate kallikrein-mediated activation of scuPA bound to uPAR (22). Recent investigations indicate that cytokeratin 1 complexes with uPAR and/or gC1qR, but that uPAR does not bind to gC1qR directly on endothelial cells (6,30). Cytokeratin 1 may closely associate with uPAR, facilitating localization of PK on HK for activation by prolylcarboxypeptidase while allowing scuPA to bind to uPAR where it becomes a plasma kallikrein substrate.

The present investigations also indicate that each domain of suPAR has the capacity to bind at least one portion of HK and that interdomain cooperativity optimizes binding. Additional studies are needed to determine whether the presence of domain 1 is necessary to stabilize high affinity binding sites in domain 2 and 3 or whether each domain in uPAR recognizes a different epitope on HK forming a composite binding surface that allows HK to contact each domain of uPAR simultaneously (Figure 10). These data suggest that HK or HKa interacts with uPAR differently than it does with cytokeratin 1. Unlike the HK-cytokeratin 1 interaction where HK
binds only to one portion of cytokeratin 1 encoded by exon 1, HK and uPAR each express multiple potential sites of interactions sites. Presently it is not known whether HK must interact with all of these sites to bind, or if a portion of HK can bind to uPAR, leaving other HK regions available to bind to the same or other uPARs or other components of the multiprotein HK binding complex.

In sum, these investigations provide insight into the function of the vascular multireceptor complex for HK/HKa. Interaction of HK/HKa with one or more members of this complex likely helps to regulate its biological activity. For example, the ability of HK to function as an adhesive protein binding by its domain 3 to GPIbα and domain 5 to Mac-1 regulates platelet-leukocyte interactions (31). Interactions with its other binding proteins may be important for activation of the fibrinolytic and complement systems and expression of anticoagulant activity. uPAR may function as a shuttle between scuPA and HK binding to modulate plasma kallikrein-mediated fibrinolysis and HK's angiogenic activities.
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Footnotes

HK: high molecular weight kininogen

CK1: cytokeratin 1

uPAR: urokinase plasminogen activator receptor

PK: prekallikrein

suPAR: soluble urokinase plasminogen activator receptor

56HKa: 56 kDa kallikrein-cleaved light chain of high molecular weight kininogen.

46HKa: 46 kDa kallikrein-cleaved light chain of high molecular weight kininogen

scuPA: single chain urokinase plasminogen activator
FIGURE LEGENDS

Figure 1. Characterization of various HK used in these investigations: One μg of intact 120 kDa HK (HK), 56 kDa kallikrein-treated HK (56HKa), and 46 kDa kallikrein-treated HK (46HKa) were analyzed by 8% SDS-PAGE under reduced (Panel A) and non-reduced conditions (Panel B). After electrophoresis, the protein bands were detected by Coomassie Brilliant Blue R-250.

Figure 2. Inhibition of biotin-HK binding to HUVEC and suPAR by various forms of kininogen. Biotin-HK (10 nM) in HEPES carbonate buffer was incubated with monolayers of human umbilical vein endothelial cells (Panel A) or purified suPAR (Panel B) in the absence or presence of increasing concentration of purified HK, 56HKa or 46HKa for 1 h at 37°C. Binding of biotin-HK was measured as described in the Experimental Procedures. The data are mean ± SEM of triplicate determinations from three different experiments.

Figure 3: Binding of biotinylated domain 5 or 3 peptide of HK to suPAR. Increasing concentrations of biotinylated-HKH20 from domain 5 (Panel A) or -CNA13 from domain 3 in HEPES carbonate buffer were incubated in microtiter plates coated with suPAR in the absence or presence of a 100-fold molar excess unlabeled HKH20 or CNA13, respectively. Binding of biotin-HKH20 or -CNA13 was measured as described in the Experimental Procedures. The data are mean ± SEM of triplicate determinations from three different experiments.

Figure 4. Immunoblot analysis of monoclonal antibody 3B10FC binding to suPAR and its isolated domains. One μg of suPAR, domains 1 (D1), 2 (D2), 3 (D3), and combined domains 2 and 3 (D2D3) prepared by enzymatic digestion of wild type suPAR and recombinant soluble domain 1 (S-D1), and soluble combined domains 2 and 3 (S-D2D3) were separated using 15% polyacrylamide sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electroblotted onto nitrocellulose membranes. An immunoblot was performed with monoclonal
antibody 3B10FC to uPAR followed with horseradish peroxidase conjugated sheep anti-mouse antibody as described in the Experimental Procedures. The numbers to the left of the figure are molecular mass markers in kilodaltons. The figure is a photograph of an autoradiogram showing chemiluminescence.

**Figure 5. Inhibition of biotin-HK and -46HKα binding to suPAR.** Microtiter plates were coated with 1 μg of suPAR. Ten nM biotin-HK (Panel A) or biotin-46HKα (Panel B) in HEPES carbonate buffer was incubated for 1 h in the absence or presence of 1 μM HK or 46HKα, 10 μM suPAR, soluble D1 (S-D1), or soluble domains 2 and 3 (S-D2D3) or a combination of soluble domains S-D1 and S-D2D3. After incubation, the wells were washed and the amount of residual biotin-HK or 46HKα bound was determined. The data represent the mean ± SEM of three or more experiments.

**Figure 6. Binding of biotinylated uPAR domain 2 or 3 peptides to HK.** Increasing concentrations of biotin-PGS20 peptide from domain 2 of uPAR (Panel A) or biotin-QCY21 or -DCR20 peptide from domain 3 (Panel B) in HEPES carbonate buffer was incubated in microtiter plates coated with HK in the absence or presence of 100-fold molar excess peptide PGS20 or QCY21 or 50-fold molar excess of HK or peptide DCR, respectively, for 1 h at 37°C. Binding of the biotinylated peptides was measured as described in the Experimental Procedures. The data are mean ± SEM of triplicate determinations from three different experiments.

**Figure 7. Inhibition of biotin-HK binding to suPAR by suPAR variants.** Biotin-HK (10 nM) in HEPES carbonate buffer was incubated in microtiter plate wells coated with wild-type suPAR in the absence or presence of 0.5 to 10 μM of suPAR or its variants from domain 2 (Panel A) suPAR, R159K161R164H165R167A, R159K161R164H165A, N184Q, H182A N184A, H182A D185A, or variants from domain 3 (Panel B) N222Q, E230A E231A for 1 h at 37°C. Binding of biotin-HK was measured as described in the Experimental Procedures. The data are mean ± SEM of at least triplicate determinations from three different experiments.
Figure 8: **The influence of scuPA on HK binding to suPAR.** suPAR (1 µg/well) in sodium carbonate buffer, pH 9.6 was linked overnight to microtiter plates. The wells were washed and incubated with 10 nM biotin-HK in the absence or presence of increasing concentrations of scuPA (0.01-3000 nM). The amount of biotin-HK bound to the suPAR was measured as indicated the Experimental Procedures. The data are mean ± SEM of at least triplicate determinations from three different experiments.

Figure 9: **The influence of peptides from uPAR on prekallikrein activation on endothelial cells.** HUVEC were cultured in monolayers in microtiter plates wells and 20 nM HK and PK in the absence or presence of 100 µM peptide DLC20, LRG20, YLP20, PGS20, FHN20, TKC19, QCY21, or DCR20 from domains 1 to 3 (See Table II) of uPAR were added simultaneously to the well in HEPES carbonate buffer, pH 7.4 for 1 h at 37°C. After washing 3 times with HEPES carbonate buffer, pH 7.4, 0.4 mM H-D-Pro-Phe-Arg-pNA were added to the wells and the degree of hydrolysis of the substrate was measured for 1 hr. The data represent the mean ± SEM of 3 or more experiments with each peptide or control.

Figure 10. **The HK binding domains on suPAR.** The figure is a schematic of the full length of uPAR consisting of its signal sequence, domains 1, 2, and 3 and short cytoplasmic domain. Documented uPA binding sites from this work, Bdeir et al., JBC 275:28532, 2000, and Li et al., JBC 278:29925, 2003 are shown in green. The vitronectin binding site of uPAR from Li et al., JBC 278:29925, 2003 is shown in the left to right shaded amino acids from C193-Q211. The HK binding sites is shown in the regions of the darkened circles, domain 2 from L166-T195 and domain 3 from Q215-N255. The blackened amino acids in domain 2 and 3 represent specific sites where mutagenesis results in reduction of binding of HK to suPAR.
| Peptide† | Sequenceƒ | Position‡ | HK Domain | IC₅₀ (µM)++ |
|----------|------------|-----------|-----------|-------------|
| KIC11    | KICVGPRDIP | 244-254   | 3         | No Inhibition | No Inhibition |
| NAT26    | NATFYFKIDNVVKARVQVVAGKKYFI | 276-301   | 3         | 20          | 20          |
| LDC27    | LDCNAEVVVPWEKKIYPTVNCOPLGM | 331-357   | 3         | 20          | 30          |
| CNA13    | CNAEVVVPWEKK | 333-345   | 3         | 15          | 10          |
| GKE19    | GKEQGHTRRHDWGHEKQKR | 402-420   | 5         | 300         | -           |
| HNL21    | HNLGHGHKHERDQGHHQRGHHGK | 421-441   | 5         | 20          | 10          |
| GHG19    | GHGLGHGHEQOQHGLGHGHK | 440-458   | 5         | 30          | -           |
| HVL24    | HVLDDHGHKHGGHGHGHKKNKGKK | 471-494   | 5         | 0.7         | 0.5         |
| HKH20    | HKHGHHKGHKKNGGKNGKH | 479-498   | 5         | 2           | 20          |

† Each peptide is named for the first 3 letters of the sequence using the single letter amino acid code followed the number of amino acids in the sequence.
Each letter represents the single letter amino acid code.

The numbers represent the amino acids position of the mature HK without its signal sequence (14,15).

The values given represent the micromolar IC$_{50}$ of biotin-HK or biotin-46HKa binding to suPAR by each of the peptides.
Table II
SEQUENTIAL AND OVERLAPPING SYNTHETIC PEPTIDES FROM uPAR AND THEIR INFLUENCE ON BIOTIN-HK BINDING TO SuPAR OR ENDOTHELIAL CELLS

| Peptide | Sequence | Position | uPAR Domain | IC50 (µM) |
|---------|----------|----------|-------------|-----------|
| DLC20  | DLCNQGNSGRAVTYSRSRY1 | 96-115 | 1 | 20 |
| ECI20  | ECISCSSDMSCERGRHQSL | 116-135 | 2 | >300 |
| OCR20  | QCRSPEEQCLDVTHWIQEG | 136-155 | 2 | 100 |
| EEG20  | EEGRPKDDRHLRGCGYLMGC | 156-175 | 2 | 100 |
| LRG20  | LRGCGYLMPCPGSNFHNND | 166-185 | 2 | 0.9 |
| YLP20  | YLPGCPGSNGFHNNDFHFL | 171-190 | 2 | 20 |
| PGS20  | PGSNFGHNNDFHFLKCCNT | 176-195 | 2 | 1.8 |
| FHN20  | FHNNDTFHFLKCCNTTCNE | 181-200 | 2 | 1 |
| TKC19  | TKCNEGPILELENLQNGR | 196-214 | 2 | >300 |
| QCY21  | QCYSCKGNSTHGCSEETFLI | 215-235 | 3 | - |
| DCR20  | DCRGMNQCLVATGTHERPN | 236-255 | 3 | - |
| TAS20  | TASMCQHAHLGDAFSMNHID | 265-284 | 3 | - |
| VSC20  | VSCCTKSGNHPDLLDVYR | 285-304 | 3 | - |
† Each peptide is named for the first 3 letters of the sequence using the single letter amino acid code followed the number of amino acids in the sequence.

ƒ Each letter represents the single letter amino acid code.

‡ The numbers represent the amino acids position of the sequence of full length urokinase plasminogen activator receptor including the signal sequence.

†† Values represent the micromolar IC₅₀ of biotin-HK binding to suPAR or endothelial cell (HUVEC) by each of the peptides prepared from the sequence of uPAR.
| Mutant                  | Sequence†/ƒ | Domain 2 | Domain 3 |
|------------------------|-------------|----------|----------|
| Wild-type              | 176‡        | P G S N G F H N N D T F H P L K C C N T | 215 Q C Y S C K G N S T H G C S S E E T F L I |
| H182A, N184A           |             | P G S N G F A N A D T F H P L K C C N T | Q C Y S C K G Q S T H G C S S E E T F L I |
| H182A, D185A           |             | P G S N G F A N N A T F H P L K C C N T | Q C Y S C K G N S T H G C S S A A T F L I |
| N184Q                  |             | P G S N G F H N Q D T F H P L K C C N T |                                      |

† Each peptide is named for the first 3 letters of the sequence using the single letter amino acid code followed the number of amino acids in the sequence.

ƒ Each letter represents the single letter amino acid code.

‡ The numbers represent the amino acids position of the mature uPAR that includes the amino acids of the signal sequence.
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