High Molecular Weight Kinogen Utilizes Heparan Sulfate Proteoglycans for Accumulation on Endothelial Cells*

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The structural requirements for specific cell binding of kini-

1 The abbreviations used are: BK, bradykinin; ABTS, 2,2′-azino-di-
[3-ethylbenzthiazolinesulfonate]6;diacemum salt; AEBSF, 4-(2-
aminooethyl)benzenesulfonylfluoride; BSA, bovine serum albumin; D3,
domain 3 of kininogens; D5H, histidine-rich domain 5 of HK; E-64,
L-trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; Fmoc,
N-(9-flu-roenyl)methoxy carbonyl; GAG, glycosaminoglycan; HK, high molecular
weight kininogen; HRG, histidine-rich glycoprotein; HS, heparan sul-
fate; HT, HEPES-Tyrode’s buffer; IC50, concentration at 50% inhibition;
LK, low molecular weight kininogen; MBP, maltose binding protein;
PBS, phosphate-buffered saline; PG, proteoglycans; SDS-PAGE, SDS-
polyacrylamide gel electrophoresis; β-D-xo-ylanes, p-nitrophenyl-β-
D-xo-ylanesanase.

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Kininogens, the high molecular weight precursor of
vasoactive kinins, bind to a wide variety of cells in a
specific, reversible, and saturable manner. The cell
docking sites have been mapped to domains D3 and D5H
of kininogens; however, the corresponding cellular ac-
ceptor sites are not fully established. To characterize
the major cell binding sites for kininogens exposed by
the endothelial cell line EA.hy926, we digested intact
cells with trypsin and other proteases and found a time-
and concentration-dependent loss of 125I-labeled high
molecular weight kininogen (H-kininogen) binding ca-
pacity (up to 82%), indicating that proteins are crucially
involved in kininogen cell attachment. Cell surface di-
gestion with heparinases similarly reduced kininogen
binding capacity (up to 78%), and the combined action of
heparinases and trypsin almost eliminated kininogen
binding (up to 85%), suggesting that proteoglycans of
the heparan sulfate type are intimately involved. Con-
sistently, inhibitors such as p-nitrophenyl-β-D-xo-pyr-
ranoside and chlorate interfering with heparan sulfate
proteoglycan biosynthesis reduced the total number of
kininogen binding sites in a time- and concentration-de-
pendent manner (up to 67%). In vitro binding studies
demonstrated that biotinylated H-kininogen binds to
heparan sulfate glycosaminoglycans via domains D3
and D5H and that the presence of Zn2+ promotes this
association. Cloning and over-expression of the major
endothelial heparan sulfate-type proteoglycans synde-
can-1, syndecan-2, syndecan-4, and glypicans in HEK293t
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cells significantly increased total heparan sulfate at the
cell surface and thus the number of kininogen binding
sites (up to 3.3-fold). This gain in kininogen binding
capacity was completely abolished by treating trans-
fected cells with heparinases. We conclude that heparan
sulfate proteoglycans on the surface of endothelial cells
provide a platform for the local accumulation of kinino-
gens on the vascular lining. This accumulation may al-

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sites of action.
their precursor molecules in close proximity to their
low the circumscribed release of short-lived kinins from
sulfate proteoglycans on the surface of endothelial cells
fected cells with heparinases. We conclude that heparan
capacity was completely abolished by treating trans-
numbers (up to 107) of specific "docking" sites to which kinino-
gens bind with high affinity (7–52 nM) in the presence of Zn2+
(9, 10). Kininogen binding to cellular surfaces does not trigger
any of the known intracellular signaling cascades of the accep-
tor cells; therefore, it has been postulated that cell binding of
kininogens serves to accumulate kinin precursors on the sur-
face of their target cells (1, 11). As yet ill defined stimuli
propagate the spatially and temporally controlled liberation of
short-lived kinin agonists in proximity to their corresponding
receptors on the surface of vascular endothelial or smooth
muscle cells.

Kinins are potent peptide hormones involved in the regula-
tion of local blood flow, formation of edema, and mediation of
pain sensations. The nonapeptide bradykinin (BK)1 is released
from its precursor, high molecular weight kininogen (H-kinino-
gen, HK), by the action of plasma kallikrein, whereas lysyl-BK
(kallidin) is liberated from low molecular weight kininogen
(t-kininogen, LK) by tissue kallikrein (1). Because of their
extremely short half-lives in plasma (<15 s), kinins are
thought to operate strictly locally; however, the molecular
mechanisms underlying the circumscribed release of kinin hor-
mones are rather poorly understood (2). Two major modes of
locally acting hormone systems exist. (i) In the paracrine mode,
the production, release and action of hormones is limited to
a set of neighboring cells. This mode is exemplified by chemoo-
kines (3) and growth factors such as basic fibroblast growth
factor (4), hepatocyte growth factor (5), and epidermal growth
factor (6). Chemokines are “caught” by the cell surface proteo-
glycan (PG) layer of their target cells, which restricts their
diffusion and eventually delivers them to their cognate recep-
tors on adjacent cells (7). (ii) In the endocrine mode, cells
produce hormones at sites distant from their target cells, and
the effectors are transported to and taken up at their site of
action. A combined endo-/paracrine mode is exemplified by the
kinin system in which hepatocytes produce and secrete large
quantities of prohormones; the total plasma concentration of
kininogens approaches 2 µM (1). Previous studies have demon-
strated that kininogens and their complexes with plasma prek-
allikrein or factor XI (8) associate with the surface of cardio-
vascular cells such as endothelial cells (9), exposing vast
numbers (up to 107) of specific "docking" sites to which kinino-
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The structural requirements for specific cell binding of kini-
nogens have been explored in some detail, e.g. the kininogen segments docking to cell surfaces were precisely mapped. One site is represented by a sequence of 27 amino acid residues, LCD27, located in domain D3 of the common heavy chain of HK and LK (12). A second cell binding site comprises a sequence of 20 residues, HKH20, located in domain D5H of the unique light chain of HK. This latter segment, forming the major HK cell attachment site, is part of a highly basic region with clusters of histidine, lysine, and glycine residues (13). The two sites in D3 and D5H flank the kinin sequence in domain D4, which further modulates the association of HK with cell surfaces (14). Unlike the cell attachment sites of kinogens, the corresponding acceptor structures of target cells are still poorly defined. Using affinity isolation techniques and/or antibody competition experiments, six potential HK binding proteins of cardiovascular cells have been characterized: the integrin receptor Mac-1/αMβ2 (15); the purative receptor for the globular domains of complement factor C1q, p33/α1qR (11, 16); urorosine receptor (17); cytokertin-1 (18); thrombospondin-1 (19); and glycoprotein-Ib (20). Although all of these proteins specifically bind kinogens, they do not share any known sequence motifs in their protein portions that may serve as a common binding structure for kinogens. Further, none of the candidate docking proteins can fully account for kinogen cell binding because of cell type-specific expression (Mac-1, glycoprotein-Ib, thrombospondin-1) association with intracellular compartments (p33/α1qR, cytokertin-1), and/or low copy number (urokinase receptor (21)).

Given the unexpected heterogeneity of candidate acceptors, the high number of kinogen binding sites, and the apparent ubiquity of kinogen-loaded cell surfaces in the cardiovascular system, we set out to study systematically the nature of the kinogen docking site(s) exposed by the endothelium-derived cell line EA.hy926. Using a combined strategy of enzymatic digestion, metabolic inhibition, and recombinant over-expression, we demonstrate that proteoglycans such as HS-PG account for the vast majority of cell surface-associated kinogen binding sites in vitro and in vivo. Our data point to a novel role of cell surface proteoglycans in the local accumulation of prohormones prior to the controlled release of short-lived peptide hormones at or in proximity to their target cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—HK was isolated from human plasma (14). Peptides LCD27 (positions 331–357 of human kinogens; LCDNAEYVVYVPEKIKYPTVNCPLGM), HKH20 (positions 479–498 of human HK; HKHGGHHGKHKNGKKNGKH), and TLP28 (positions 268–295 of human plasma prekallikrein; TLPEPCHSKYPGVDFGGEELNVTFTYRG) were synthesized in their amide form on a 9050 Pep-Synthesizer (F. heparinum), heparinase III (EC 4.2.2.8, from Flavobacterium heparinum), heparinase III (EC 4.2.2.8, from F. heparinum), glycosidases, and proteases were purchased from Roche Molecular Biochemicals. The protease inhibitors, soybean trypsin inhibitor, benzamidine, leupeptin, and Pefabloc SC target matrix metalloproteinase 2 (Karlsruhe, Germany), and pepstatin A, bestatin, aprotonin, t-transpycysuccinyl-leucylamido(4-guanidino)butane (E-64), and AEBSF were from Calbiochem. All other reagents were from Sigma unless otherwise stated.

**Cell Culture**—EA.hy926 (11) and HEK293t cells (37) were cultured under standard conditions in Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Inc.) containing 4.5 g/liter glucose, 10% (v/v) fetal bovine serum, 0.01% (w/v) penicillin-streptomycin in a humidified CO2 atmosphere at 37 °C. For EA.hy926 cells, the medium was supplemented with HAT (100 μM hypoxanthine, 0.4 μM amionopterin, and 10 μM thymidine) according to established protocols (11).

**Labeling of Proteins**—Purified HK and monoclonal antibodies 10E4 and 3G10 were radiolabeled by the method of Praker and Speck (28) with minor modifications. Ten μg of protein dissolved in 20 μl of 6.5 mM NaHPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 150 mM NaCl, pH 7.4 (PBS), was applied to a reaction tube coated with 100 μg of IODO-GEN (Pierce, St. Augustin, Germany), and 1 μCi of Na125I in a 10-μl volume was added. The reaction was allowed to proceed for 10 min at room temperature, and unreacted iodine was separated from radiolabeled protein by incubation for 30 min in a Sephadex G-10 column (Pharmacia Biotech) at 4 °C to minimize the loss of radiolabeled protein. 125I-labeled HK had a specific activity of approximately 1 Ci/μmol and appeared as a single band of 120 kDa in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. Monoclonal antibodies 10E4 and 3G10 were 125I-labeled to a specific activity of 0.8 Ci/μmol, appearing as single bands of 150 kDa in nonreducing SDS-PAGE. For biotinylation, 100 μg of HK was incubated with 10 μg of biotin-e-aminocaproyl-N-hydroxysuccinimide (biotin-X-NHS, Pierce) for 4 h at 4 °C in 0.1 mM NaHCO3. Unreacted biotin-X-NHS was separated by centrifugation of the reaction mixture over a Microcon-10 column (Amicon, Beverly, MA), and biotinylated HK was used without further purification.

**Degradation of Kinogen Binding Sites Exposed by EA.hy926 Cells**—A confluent monolayer of EA.hy926 cells was washed three times with PBS. Cell surface proteins were proteolyzed for 3 min at 37 °C with 0.05, 0.25, or 1.0% (w/v) trypsin, chymotrypsin, papain, or proteinase K in PBS including 0.5 mM EDTA (29). The reaction was stopped by washing the detached cells four times with a protease inhibitor cocktail containing 1 μg/ml each soybean trypsin inhibitor, leupeptin, and 0.1 mM Pefabloc SC in PBS. EA.hy926 cells (approximately 106/assay) were incubated in suspension for 1 h with HEPES-Tyrode’s buffer (HT; 0.135 mM NaCl, 2.7 mM KCl, 11.9 mM NaHCO3, 0.36 mM Na2HPO4, 14.7 mM HEPES, 3.5 mM glucose, 50 μM ZnCl2, pH 7.4) supplemented with 1% (w/v) bovine serum albumin (BSA) and 20 μM 125I-HK under gentle shaking at 37 °C for 60 min. Unbound 125I-HK was removed by centrifugation through a cushion of 47% (v/v) dibutyolphalate and 53% 1-bis-2-ethyl-butyl-phthalate at 20,000 × g for 1 min (9). Cell-bound 125I-HK was quantified in a γ-counter. Unspecific binding in the presence of a 200-fold molar excess of unlabeled kinogen was <15% of the total binding (9, 10). No significant differences in the amount of cell-bound 125I-HK were found when the binding assay was done at 4 °C. Deglycosylation of cell surface carbohydrates was performed by incubating intact EA.hy926 cells with 0.01, 0.1, or 1 unit/ml of sialidase, N-glycosidase F, N-acetyl-β-D-glucosaminidase, or a combination of sialidase and N-glycosidase F (enzymes from Calbiochem throughout). Digestion was done for 30 min at 37 °C in PBS supplemented with soybean trypsin inhibitor, benzamidine, leupeptin (10 μg/ml each), 0.1 mM Pefabloc SC, 0.5 mM EDTA, 500 μM AEBSF, 150 mM aprotin, 1 μM E-64, 25 μM bestatin, and 2 mM pepstatin A. Cell-associated HS-type glycosaminoglycan (GAG) was degraded by incubating the cells for 30 min at 37 °C with 0.01, 0.1, or 1 unit/ml heparanase I or heparanase III in PBS including the protease inhibitor mixture. After extensive washing of the cells with PBS including protease inhibitors, the 125I-HK binding assay was performed as detailed above except that the cells remained attached to the culture dish. Unbound radiolabeled 125I-HK was removed by washing the cells three times with PBS; then the cells were lysed in 2 N NaOH, and cell-associated 125I-HK was measured. Total cellular protein was determined by the Bradford test (Bio-Rad). For control, cells were incubated with buffer alone.

**Cloning of PG cDNAs and Recombinant Expression in HEK293t Cells**—For RNA isolation, 106 cells of EA.hy926 (syndecan-2) or human
umbilical vein endothelial cell cultures (syndecan-4) were washed with ice-cold PBS and lysed with 4 M guanidinium isothiocyanate, 0.5% (w/v) sarcosy1, 25 mM sodium citrate, and 0.1 M 2-mercaptoethanol and extracted by the phenol/chloroform method (30). One µg of total cellular RNA was reverse-transcribed with 200 units of Moloney murine leukemia virus reverse transcriptase (New England Biolabs, Schwalbach, Germany) using 1 µM dNTPs, 10 units of RNasin (Roche Molecular Biochemicals), and 100 ng of oligo(dT)16 in 20 µl of reverse transcriptase buffer at 37 °C for 2 h (31). The reaction mixture was diluted 50-fold with 10 µl Tris-HCl, pH 8.0, 1 µM EDTA. 5 µl of this template mixture was transferred to 95 °C for 3 min with 5 µl of this template mixture was transferred to 95 °C for 3 min, cooled on ice, and 5 µl of cDNA was amplified with the 5'-primer 5'-CGCAATTTCAATATGCCGCTG-3' and 5'-primer 5'-GTTCTAGATTTTACCGATAAA-CTCCTTAG-3' (22). For full-length syndecan-4 cDNA, 5'-primer 5'-TCGGAATTCGTAAGTCCACCCCACACTTCC-3' and 5'-primer 5'-AAGATTCTTCCAGCGTAGAATCTGGG-3' (24) were used. The 5'-primers introduced an EcoRI site 6 bases upstream of the ATG start codon, and the 3'-primers contained an XhoI and an EcoRI site, respectively. The polymerase chain reaction (PCR) products were ligated into the corresponding restriction en- zymes and ligated into the pcDNA3 vector (Invitrogen, Leek, The Netherlands) utilizing the same sites. Constructs that had been confirmed by full-length sequencing were transiently transfected in HEK293t cells using the LipofectAMINE method (32). The transfection efficiency was ≥40% as revealed by transfection with a vector encoding green fluores- cent protein (31).

Quantification of Cell Surface GAG—To quantify HS-type GAG at the cell surface following over-expression of HS-PG in HEK293t cells or enzymatic digestion of EA.hy926 cells (see above), a direct binding assay using specific antibody probes was employed. The cells were washed extensively with PBS including a protease inhibitor mixture, treated with 0.5% (w/v) casin in PBS, and incubated at 37 °C for 45 min with 10 µg/ml 125I-labeled monoclonal antibody 10E4 in the same buffer. Antibody 10E4 specifically recognizes HS-type GAG exposed by proteoglycans such as syndecans and glypicans (26). Cells were washed five times with PBS to remove unbound 125I-labeled 10E4 and lysed in 2 N NaOH, and cell-associated 125I-labeled 10E4 was quantified in a gamma-counter. Total cellular protein was determined by the Bradford test (Bio-Rad).

RESULTS

Role of Glycoproteins in Kininogen Binding to EA.hy926 Cells—Because candidate kininogen binding proteins do not share common protein sequence motif(s) that could serve as prohormone docking sites, we set out to determine whether their carbohydrate moieties may be responsible for kininogen attachment. Initially, we studied the effect of various glycosi- dases on the HK binding capacity of endothelial cells. We incubated intact EA.hy926 cells with 0.01–1.0% glycosidases such as sialidase, N-glycosidase F, and N-acetyl-α-D-glu- cosaminidase; cell surface digestion was done in the presence of an inhibitor mixture to prevent accidental loss of HK binding sites by proteolytic degradation (Fig. 1). Sialidase, N-glycosidase F, and a combination of both inhibited the specific HK binding capacity to 70, 68, and 64% of untreated cells (set 100%), whereas N-acetyl-α-D-glucosaminidase had no effect (101%). The loss of HK binding sites was concentration-dependent and increased over time (shown for sialidase, Fig. 1, upper left inset). Unspecific binding of 125I-HK to the treated cells measured in the presence of a 200-fold excess of unla-

Direct and Indirect Binding Assays—A direct binding assay was employed to analyze the binding of HK to various carbohydrates. Microtiter plates (MaxiSorp, Nunc, Wiesbaden, Germany) were incubated with 20 mg/ml poly-ι-l-lysine at 4 °C overnight, then washed six times with PBS, and coated with serial dilutions (2°) starting from 100 µg/ml (15) of HS, heparin, MBP, dextran sulfate 5000, and glucose, respectively; in 100 mM sodium acetate, 100 mM NaCl, pH 6.5 (coating buffer). The plates were washed six times with HT and blocked with 1% BSA in HT for 45 min, and 10 µl biotinylated HK in HT containing 1% BSA was applied for 45 min at 37 °C. After washing with HT, bound biotinylated HK was incubated by the preformedstreptavidin-peroxidase complex (2 µg/ml; Roche Molecular Biochemicals) followed by the substrate solution. To analyze the interaction between HK and HS, GAG was covalently bound to CovaLink® plates (Nunc). To this end, 250 µg/ml HS were preincubated for 10 min at 37 °C with 0.05 M N-hydroxysuccinimide (Pierce) and 0.2 M 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (Pierce) in H2O. The mixture was applied to a CovaLink® plate and incubated for 60 min at 37 °C. Following extensive washing, free bind- ing sites on the plate were blocked with 100 mM ethanolamine, pH 8.5, for 30 min at 37 °C. The HS-coated CovaLink® microtiter plates were regenerated by washing six times with 20 mM HCl after each experiment. The efficiency of covalent coupling was controlled by quantifying immobilized GAG by the digoxigenin glycan detection kit (Roche Molecular Biochemicals). To test the effect of Zn2+ on HK, binding plates (HT) were preincubated with a serial dilution of ZnCl2 (100 mM) of biotinylated HK in the absence or presence of 50 µM ZnCl2 in HT. For competition experiments, serial dilutions (2°) of the following competitors were prepared in HT containing 1% BSA, 10 µl biotinylated HK (starting concentrations are given in parentheses): fusion proteins MBP-D3, MBP-D6 (5 µM), and unfused MBP (2 µM); peptides LCD27, HKH20, or PK31 (100 µM); anti-peptide antibodies α-BK, α-LDC27, or α-HKH20 (500 µM); saccharides HS, heparin, dextran sulfate, MBP, or glucose. For control, HT buffer alone was applied. Bound biotinylated HK was probed as above.

Immunofluorescence Studies—EA.hy926 cells were grown on 6-well plates (Nunc). The cells were washed five times with ice-cold PBS and incubated for 45 min at 37 °C with 40 µg/ml HK protein in HT including 1% (w/v) BSA. After washing five times with HT, the cells were fixed with 4% (w/v) formaldehyde in PBS for 30 min at 37 °C, washed again, and incubated with 10% (w/v) ammonium chloride for 10 min. Cells were washed and incubated for 30 min at 37 °C with 20 µg/ml antibody 1107 (directed to HK light chain) in PBS, 1% BSA. Cells were washed three times with HT and incubated with fluorescein isothiocyanate-conju- gated rabbit anti-goat immunoglobulin (Sigma) at 1:100 in the same buffer for 30 min. After three washes, the cells were incubated for 1 min with 1% (v/v) 4,6-diamidino-2-phenylindole in PBS, rinsed again, and embedded in n-propyl gallate. The fluorescence was examined using an Axioskop microscope (Zeiss, Oberkochen, Germany).

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beled kininogen was <10% of the total binding of HK to intact endothelial cells. Thus, glycoproteins may directly or indirectly contribute to HK binding of endothelial cells; however, they cannot fully account for the total kininogen binding capacity of EA.hy926 cells.

We also monitored HK binding by immunofluorescence analysis. EA.hy926 cells were incubated with unlabeled HK, and cell-bound HK was detected by antibody I107 directed to the light chain of HK, which does not interfere with cell binding. Bound I107 was detected by a fluorescein isothiocyanate-labeled secondary antibody. A strong pericellular staining was observed (Fig. 1, right inset), which was most prominent at cell-cell contacts and lamellipodia-like structures extending into the intercellular spaces. This staining pattern prompted the question of whether components of the pericellular matrix such as proteoglycans (PG) might mediate kininogen binding.

Role of PG for Cell Binding of Kininogens—As PG are composed of core proteins and GAG side chains, we first studied the effect of proteases on the HK binding capacity of intact cells. To this end, we incubated EA.hy926 cells with 0.05–1% trypsin, chymotrypsin, papain, or proteinase K for 3 min at 37°C and tested the treated cells for their residual HK binding capacity (Fig. 2). Enzymatic degradation of cell surface-associated proteins maximally reduced the specific HK binding capacity to 18% of untreated cells. HK binding sites were progressively lost with increasing enzyme concentration. The loss of HK binding capacity occurred rapidly over 2 min and leveled off thereafter (exemplified for papain, Fig. 2, inset). Next, we explored the role of the long, unbranched, and highly negatively charged GAG side chains that are attached to core proteins of cell surface PG. EA.hy926 cells were treated with 2.5–5 μM p-nitrophenyl-β-D-xlyropyranoside (β-D-xylanoside), an inhibitor of GAG attachment to core proteins, and the 125I-HK binding capacity was followed. Treatment with β-D-xylanoside resulted in a time- and concentration-dependent reduction of 125I-HK binding to 33% of control cells grown in the absence of the inhibitor, whereas vehicle without inhibitor was ineffective (Fig. 3A). Likewise, the application of 5–10 mM chlorate, an inhibitor of intracellular sulfation, reduced 125I-HK cell binding to 39% of the control in a time- and concentration-dependent fashion (Fig. 3B). The loss of HK binding capacity was attenuated when the culture medium was supplemented with 10 mM chloride to overcome chlorate inhibition (Fig. 3B).

To correlate the loss of HK binding capacity with the inhibition of GAG synthesis, we employed 125I-labeled antibody 10E4 directed to native HS-type GAG (26). Treatment of EA.hy926 cells with 10 μM chlorate or 5 μM β-D-xylanoside reduced 125I-10E4 binding to 33 and 27%, respectively, of the untreated control. By contrast, surface digestion of EA.hy926 cells with various glycosidases (cf. Fig. 1) did not significantly change total 125I-10E4 binding (95–103%). We tentatively conclude that PG-associated GAG contributes to the HK binding capacity of endothelial cells and that negatively charged sulfate groups attached to the GAG backbone may play an important role in this interaction, although the effect of chlorate could also reflect a critical role of other sulfated components of the cell.

Effect of Enzymatic Degradation of HS-PG on the HK Binding Capacity—Because HS-PG dominate on endothelial cell surfaces (6, 34) we asked whether enzymatic degradation of HS could affect their HK binding capacity. EA.hy926 cells were incubated with heparinases I or III, which efficiently break down HS-type GAG at the cell surface. Incubation was performed in the presence of a protease inhibitor mixture to prevent loss of HK binding sites because of traces of contaminating proteases. Heparinase action diminished the 125I-HK binding to 33% of control cells (Fig. 4, inset). The HK binding capacity of EA.hy926 cells to 29–35% of control in a concentration- and time-dependent manner (Fig. 4). The HK binding capacity was further decreased to 22% of the control by combining heparinases I and III; a minimum binding capacity was observed after 25 min of continuous incubation (Fig. 4, inset).
The combined action of both heparinases and trypsin reduced the number of HK binding sites to 15% of the control, i.e. the capacity of EA.hy926 cells to bind HK was almost abrogated under these conditions. Collectively, these data lend strong support to the hypothesis that HS-PG are intimately involved in HK binding to endothelial cells.

Binding of HK to HS-type GAG—To analyze the interaction of HK with HS-type GAG in vitro, we employed a direct binding assay. Microtiter plates coated with polylysine were incubated with increasing concentrations of HS, heparin, and dextran sulfate and subsequently probed with biotinylated HK followed by the streptavidin-peroxidase detection system. For control, glucose and MBP were applied. Biotin-HK bound most efficiently to HS and with almost the same affinity to heparin, whereas it bound moderately to dextran sulfate but not to MBP or glucose (Fig. 5). Probes such as biotin-MBP or biotin-BSA did not produce specific binding (data not shown). Hence, HK attaches to immobilized HS-type GAG in vitro. To further analyze the interaction of HK with HS, we performed competition experiments with HS covalently linked to microtiter plates.

The efficiency of the coating procedure was monitored by a digoxigenin-based enzyme-linked immunosorbent assay quantifying chemically cross-linked HS (data not shown). HS-coated plates were incubated with biotinylated HK in the presence of serial dilutions of various competitors (Fig. 6A). Unlabeled HK efficiently blocked binding of biotinylated HK to HS with an apparent IC50 of 70 nM. HS-type GAG, heparin, and dextran sulfate inhibited with an IC50 of 2, 3, and 20 μM, respectively, whereas MBP and glucose failed to interfere (IC50 ≥ 100 μM). Pretreatment of HS-coated plates with heparinases completely abrogated their HK binding capacity (data not shown). Next, we explored whether HS-type GAG competes with HK binding to endothelial cells in vivo. Thus, confluent EA.hy926 cells were incubated with serial dilutions of biotinylated HK in HT buffer in the presence of various competitors. HS, heparin, and unlabeled HK efficiently inhibited biotinylated HK binding to EA.hy926 cells compared with control (HT buffer alone) as evidenced by a shift of the binding curves to the right (Fig. 6B). Dextran sulfate was a moderate competitor, whereas glucose and MBP failed to interfere significantly. Together, these experiments demonstrate the specificity of interaction between HK and HS-type GAG in vitro as well as in vivo on cultured endothelial cells.

Interaction Sites of HK for HS Binding—Because previous mapping studies have localized the cell binding sites to
epitopes LDC27 and HKH20 of domains D3 (12) and D5H (13), respectively, of human HK, we tested whether the same sites mediate HK binding to HS-type GAG. HS-coated microtiter plates were incubated with biotinylated HK in the presence of increasing concentrations of MBP fusion proteins of HK domains D3 and D5H, MBP-D3 and MBP-D5H inhibited biotinylated HK binding to immobilized HS with IC_{50} values of 1050 and 200 nM, respectively, whereas unfused MBP was without effect (Fig. 7A). Synthetic peptides LDC27 and HKH20 inhibited biotin-HK binding to HS with IC_{50} values of 90 and 12 μM, respectively. Unrelated peptide TLP28, derived from prekallikrein, did not compete (Fig. 7B). Affinity-purified antibodies α-LDC27 and α-HKH20 directed to the relevant cell binding sites of HK were efficient competitors with IC_{50}' values of 470 and 110 nM, respectively, whereas α-BK, an antibody to the kinin sequence of kinogen D4, failed to interfere (Fig. 7C). These findings demonstrate that HK binds to HS via its cell binding sites used in vivo, namely domain D3 of the heavy chain and domain D5H of the light chain of HK.

One of the hallmarks of kininogen-cell interactions is the presence of Zn^{2+} ions on the binding of biotinylated HK to HS by a direct binding assay in which serial dilutions of biotinylated HK were applied to HS-coated microtiter plates in the absence or presence of 50 μM Zn^{2+}. The apparent K_{d} for biotinylated HK binding to HS was 5 nM in the presence of Zn^{2+} and rose to 26 nM in the absence of Zn^{2+} (Fig. 7D). This 5.2-fold increase in biotinylated HK binding to HS in the presence of Zn^{2+} reproduces the findings reported for endothelial cells (9, 10, 13, 35) and further stresses our hypothesis that HS-type PG represent the major HK docking structures on endothelial cells.

**Transient Over-expression of HS-type PG**—If correct, our notion would predict that up-regulation of HS-PG should significantly increase the HK binding capacity of corresponding cells. Thus, we transiently over-expressed prototypic endothelial cell surface HS-type PG syndecan-1, syndecan-2, syndecan-4, and glypican in HEK293t cells, and examined the ^{125}I-HK binding capacity of transfected versus mock-transfected cells. First, functional expression of the constructs was demonstrated. Total cell membranes were prepared and analyzed by SDS-PAGE and Western blotting using antibodies to PG core proteins. Highly glycosylated PG forms in the ranges of 100 to 220 kDa (syndecan-1), 80 to 210 kDa (syndecan-2), and 50 to 140 kDa (syndecan-4) and 140 to 200 kDa (glypican) were found. In mock-transfected cells, a faint band indicative of wild-type syndecan-1 was visible (not shown). For control, cells were transfected with the cDNA of p33/gC1qR (30). Over-expression of syndecan-1, syndecan-2, syndecan-4, and glypican raised the ^{125}I-HK binding capacity by a factor of 2.9, 2.7, 3.3, and 1.9, respectively (Fig. 8A). Cell-surface associated HS-type GAG increased by a factor of 3.5, 2.8, 4.0, and 2.2, respectively, over mock-transfected cells, as probed by 125I-labeled 10E4 antibody (Fig. 8C). Interestingly, over-expression of p33/gC1qR, one of the previously identified kininogen acceptor proteins, did not increase HK binding capacity over basal levels. Treatment of HS-type PG over-expressing cells with heparinases I and III converted the broad bands of HEK293t into distinct bands of 30–80 kDa (syndecan-1), 80 to 210 kDa (syndecan-2), and 50 to 140 kDa (syndecan-4), and 140 to 200 kDa (glypicans) were found. In mock-transfected cells, a faint band indicative of wild-type syndecan-1 was visible (not shown). For control, cells were transfected with the cDNA of p33/gC1qR (30). Over-expression of syndecan-1, syndecan-2, syndecan-4, and glypican raised the ^{125}I-HK binding capacity by a factor of 2.9, 2.7, 3.3, and 1.9, respectively (Fig. 8A). Cell-surface associated HS-type GAG increased by a factor of 3.5, 2.8, 4.0, and 2.2, respectively, over mock-transfected cells, as probed by ^{125}I-labeled 10E4 antibody (Fig. 8C). Interestingly, over-expression of p33/gC1qR, one of the previously identified kininogen acceptor proteins, did not increase HK binding capacity over basal levels. Treatment of HS-type PG over-expressing cells with heparinases I and III converted the broad bands of untreated PG into distinct bands of 30–80 kDa (Fig. 8B). At the same time, heparinase treatment decreased the HK binding capacity of transfected HEK293t cells even below the level of untreated mock-transfected cells, indicating that enzymatic cell surface digestion had also destroyed the endogenous HK binding sites of
HEK293t cells (Fig. 8A). The loss of HK binding capacity following degradation of HS-type GAG was mirrored by an increased binding of 125I-labeled antibody 3G10, which recognizes a neo-epitope on heparinase-treated HS-type GAG, by a factor of 2.2 (syndecan-1 over-expressing cells), 2.3 (syndecan-2), 2.5 (syndecan-4), 1.8 (glypican), and 1.0 (p33), respectively (Fig. 8D). Thus, over-expression of HS-PG significantly increases the binding capacity of HEK293t cells for kininogens, and heparinase treatment reverses this effect, even below basal levels. Together, these data demonstrate that HS-type PG exposed on cellular surfaces mediate HK binding to endothelial cells via their GAG side chains.

**DISCUSSION**

Understanding the molecular mechanisms by which hormone systems finely tune the human body's homeostasis is a major goal of molecular endocrinology. One prototypical peptidergic effector system that has been studied in great detail is the kallikrein-kinin system (1). Over the past years, much effort has been put into the identification of cellular docking structures recruiting the kinin precursors from the plasma and assembling the critical components that trigger the release of kinins from kininogens in proximity to their target cells (1, 11, 16). The salient features of these binding sites are (i) affinity and specificity for kininogens, (ii) abundance, (iii) ubiquity, and (iv) availability on cell surfaces. Six candidate proteins have as yet been identified: the integrin Mac-1/αMβ2, p33/gC1qR (11, 16), urokinase receptor (17), cytokeratin-1 (18), thrombospondin (19), and glycoprotein-Ib (20). However, none of the candidates meets the full range of criteria defining kininogen acceptors. For instance, the urokinase receptor is present in a limited copy number per cell (<5% of the HK binding sites) and therefore does not fulfill the criterion of abundance (21). Mac-1, thrombospondin-1, and glycoprotein-Ib are cell typespecific proteins (36, 37), which do not meet the criterion of ubiquity; and p33/gC1qR, a component of the mitochondrial matrix (31, 38), and cytokeratin-1, a typical cytoskeletal protein (39), are unlikely to comply with the criterion of cell surface availability under physiological conditions. Thus, high affinity binding to HK, a feature shared by all candidate proteins, does not necessarily indicate a biologically relevant interaction (31, 38). Given the heterogeneity of the candidate proteins, we felt that components of the cell surface other than proteins might operate in vivo as kininogen acceptors and that a systematic approach could help to determine the nature of the illusive kininogen binding sites. Here, we have used a combined strategy of enzymatic degradation, metabolic inhibition, and recombinant over-expression to identify heparan sulfate-type proteoglycans as the major HK binding sites on the surface of the endothelial cell line EA.hy926.

The notion that HS-PG represent cellular docking structures for HK offers intuitive solutions to some of the conundrums associated with putative kininogen binding sites (1). First, the repetitive nature of the HS chain structure of PG can easily account for the huge number of kininogen binding sites that have been reported, e.g. for endothelial cells (9, 10, 13, 35), and thus they meet the criterion of abundance. One may envisage that stacks of kininogen molecules are fixed to cell surfaces via the extended HS chains (40). Also, the large variation in the number of kininogen binding sites per cell (9, 10) may well reflect gross differences in the GAG content of PG expressed by the various cell types (6, 41). Second, the apparent ubiquity of kininogen binding sites is easily explained by the fact that most cell surfaces are furnished with a layer of PG-anchored HS (6, 42). A notable exception to this rule are erythrocytes, which lack a HS shell, and are correspondingly poor binders of kininogens (9, 31). Third, the association of kininogens with HS-PG may offer a simple mechanism for the local release of kinins close to their site of action (43). Thus, cell surface HS-PG serve locally to accumulate intact kininogens in proximity to the kinin receptors. A wasteful release of kinins, for instance in the circulatory system, is thereby avoided (note that the molecular ratio of kinins versus kininogens is approximately 10⁻⁶ in human plasma). This notion is reminiscent of the well-established role of PG as coreceptors for chemokines (3) and growth factors (44), where they restrict the diffusion of active ligands by tethering them to flexible glycosaminoglycan chains (7).
contrast, kininogens are completely inactive on kinin receptors, and proteolytic processing is mandatory for triggering physiological effects.

Although our results clearly identify HS-PG as the major cellular docking sites for kininogens, they do not refute the possibility that other PG, such as dermatan and chondroitin sulfate-type proteoglycans, may function as kininogen acceptors. In fact, our preliminary studies indicate that cell surface digestion with chondroitinase ABC or testicular hyaluronidase moderately reduces the kininogen binding capacity of EA.hy926 cells by 10–35%. Thus, HS-PG are probably not the sole docking structures for kininogens, and other PG or even glycoproteins may contribute directly or indirectly. For instance, the binding sites of the reported HK binding proteins (15–20) could be glycosidic in nature (45–49), or the HK binding proteins could indirectly dock to cells via HS-type PG; we have not further explored these latter possibilities.

We note limitations in our present study in two respects. First, we have focused on endothelium-derived EA.hy926 cells because the bulk of plasma-borne kininogen associates with endothelial cells (9, 10). Our preliminary results indicate that human umbilical vein endothelial cells, known to be rich in HS-PG, utilize the same docking sites as EA.hy926 cells. Kininogen biosynthesis has also been reported for the kidney where kininogens associate with epithelial cells lining the renal tubulus system (50). Consistently, we find that HEK293t, a renal epithelium-derived cell line, uses HS-PG for kininogen sequestration. However, as most cells express HS-PG on their surfaces (6, 42), the role of HS-PG as kininogen docking sites may apply to many different cell types and represent not merely a peculiar phenomenon of engineered cells. Second, we have focused on the major type of human kininogen, i.e., H-kininogen, known to participate in the contact phase system that promotes kinin release on non-endothelial surfaces (1). The second type of human kininogen, LK, shares the entire heavy chain, including domain D3, with HK but lacks the unique light chain domain of D5H. Our preliminary studies indicate that LK also binds to HS-type GAG in vitro and therefore may use the same docking sites in vivo. However, differences in the particular interaction mode must exist between the two kininogen types. Likewise, T-kininogen the major acute phase reactant of the rat, which is not present in man, may dock to various cells; we have not experimentally addressed this possibility.

The fact that optimum binding of kininogen to GAG requires Zn2+ (this study) and depends on the pH of the medium points to a critical role of histidine residues in the protein-carbohydrate interaction. Recently, the involvement of histidine residues in the binding of histidine-rich glycoprotein (HRG) to heparin has been demonstrated (51). Tight binding between HRG and heparin occurs only under acidic pH conditions (<6.5), and the association is markedly enhanced in the presence of transition metal ions such as Zn2+ and Cu2+. This finding may suggest the involvement of protonated histidine side chains (51, 52). HRG consists of two cystatin domains and a unique His-rich region and is thus classified as a member of the cystatin superfamily, as are the kininogens. As HRG is considered an evolutionary ancestor of the kininogens (1), it is tempting to speculate that HK may accommodate HS-type GAG and heparin in a similar fashion as HRG. This notion is supported by the finding that HK efficiently antagonizes the enhancing effects of heparin on thrombin-antithrombin III complex formation (52). The high density of negatively charged groups in heparin and HS-type GAG could explain why the histidine-rich domain D5H is the major cell docking site of HK (13). Accordingly, other negatively charged carbohydrates such as dextran sulfates, shown to function as surrogates of the contact phase, provide an ideal in vitro platform for assembly of the various components of the kinin-generating complex, including kininogens (8, 53). By analogy, one may postulate that HS-PG represent the biological substratum on the surface of cardiovascular cells, which controls kininogen accumulation and processing (1, 35). The biological stimuli and molecular mechanisms driving kinin liberation on cellular surfaces are presently obscure (54), however, one reasonable candidate trigger is α-granule release from activated platelets, causing a burst of the local Zn2+ concentration (55). Future definition of the molecular events underlying the stimulus-promoted kinin release from HS-PG-bound kininogens will enhance our understanding of endocrine hormone systems that target inactive precursors to the site of action before releasing their potent cargo in situ.

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