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# Cell surface-associated chondroitin sulfate proteoglycans bind contact phase factor H-kininogen

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**Abstract The kinin system has been recognized as a locally operating hormone system of cardiovascular cells, however, the molecular mechanisms regulating circumscribed kinin release on cell surfaces are not fully understood. In particular, the principal cell docking sites for the kinin precursor, high molecular weight kininogen (HK), are not fully explored. Here we demonstrate by enzymatic digestion, recombinant overexpression, and affinity cross-linking studies that cell surface chondroitin sulfate (CS) chains of proteoglycans (PGs) serve as major HK binding sites on platelet, fibroblast, liver, and endothelial kidney cells. In this way, CS-type PGs may contribute to a local accumulation of kinin precursors on cell surfaces and modulate circumscribed release of short-lived kinin hormones at or next to their site of action.** 

*Key words:* Bradykinin; Kininogen; Contact phase system; Hypertension; Chondroitin sulfate; Heparan sulfate; Proteoglycan

## 1. Introduction

Kinins are short-lived peptide hormones of 9–11 amino acids that serve as key regulators of local blood pressure and inflammatory responses by increasing intracellular $Ca^{2+}$ levels, stimulating nitric oxide production, and enhancing prostaglandin biosynthesis [1]. Three major components regulate kinin production on surfaces of cardiovascular cells: factor XII (Hageman factor), plasma prokallikrein and high molecular weight kininogen (HK). These factors attach directly (factor XII, HK) or indirectly (prokallikrein via HK) to physiological cell surfaces, as well as to artificial surfaces such as kaolin or certain polymers. Exposure of blood to subendothelial structures ('contact phase') following disruption of the endothelial barrier, or secretion of proteases from neutrophil granulocytes that attach to the endothelium, initiate the conversion of small amounts of factor XII into its active form, factor XIIa. Factor XIIa activates prokallikrein to the active enzyme kallikrein, which, in turn, activates additional factor XII. Active kallikrein rapidly cleaves the tightly associated HK with a resulting burst of bradykinin liberation in proximity to its receptor [1].

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*Abbreviations:* CS, chondroitin sulfate; HK, high molecular weight kininogen; HS, heparan sulfate; PBS, phosphate-buffered saline

Contact factors have been immunolocalized to the surface of endothelial cells, neutrophils, platelets, vascular smooth muscle cells, kidney epithelial cells, glandular duct cells, neurons, and fibroblasts [2]. HK binds to cell surfaces via two segments on its D3 and $D5_H$ domains, and at the same time it tethers prokallikrein to cells via a specific binding site exposed on domain $D6_H$. Thus, HK serves as an adapter protein that links proteolytic activity and kinin generation to the surface of target cells. A search for HK acceptors has identified the proteins Mac-1 [3], p33/gC1qR [4], thrombospondin-1 [5], glycoprotein-Ib [6], cytokeratin-1 [7], and urokinase receptor (binds only to kinin-free HK) [8] as candidate binding proteins. Though all of these proteins bind to HK in vitro, none of them accounts for the high number of cellular HK binding sites on eukaryotic cell surfaces (up to $10^7$/cell) and their ubiquitous occurrence [4,7–11].

We have recently identified heparan sulfate (HS) glycosaminoglycans (GAGs) of proteoglycans (PGs) as major HK acceptors on endothelial cells [11]. Here, we demonstrate that HK binds to yet another type of GAG, chondroitin sulfate (CS). CS is the predominant GAG on most cell types other than endothelial cells, and could provide a platform for contact phase factors on most eukaryotic cells.

## 2. Materials and methods

### 2.1. HK binding assays

Confluent cell monolayers were washed three times with phosphate-buffered saline (PBS). To degrade cell-bound HS- and CS-type GAG, cells were incubated (30 min, 37°C) with 1 U/ml of chondroitinase ABC and/or a mixture of heparinase I and heparinase III (0.5 U/ml each) in PBS supplemented with a protease inhibitor cocktail. Washed cells were incubated (1 h, 37°C) in 0.135 M NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.36 mM $NaH_2PO_4$, 14.7 mM HEPES, 3.5 mg/ml dextrose, 50 μM $ZnCl_2$, pH 7.35 (HEPES-Tyrode's buffer, HT) containing 1% (w/v) bovine serum albumin and 20 nM [$^{125}$I]HK. Following six washes, cell-associated [$^{125}$I]HK was measured and normalized for total cellular proteins [4]. Alternatively, binding of [$^{125}$I]HK was carried out in cell suspension [9]. To study the effect of GAG on [$^{125}$I]HK binding to HEK293t cells, confluent cells were incubated with serial 1:2 dilutions of biotinylated HK ('biot-HK') in HT (starting from 10 nM) supplemented with 500 nM HK, CS-A, HS, glucose or HT (control). In vitro binding of 10 nM biot-HK to serial 1:2 dilutions (starting from 100 μg/ml) in HT of HS, CS-A, -B or -C (Sigma) was performed on poly-L-lysine-coated MaxiSorp™ microtiter plates (Nunc). For competition experiments, CS-A (250 μg/ml) was chemically linked to CovaLink™ plates (Nunc) by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide [11]. Binding of 10 nM biot-HK in HT to immobilized CS-A was quantified by the streptavidin–peroxidase method (Roche) in the presence of serial 1:2 dilutions of maltose binding protein (MBP) fused to HK domain D3 ('MBP–D3'), MBP–$D5_H$ or unfused MBP (starting concentration 2 μM);


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antibodies to bradykinin ('α-BK'), D3 ('αLDC27') or $D5_H$ ('αHKH20') (2 μM); GAG (CS-A, -B, -C, HS), glucose or unlabeled HK (200 μg/ml).

*2.2. Transient transfection of decorin and biglycan cDNAs*

Decorin cDNA was cloned by reverse transcription-PCR [11] from HF15 fibroblast mRNA using 5′-primer, 5′-ATGGGATCCATCATGAAGGCCACTATCATCCTCC-3′ (nucleotide positions 214–247 referring to cDNA accession no. J02814) and 3′-primer, 5′-GAACCCGGGATAGTTTCCGAGTTGAATGGCAGAGCG-3′ (positions 1273–1308). For cloning of the biglycan cDNA from mRNA of EA.hy926 cells, we used 5′-primer, 5′-GGTGGATCCGCCATGTGGCCCCTGTGGCGCCTCG-3′ (positions 109–142, accession no. J04599) and 3′-primer, 5′-CTGCCCGGGCTTTTTGTAGTTGCCAAACTGGATGG-3′ (positions 1199–1233). The 5′-primers introduce a *Bam*HI site 6 bp upstream of the AUG start codon, and the 3′-primers replace the stop codon by a CCC triplet for an additional *Sma*I site. An oligonucleotide encoding a double strand myc epitope was ligated onto the 3′-end of the PCR product using the *Sma*I sites. The resulting cDNAs were cloned into the pcDNA3.0 vector (Invitrogen) using *Bam*HI and 3′ *Eco*RI sites from the myc oligonucleotide. The cDNA constructs (correct sequence verified by full-length sequencing) were used to transfect COS-7 cells by DEAE-dextran or HEK293t cells by lipofectamine (Life Technologies) techniques. HK binding assays were performed 60 h after transfection. Transfection efficiency was >50% as determined by co-transfection with a vector encoding green fluorescent protein.

*2.3. Immunofluorescence*

HK bound to HUVEC, KINS fibroblasts and HEK293t cells was immunolocalized with 20 μg/ml of antibody I107 directed to the light chain of HK as described [11]. Nuclear staining was done with 1% (w/v) 4,6-diamidino-2-phenylindole (Sigma) before embedding in *N*-propyl gallate.

*2.4. Cross-linking and immunoprecipitation*

COS-7 cells overexpressing myc-tagged decorin or biglycan were three times PBS-washed, incubated (1 h, 4°C) with 10 mM sodium metaperiodate (Sigma) in 150 mM sodium acetate, pH 5.5. Oxidation was terminated by adding glycerol to 15 mM and incubating for 5 min at 4°C. The hetero-bifunctional photoaffinity cross-linker *p*-azidobenzoyl-hydrazide (ABH, Pierce) was dissolved in DMSO at 250 mM, and 50 μl was added to the reaction mixture (final ABH concentration 5 mM) followed by incubation (30 min, 37°C) in the dark [12]. Cells were washed six times with PBS in the dark, and a cell binding assay using 20 nM [$^{125}$I]HK was performed. The cross-linker was activated with 10 flashlights, the cells were six times PBS-washed, lysed in 1 ml of RIPA buffer and centrifuged (14 000×*g*, 15 min, 4°C). The supernatant was divided and subjected to immunoprecipitation using α-myc or α-HK (I107) antibodies bound to protein G-Sepharose (Pharmacia), respectively. Following centrifugation and washing three times, immunoprecipitates were dissolved in SDS buffer, and analyzed by SDS–PAGE and autoradiography [13].

## 3. Results

*3.1. HK binding to various cell types*

To analyze the interaction of HK with various cell types, we employed a direct binding assay using [$^{125}$I]HK in the presence of 50 μM $Zn^{2+}$ (Fig. 1). Specific binding of [$^{125}$I]HK to cells was defined as the difference between the total binding and the binding in the presence of a 250-fold molar excess of HK cell binding domains D3 and $D5_H$. The following rank order for HK binding was obtained: HUVEC (set to 100%) > EA.hy926 (89%) > HEK293t (83%) > KINS fibroblasts (81%) > platelets (77%) > HepG2 (61%) > COS-7 (52%) > erythrocytes (35%). In the absence of $Zn^{2+}$, specific binding was reduced 2–3-fold, for all cell types tested. Immunofluorescence analysis with anti-HK antibodies revealed similar staining patterns for HUVEC, fibroblasts and HEK293t cells (Fig. 1, inserts). Cell-bound HK was prevalent at the cell surface and staining extended pericellularly into the extracellular matrix. These results may indicate that various cell types expose similar HK binding sites, albeit at varying copy numbers.

*3.2. Docking of HK to the cell surface via CS-type PG*

As we have previously identified the HS type of GAGs as the major HK binding sites on endothelial cells, we wanted to determine if the same structure may account for HK binding by other cell types, and whether the most abundant GAG type, CS [14], may be involved. To this end, we surface-digested confluent cells with chondroitinase ABC alone, or in

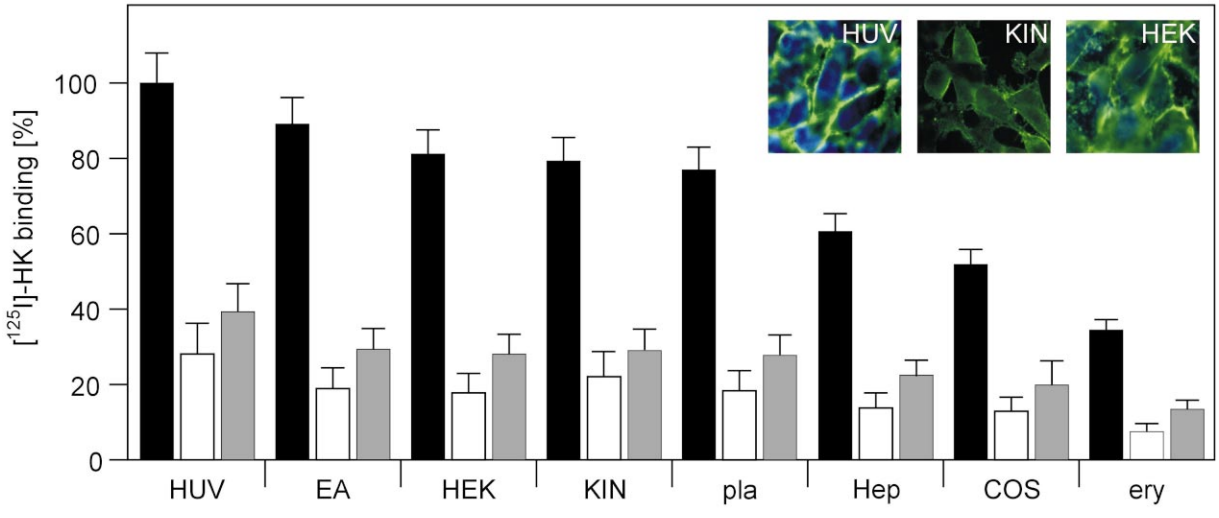


Fig. 1. Binding of HK to cell surfaces. Relative binding capacity for 20 nM [$^{125}$I]HK on HUVEC (HUV), EA.hy926 (EA), platelets (pla), erythrocytes (ery), HepG12 (Hep), KINS fibroblasts (KIN), HEK293t (HEK), and COS-7 (COS) cells in the presence of 50 μM $Zn^{2+}$. Total HK binding (dark columns) was arbitrary set at 100% for HUVEC. Unspecific binding was determined in the presence of 500 nM MBP–D3 and MBP–$D5_H$ (light columns) or in the absence of $Zn^{2+}$ (gray columns). Means ± S.D. of three independent binding assays standardized to 1 μg of cell protein are presented. Inserts: immunolocalization of HK bound to cultured HUVEC, KINS fibroblasts and HEK293t cells. Magnification ×100.

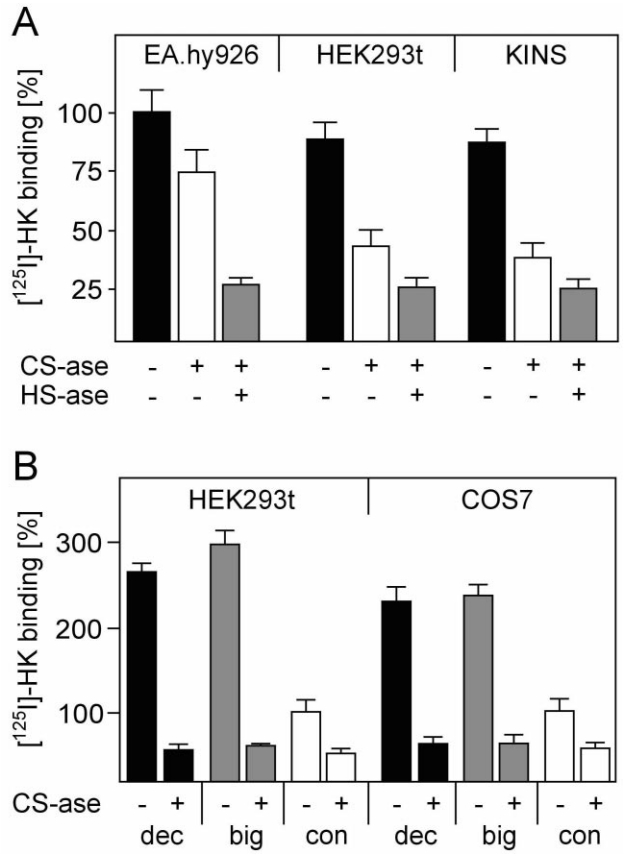


Fig. 2. Correlation of HK binding capacity and CS expression. A: Relative binding of 20 nM [$^{125}$I]HK to EA.hy926, KINS fibroblasts and HEK293t cells with or without prior treatment with chondroitinase ABC (CS-ase) and/or heparinase I/III (HS-ase); untreated EA.hy926 cells are arbitrary given a binding capacity of 100%. B: [$^{125}$I]HK binding to COS-7 and HEK293t cells transiently overexpressing decorin (dec) or biglycan (big) or mock-transfected cells (con), with or without prior treatment with chondroitinase ABC (CS-ase). Means ± S.D. of three independent experiments are presented.

combination with heparinases I/III, and measured the [$^{125}$I]HK binding capacity of the treated cells (Fig. 2A). Controls were done in the absence of enzyme (set at 100%). Chondroitinase treatment reduced HK binding to 43%, 50% and 73% of the control for KINS fibroblasts, HEK293t, and EA.hy926 cells, respectively. Co-incubation with heparinases further lowered the binding capacity to 27%, 28% and 25%, respectively, of the control. The chondroitinase-induced loss of kininogen binding capacity was minor for endothelial cells (27% decrease of binding), while the effect of heparinase was most pronounced on this cell type (48%). In contrast, chondroitinase treatment of KINS fibroblasts resulted in a major loss of HK binding capacity (57% decrease of binding), whereas the effect of heparinase was relatively minor (16%). Similar results were obtained for HEK293t cells (Fig. 2A). Thus, CS-type PG plays a major role as HK docking structures on fibroblasts and embryonic kidney cells, but are of minor importance for binding to endothelial cells.

To further substantiate a role for CS in HK binding, we tested whether upregulation of cell surface CS-PG is paralleled by an increase in HK binding capacity. The prototypical CS-type PG decorin and biglycan were transiently overexpressed in HEK293t and COS-7 cells. After 60 h, [$^{125}$I]HK binding capacity was tested (Fig. 2B). Mock-transfected cells (set to 100% HK binding) were used as control. Overexpression of decorin raised HK binding capacity to 271% and 228% for HEK293t and COS-7 cells, respectively, while overexpression of biglycan increased the HK binding capacity to 296% and 235%, respectively (Fig. 2B). Surface digestion of PG overexpressing cells using CS-ase resulted in a decrease in HK binding of up to 39% compared to non-digested, mock-transfected control cells. Thus, both loss-of-function and gain-of-function experiments clearly point to CS GAG as major HK acceptor sites on various cell types.

### 3.3. Specificity of HK binding to CS in vitro

To test the specificity of HK binding to CS, we used a direct binding assay in which GAGs were covalently bound to microtiter plates. Biot-HK specifically bound to various CS types with high affinity (Fig. 3A). The apparent $K_D$ values were 2.5 nM (CS-A), 3.5 nM (CS-B), and 10 nM (CS-C). Binding to HS was used as a positive control ($K_D = 8$ nM). To test the specificity of HK binding to CS-A, we applied competitors with apparent $IC_{50}$ values of 3–14 nM (CS-A, -B and -C), 8 nM (HS) and 2 nM (unlabeled HK) (Fig. 3B). Recombinant fusion proteins MBP–D3 ($IC_{50} = 1.1$ μM) or MBP–$D5_H$ (200 nM), as well as antibodies αLDC27 (750 nM) and αHKH20 (85 nM) directed against the cell binding segments of HK domains D3 and $D5_H$, respectively, competed with HK for CS binding (Fig. 3C). We also tested the specificity of biot-HK binding to confluent HEK293t cells. Soluble CS at 500 nM reduced HK cell binding by $>60\%$ at 10 nM of biot-HK, as did HS and unlabeled HK but not glucose (Fig. 3D).

### 3.4. Cross-linking of HK to CS GAG

To demonstrate the physical interaction of HK with cell surface-bound CS, we employed COS-7 cells that overexpress myc-tagged decorin or myc-tagged biglycan CS-PG. Following oxidation of the GAG the hetero-bifunctional photoaffinity cross-linker ABH was attached to oxidized carbohydrates. ABH-conjugated cells were incubated with 20 nM [$^{125}$I]HK, and cross-linking was induced with light. Cell lysates were subjected to immunoprecipitation with anti-HK or anti-myc antibodies. Autoradiography of the precipitates showed bands migrating at 270–290 kDa, corresponding to the combined molecular masses of [$^{125}$I]HK bound to decorin-myc or biglycan-myc (Fig. 4). The heterogenity of the signals, typically for PG, most likely reflects heterogenity in the CS chains. Cells that had been treated with CS-ase failed to produce any specific bands (not shown). Additional high molecular weight bands (migrating at $>500$ kDa) may be cross-linked multimeric complexes of HK-decorin or HK-biglycan, and/or high molecular mass PG bound to HK, however, we have not experimentally addressed these possibilities. Altogether, our data demonstrate a crucial role for CS-type PG in HK binding to cells.

## 4. Discussion

The binding of HK to cell surfaces is a crucial step in the kinin-generating pathway since (i) it assembles two of the three contact phase factors onto endothelial and epithelial linings [1]; (ii) docking to the cell surface protects HK from proteolysis by kallikreins, preventing a constitutive release of

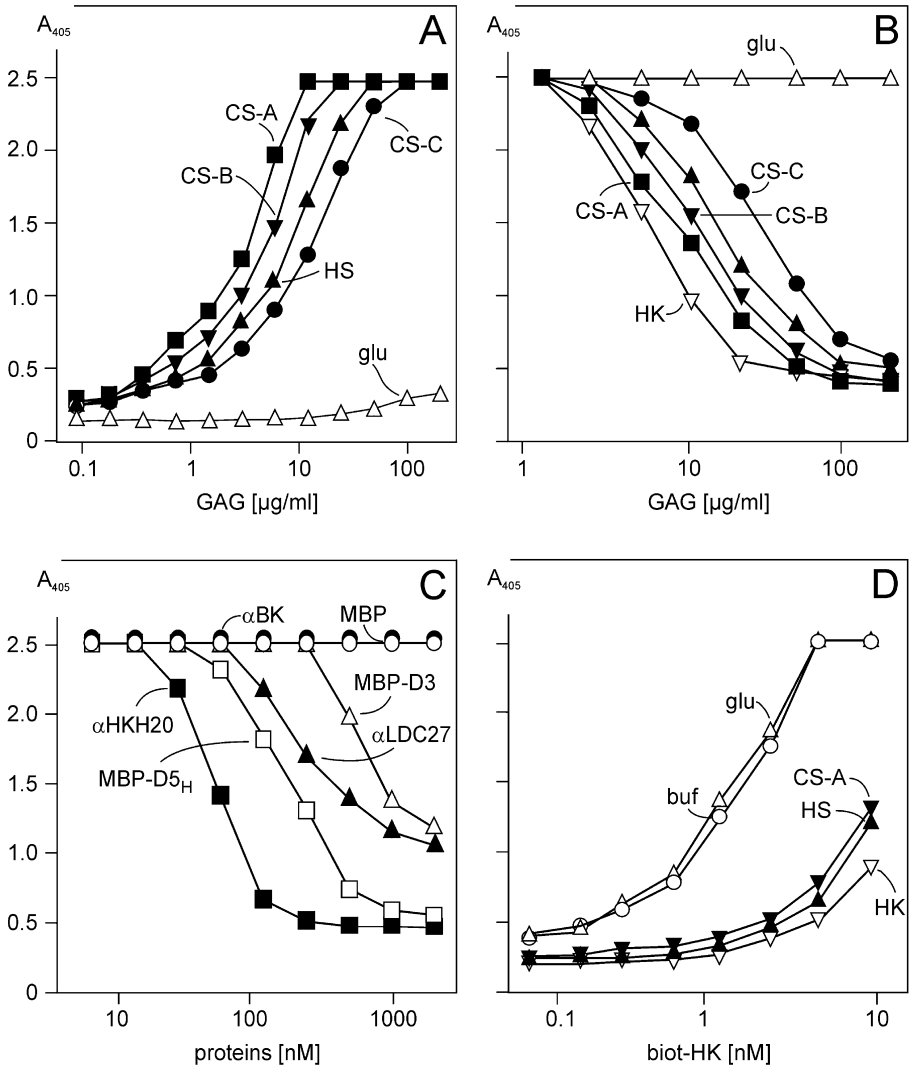


Fig. 3. HK interaction with CS in vitro. A: Direct binding of 10 nM biot-HK to CS-A (■), CS-B (▼), CS-C (●), HS (▲), and glucose (glu, △). B: Specificity of HK binding to covalently immobilized CS-A was probed by incubating 10 nM biot-HK with serial 1:2 dilutions of competitors CS-A (■), CS-B (▼), CS-C (●), HS (▲), glu (△), HK (▽). The starting concentration for each competitor is 200 μg/ml. C: Specificity of HK binding to covalently immobilized CS-A was probed by incubating 10 nM biot-HK with serial 1:2 dilutions of HK domains MBP–D3 (△), MBP–D5$_H$ (□) or MBP (○); antibodies αLDC27 (▲), αHKH20 (■), or αBK (●) (down from 2 μM each). D: Binding of biot-HK (serial 1:2 dilutions start at 10 nM) to confluent HEK293t cells in the absence (buffer alone, buf) or presence of 500 nM HK (▽), HS (▲), CSA (▼) or glu (△). Bound biot-HK was quantified by the streptavidin–peroxidase method.

bradykinin [10]; (iii) attachment to cell surfaces locates the prohormone in close proximity to its receptors, increasing the probability that short-lived bradykinin will trigger cellular responses [15]. We have recently identified HS-type GAG as the major HK docking structures on endothelial cells [11]. Our present finding that CS are important HK acceptors for many non-endothelial cells broadens and generalizes the concept that PG anchors HK to most cells. Although the mode of HK interaction with PG has not been addressed in molecular detail, it appears reasonable to postulate that both CS- and HS-PG bind to HK through their negatively charged GAG portion. Most likely these carbohydrates interact electrostatically with charged amino acid side chains that are abundant in the His/Lys-rich region of the HK D5$_H$ domain [16].

Binding of (pre)hormones to GAG chains of PG appears to be a common biological phenomenon. Basic fibroblast growth factor (bFGF), interferon-γ, and interleukins 7, 8, 10 and 12 attach to GAG on cell surfaces [17]. Based on these observations a functional model of PG as modulator of hormone action is emerging. GAGs provide cell-bound reservoirs of cytokines and other potent hormones that are presented to receptors on target cells [18]. Some cytokines are protected from proteolytic degradation when bound to GAG, as is the case for cell-bound HK [10]. Furthermore, GAG-bound cytokines can be detached from cell surfaces by heparin released from mast cells during inflammation [19]. Additionally, shedding proteases or heparinases cleave PG, creating soluble PG fragments with hormones (e.g. bFGF) still attached [20].

The contact phase factors HK, plasma prekallikrein and factor XII comprise a plasma kinin-generating system that complements the tissue-based system comprised of tissue kallikrein, low molecular weight kininogen (LK) and kallistatin [21]. While the cell binding of LK through D3 is well established, the recent identification of a major heparin binding site

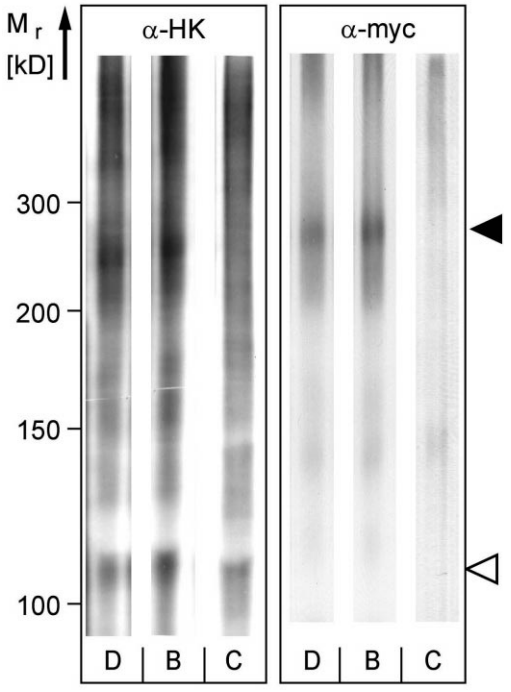


Fig. 4. Cross-linking of [$^{125}$I]HK to the CS portion of PG. GAG chains of transiently overexpressed decorin-myc (D) or biglycan-myc (B) PG were photoaffinity cross-linked to [$^{125}$I]HK using ABH. After lysis of the cells, immunoprecipitation with α-HK (left panel) or α-myc (right panel) was performed, followed by SDS–PAGE and autoradiography. Mock-transfected cells served as control (C). Relative positions of cross-linked products ([$^{125}$I]HK-PG) and free ligand ([$^{125}$I]HK) are indicated by solid and open arrowheads, respectively. Positions of molecular mass standards are shown on the left.

in the proteinase inhibitor kallistatin may shed new light on the role of PG in the tissue-bound system [22]. Indeed CS- and HS-PG in the extracellular matrix and on surfaces of (for example) smooth muscle cells may bind to, and assist, kallistatin by bringing the inhibitor in close apposition to its target protease, tissue kallikrein. Thus, similar to the plasma kinin, the tissue kinin system may use a PG-based platform to control its powerful actions in glands and neurons.

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