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## Proteoglycans modulate renal inflammation

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## Chapter 2

### **Identification of Tubular Heparan Sulfate as a Docking Platform for the Alternative Complement Component: Properdin in Proteinuric Renal Disease**

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## ABSTRACT

Properdin binds to proximal tubular epithelial cells (PTEC) and activates the complement system via the alternative pathway *in vitro*. Cellular ligands for properdin in the kidney have not yet been identified. Because properdin interacts with solid phase heparin, we investigated whether heparan sulfate proteoglycans (HSPG) could be the physiological ligands of properdin. Kidneys from proteinuric rats showed colocalization of syndecan-1, a major epithelial HSPG, and properdin in the apical membranes of PTEC, which was not seen in control renal tissue. *In vitro*, PTEC did not constitutively express properdin. However, exogenous properdin binds to these cells in a dose-dependent fashion. Properdin binding was prevented by heparitinase pretreatment of the cells and was dose-dependently inhibited by exogenous heparin. ELISA and surface plasmon resonance spectroscopy (BIAcore) showed a strong dose-dependent interaction between heparan sulfate (HS) and properdin ( $K_D$ : 128 nM). Pretreatment of HSPG with heparitinase abolished this interaction in ELISA. Competition assays, using a library of HS-like polysaccharides, showed that sulfation pattern, chain length, and backbone composition determine the interaction of properdin with glycosaminoglycans. Interestingly, two non-anticoagulant heparin derivatives inhibited properdin-HS interaction in ELISA and BIAcore. Incubation of PTEC with human serum as complement source led to complement activation and deposition of C3 on the cells. This C3 deposition is dependent on the binding of properdin to HS as shown by heparitinase pretreatment of the cells. Our data identify tubular HS as a novel docking platform for alternative pathway activation via properdin, which might play a role in proteinuric renal damage. Our study also suggests non-anticoagulant heparinoids may provide renoprotection in complement-dependent renal diseases.

## 2.1 Introduction

The complement system plays an important role in glomerular injury and the development of tubulointerstitial scarring in several progressive renal diseases (1, 2). One of the three complement activation pathways is the alternative pathway (AP), and there is growing evidence of activation of AP on renal tubular cells in proteinuric renal diseases (3–7).

The AP is controlled by a number of inhibitory regulators, but there is only one known positive regulator, properdin (8–10). This protein was first discovered in 1954 but received renewed interest in the 1970s (9). Properdin is a highly positively charged protein. It is composed of identical subunits that associate together to make dimers, trimers, tetramers, and even higher oligomers. Properdin oligomerization is known to be essential for its function (9). AP activation can be amplified following formation of the C3 convertase complex (C3bBb). Nascent C3bBb is known to be unstable in plasma. Properdin can bind to this complex and stabilizes it 5–10- fold, protecting the complex partially from inhibition by factors I and H (8–10). Recent data indicate that extending the half-life of the C3bBb complex is not the only role of properdin. Properdin also may initiate AP activation by binding to the target surface and providing a platform for convertase assembly (11, 12). In proteinuria, properdin is found in the urine and may bind to proximal tubular cells. *In vitro* it was demonstrated that this properdin activates the AP on renal cells (6, 13). Furthermore, the excretion of properdin in urine is correlated with increased urinary levels of the terminal complement cascade complex C5b-9 and with poor renal function (6, 13).

Although properdin activates the AP on renal tubular cells, the ligand for properdin to bind to these cells is not yet known. A previous study showed that properdin can bind to immobilized heparin (14). In another study comparing wildtype and glycosaminoglycan (GAG)-deficient Chinese hamster ovary (CHO) cells, it was shown that properdin binds to these cells via heparan sulfate and chondroitin sulfate proteoglycan chains and that this binding is dependent on the sulfation pattern of these GAG chains (15). It has also been shown that properdin binds to apoptotic T cells via GAGs (15).

GAGs are linear polysaccharides covalently bound to a core protein, forming a proteoglycan. Based on the composition of GAG chains, proteoglycans are categorized as heparan sulfate (HS), chondroitin sulfate (CS), keratan sulfate, or dermatan sulfate proteoglycans (16, 17). The sulfation pattern on these GAG chains affects activities of proteoglycans (18). Proteoglycans are found in the extracellular matrix and on almost all mammalian cell types, and they can interact with many factors among which are growth factors, cytokines, and chemokines (19). Proteoglycans are involved in cell proliferation, differentiation, inflammation, development, cell-cell adhesion, and signaling (19–22). Although proteoglycans play a role in mammalian physiology, under certain conditions they can also be involved in the pathophysiology of certain diseases (19). The most abundant form of GAGs found in renal tissue is HS (23). These HS polysaccharide side chains display variations in sulfation and the expression pattern in renal tubulointerstitium of various renal diseases (24).

To clarify the mechanism of AP activation by properdin on renal tubular cells, we studied the possibility of tubular GAGs acting as ligands for properdin. To this end, we searched for the presence of properdin in several proteinuric rat models and investigated the interaction of properdin with heparan sulfate proteoglycans *in vitro*. Our results

showed tubular heparan sulfate proteoglycan to be a platform for properdin binding. We therefore suggest that, upon leakage of proteins from plasma into the primary urine, properdin binds to tubular cells and subsequently leads to complement activation on tubular cells ultimately leading to renal injury.

## 2.2 Experimental procedures

*Tissues*—Experimental renal disease was induced in male Wistar rats with a single injection of adriamycin as described previously (25). Protein-overload nephropathy was also induced in male Wistar rats as described previously (26). Healthy male Wistar rats were used as source of normal control rat renal tissue. Kidneys of rats were obtained, quickly frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Sections were cut and used for immunofluorescent staining (see below).

*Immunofluorescent Staining*— Human properdin and polyclonal rabbit anti-human properdin antibodies were prepared as described previously (6). Rabbit anti-human syndecan-1 was purchased from Invitrogen, Zymed Laboratories Inc.

Frozen sections (5  $\mu\text{m}$  thickness) were cut from rat renal tissue with cryostat Leica CM1950. First, the sections were fixed with acetone for 10 min. The sections were then washed with TBS (20 mM Tris, 150 mM NaCl, 5 mM  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 5 mM  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ , pH 7.4), followed by blocking endogenous peroxidase activity with 0.1%  $\text{H}_2\text{O}_2$ . After washing with TBS, sections were blocked with 5% bovine serum albumin (BSA) for 15 min. After washing with TBS, the slides were incubated with 10  $\mu\text{g}/\text{ml}$  polyclonal rabbit anti-human properdin antibody diluted in 1% BSA for 1 h. After washing with TBS, sections were incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) (1:100) for 45 min. The HRP antibody was detected by the TSA<sup>TM</sup> tetramethylrhodamine system (PerkinElmer Life Sciences) (10 min, 1:50). Slides were washed again with TBS and mounted in VECTASHIELD mounting medium with DAPI for nuclear staining (Brunschwig Chemie, Amsterdam, The Netherlands). The whole staining procedure was done at room temperature.

Double staining of properdin and syndecan-1 was done on adriamycin-induced proteinuric rat renal tissue. The double staining was done by Zenon rabbit IgG labeling kit (Molecular Probes). Rabbit anti-human syndecan-1 was labeled with Alexa 647 with a molar ratio 1:6 of antibody/ labeling reagent. Rabbit anti-human properdin was labeled with the same molar ratio with Alexa 488. The antibodies were incubated with labeling reagents for 5 min and then blocked with the kit's blocking reagent for an additional 5 min, and the mixtures were applied to the sections and incubated for 1 h. The fixation, washings, and blockings were done as described above. An additional fixation was done with 4% formaldehyde at the end of the process just before mounting.

In addition, a binding assay was performed to evaluate the localization and identity of the binding sites for properdin on renal tissue. To this end, sections were incubated with 10  $\mu\text{g}/\text{ml}$  purified human properdin before detection with anti-properdin antibody.

To cleave HS, pretreatment of the sections was done with heparitinase I (from flavobacterium, 0.05 unit/ml; purchased from Seikagaku Corp., Tokyo, Japan) for 1 h at  $37^{\circ}\text{C}$ . Heparitinase was diluted in acetate buffer (50 mM  $\text{C}_2\text{H}_3\text{O}_2\text{Na}$ , 5 mM  $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ , 5 mM  $\text{MgCl}_2\cdot 6\text{H}_2\text{O}$ , pH 7.0).

Fluorescence microscopy was performed using a Leica DMLB microscope (Leica Microsystems, Rijswijk, The Netherlands) equipped with a Leica DC300F camera and LeicaQWin 2.8 software. Photos were taken from cortical regions of the tissue in random order.

Quantification of the stainings was done in five animals for each group (normal rat renal tissue, adriamycin-induced nephropathy renal tissue, and protein overload-induced nephropathy renal tissue) on 10 randomly taken photos of cortical tubular regions per sample (magnification  $\times 400$ ). In heparitinase I pretreated sections of normal rat, 10 random photos were taken per field for each group (magnification  $\times 200$ ). The total area stained was quantified using ImageJ 1.41 (rsb.info.nih.gov).

The difference between groups in total area stained for properdin was tested with a Mann-Whitney U test;  $p < 0.05$  was considered statistically significant. Statistics were performed using GraphPad Prism 5.00 for Windows (GraphPad Software Inc.).

*HK-2 Cells and Renal Tissue*—The immortalized human kidney proximal epithelial cell line HK-2 was provided by M. van der Toorn (Laboratory of Allergology and Pulmonary Diseases, University Medical Center, Groningen). Cells were cultured in DMEM/F-12 medium (Invitrogen), supplemented with 2 mM L-glutamine, 25 mM HEPES, 50 units/ml penicillin, 50  $\mu\text{g}/\text{ml}$  streptomycin (all purchased from Invitrogen), and also 5  $\mu\text{g}/\text{ml}$  insulin, 5  $\mu\text{g}/\text{ml}$  transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, and 10 ng/ml epidermal growth factor (EGF) (all purchased from Sigma).

For properdin staining on HK-2 cells, the cells were grown on cover glass in wells in medium as described above. The medium was removed, and the cells were washed with TBS and incubated with 5% BSA for 15 min. After washing with TBS, the cells were incubated with 1  $\mu\text{g}/\text{ml}$  anti-human properdin antibody. Bound anti-properdin antibody was detected by HRP-labeled goat anti-rabbit immunoglobulins. The signal was visualized by using the TSA<sup>TM</sup> tetramethylrhodamine system. The whole staining procedure was done on ice without fixation and permeabilization. For evaluating the binding sites for properdin on HK-2 cells, the binding assay was performed following incubation of the cells with 5  $\mu\text{g}/\text{ml}$  human properdin before incubation with anti-properdin antibody.

Pretreatment of the cells with heparitinase I (from flavobacterium, 0.05 units/ml) was done for 1 h at 37 °C, to cleave HS side chains of proteoglycans on HK-2 cells. The heparitinase was diluted in acetate buffer (50 mM  $\text{C}_2\text{H}_3\text{O}_2\text{Na}$ , 5 mM  $\text{CaCl}_2\text{H}_2\text{O}$ , 5 mM  $\text{MgCl}_2\text{6H}_2\text{O}$ , pH 7.0). The microscopy and statistics were done in the same fashion as described above.

*FACS Analysis*—C3 recruitment from serum by HK-2 bound properdin and its dependence on tubular heparan sulfates was tested by FACS staining. HK-2 cells were cultured in 48-well tissue culture plates. Cells were incubated with heparitinase I (from flavobacterium, 0.05 units/ml) and chondroitinase ABC (5 units/ml) diluted in medium without serum at pH 7.2 for 30 min at 37 °C. Both enzymes were purchased from Seikagaku Corp., Tokyo, Japan. After washing the cells with medium, human properdin (10  $\mu\text{g}/\text{ml}$ ) was added, and incubation was followed for 30 min at 37 °C. Cells were washed again and incubated further with 5% normal human serum for 1 h at 37 °C. Thereafter, the cells were washed with medium and harvested with nonenzymatic cell dissociation solution (Sigma). The cells were then transferred to FACS tubes and incubated with rabbit anti-human properdin (to detect properdin binding on cells) or with mouse antihuman C3 (to detect C3 deposition and AP activation on cells) (RFK-22; Laboratory of Nephrology,

Leiden, The Netherlands) for 30 min on ice. Cells were washed with PBS, centrifuged three times, and incubated with goat anti-mouse IgG-APC (Beckman Coulter, Woerden, The Netherlands) or goat anti-rabbit IgG-PE (Southern Biotech Associates, Birmingham, AL) for 30 min on ice. Annexin V (VPS Diagnostics, Hoeven, The Netherlands) was also included for staining of apoptotic and dead cells. Propidium iodide (1  $\mu\text{g}/\text{ml}$ , Molecular Probes, Leiden, The Netherlands) was used for exclusion of apoptotic cells at the last step. Properdin binding and C3 deposition on cells were assessed using a FACSCalibur flow cytometer (BD Biosciences).

*Competition ELISAs*—Maxisorp 96-well plates (U96 from VWR International, Amsterdam, The Netherlands) were coated overnight in PBS with 5  $\mu\text{g}/\text{ml}$  heparan sulfate proteoglycan (HSPG/mouse perlecan, Sigma). After washing in PBS with 0.05% Tween 20, wells were blocked with 2% BSA in PBS for 1 h. In a separate microtiter plate, properdin (0.75  $\mu\text{g}/\text{ml}$  diluted in PBS) was incubated with a dilution range of different HS-like heparinoids (see below) for 30 min and transferred to the ELISA plate after the wells were washed again. Incubation in the wells took 1 h. The wells were washed again, and polyclonal rabbit anti-human properdin antibody (10  $\mu\text{g}/\text{ml}$ ) diluted in PBS was added for 1 h. Secondary antibody was added after a washing step (HRP-labeled goat anti-rabbit immunoglobulins, 1:2000 DAKO, Glostrup, Denmark). Secondary antibody was detected with 3,3',5,5'-tetramethylbenzidine substrate (Sigma) for 15 min in the dark, and the reaction was stopped by adding 1.5 N  $\text{H}_2\text{SO}_4$ . Absorbance was measured at 450 nm in a microplate reader. All incubations were done at room temperature in a volume of 100  $\mu\text{l}/\text{well}$ .

*Polysaccharides*—Heparin from ovine intestinal mucosa, heparin from bovine lung, heparin from porcine intestinal mucosa, CS-A, CS-B, CS-C, dextran, dextran sulfate, fucoidan, *N*-acetylated heparin porcine intestine, and perlecan HSPG were from Sigma. Fraxiparin (molecular weight  $\approx 4500$ ) was purchased from Sanofi Winthrop (Maassluis, The Netherlands); Fragmin (molecular weight  $\approx 6000$ ) was purchased from Pharmacia & Upjohn, and enoxaparin or clexane (molecular weight  $\approx 4500$ ) were purchased from Rhone-Poulenc Rorer (Paris, France). Keratan sulfate, isolated from bovine cartilage, was obtained from Fluka Biochemicals (Buchs, Switzerland). HS isolated from bovine kidney or from Engelbreth-Holm-Swarm sarcoma and *N*-sulfated K5 were obtained from Seikagaku Corp. *Escherichia coli* capsular polysaccharide K5, with the same (GlcUA  $\rightarrow$  GlcNAc)<sub>n</sub> structure as the nonsulfated HS/heparin precursor polysaccharide (27); *O*-sulfated K5; low molecular weight *O*-sulfated K5; and *N*-desulfated reacylated heparin were kindly provided by Dr. G. van Dedem (Diosynth, Oss, The Netherlands). HS from bovine intestine was kindly provided by Marco Maccarana (Department of Experimental Medical Science, Biomedical Center, University of Lund, Sweden) (28).

HS from human aorta was isolated essentially as described by Iverius (29). *N*-deacetylation was performed by hydrazinolysis as described previously (30). *N*-acetylation of polysaccharides was performed by treatment with acetic anhydride as described by Danishefsky and Steiner (31). Completely (*N* and *O*-) desulfated heparin was prepared according to Bäckström *et al* (32) and reacylated as described above. *N*+*O*-sulfated K5 was produced by *N*-deacetylation (hydrazinolysis), subsequent *N*-sulfation with sulfur trioxide-trimethylamine (33), followed by *N*-acetylation. The same procedure was followed for HS from bovine kidney to make a fully *N*-sulfated HS and for completely desulfated heparin to make *O*-desulfated, *N*-sulfated heparin (34).

*Surface Plasmon Resonance-based Binding Assay*—Size defined heparin (6 kDa) and HS (15 kDa) were biotinylated at the reducing end and immobilized on a BIAcore sensor chip (BIAcore AB) in different flow cells as described previously (35). For this purpose, three flow cells of a CM4 sensor chip on a BIAcore 1000 were activated with 50  $\mu$ l of 0.2 M 1-ethyl-3-(dimethylaminopropyl) carbodiimide, 0.05 M *N*-hydroxysuccinimide before injection of 50  $\mu$ l of streptavidin (0.2 mg/ml in 10 mM acetate buffer, pH 4.2). Remaining activated groups were blocked with 50  $\mu$ l of 1 M ethanolamine, pH 8.5. Typically, this procedure permitted coupling of 2000–2500 resonance units of streptavidin. Biotinylated heparin (5  $\mu$ g/ml) in HBS (10 mM HEPES, 150 mM NaCl, pH 7.4) containing 0.3 M NaCl was then injected over the second flow cell to obtain an immobilization level of 60 resonance units. Similarly, an HS surface was prepared on the third flow cell (70 resonance units). The first flow cell was left with streptavidin only, as a negative control. Flow cells were then conditioned with several injections of 1 M NaCl.

For binding assays, different concentrations of purified human properdin (ranging from 0 to 200  $\mu$ g/ml) in HBS-P buffer (HBS plus 0.005% surfactant P20) were injected over the streptavidin only, heparin, and HS surfaces for 5 min at a flow rate of 10  $\mu$ l/min, followed by a 5-min washing with HBS-P buffer (BIAcore AB) and a 2-min regeneration step with 3 M MgCl<sub>2</sub>. For competition experiments, different polysaccharides (at 2.5  $\mu$ g/ml, final concentration) were incubated with purified human properdin (12.5  $\mu$ g/ml, final concentration) for 30 min and then injected on the heparin coated flow cell, at a flow rate of 10  $\mu$ l/min. Sensorgrams were fitted to a 1:1 Langmuir binding model using the BIAevaluation 3.1 software to determine the on and off rate constants ( $k_{\text{on}}$  and  $k_{\text{off}}$ ) from which the affinity  $K_D$  value was calculated ( $K_D = k_{\text{off}}/k_{\text{on}}$ ).

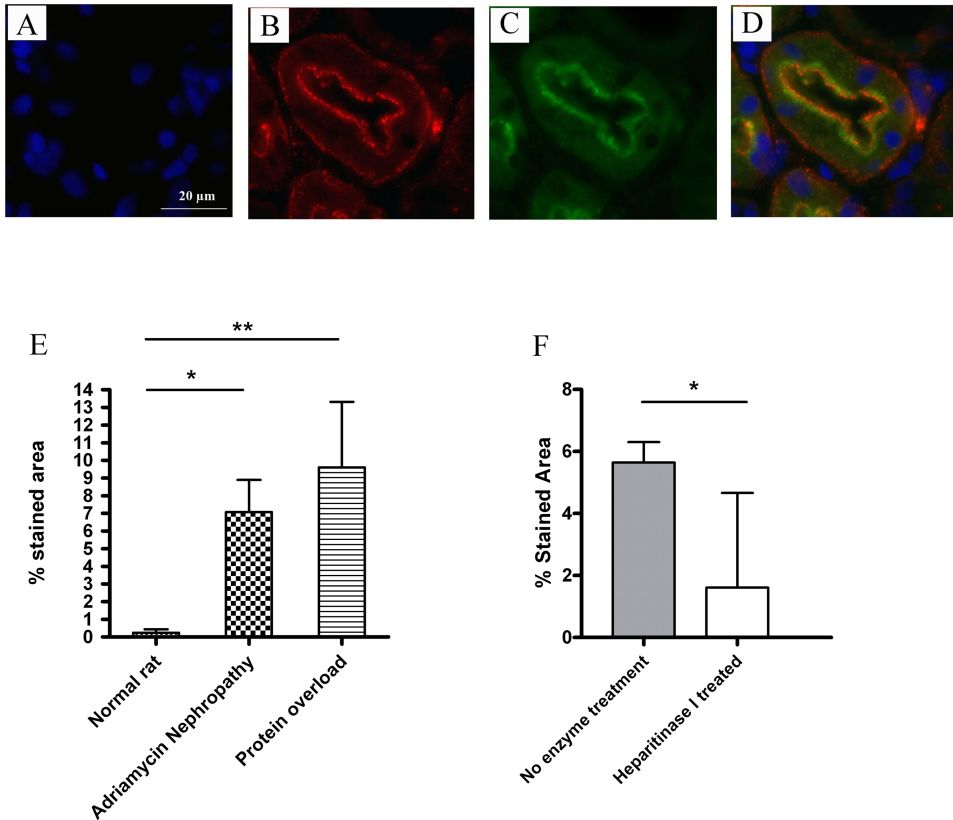
## 2.3 Results

*Properdin Colocalizes with Syndecan-1 on Apical Side of Tubular Epithelial Cells* — Syndecan-1 is a major cell surface HSPG on tubular cells. By a double staining approach in adriamycin nephrotic rats, we demonstrated the presence of syndecan-1 at basal and apical cell membranes, whereas properdin was only present at the apical cell membranes and nicely colocalized with syndecan-1 (Fig. 2.1, A–D). Based on the location of these tubules (mostly cortex), the diameter and the presence of a brush border, we identified these tubules as being proximal tubules. In protein overload nephropathy, properdin exactly localized in the same apical tubular membranes, which was not found at all in control renal tissue. Results of quantification showed a significant increase in the presence of tubular properdin in protein overload-induced nephropathy and adriamycin-induced nephropathy as compared with normal rat renal tissue (Fig. 2.1 E).

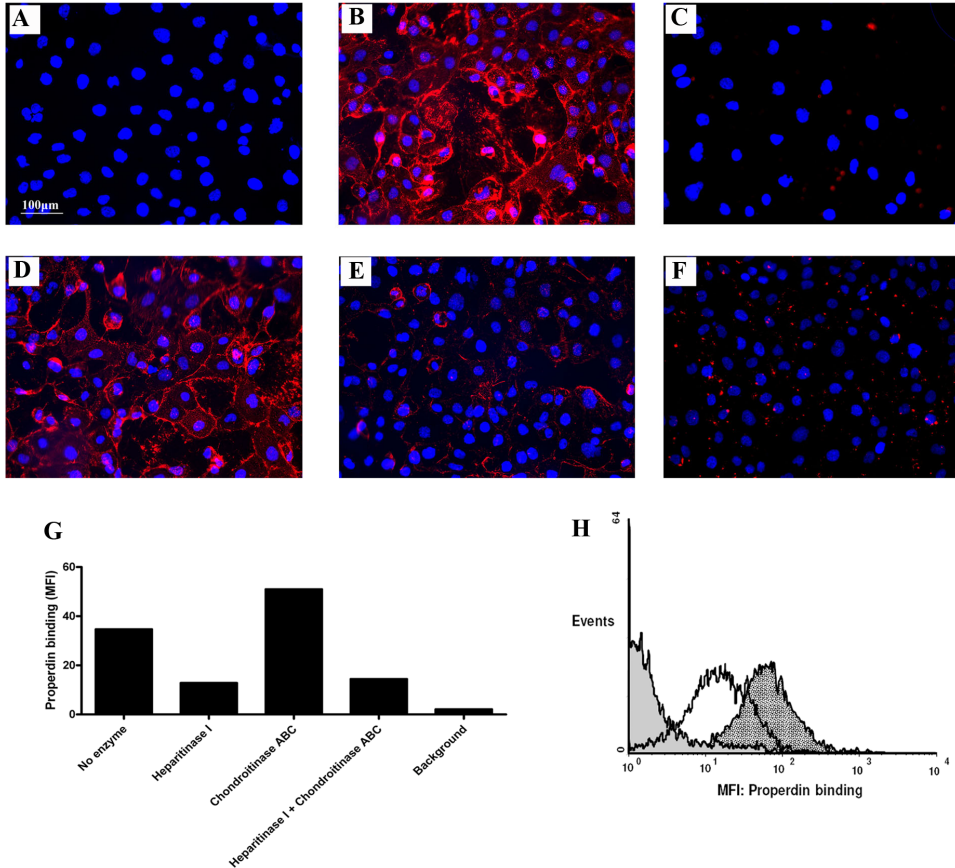
Addition of exogenous properdin (10  $\mu$ g/ml) to normal rat renal tissue sections resulted in properdin binding to tubular cells. This binding could be reduced significantly following pretreatment of the tissue by means of the HS-degrading enzyme, heparitinase I (Fig. 2.1 F).

*Properdin Binding to Proximal Tubular Cells Is Dependent on HS*—We found that proximal tubular epithelial cells (HK-2 cells) did not show a positive staining for properdin under normal culture conditions (Fig. 2.2 A). However, incubation of unfixed, living cells with exogenous properdin on ice resulted in the binding of properdin to these cells as





**Figure 2.1: Renal binding and expression of properdin.** Properdin colocalizes with syndecan-1 on the apical side of renal tubular epithelial cells in adriamycin- induced proteinuric rat tissue (A, DAPI; B, syndecan-1; C, properdin; D, merge). Scale bars, 20  $\mu\text{m}$ . E, quantitative analysis shows a statistically significant increase in tubular stained area positive for properdin both in adriamycin-induced nephrosis and protein-overload nephropathy. \* and \*\*, p value 0.007. F, binding of exogenous properdin (10  $\mu\text{g}/\text{ml}$ ) to normal rat renal tubular tissue (gray bar) is significantly reduced upon pretreatment of the tissue with heparitinase I (open bar). Results are calculated as percentage of surface area that is positively stained for bound properdin. \*, p value < 0.001.



**Figure 2.2: Properdin binding to HK-2 cells.** A, cultured HK-2 cells do not produce properdin by themselves. B, exogenous properdin (5  $\mu\text{g/ml}$ ) binds to HK-2 cells. C, heparitinase I pretreatment of the cells before properdin incubation abolishes the binding. D–F, incubation of 5  $\mu\text{g/ml}$  properdin with 1.6  $\mu\text{g/ml}$  (D), 6.2  $\mu\text{g/ml}$  (E), and 25  $\mu\text{g/ml}$  (F) heparin prior to incubation on HK-2 cells impairs the binding. Scale bars represent 100  $\mu\text{m}$ . G, in a representative experiment, HK-2 cells were incubated either with heparitinase I, chondroitinase ABC, or both heparitinase I and chondroitinase ABC before properdin incubation (10  $\mu\text{g/ml}$ ). Properdin binding was detected on the cells by FACS staining. Background represents HK-2 cells without properdin incubation. H, histogram also showed the same results. Properdin binding to HK-2 cells (dotted histogram) decreased after heparitinase treatment of the cells (open histogram). Properdin binding was not detected on HK-2 cells without properdin incubation (gray histogram). Results are expressed as the mean fluorescence intensity (MFI).

shown by immunofluorescent staining (Fig. 2.2 B). Before incubation with properdin, pretreatment of HK-2 cells with heparitinase I (which cleaves HS polysaccharide chains from these cells) resulted in a significant reduction of properdin binding to the cells (Fig. 2.2 C). Preincubation of properdin with increasing concentrations of heparin dose-dependently reduced the binding of properdin to HK-2 cells (Fig. 2.2, D–F).

We next treated HK-2 cells with GAG-degrading enzymes, followed by properdin incubation and detection of cell-bound properdin by FACS analysis. In control cells without enzyme pretreatment, properdin binding to HK-2 cells was observed as seen previously. However, after cleaving the HS side chains from the cells with heparitinase I, a strong reduction in properdin binding was observed (Fig. 2.2, G and H). This was not the case after chondroitinase ABC treatment of the cells, which cleaves CS and dermatan sulfate chains. A combination of both enzymes reduced properdin binding to HK-2 cells in a fashion comparable with use of heparitinase alone (Fig. 2.2, G and H). These enzyme treatments provide evidence that properdin specifically binds to HS present on HK-2 cells.

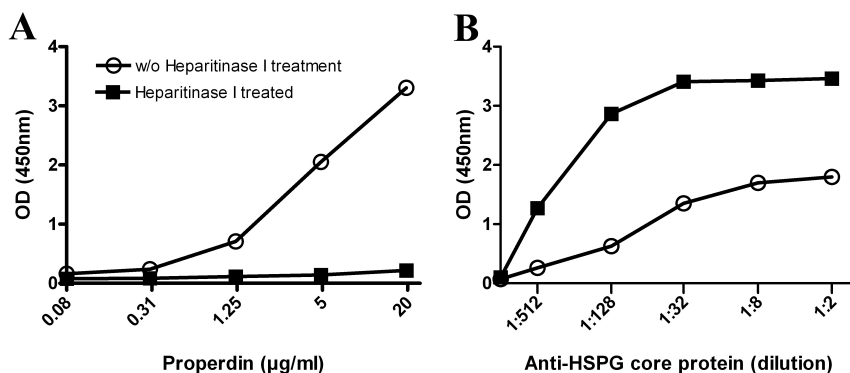
*GAG Epitope Requirements for Binding to Properdin*—In ELISA, properdin dose-dependently interacted with immobilized HSPG as shown in Fig. 2.3. Pretreatment of plate-bound HSPG with heparitinase I before properdin incubation completely abolished any properdin-HSPG interaction, whereas an antibody against the core protein of this HSPG remained bound after heparitinase digestion. The HSPG binding even increased, most probably due to reduced steric hindrance resulting from removal of the HS chains. Based on these experiments, we concluded that properdin binding to HSPG occurs via the HS GAG side chains.

The binding of properdin to solid-phase HSPG was investigated using competition experiments with a number of different HS preparations from various sources (see Table 2.1). Table 2.1, part A, shows that only one out of four HS preparations was able to bind to properdin, namely HS isolated from human aorta. This suggests that the epitope for properdin binding is apparently only present in a subset of HS isoforms. Interestingly, HS from bovine kidney was unable to bind to properdin. However, by increasing the amount of *N*-sulfation from ~40 to ~100% (*N*-sulfated HS from bovine kidney), properdin binding was induced.

Next, we performed a series of competition experiments with a number of HS-like polysaccharides. Various chemically modified K5 polysaccharides were tested. The *E. coli* K5 capsular polysaccharide has the same (Glc-GlcNAc)<sub>n</sub> structure as the unmodified biosynthetic precursor of heparin/HS. No properdin binding was observed with native and *N*-sulfated K5 preparations. However, *O*-sulfated K5 and *N+O*-sulfated K5 were able to bind to properdin (Table 2.1, part B). Apparently, a certain density of sulfate groups is important, whereas *N*-substitution and iduronate residues (not present in *O*-sulfated K5) are not critical. We then evaluated a series of other natural and chemical GAGs. No properdin binding was found to chondroitin sulfate A or C, dermatan sulfate, keratan sulfate, and dextran. However, clear inhibition of the HSPG-properdin binding was observed for dextran sulfate and fucoidan (Table 2.1, part C). These data indicate that a certain degree of sulfation is important; however, the exact backbone on the polysaccharide might vary to some extent. We then tested a number of heparin-derived GAGs (Table 2.1, part D). Unfractionated heparin from ovine intestinal mucosa, bovine lung, and porcine intestinal mucosa effectively dose-dependently inhibited the properdin-HSPG interaction. *N*-desulfation of heparin did not influence properdin binding. Subsequent *N*-acetylation

**Table 2.1: Fluid phase inhibition of properdin binding to immobilized HSPG by A, HS from different sources; B, K5-derived polysaccharides; C, different GAGs; D, (chemically modified) heparins** Dose-response inhibition curves were generated as described under “Experimental Procedures,” and IC50 was calculated.

<b>A. Heparan Sulfates (HS)</b>	
<i>HS human aorta</i>	12.5 $\mu\text{g/ml}$
HS Engelbreth-Holm-Swarm tumor cells (EHS)	> 25 $\mu\text{g/ml}$
HS bovine intestine	> 25 $\mu\text{g/ml}$
HS bovine kidney	> 25 $\mu\text{g/ml}$
<i>N-deacetylated, N-sulfated HS-bovine kidney</i>	12.5 $\mu\text{g/ml}$
<b>B. K5 derived polysaccharides</b>	
K5	> 25 $\mu\text{g/ml}$
<i>O-sulfated K5</i>	0.160 $\mu\text{g/ml}$
N-sulfated K5	> 25 $\mu\text{g/ml}$
LMW-O-sulfated K5	> 25 $\mu\text{g/ml}$
<i>N + O-sulfated K5</i>	0.195 $\mu\text{g/ml}$
<b>C. Glycosaminoglycans</b>	
Keratan sulfate	> 25 $\mu\text{g/ml}$
Chondroitine sulfate A	> 25 $\mu\text{g/ml}$
Chondroitine sulfate B	> 25 $\mu\text{g/ml}$
Chondroitine sulfate C	> 25 $\mu\text{g/ml}$
Dextran T40	> 25 $\mu\text{g/ml}$
<i>Dextran sulfate</i>	0.020 $\mu\text{g/ml}$
<i>Fucoidan</i>	0.025 $\mu\text{g/ml}$
<b>D. Heparins</b>	
<i>Heparin porcine intestinal mucose</i>	3.1 $\mu\text{g/ml}$
<i>Heparin ovine intestinal mucose</i>	3.1 $\mu\text{g/ml}$
<i>Heparin bovine lung</i>	3.1 $\mu\text{g/ml}$
Enoxaparin	> 25 $\mu\text{g/ml}$
Fragmin	> 25 $\mu\text{g/ml}$
Fraxiparine	> 25 $\mu\text{g/ml}$
O-desulfated heparin	> 25 $\mu\text{g/ml}$
<i>N-desulfated heparin</i>	3.15 $\mu\text{g/ml}$
<i>N-acetylated heparin porcine intestine</i>	25 $\mu\text{g/ml}$



**Figure 2.3: Properdin interacts with the HS side chains of HSPG in a dose-dependent manner.** A, properdin binds dose-dependently to intact HSPG (open circle), and the binding is completely abolished upon pretreatment of HSPG with heparitinase I (closed square). B, anti-mouse perlecan monoclonal antibody recognizes core protein before (open circle) and after (closed square) cleaving HS side chains of HSPG.

of the *N*-unsubstituted Glc units reduced the binding to properdin somewhat, but this *N*-acetylated heparin was still able to interrupt the HSPG-properdin interaction. Completely *O*-desulfated heparin did not bind to properdin (Table 2.1, part D). Importantly, both *N*-desulfated heparin and *N*-acetylated heparin are non-anticoagulant heparin derivatives, which might be important from a future treatment perspective (Table 2.1, part D). Comparison of heparin with *N+O*-sulfated K5 (~80% IdoUA units versus 0% IdoUA units, respectively, with degree of *N+O*-sulfation being comparable), as well as *N*-acetylated heparin with *O*-sulfated K5, strongly suggests that GlcUA to IdoUA conversion hampers properdin interaction to some extent (see Table 2.1).

Finally, we evaluated the effect of depolymerization of the GAG chains on properdin binding. We evaluated three different low molecular weight heparin preparations (fragmin, enoxaparin, and fraxiparin). All three preparations did not bind to properdin, whereas the intact heparin was a potent inhibitor for properdin. The same result was obtained from comparing *O*-sulfated K5 with low molecular *O*-sulfated K5. These experiments clearly and uniformly showed that depolymerization of the GAG chains abolishes effective properdin interaction.

We used the surface plasmon resonance (BIAcore) technique to study direct properdin-heparin and properdin-HS interaction in more detail. BIAcore sensorgrams showed a slow association rate ( $k_{on}$ ,  $2.91 \times 10^3 \text{ M}^{-1} \times \text{s}^{-1}$ , and  $k_{on}$ ,  $2.25 \times 10^3 \text{ M}^{-1} \times \text{s}^{-1}$  for heparin and HS, respectively) as well as a slow dissociation rate of properdin with immobilized heparin and heparan sulfates ( $k_{off}$ ,  $3.37 \times 10^4 \times \text{s}^{-1}$ , and  $k_{off}$ ,  $2.88 \times 10^4 \times \text{s}^{-1}$  for heparin and HS, respectively).  $K_D$  values, which are  $k_{off}/k_{on}$  and represent the affinity of heparin and HS for properdin binding, showed that properdin has a relatively high affinity for binding to heparin and HS compared with other heparin-binding molecules ( $K_D$ , 116 nM, and  $K_D$ , 128 nM for heparin and HS, respectively) (Fig. 2.4). Most effective properdin binding GAGs, as described above in the ELISA experiments, were tested in the BIAcore system as well and nicely confirmed the inhibition of the heparin-properdin interaction

(data not shown).

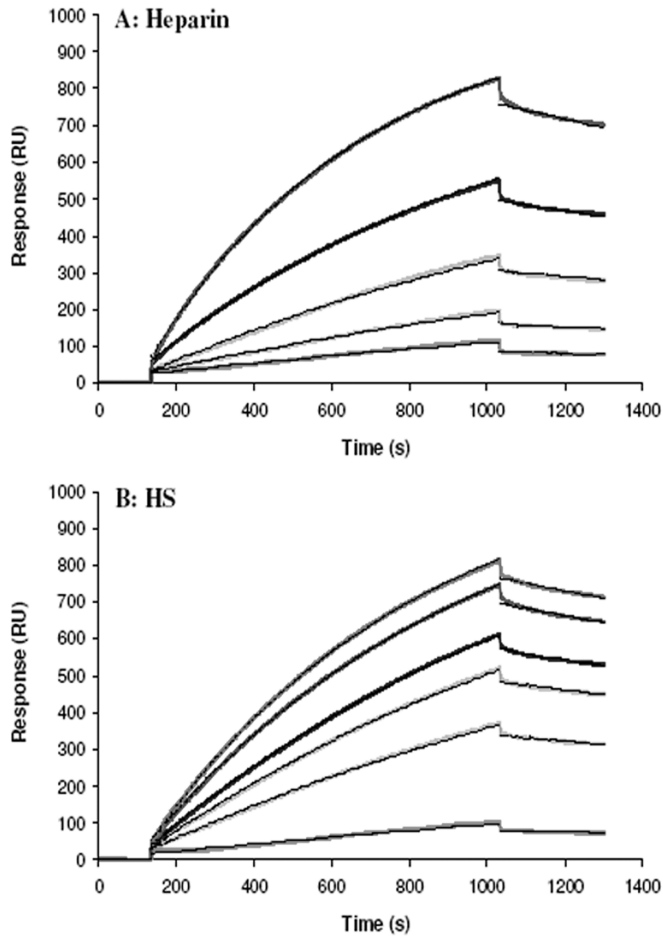
*Tubular HS Acts as a Platform for Properdin Binding and Activates the AP of Complement* — In a final experiment, we aimed to determine whether HS-bound tubular properdin was functional in activation of the AP of complement. We therefore pretreated HK-2 cells with heparitinase I and/or chondroitinase ABC followed by properdin incubation. The cells were then incubated at 37 °C with normal human serum as a source of complement and assessed for C3 deposition by FACS analysis. The results showed that untreated HK-2 cells can activate the AP in the presence of properdin (Fig. 2.5). HK-2 cells without enzymatic pretreatment, followed by properdin and serum incubation, initiated AP of complement by proper C3 deposition on the cells. In contrast, pretreatment of the cells with heparitinase I showed a strongly reduced C3 deposition. Pretreatment of the cells with chondroitinase ABC did not result in reduction of C3 deposition. We included positive controls for both enzymes, which showed that both of them were working properly (data not shown). These data indicate that properdin can initiate the AP of complement on HK-2 cells, and this complement activation is dependent on cellular HS polysaccharide chains.

## 2.4 Discussion

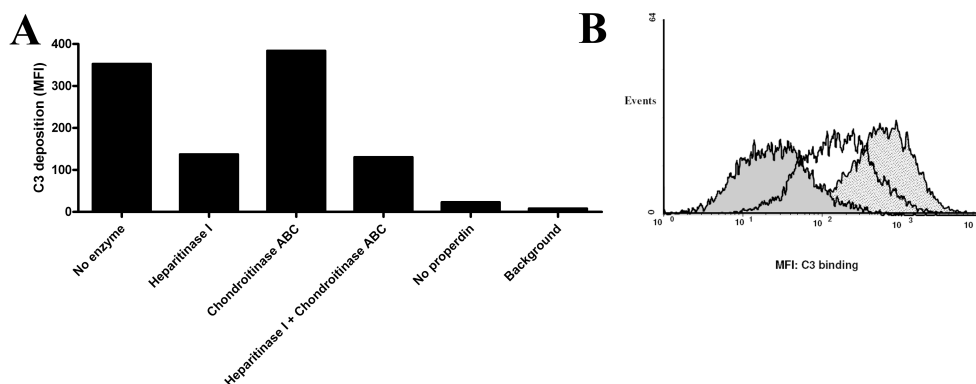
Our study shows renal tubular HSPG acting as a ligand for filtered properdin during proteinuric conditions. Our data also identify tubular HS as a docking platform for the AP activation via properdin.

The importance of complement activation in tubulointerstitial toxicity was already demonstrated years ago (13, 36, 37). In vitro and in vivo studies have implicated activation of the AP of complement on tubular epithelial cells and its linkage to renal fibrosis (3, 5, 38, 39). It has been described that properdin stabilizes the C3 convertase C3bBb and renders this complex relatively resistant to dissociation and inactivation by inhibitory complement factors. Recently, it has been shown that properdin is also an initiator of the AP by binding to the target and serving as a platform for C3. Consequently, this has raised new interest in properdin (11, 12). Because properdin is a cationic protein, and because heparin and other GAGs have been shown to bind to properdin, we tested GAG-dependent binding of properdin to HK-2 cells. Our study shows properdin binds to HK-2 cells via HS present on these cells, and upon its binding on HK-2 cells, it can recruit C3 for further activation of AP.

Recent data show the presence of properdin in urine in proteinuria and its association with more excretion of late complement components and worse renal function (13). It has also been reported that in proteinuric patients, properdin is present on tubular brush borders, although in vitro properdin binds to tubular cells and serves as a focal point for the AP amplification on these cells (6). It has been shown that properdin does not bind to endothelial cells (human umbilical vein endothelial cell) under normal conditions, although it does bind to HK-2 cells (6). One of the possible reasons for this might be the presence of a glycocalyx on endothelial cells of the vessel walls, which prevents properdin from binding to GAGs. Tubular epithelial cells do not need a protecting layer of glycocalyx, because these cells are normally not exposed to complement components. In proteinuria, unprotected tubular epithelial cells are exposed to complement components (including



**Figure 2.4: BIAcore analysis of properdin binding to heparin and HS.** Sensorgrams corresponding to the following: A, injection of (from bottom to top) 1.6, 3.1, 6.25, 12.5, and 25  $\mu\text{g}/\text{ml}$  properdin on a heparin-coated sensor chip; B, 1.6, 6.25, 10, 12.5, 20, and 25  $\mu\text{g}/\text{ml}$  properdin on an HS-coated sensor chip. A and B, data were fitted to a Langmuir 1:1 model. Overlays of fitting curves are shown in thin black lines.



**Figure 2.5: C3 recruitment by HK-2 cells after preincubation with properdin is dependent on HS.** A, HK-2 cells were incubated either with heparitinase I, chondroitinase ABC, or both heparitinase I and chondroitinase ABC before properdin incubation (10  $\mu$ g/ml). Five percent normal human serum was incubated on cells after properdin, and C3 deposition was detected with mouse anti-human C3. Background represents HK-2 cells without properdin and normal human serum incubation. B, histogram also showed the same results. C3 deposition upon properdin incubation on HK-2 cells (dotted histogram) decreased after heparitinase treatment of the cells (open histogram). Providing HK-2 cells with normal human serum without properdin incubation results in a decreased C3 deposition on these cells (gray histogram). Results are expressed as the mean fluorescence intensity (MFI).

properdin) on their apical side. The difference in properdin binding to cells of different origin can also be explained by the GAG patterns on the surface of the cells. We speculate that not all the cell types express GAGs capable of binding to properdin. Indeed, our results show that certain characteristics of GAGs are important for properdin binding.

Our data, in addition to those of other studies (6, 13), show that properdin can be an initiator of AP activation during proteinuria on tubular epithelial cells. However, the cellular ligand on tubular epithelial cells for properdin was not identified. It was reported earlier that properdin interacted with heparin. Recently, it has been discovered that properdin binds to apoptotic T cells via GAG chains, mostly CS (14, 15). The required epitope in GAG chains for binding to properdin is not known in detail, although previous studies have shown the dependence of the interaction on the sulfation pattern of GAGs (15, 40). Our GAG-profiling data confirm previous reports on properdin interactions with heparin (14). Another study has suggested that apoptotic T cells are recognized by properdin binding to the CS and HS on the cell surfaces (15). It is reasonable to have a mechanism for target recognition on those apoptotic cells that are self-altered cells and that should be removed by the complement system and phagocytes. However, on proximal tubular epithelial cells, we demonstrated that the interaction was among viable cells (by excluding apoptotic cells with propidium iodide and annexin V staining in the FACS experiments) and through their cell surface HS side chains. Although there are some controversies in terms of the binding of properdin to viable Jurkat cells (6, 15), properdin binding to CHO cells via GAG chains has already been shown (15). Our ELISA and BIAcore results showed the involvement of HS chains in the interaction with properdin



and not that of CS. We also clearly demonstrated HSPG on tubular epithelial cells to be a major ligand for properdin, which is present in urine during proteinuria. We also showed that this interaction leads to activation of the AP on proximal tubular epithelial cells.

Using ELISA competition assays, we showed that the binding of properdin to GAGs is mediated by more than just electrostatic interaction. For example, HS from human aorta contains on average  $\sim 0.6$  sulfate groups/disaccharide and binds to properdin. HS from bovine intestine, however, contains more sulfate groups ( $\sim 0.98$  sulfate groups/disaccharide), and it does not bind to properdin. This indicates that certain motifs are involved in properdin binding. As we demonstrated, HS from bovine kidney does not bind to properdin, whereas HS on human tubular cells is able to bind to properdin as shown by FACS experiments. This might be explained by the fact that upon isolation of HS from whole kidney, the majority of HS originates from basement membranes and the extracellular matrix. These two kinds of HS (matrix versus cell surface HS) can differ in their structure and sulfation patterns, and this confirms that properdin-HSPG interaction needs a specific structure of HS. The backbone of GAG plays a role in the specificity of properdin binding, because CS-A and CS-C along with dermatan sulfate did not bind to properdin. This, however, could be overruled when the GAG has sufficient density of sulfation, as in highly sulfated dextran sulfate and fucoidan, both of which are effective binders to properdin. CS-E has been shown to be involved in properdin binding to apoptotic T cells (15). Because CS-E is also a highly sulfated CS, this finding was in complete agreement with our results. Our data indicate that a dense patch of (*N*) *O*-sulfate groups enables GAGs to interact with properdin as evidenced by the induction of properdin binding to bovine kidney HS after increasing *N*-sulfate content. The length of the GAG chains is also an important requirement of interaction between GAG chains and properdin, at least when GAG is not immobilized (as discussed below). From the ELISA competition experiments, we concluded that a critical density of sulfation (mostly a combination of *N*- and *O*-sulfation), along with sufficient chain length, is crucial for properdin interaction; the polysaccharide backbone and *N*-substitution is not critical, whereas the conversion of GlcUA into IdoUA did reduce properdin binding.

Although we cannot exclude that neutrophil-derived properdin might be available in renal tissue, the presence of properdin on the apical side of tubular cells, besides the fact that monomeric properdin in supernatant from polymorphonuclear neutrophils did not interact with HSPG (A. Zaferani and J. van den Born, unpublished results) strongly suggests that properdin interacting with tubular HSPG is derived from the ultrafiltrate. Moreover, tubular binding of properdin has also been shown in proteinuric patients (6) and rats (our study) and not in control nonproteinuric tissues. GAGs are known to be involved in multimerization of some chemokines and growth factors. The repetitive disaccharides in GAG chains serve as multiple binding sites for their binding partners on the cells, allowing amplification of the signal of the binding partner. Because multimerization of properdin is important for its function, we speculate about the interaction of multimeric properdin with repetitive binding sites on a HS chain, leading to more effective and stronger interaction.

Interestingly, the competition ELISA results illustrated two heparin preparations (*N*-desulfated heparin and *N*-acetylated heparin) that are non-anticoagulant heparinoids but are still able to prevent properdin from binding to HSPG. Considering the fact that properdin is the only positive regulator of AP, non-anticoagulant heparinoids might be important for future treatment prospects. It is already known that non-anticoagulant

heparins have a beneficial effect on proteinuria and on structural changes in diabetic animals (41, 42). In addition, the beneficial effects of these heparinoids on renal tissue, renal outcome, and graft rejection have been studied in several animal models (42–47). The effects of non-anticoagulant heparins have also been investigated in ischemia-reperfusion, inflammation, and cancer treatment (48–50). Despite extensive research, their mechanism of action is still not fully understood. Our data have identified one of their mechanisms of action by interfering in the properdin-HS interaction. Nevertheless, more research is needed to figure out whether or not these heparinoid preparations could be clinically used to inhibit the inflammatory signals raised by properdin, by targeting the interaction of properdin with tubular heparan sulfate. Our findings might be of great importance for preventing progressive renal function loss due to proteinuria-induced AP activation in renal tissue.

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