STRUCTURAL REQUIREMENTS FOR HEPARIN/HEPARAN SULFATE BINDING TO TYPE V COLLAGEN

Sylvie Ricard-Blum¹, Mickael Beraud¹, Nicolas Raynal², Richard W. Farndale² and Florence Ruggiero¹

From the ¹Institut de Biologie et Chimie des Protéines, UMR CNRS 5086, Université Claude Bernard Lyon 1, IFR 128 BioSciences Gerland, 7 passage du Vercors, 69367 Lyon Cedex 07, Lyon, France; ²Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, United Kingdom

Running title: Heparin/heparan sulfate binding to collagen V

Collagen-proteoglycan interactions participate in the regulation of matrix assembly and in cell-matrix interactions. We reported previously that a fragment (I¹824-P¹950) of the collagen α¹(V) chain, HepV, binds to heparin via a cluster of three major basic residues R⁹¹2, R⁹¹⁸, R⁹²¹ and two additional residues, K⁹⁰⁵ and R⁹⁰⁹ (Delacoux et al., 2000 J. Biol. Chem. 275: 29377-82). Here, we further characterized the binding of HepV and collagen V to heparin and heparan sulfate by surface plasmon resonance assays. HepV bound to heparin and heparan sulfate with a similar affinity (K_D ~ 18 and 36 nM respectively) in a cation-dependent manner and 2-O-sulfation of heparin was shown to be crucial for the binding. An octasaccharide of heparin and a decasaccharide of heparan sulfate were required for HepV binding. Studies with HepV mutants showed that the same basic residues were involved in the binding to heparin, to heparan sulfate and to the cell surface. The contribution of K⁹⁰⁵ and R⁹⁰⁹ was found to be significant. The triple-helical peptide GPC(GPP)₅G⁹⁰⁴-R⁹¹⁸(GPP)₅GPC-NH₂ and native collagen V molecules formed much more stable complexes with heparin than HepV and collagen V bound to heparin/heparan sulfate with a higher affinity (in the nanomolar range) than HepV. Heat and chemical denaturation strongly decreased the binding, indicating that the triple helix plays a major role in stabilizing the interaction with heparin. Collagen V and HepV may play different roles in cell-matrix interactions and in matrix assembly or remodeling mediated by their specific interactions with heparan sulfate.

Proteoglycans are widely distributed at the cell surface and in the extracellular matrix. They consist of a protein core to which one or more glycosaminoglycan side chains are covalently attached conferring a strong negative charge on proteoglycans (1, 2). Interactions of proteoglycans with a number of matrix proteins including collagens are important in regulating cell behaviour and fibril formation during development and pathophysiological events. Interactions with collagens can be dependent on the proteoglycan core protein or on the glycosaminoglycans chains. The heparan sulfate chain has various important biological properties and influences cell behavior through interactions with a variety of matrix proteins. Heparin-binding sites have been identified and characterized in a wide range of matrix proteins including collagens, but the structural
requirements for heparan sulfate to bind these proteins is not fully understood.

Collagen V, although a quantitatively minor component of connective tissues plays a crucial role in matrix organization. Several isoforms of collagen V, including hybrid molecules containing collagen XI chains (3), occur in tissues, but the predominant molecular form is the heterotrimer $[\alpha_1(V)]_2\alpha_2(V)$, whereas the $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ molecule and the $[\alpha_1(V)]_3$ homotrimer are minor isoforms with a more restricted tissue distribution. Collagen V interacts with proteoglycans, which are widely distributed at the cell surface and in the extracellular matrix. It binds to the two small proteoglycans, decorin and biglycan, to the proteoglycan form of macrophage colony-stimulating factor (4), to the membrane chondroitin sulfate proteoglycan NG2 (5) and to syndecan-1 (6, 7). Binding to collagen V involves either the core protein or the glycosaminoglycan chains. Collagen V was shown to bind heparin (HP) through a 12kDa fragment of the $\alpha_1(V)$ chain referred to as HepV. Interestingly, we also showed that the recombinant HepV fragment supports heparin-dependent cell adhesion (8). The binding site for heparin is found in the $\alpha_1$ chain of collagen V, whilst the two other chains of collagen V, $\alpha_2(V)$ and $\alpha_3(V)$, do not bind to HP.

Sequence alignment of alpha chains of collagens V and XI indicated that these two collagen types may use a common sequence motif to interact with heparin. This hypothesis was confirmed experimentally by others (9). The recombinant production of HepV in E. coli has set the stage for identifying the heparin-binding site by site-directed mutagenesis and affinity chromatography. We have previously identified within the Lys$^{905}$-Arg$^{921}$ sequence of the $\alpha_1(V)$ chain five basic residues participating in the binding of HepV to heparin (10), but the molecular features of heparin responsible for the specific recognition of HepV have not been characterized, although the characteristics of heparin/heparan sulfate-protein interactions have been elucidated for several other proteins (for reviews see 11, 12). Furthermore, previous studies were performed with heparin, a chemical analog of heparan sulfate (HS), but not with HS. However, HS, which is ubiquitously distributed on the surface of cells and in the extracellular matrix, is the physiological glycosaminoglycan ligand of collagen V, and a number of biological roles have been reported for HS through its interaction with extracellular matrix proteins (for reviews see 13, 14). In our first studies (8, 10), the affinity of the binding of HepV and its mutants to heparin was qualitatively evaluated by affinity chromatography using heparin-Sepharose. HepV and its mutants were eluted with sodium chloride, the concentration of which was taken as an estimate of the binding affinity. However, this commonly-used technique measured only the ionic component, the most prominent interaction between heparin and a protein, but failed to measure the hydrophobic and hydrogen-bonding contribution to the binding (11).

In this study, we further characterized the interactions between collagen V and heparin by surface plasmon resonance (SPR) assays i) to take into account the whole set of non-covalent interactions participating in the interactions, ii) to study the influence of divalent cations, and iii) to measure rate constants and to calculate the affinity constant governing those interactions by kinetic analysis. The amino acids crucial for the binding of HepV to heparin/heparan sulfate and to cells were precisely mapped within the heparin-binding site. We also determined the chemical structures of the glycosaminoglycan chains (size, sulfate substitution pattern) required for binding, because subtle changes in heparan sulfate structure, and especially the fine structure of heparan sulfate with specific monosaccharide sequences characterized by the sulfation pattern (11, 12, 15-17), can
modulate HS-binding and proteoglycan activity (18). Those experiments were carried out using the monomeric, non-triple-helical fragment HepV, and full-length, triple-helical isoforms of collagen V, namely the [α1(V)]2α2 heterotrimer and the [α1(V)]3 homotrimer. The dependence of the interaction between collagen V and heparin/heparan sulfate upon the triple-helical conformation of collagen V was also investigated with a triple-helical synthetic peptide containing the fifteen amino acid residues of the previously-defined heparin binding site (10). Taken altogether, these results emphasize differences between the binding of the HepV fragment and of the full-length collagen V molecules to heparan sulfate. This suggests that a different network of interactions between collagen V molecules and HepV with their respective glycosaminoglycan ligands may modulate biological functions in vivo during remodeling of the extracellular matrix for HepV or in the organization and assembly of extracellular matrix for the collagen V isoforms.

**Experimental Procedures**

*Construction, expression and purification of HepV and its mutants* - The recombinant HepV fragment and mutant constructs were prepared as previously described and inserted into the EcoRI and PstI sites of the pT7/7 expression vector (8, 10). The recombinant wild type plasmid named pHepV and the mutants named after the mutation, e.g. pR909A and R909A were transformed in an E. Coli strain (BL21 SI-GJ1158) that carries the T7 RNA polymerase gene under the control of the lac promoter/operator and that makes use of NaCl as an inducer (19). Cells were harvested by centrifugation 6 after 0.2 M NaCl induction, resuspended in 50 mM Tris/HCl, pH 7.4 and then sonicated. After centrifugation and filtration, bacterial supernatants were first subjected to cation-exchange chromatography using a HiTrapSP column (Pharmacia, Biotech) to remove most contaminant bacterial proteins and were purified to homogeneity using a monoQ column (Pharmacia, Biotech) as previously described (8). Recombinant protein-containing fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 15% gel and dialyzed against a 50 mM Tris-HCl, pH 7.5. The recombinant Hep V fragment and its mutants were stored at −20°C until use.

*Collagens I and V and synthetic peptides* - Collagen I and V heterotrimers were extracted from embryonic calf bones by pepsin digestion in 0.5 M acetic acid, 0.2 M NaCl at 4°C for 20 ho. They were purified by repeated salt fractionation in acetic acid as previously described (20). Purified collagens were analyzed by 6% SDS-PAGE and Coomassie Blue staining using human placental collagen V (Sigma) as a standard. Collagens were dialyzed against 0.1 M acetic acid, then lyophilised and stored at -20°C. Production and purification of the recombinant collagen V homotrimer was performed as previously described (21). Briefly, 293-EBNA cells were transfected by electroporation with an expression vector containing full-length cDNA encoding the human pro-α1(V) chain. The purified homotrimer was dialyzed against 0.5 M acetic acid containing 0.2 M NaCl and digested with pepsin. The digestion product was dialysed against 0.1 M acetic acid and stored at -20°C until use. The concentration of the pepsinized homotrimer was determined after hydrolysis under vacuum (6 N HCl, 115°C, 24 h) in an amino acid analyser (Beckman). The peptide 901GTPGKPGRGPTGPRGERG923 referred to HepP was prepared using methodology previously described (8). The corresponding triple-helical peptide, G904-R918, GPC-(GPP)5-GKOGPRQGPRGPRGERG-(GPP)5-GPC-NH2, was prepared as previously described, and its triple-helical state was checked by polarimetry (22).
demonstrated a sigmoidal transition in optical rotation, confirming the triple-helical structure, and gave a melting point of 53.46°C for the peptide.

Surface plasmon resonance (SPR) binding assays - The SPR measurements were performed on a BIAcore Upgrade and on a BIAcore 3000 instruments (BIAcore AB, Uppsala, Sweden). Streptavidin (100 µg/ml in 10 mM acetate buffer pH 4.0) was covalently immobilized to the dextran matrix of a CM4 sensor chip via its primary amine groups (amine coupling kit, BIAcore AB) at a flow rate of 5 µl/min. Activation and blocking steps were performed as previously described (23, 24). Streptavidin surfaces were used to capture biotinylated heparin and heparan sulfate. Heparin (from porcine intestinal mucosa, 16 kDa, Sigma) and heparan sulfate (from porcine intestinal mucosa, Celsius, OH, USA) were biotinylated at their reducing end (24) and injected over streptavidin in 10 mM Hepes pH 7.4, 0.3 M NaCl and 0.005% P20 surfactant at a flow rate of 10 µl/min. An immobilization level ranging between 80 and 250 resonance units (RU) was obtained. A control flow cell was prepared by immobilizing only streptavidin. Control sensorgrams were automatically subtracted from the sensorgrams obtained with immobilized heparin or heparan sulfate in order to yield specific binding responses. Binding assays were performed at 25°C in 50 mM Tris buffer pH 7.5, containing or not 0.15 M NaCl, and 0.005% (v/v) P20 surfactant. Collagen V, HepV and mutants, and synthetic peptides were injected at several concentrations and different flow rates over immobilized glycosaminoglycans. When indicated, the triple-helical peptide was reduced with dithiothreitol and heated at 100°C for 15 min before injection. The surface was then regenerated with a pulse of 1 M sodium chloride. The kinetic parameters ka and kd (association and dissociation rate constants, respectively) were analyzed simultaneously using a global data analysis program (BIAevaluation 3.1 software). This software also fitted simultaneously the sensorgrams obtained at different concentrations of HepV, constraining the kinetic rate constants to a single value for each set of curves. Apparent equilibrium dissociation constants (K_D) were calculated as the ratio of k_d/k_a. R_max, the maximal capacity of the surface was calculated during the fitting procedure.

Heparin and heparan sulfate oligosaccharides and modified heparins - Heparin oligosaccharides of defined size were prepared by digestion of heparin with heparinase I followed by gel filtration chromatography on a Bio-Gel P-10 column (25). Heparan sulfate oligosaccharides were prepared by digestion of heparan sulfate by heparinase III. Glycosaminoglycan oligosaccharides were a generous gift from Rabia Sadir (Institut de Biologie Structurale, Grenoble, France). Selectively desulfated heparins were kindly provided by Professor John Gallagher (Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Manchester, U.K.) (26).

Ligand Blotting - Calcium binding was studied by ligand blotting and 45Ca autoradiography (23) using a nitrocellulose membrane (Schleicher and Schuell). Different concentrations of purified proteins were applied on the membrane using a dot-blot apparatus. Each well was washed twice (2 x 200 µl) with 10 mM imidazole buffer pH 6.8 containing 60 mM KCl and 5 mM MgCl_2. Membranes were incubated with 45CaCl_2 (Amersham Biosciences) at a final concentration of 2 µCi/ml for 15 min, rinsed with 50% ethanol for 5 min, dried, and exposed overnight to a BioMax MS film in a cassette with an intensifying screen. The C-terminal propeptide of procollagen III (gift from D. Hulmes, Institut de Biologie et Chimie des Protéines, Lyon, France) was used as a positive control (23).

Circular dichroism (CD) spectroscopy - The CD spectra were recorded on a Jasco J-800 dichrograph. HepV (5 µM) was
dialyzed against 10 mM potassium phosphate buffer pH8.
Glycosaminoglycans were either added to HepV prior to analysis or incubated overnight with HepV at 4°C with gentle mixing before recording the CD spectra. Spectra were collected from 190 nm to 260 nm with a step size of 1 nm and a bandwidth of 1 nm at 20°C. They were corrected for buffer baseline measured in the same cells of path length 0.1 cm. Five spectra of each sample were averaged. The circular dichroism signal of heparin and heparan sulfate was also subtracted to determine genuine changes in HepV conformation. The results were normalized to mean molar residue ellipticities.

Cell adhesion assays - Chinese Hamster Ovary (CHO) cells were maintained in monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, glutamine, non-essential amino-acids and a cocktail of antibiotics. Prior to the adhesion assay, cells were harvested with 1% EDTA in phosphate-buffered saline (PBS) pH 7.4. 96-well plates (Costar, France) were coated overnight at 4 °C with HepV and mutants with 1 µg per well. After blocking the wells with 1% bovine serum albumin (BSA), cells freshly suspended in serum free medium (5 x 10^5 cells/ml) were plated onto wells (0.1 ml/well), and allowed to attach for 45 min at 37°C. Then, the wells were gently washed with PBS, fixed with 1% glutaraldehyde and stained with 0.1% crystal violet. After extensive washing, the dye adsorbed to the cells was solubilized in 0.2% Triton X-100 and the absorbance was read at 570 nm with an ELISA plate reader (SLT Lab Instrument, Austria). Cell adhesion experiments were performed three times, each assay point being carried out in duplicate.

RESULTS

HepV binds to heparin and heparan sulfate with a high affinity

The expression of the wild-type HepV fragment and its mutants was markedly increased (by up to 20-fold) using the BL21 SI E. Coli strain that used NaCl as an inducer. Hep V (Figure 1A) and mutants were efficiently purified by successive anion- and cation-exchange chromatography steps. The binding of HepV to heparin and heparan sulfate was inhibited in a dose-dependent manner by heparin and heparan sulfate (Table 1) in SPR inhibition assays, thus assessing the specificity of the binding. Kinetic analysis of those interactions was performed by injecting different concentrations of HepV over immobilized heparin (Figure 1B) and heparan sulfate to determine the affinity constant K_D, which was calculated as the ratio of the rate constants k_a/k_d using the BIAevaluation software. The 1:1 Langmuir model was found to fit the kinetic data and might account for the mechanism of HepV binding to glycosaminoglycans. The affinity constants were K_D ~ 18 nM for the interaction of HepV with heparin (k_a ~ 3.69 x 10^4 M^-1 s^-1, k_d ~ 6.56 x 10^-4 s^-1, chi^2: 0.67) and K_D ~ 36 nM (k_a ~ 3.43 x 10^4 M^-1 s^-1, k_d ~ 1.23 x 10^-3 s^-1, chi^2: 1.61) for the interaction of HepV with heparan sulfate. Binding data to heparin also fitted well to the heterogeneous ligand model predefined in the BIAeval 3.1 software. The two sets of calculated affinity constants were K_D1: 12.5 nM and K_D2 22 nM for heparin (chi^2: 0.519). These values were similar to the values obtained when data were fitted to the 1:1 Langmuir model. The good fit to the heterogenous ligand model can be explained by the natural heterogeneity of heparin. Heparin undergoes extensive modification after the initial polymer synthesis, and binding to subpopulations of the glycosaminoglycan heparin has been reported by others (7, 24). Fibronectin, collagen I and laminin fractionate heparin into subpopulations that differ substantially in binding affinity and the same phenomenon might occur for
HepV (7). Furthermore, we have shown by dual polarization interferometry that the binding stoichiometry for HepV-heparin complex was 1.7:1 (37).

**Mapping of heparin- and cell-binding sites within the HepV fragment: the importance of the triple helical conformation**

Previous mutagenesis studies demonstrated that three arginine residues, Arg₁⁹¹₂, Arg₁⁹¹₈ and Arg₀⁹₂¹, were crucial for binding to heparin whereas two additional neighbouring basic residues, Lys₀⁹₀⁵ and Arg₀⁹₀⁹, were less critical for HepV binding to heparin-Sepharose (10). To investigate in details the contribution of each basic residues to the HepV-heparan sulfate interaction, SPR binding assays to heparin and heparan sulfate were performed with several mutants of HepV. Mutations of Arg₀⁹₀⁰ and Arg₂⁴ to alanine did not affect the binding of HepV to heparin and heparan sulfate. In contrast, a significant decrease in binding was observed when Arg₁⁹¹₂, Arg₁⁹¹₈ and Arg₀⁹₂¹ were mutated to alanine (Figure 2A). Contrary to our previous results (10), the binding level of K₉₀⁵₉ and R₉₀⁹₉ mutants to heparin/heparan sulfate was significantly reduced, indicating that these two residues are also essential for HepV binding to heparan sulfate. Those two particular residues were also critical for CHO cell adhesion to HepV, whereas R₉₁₂₉ and R₉₁₈₉, but not R₉₂¹₉, mutants decreased CHO cell adhesion to HepV (Figure 2B). These results suggested that the cell adhesion site to HepV was somewhat shorter than the HepV binding site to the glycosaminoglycans. Indeed, mutation of Arg₀⁹₂¹ did not affect cell attachment to HepV, whereas it decreased the binding of HepV to heparin by two-third. Furthermore, although the amino acid sequence located between Lys₀⁹₀⁵ and Arg₀⁹₂¹ was necessary for heparin binding, it was not sufficient because a synthetic peptide (called HepP) comprising this sequence did not bind to heparin, either in affinity chromatography on heparin-Sepharose or in SPR assays (Figure 3A). The sequences flanking the heparin binding site might play a role in heparin binding or alternatively the short HepP peptide (23 amino acid residues) might fail to adopt a conformation suitable to bind heparin. A triple-helical synthetic peptide comprising the G₉₀⁰⁴-R₉₁₈ sequence (GPC(GPP)₃-GKOGPGRQGPTGPR-(GPP)₂GPC-NH₂), which contains the heparin binding site, was found to bind heparin/heparan sulfate to a higher extent than HepV as measured in resonance units. In addition it forms much more stable complexes with the glycosaminoglycans than HepV, demonstrated on the sensorogram by a slower dissociation rate (Figure 3B). This suggests a strong stabilizing role of the triple helix in HepV binding to heparin/heparan sulfate. When the triple-helical conformation of the synthetic peptide GPC(GPP)₃G₉₀⁰⁴-R₉₁₃(GPP)₂GPC-NH₂ was lost upon reduction by 1,4-dithiothreitol followed by heat-denaturation, its binding to heparin was inhibited by 98.18%, further emphasizing the role of the triple helix in the binding of collagen to heparin. The extremely slow dissociation rate of the peptide/heparin-heparan sulfate complexes prevented the calculation of reliable kinetic constants using the BIAevaluation software.

**The binding of HepV to heparin and heparan sulfate depends on divalent cations**

It has been reported that cations may affect binding of glycosaminoglycans to proteins (24, 29-31). This prompted us to investigate the role of divalent cations in HepV binding to heparin or heparan sulfate. The addition of EDTA, which chelates several divalent cations including Ca²⁺ and Zn²⁺, strongly inhibited the binding of HepV to immobilized glycosaminoglycans (Table 2). EGTA, a Ca²⁺ and Mg²⁺ chelator, was also effective in inhibiting the binding of HepV to heparin and heparan sulfate, whereas 1,10
phenanthroline, a specific chelator of Zn$^{2+}$, inhibited the binding of HepV to heparan sulfate to a far lower extent than the binding to heparin (Table 2). The binding of the triple-helical synthetic peptide G$^{904}$-R$^{918}$ to heparin was nearly abolished (94.4 % inhibition) in the presence of 20 mM EDTA, 10 mM being the concentration we previously used to demonstrate the involvement of divalent cations in the binding of endostatin to heparin (24). The binding of HepV to heparin and heparan sulfate was thus dependent upon divalent metal cations, which might bind glycosaminoglycans and/or HepV. To determine whether cations bound to the immobilized glycosaminoglycans contributed to their interactions, 20 mM EDTA, EGTA or 1,10 phenanthroline were injected over immobilized heparin and heparan sulfate to remove divalent cations from the surface of the sensor chip before injecting HepV. This did not prevent HepV binding to heparan sulfate but EDTA and EGTA induced a small decrease in binding to heparin (~ 10% for EDTA and up to 20% for EGTA) in some experiments, suggesting that some cations participating in the binding of HepV to heparin might associate with heparin. However, the results were of borderline significance and no firm conclusion could be drawn from these experiments. We then checked by ligand blotting if the homotrimer of collagen V and several of its domains, including HepV, were able to bind calcium ions. Using this technique, HepV failed to bind calcium, whereas collagen V homotrimer and the C-terminal propeptide of procollagen III used as a positive control (23) bound calcium in those conditions (Figure 4).

We added Ca$^{2+}$, Mg$^{2+}$ and Zn$^{2+}$ to HepV before its injection over immobilized glycosaminoglycans to determine if they influenced HepV binding. We observed a limited increase in HepV binding to heparin and heparan sulfate in the presence of calcium ions (17.5 and 19.8% respectively) and of magnesium ions (20.9 and 29.9% respectively). The lower effect was observed upon the addition of Zn$^{2+}$, which did not change the binding level of HepV to heparin but increased its binding to heparan sulfate by 16.7%. Cations mostly increased HepV binding to heparan sulfate, their influence on the binding level to heparin being more limited. Complexes formed between HepV and immobilized glycosaminoglycans in the presence of cations were more stable as evidenced by their slower dissociation rate recorded during SPR assays (data not shown).

A decasaccharide of heparan sulfate is required for HepV binding

The minimum size of heparin and heparan sulfate required for HepV binding was evaluated by performing SPR inhibition assays with heparin and heparan sulfate oligosaccharides of defined size. Heparin (dp2–18, where dp represents the degree of polymerization) and heparan sulfate (dp4–18) oligosaccharides were screened to examine their ability to compete with immobilized heparin or heparan sulfate for binding to HepV. The smallest size of heparin inhibiting at least 50% of HepV binding to heparin was an octasaccharide (Figure 5A), whereas a longer oligosaccharide of heparan sulfate (a decasaccharide) was required to achieve a similar level of inhibition (Figure 5B). No significant inhibition was observed with heparan sulfate tetrasaccharide and hexasaccharide, whereas a heparin hexasaccharide inhibited the binding of HepV to heparin and heparan sulfate by 42.7% and 55.9% respectively.

2-O sulfation of heparin strongly contributes to the binding of HepV

Inhibition experiments were carried out with selectively desulfated heparins (26) to identify the sulfate groups of heparin involved in HepV interaction. Desulfated heparins were incubated for 1 h with HepV before injection onto immobilized heparin or heparan sulfate. If a particular desulfated heparin retains the capacity to
interact with HepV, the injection of the complex will result in inhibition of HepV binding to the immobilized glycosaminoglycans. Hence, strong inhibition by a modified heparin indicates that the (removed) sulfate groups did not prevent the binding of modified heparin to HepV during the preincubation step and consequently, that they are not involved in HepV recognition. Selectively 2-O desulfated heparin did not inhibit the binding of HepV to heparin or heparan sulfate indicating that 2-O-sulfation was required for heparin binding to HepV (Table 3). N-desulfation inhibited the binding of HepV to heparin and heparan sulfate by 30 and 40% respectively, whereas the removal of 6-O sulfates impaired 50% and 60% of the binding to heparin and heparan sulfate respectively (Table 3). 2-O-sulfation contributed the most to the binding of HepV, whereas N- and 6-O-sulfation participated to a lesser extent.

No significant structural change is induced in HepV upon its binding to heparin and heparan sulfate

To determine whether structural changes could be induced in HepV by its binding to heparin and heparan sulfate, HepV was analyzed by circular dichroism spectroscopy with and without added glycosaminoglycans. The CD signals exhibited a minimum at 198-200 nm (data not shown). A typical spectrum of triple-helical collagen exhibited a minimum at 195-200 nm and a maximum at 220-223 nm. No maximum was observed on the CD spectrum of HepV (data not shown) suggesting that this fragment did not adopt a triple helical conformation. We reported previously that HepV can fold into a triple helical structure after in vitro renaturation at high concentration (1 mg/ml) using an annealing procedure (32). However, as shown by our circular dichroism data, HepV does not fold spontaneously into a triple-helical conformation at the concentration used for our experiments. No significant change in molar ellipticity of HepV was observed at the minimum either when the spectra were recorded 15 min after addition of glycosaminoglycans at 5, 25 and 50 µM or when the spectra were recorded after overnight incubation at 4°C with several concentrations of heparan sulfate (heparan sulfate/HepV molar ratios: 1:1, 5:1 and 10:1). Indeed, changes in molar ellipticities were limited to 7% for the samples analyzed 15 min after glycosaminoglycan addition and to 4.5% for the samples incubated overnight. This suggests that heparin or heparan sulfate did not induce significant changes in HepV conformation upon binding.

The collagen V homotrimer and heterotrimer form very stable complexes with heparin and heparan sulfate

Recombinant collagen V homotrimer, which comprised three α1 chains, and heterotrimer both bound to heparin and heparan sulfate to a higher extent than HepV. The highest binding level, measured in resonance units, was obtained for the heterotrimer (Figure 6). Collagen V heterotrimer bound to heparin and heparan sulfate with a higher affinity (K_D ~ 5.62 nM and 2.0 nM respectively, 1:1 Langmuir model) than HepV. Collagen V homotrimer formed more stable complexes with heparan sulfate than HepV as demonstrated by a lower dissociation rate on the sensorgram (Figure 6). This extremely low dissociation rate prevented the calculation of reliable kinetic constants using the BIAevaluation software and thus of the affinity constant. Heat denaturation of the homotrimer for 15 min at 100°C decreased its binding to heparin by 71% indicating that the triple helix conformation participated in its recognition.

Collagen I, which is known to bind heparin through a basic triple helical domain (7), exhibited an intermediate binding level between the two forms of collagen V. It binds to heparin and heparan sulfate with a similar affinity (K_D
~3.4 nM and 3.5 nM respectively). Our results show that collagen I and collagen V heterotrimer exhibit similar affinities. It has been previously reported that collagen I has a lower apparent affinity for heparin/heparan sulfate than collagen V as determined by solid phase assays (33) and by affinity co-electrophoresis (34). This discrepancy is likely due to difference in the experimental procedure and in collagen sources.

Molecular characterization of the interaction of the collagen V homotrimer with heparin
The specificity of the binding of the collagen V homotrimer to heparin/heparan sulfate was assessed by SPR inhibition experiments. Heparin inhibited the binding of HepV to heparin and to heparan sulfate (Table 4). A higher concentration of heparan was required to achieve inhibition levels similar to those obtained with heparin (Table 4). The minimal size of heparin that promoted a significant binding of the homotrimer to heparin was found to be a decasaccharide by SPR inhibition assays (Figure 7). SPR inhibition experiments were also performed with modified heparins as described above for HepV. De-N-sulfated and re-N-acetylated heparin did not inhibit the homotrimer binding to glycosaminoglycans, suggesting that N-sulfation of heparin was crucial for the binding, whereas 2-O and 6-O sulfation contributed to the binding to a lower extent (Table 5).

DISCUSSION
The investigation of the functional relevance of glycosaminoglycan-collagen V interactions required first their characterization at the molecular level. The identification and characterization of the heparin binding site of the \( \alpha1(V) \) chain demonstrated that trimeric collagen V molecules and a monomeric recombinant fragment, referred to as HepV, bind efficiently to heparin and heparan sulfate through a common motif containing a cluster of 5 basic residues, which was identified by site-directed mutagenesis (8, 10). The binding of HepV and its mutants to heparin was assessed by affinity chromatography on heparin-sepharose and the relative strength of the binding was determined by the salt concentration required to elute them from the resin (10). In those conditions, the contribution of Lys\(^{905} \) and Arg\(^{909} \) to heparin binding was found to be limited compared to Arg\(^{912} \), Arg\(^{918} \) and Arg\(^{921} \) (10). This finding was at odds with the fact that the human \( \alpha3(V) \) chain does not bind to heparin despite the presence of the three arginine residues, Arg\(^{912} \), Arg\(^{918} \) and Arg\(^{921} \). The main difference between the primary sequences of the \( \alpha1(V) \) and \( \alpha3(V) \) chains resides in the substitution in the \( \alpha3(V) \) chain of Lys\(^{905} \) and Arg\(^{909} \) by glutamine and glutamic acid respectively. Interestingly, the SPR and cell adhesion assays reported here highlighted the crucial role of Lys\(^{905} \) and Arg\(^{909} \) in the binding of the HepV fragment to heparin/heparan sulfate and to the cells. This discrepancy between the affinity chromatography assays and the SPR experiments might be due to the fact that heparin-sepharose acts as a cation-exchanger because of the highly negative charge of heparin. In this approach, the amount of salt required for elution of HepV and its mutants from the affinity column is a quantitative measure of the ionic component of the binding, but this technique fails to measure the hydrophobic and hydrogen-bonding contributions to binding (11), whereas SPR assays take into account all the possible bonding mechanisms. Furthermore, deviations between the relative ionic strength elution of proteins from heparin-sepharose and direct affinity measurements have been observed (17), suggesting that differential ionic strength elution of HepV and its mutants may not reflect their true relative affinities.

In addition, similar results were obtained by assessing CHO cell...
attachment to Hep V mutants. This confirms that most of the basic residues involved in heparin/heparan sulfate binding are also crucial for cell binding. CHO cells express a great variety of proteoglycans at their surface including syndecan-1 that carries heparan and chondroitin sulfate chains (35). Chondroitin sulfate has been shown to bind collagen V (36, 37) with a high affinity constant, $K_D = 5$ nM, as determined by SPR (36) and we have previously shown that NG2, a chondroitin sulfate membrane proteoglycan, binds efficiently to collagen V (5). The binding site of chondroitin sulfate on collagen V has not yet been determined but, from our previous data, it is unlikely that it binds to collagen V through the heparin binding site since we previously showed that (i) heparan sulfate, but not chondroitin sulfate, efficiently inhibits heparin binding to HepV, and (ii) CHO cell attachment to HepV is inhibited by heparin and heparan sulfate but not by chondroitin sulfate (8). Collagen V is a fibrillar collagen that generally co-polymerizes with collagen I to form heterotypic fibrils. However, it has also the property of forming fibrils that show a preferential pericellular localization (8). Since collagen V can interact with cells through its heparin binding site and is located pericellularly (8), heparan sulfate proteoglycans associated with the cell surface, such as syndecans and glypicans or to the basement membrane such as perlecan are likely good candidates for collagen V binding partners.

HepV binds to heparin and heparan sulfate with a lower affinity ($K_D \sim 18$ and 36 nM respectively) than the collagen V heterotrimer ($K_D \sim 5.6$ and 2.0 nM respectively). This does not mean that HepV binding to heparin is not physiologically relevant. Hep V might be generated by proteolytic cleavage of collagen V in physio-pathological situations where extensive extracellular matrix remodeling occurs, whereas interactions between heparan sulfate and native collagen molecules could play a role in the cohesion and assembly of the extracellular matrix. Moreover, we showed previously (8), and in the present report, that HepV can promote cell adhesion.

Triple-helical molecules such as the GPC(GPP)$_5$G$_{904}$-R$_{915}$(GPP)$_5$GPC-NH$_2$ peptide and the homotrimeric and heterotrimeric collagen V molecules form very stable complexes with heparin and heparan sulfate. The extremely slow dissociation rate of the corresponding complexes observed in SPR assays demonstrates that the triple helix strongly stabilizes the complexes formed between the triple helical peptide, or native, triple-helical collagen V molecules, and the glycosaminoglycans. The increased level in binding of triple helical peptides and of collagen molecules to heparin/heparan sulfate might be due to the association of three identical (homotrimer) or different (heterotrimer) $\alpha$ chains into a triple helix that can generate new combinations of basic amino acid residues. This could modify the affinity for heparin by clustering a higher number of basic amino acid residues. Concerning the heterotrimer, the triple helix formation could reinforce the efficiency of heparin binding by contributing basic aminooacids of the $\alpha_2$(V) sequence. Two arginine residues of the $\alpha_2$ chain can contribute to the heparin binding site because they are in close proximity with the basic amino acid K905 and R912 of the $\alpha_1$ chain, as shown in our previously established molecular model (8). The affinity of glycosaminoglycan-collagen V inter-actions in tissues might be modulated in function of the collagen V chain associations, $[\alpha_1(V)]_2[\alpha_2(V),$ $[\alpha_1(V)]_3$ and $\alpha_1(V)\alpha_2(V)\alpha_3(V)$ as previously suggested (8).

Besides difference in binding affinities, we also showed distinct structural requirement for heparin/heparan sulfate binding to HepV and collagen V molecules. This
strengthens the idea that collagen V and HepV may interact with distinct sequences of glycosaminoglycans in the extracellular matrix or at the cell surface. The minimal size of heparin required for efficient binding of HepV and of the homotrimer to heparin was an octasaccharide and a decasaccharide respectively. Heparin forms a right-handed helix and successive disaccharide units in the heparin chain are related by a rotation of about 180° and a translation of 0.8–0.87 nm (38). This leads to a length ranging between 3.2 and 3.5 nm for a heparin octasaccharide and between 4.0 and 4.35 nm for a decasaccharide. A decasaccharide was the smallest heparan sulfate oligosaccharide able to bind significantly to HepV. Given that heparan sulfate chains adopt an extended helical coil in solution, with a disaccharide unit length of approximately 0.75 nm (39), the length of a heparan sulfate decasaccharide is about 3.75 nm. In all cases, the minimal length of glycosaminoglycan required for HepV or collagen V binding to heparin/heparan sulfate is shorter than the distance separating the two extreme amino acid residues of the heparin binding site Lys^{905} and Arg^{921}, which has been evaluated as 4.5 nm (8).

Although it is difficult to attribute a biological function to a specific sulfation motif of heparan sulfate, the sulfation pattern can modulate its biological activity and its binding to proteins (11, 12, 17). 2-O-sulfation of heparin, which occurs at the C-2 position of the uronic acid residues, is critical for HepV binding and 6-O-sulfation of glucosamine residues significantly contribute to the binding. In contrast, the contribution of 2-0 and 6-O sulfation to the binding of the triple helical collagen V homotrimer was low, whereas N sulfate groups played a prominent role. The distinct sulfation preferences of HepV and of the homotrimer suggest that they did not bind to the same residues on heparin and indicate that different sulfation motifs carry instructions for protein binding. Information in the form of a sulfation code could be a means of molecular-level control as suggested by Habuchi et al. (16). It should also be noted that we characterized interactions in terms of the sulfate groups required but not in terms of the conformation adopted by each glycosaminoglycan sequence, and it has been suggested that substitution pattern may influence the conformation around the glycosidic linkages (17). Furthermore, the homotrimer has a triple-helical conformation, whereas HepV does not, as demonstrated by circular dichroism, and that might contribute to their different requirements for sulfate group binding. It has been previously reported for the heparin binding domains of the acetylcholinesterase collagen tail that addition of increased amounts of heparin can increase the triple-helix content and the thermal stability of triple-helical peptide models (40, 41). However, addition of heparin and heparan sulfate at different concentrations failed to induce significant structural change in HepV even during an overnight incubation.

A number of protein-glycosaminoglycan interactions have been reported to involve cations. The interaction of endostatin (24) and heparin cofactor II (31) with heparin/heparan sulfate depend upon zinc. The interaction of fibroblast growth factor receptor with heparin requires divalent cations at levels present in extracellular fluids (29), and copper bridges promote a tighter interaction of prion protein with heparin and heparan sulfate (29). We showed in this study that the binding of HepV to heparin/heparan sulfate is also cation-dependent and involved Ca^{2+} and Mg^{2+}, whereas the contribution of Zn^{2+} was not significant. Complexes formed between HepV and immobilized glycosaminoglycans in presence of cations exhibited a lower dissociation rate suggesting that cations stabilize the complexes between HepV and glycosaminoglycans. Calcium might associate with heparin/heparan sulfate since HepV did not bind calcium in ligand
blotting experiments. Binding of calcium to collagen might require a triple-helical structure since collagen V molecules bind calcium.

In summary, our results provide a more precise understanding of the molecular mechanisms whereby heparan sulfate interact with collagen V. Collagen V is able, as collagen I, to interact with heparin and heparan sulfate in a trimeric, triple-helical, form. Although, collagens are present in normal tissues as trimeric molecules, collagen fragments might be proteolytically released from parent molecules in pathophysiological situations where extensive extracellular matrix remodeling occurs and HepV could thus be released from collagen V. The structural requirements for the binding of the HepV fragment and the native, triple-helical, collagen V molecules differ in several ways including affinity, minimal heparin size, and sulfation pattern. Interactions mediated by heparin/heparan sulfate could modulate biological activities of monomeric HepV and of trimeric collagen V molecules.

Acknowledgements
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Abbreviations

REFERENCES
**FIGURE AND TABLE LEGENDS**

**Figure 1:** A. Purification of recombinant HepV with two steps of ion-exchange chromatography. The different fractions were subjected to electrophoresis on a 15% SDS polyacrylamide gel and stained with Coomassie blue. Lanes 1. crude bacterial lysate, 2. after cation-exchange chromatography, 3. after anion-exchange chromatography. Molecular weight standards are shown on the left.

B. Overlay of sensorgrams resulting from the injection of different concentrations (3 - 50 nM) of recombinant HepV over immobilized 16 kDa heparin (flow rate: 40 µl/min - injected volume: 160 µl).

**Figure 2:** Mapping of the amino acid residues of HepV involved in A) glycosaminoglycans binding using SPR assays and B) CHO cell binding using colorimetric cell adhesion assays. Mutated basic amino acids are in black and the residues involved in heparin and cell binding are underlined. Heparin sulfate (grey bars), Heparin (black bars). Percentage of binding is shown relative to binding to wild type HepV taken as 100%.

**Figure 3:** SPR assays. Injection of HepP (A) and of the triple-helical synthetic peptide G904-R918 encompassing the amino acid sequence involved in heparin binding (B) over immobilized heparin.

**Figure 4:** Calcium binding to the collagen V molecules and their domains. 5 µg (1), 10 µg (2), 20 µg (3) and 50 µg (4) of collagen V homotrimer (a), HepV (d) were applied to the nitrocellulose membrane. Acetic acid (b) and Tris buffer (c) were used as negative controls and the C-terminal propeptide of procollagen III (e) was used as a positive control (23). Following incubation with 45CaCl2, binding was visualized by autoradiography as described under “Materials and Methods.”

**Figure 5:** A. SPR inhibition assays. HepV (200 nM) was preincubated for 1h at room temperature with heparin oligosaccharides of different size (dp2–18, 5 µg/ml) before injection over immobilized heparin (□) and heparan sulfate (■) (flow rate: 20 µl/min, injected volume: 80 µl). Binding was expressed as a percentage of the amount of HepV bound in the absence of competing oligosaccharides. B: SPR inhibition assays. HepV (200 nM) was preincubated for 1h at room temperature with heparan sulfate oligosaccharides of different size (dp4–18, 5 µg/ml) before injection over immobilized heparin (□) and heparan sulfate (■) (flow rate: 20 µl/min, injected volume: 80 µl). Binding was expressed as a percentage of the amount of HepV bound in the absence of competing oligosaccharides.

**Figure 6:** SPR binding assay of HepV, collagen V homotrimer and heterotrimer to heparan sulfate (flow rate: 40 µl/min, injected volume: 160 µl)

**Figure 7:** SPR inhibition experiments. The homotrimer of collagen V (20 µg/ml) was preincubated with heparin oligosaccharides of different size (dp: degree of polymerisation) for 1 h at room temperature before its injection over immobilized heparin (□) and heparan sulfate (■). Results were expressed as percentage of inhibition of the binding in the absence of competing oligosaccharides.
### TABLES 1-5

<table>
<thead>
<tr>
<th>Concentration of glycosaminoglycans (µg/ml)</th>
<th>Inhibition of HepV binding to immobilized heparin</th>
<th>Inhibition of HepV binding to immobilized heparan sulfate</th>
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</thead>
<tbody>
<tr>
<td>Heparin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.25</td>
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<tr>
<td>5</td>
<td>59.4</td>
<td>67.6</td>
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</table>

**Table 1: SPR inhibitions experiments.** HepV (200 nM) was preincubated for 1 h with different concentrations of heparin and heparan sulfate before injection over heparin and heparan sulfate immobilized on the sensor chip. The results were expressed as percentage of inhibition of the binding measured in the absence of competing glycosaminoglycans.

<table>
<thead>
<tr>
<th>Chelators</th>
<th>Inhibition of HepV binding to heparin</th>
<th>Inhibition of HepV binding to heparan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>10 mM</td>
<td>44.0</td>
</tr>
<tr>
<td></td>
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<td>75.4</td>
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<tr>
<td>EGTA</td>
<td>10 mM</td>
<td>36.4</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>70.7</td>
</tr>
<tr>
<td>1,10 phenanthroline</td>
<td>10 mM</td>
<td>31.3</td>
</tr>
<tr>
<td></td>
<td>20 mM</td>
<td>63.6</td>
</tr>
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</table>

**Table 2: SPR assays.** Chelators was added to HepV (200 nM) before its injection over heparin and heparan sulfate immobilized on the sensor chip. Results were expressed as percentage of inhibition of the binding measured in the absence of chelators.
Modified heparins & Inhibition of HepV binding to heparin & Inhibition of HepV binding to heparan sulfate \\
De-O-Sulfated & 8.1 & 12.5 \\
De-N-Sulfated & 30.2 & 39.5 \\
De-6-O-Sulfated & 49.3 & 60.3 \\
De-2-O-Sulfated & 0 & 7.5 \\

Table 3: SPR inhibition assays. Recombinant HepV (200 nM) was preincubated for 1h at room temperature with selectively desulfated heparins (1µg/ml) before injection immobilized heparin or heparan sulfate. The results were expressed as percentage of inhibition of HepV binding in absence of competitors.

<table>
<thead>
<tr>
<th>Glycosaminoglycans µg/ml</th>
<th>Inhibition of collagen V homotrimer binding to heparin (%)</th>
<th>Inhibition of collagen V homotrimer binding to heparan sulfate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heparin</td>
<td></td>
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<tr>
<td>0.2</td>
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</tr>
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<td>0.5</td>
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<td>96.3</td>
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<tr>
<td>50</td>
<td>85.9</td>
<td>94.1</td>
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</table>

Table 4: SPR inhibition experiments. The homotrimer of collagen V (20 µg/ml) was preincubated with different concentrations of heparin or heparan sulfate for 1h at room temperature before its injection over heparin and heparan sulfate immobilized on the sensor chip. Results were expressed as percentage of inhibition of the binding in absence of competing glycosaminoglycans.
<table>
<thead>
<tr>
<th>Modified heparins</th>
<th>Inhibition of the binding of homotrimer V to heparin</th>
<th>Inhibition of the binding of homotrimer V to heparan sulfate</th>
</tr>
</thead>
<tbody>
<tr>
<td>De-N-sulfated and re-N-acetylated heparin</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>De-6-O-sulfated heparin</td>
<td>50.2</td>
<td>42.4</td>
</tr>
<tr>
<td>De-2-O-sulfated heparin</td>
<td>44.8</td>
<td>32.9</td>
</tr>
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</table>

Table 5: SPR inhibition experiments. The homotrimer of collagen V (20 µg/ml) was preincubated with modified heparins (0.7 µg/ml) for 1 h at room temperature before its injection over heparin and heparan sulfate immobilized on the sensor chip. The results were expressed as percentage of inhibition of the binding in absence of modified heparins.
Figure 1

A

KDa

66
31
21.5
14.5

1 2 3

HepV

B

RU

Time (s)

50 nM
37.5 nM
25 nM
18.7 nM
12 nM
9 nM
6 nM
3 nM
Figure 3

A

B

Triple helical Peptide 5 µg/ml

Time (h)

0 400 800 1200

0 100 200 300

Heat 2 µg/ml

Heat 400 µg/ml

Time (h)

0 30 300 500
Structural requirements for heparin/heparan sulfate binding to type V collagen
Sylvie Ricard-Blum, Mickael Beraud, Nicolas Raynal, Richard W. Farndale and Florence Ruggiero

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