Molecular Features of the Collagen V Heparin Binding Site*

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A heparin binding region is known to be present within the triple helical part of the α(V) chain. Here we show that a recombinant α(V) fragment (Ile\textsuperscript{824} to Pro\textsuperscript{950}), referred to as HepV, is sufficient for heparin binding at physiological ionic strength. Both native individual α(V) chains and HepV are eluted at identical NaCl concentrations (0.35 M) from a heparin-Sepharose column, and this binding can be inhibited specifically by the addition of free heparin or heparan sulfate. In contrast, a shorter 23-residue synthetic peptide, containing the putative heparin binding site in HepV, fails to bind heparin. Interestingly, HepV promotes cell attachment, and HepV-mediated adhesion is inhibited specifically by heparin or heparan sulfate, indicating that this region might behave as an adhesive binding site. The same site is equally functional on triple helical molecules as shown by heparin-gold labeling. However, the affinities for heparin of each of the collagen V molecular forms tested are different and increase with the number of α(V) chains incorporated in the molecules. Molecular modeling of a sequence encompassing the putative HepV binding sequence region shows that all of the basic residues cluster on one side of the helical face. A highly positively charged ring around the molecule is thus particularly evident for the α(V) homotrimer. This could strengthen its interaction with the anionic heparin molecules. We propose that a single heparin binding site is involved in heparin-related glycosaminoglycans-collagen V interactions, but the different affinities observed likely modulate cell and matrix interactions between collagen V and heparan sulfate proteoglycans in tissues.

Collagen V is a fibrillar collagen that plays an important role in fibrillogenesis, and it also acts as an adhesive substrate for a large variety of cells and binds to a number of extracellular components through its major triple helical domain (1). Collagen V interacts with matrix proteoglycans such as the two small proteoglycans decorin and biglycan (2), the proteoglycan form of macrophage colony-stimulating factor (3), the cell surface proteoglycan syndecan-1 (4, 5), and as shown recently, the membrane spanning proteoglycan NG2 (6). Some of these interactions are mediated by the core proteins, but others depend on the glycosaminoglycan chains such as the heparan sulfate chains.

Apart from in vitro binding of collagen V to membrane-spanning proteoglycans, the suggestion that heparan sulfate interacts with collagen V was supported by inhibition experiments showing a reduction of cell attachment to collagen V in the presence of heparin (7). It has been shown already that cell focal adhesion on fibronectin requires the cooperation of both cell transmembrane proteoglycans and integrin receptors (8). Because we have demonstrated already that cell-collagen V interactions involved integrins (9, 10), the binding of membrane-spanning proteoglycans could reinforce cell attachment to collagen V and, in that sense, would be of physiological importance. Therefore, to understand the role of collagen V-heparan sulfate proteoglycan interactions, it appears essential to characterize the specific domain(s) of the molecule responsible for binding to heparin, a glycosaminoglycan related to heparan sulfate.

Collagen V is a typical fibrillar collagen containing a 300-nm-long triple helical domain that presents different molecular forms in tissue. The predominant molecular form found in most tissues is the heterotrimer [α1(V)\textsubscript{2}α2(V)], whereas the α1(V)α2(V)α3(V) molecule is only extracted from human placenta (1). The homotrimer [α1(V)\textsubscript{3}] occurs in cultures of hamster lung cells and was suggested to be present in embryonic tissue (11–13). It was produced recently as a recombinant molecule (14). The heterotrimeric [α1(V)\textsubscript{2}α2(V)] molecular form was shown previously to bind to heparin at physiological salt concentrations. This activity was attributed to a proteolytic NH\textsubscript{2}-terminal 30-kDa fragment of the α1(V) chain (15).

Aside from a requirement for the α1(V) chain, the features of the different stoichiometries of the collagen V triple helix necessary for heparin binding remain unknown. Such studies have been hampered by the fact that determination of minimal sites on the collagenous triple helix is difficult. Thus in this manuscript, to locate more precisely the heparin binding site, we have combined several approaches: (i) recombinant technology, in which collagenous domains and the molecule isotypes can be engineered and generated in quantities sufficient for biochemical and functional analysis; (ii) a synthetic peptide to narrow the sequence involved in heparin-collagen V recognition; (iii) electron microscopy to visualize the site on triple helical molecules. Using these approaches it has been possible to establish that a recombinant fragment of the α1(V) chain (Ile\textsuperscript{824} to Pro\textsuperscript{950}) but not a synthetic peptide encompassing the putative heparin binding site binds to heparin and heparan sulfate. Moreover, we provide evidence for a cell adhesive function of this fragment through a cell surface heparan sulfate proteoglycan. This region corresponds to a heparin binding site common to the three known collagen V molecular forms, although increasing heparin affinities were correlated positively with the

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The atomic coordinates and structure factors (codes 1a89 and 1a9a) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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number of α1(V) chains incorporated in the molecule. Based on molecular modeling of the portion of the α chain which includes the putative binding sequence, we propose a model to explain the different affinities observed for the distinct collagen V molecular forms.

**EXPERIMENTAL PROCEDURES**

**Construction, Expression of HepV**—The HepV module cDNA (nucleotides 2596–2976) was generated by polymerase chain reaction using as template the clone 302 kindly provided by Dr. Takahara (16). Two oligonucleotide primers flanking the desired sequence were designed to correspond to the 5′- and 3′-ends of the module carried an EcoRI site (5′-TATGATTTCCATCAAGGTTGATCAGGGGGA-3′), and the second, corresponding to the 3′-end of the module, introduced a PstI site and a stop codon (5′-TATTCGCAATTGATCCGCACCCAGGAGG- CCGACTG-3′). The resulting polymerase chain reaction product of 399 base pairs was subcloned in the EcoRI and PstI sites of a pT7-T expression vector (17). The plasmid obtained, named pHepV, thus encoded the heparin binding site of α1(V) under the control of the Escherichia coli phage T7 promoter. The sequence of the recombinant DNA was checked thoroughly.

To obtain the recombinant protein, pHepV was transformed in the E. coli host strain BL21 (DE3). This strain carries the T7 RNA polymerase gene under the control of the lac promoter/operator region which allows induction by isopropyl-β-D-thiogalactopyranoside and subsequently the transcription of the recombinant plasmid. E. coli cells harboring the plasmid pHepV were grown at 37 °C to an A600 of 0.7 in Luria-Bertani medium containing 50 μg/ml ampicillin. The culture was then supplemented with 0.4 mM isopropyl-β-D-thiogalactopyranoside to induce protein expression. The incubation was then maintained at the same conditions for an additional period of 6 h. Cells were harvested by centrifugation at 8,920 × g for 20 min, resuspended in 10 mM Tris/HCl, 1 mM EDTA, pH 8.0, and sonicated by two 30-s pulses at intensity level 3, using a Branson Sonifier-250 (Branson Ultrasonics). After centrifugation, supernatants and pellets were analyzed by 15% SDS-PAGE according to Laemmli (18) followed by Coomassie Blue staining.

**Protein Purification**—For purification of the recombinant fragment HepV, the bacterial supernatant was dialyzed against 35 mM Tris/HCl, pH 7.4, filtered, and applied to a HPLC cation exchange chromatography on a 1 ml Resource-S column (Amersham Pharmacia Biotech) (Waters 625 LC system) and eluted by a linear NaCl gradient (0–300 mM) in the same buffer. The fraction eluted at 200 mM NaCl and containing HepV was purified further by anion exchange chromatography on a Hitrap Q (Amersham Pharmacia Biotech) column. The column was equilibrated in 200 mM NaCl, 35 mM Tris/HCl, pH 7.4. The unbound fractions contained purified HepV as analyzed by 15% SDS-PAGE.

Collagen V (α1(V))2α2(V) heterotrimer was extracted with pepsin and purified as described previously (9). Human placental pepsinized collagen V were grown at 37 °C to an OD600 of 2.0 before being applied to the resin for an additional 30 min. Unbound material was recovered by gentle centrifugation after the addition of 1 ml of starting buffer to the samples. Elution of bound material was achieved by adding 0.5 M NaCl to the starting buffer. All fractions were analyzed by 15% SDS-PAGE.

For peptide inhibition, 50 μl of the resin was incubated with 5 μg of HepV for 30 min at room temperature and washed with 1 ml of starting buffer. Bound material was then incubated with various concentrations of the synthetic peptide HepP, and the released material was recovered by gentle centrifugation. The resin was then washed with starting buffer, and the elution of the remaining bound material was achieved by adding 1 M NaCl to the starting buffer. Fractions were analyzed by 15% SDS-PAGE.

**Rotary Shadowing**—Collagen solutions (collagen V samples and collagen I as control) were dialyzed overnight against PBS at 4 °C and then incubated with heparin-BSA-gold 10-mm particles (Sigma) for 3 h at room temperature. Samples were dialyzed against 1 μM ammonium acetate and finally diluted to 10 μg/ml with the same buffer. After the addition of an equal volume of glycerol, the solutions were sprayed onto freshly cleaved mica sheet and were placed immediately on the holder of a MED 010 evaporator (Balzers). Rotary shadowing was carried out as described previously (19). Observations of replicas were performed with a Philips CM120 microscope at the CMEABG (Centre de Microscopie Electronique Apparate à la Biologie et à la Géologie, Université Claude Bernard, Lyon I).

**Cell Adhesion Assays and Inhibition Assays**—Chinese hamster ovary (CHO) cells were maintained in monolayer cultures in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, glutamine, nonessential amino acids, and a mixture of antibiotics. Before the adhesion assay, cells were harvested with 1% EDTA in PBS or with 0.02% EDTA, 0.1% trypsin, and 0.002% EDTA, pH 7.4, which changed the adhesion to 0.2% Triton X-100, and optical density was read with an enzyme immunoassay reader (Dynex MRX) at 570 nm. Each assay point was carried out in triplicate.

For inhibition assays, the coated wells were preincubated with 10 μg/ml glycosaminoglycans (heparin, heparan sulfate, or chondroitin sulfate) for 1 h at 37 °C. Freshly suspended cells were then added, and the wells were incubated for 30–40 min. For synthetic peptide inhibition, cells were first mixed with 500 μg/ml RGDS peptide before being seeded onto the coated wells. The assays were then continued as described above.

**Two-dimensional Representation and Molecular Modeling**—The two-dimensional projection used to represent the three-dimensional structure of α-helices is called a helical wheel. It allows, in the case of heparin binding consensus sequences, visualization of clusters of basic residues (20). The helical wheel representation for collagen helix differs from a true α helix and thus was represented as described previously (21). For the representation of collagen V triple helices (α1(V))α2(V)
and \([\alpha_1(V)_3]\), the helical wheels were positioned with the glycial residues at the center of the molecule.

For the molecular modeling of the peptide \([\text{Gly}^{904}\text{to Arg}^{1212}]\), the coordinates of the three NH$_2$-terminal GPP$^+$(P$^+$ for hydroxyproline) triplets of the collagen-like peptide (P$^+P^+G$) were used to calculate interproton distance (22). The Protein Data Bank code number is 1CAG. Each value was allowed to vary from $0.1$ to $0.3$ Å around the actual interproton distance. In this way a total of 683 constraints was introduced for the triple helical region, 105 of which were interchain constraints. In addition, average \((\Phi, \Psi)\) dihedral angles deduced from the crystal structure were also introduced with the following values: for Gly $\Phi = -72 \pm 15^\circ$ and $\Psi = 174 \pm 25^\circ$; for X position $\Phi = -72 \pm 15^\circ$ and $\Psi = 164 \pm 20^\circ$; for Y position $\Phi = 60 \pm 15^\circ$ and $\Psi = 150 \pm 18^\circ$ (see Table 1 in Ref. 22; note that the reported standard deviations were doubled for each angle).

Three-dimensional structures were generated from set of constraints with the X-PLOR 3.8.5 program (23) using the default parameter sets, except for some minor modifications to increase the duration of the molecular dynamic simulations and the number of energy minimization steps. Structure superimposition, three-dimensional graphic displays, and manipulations were accomplished using ANTHEPROT 2.0 software (24). The Protein Data Bank numbers are 1a89 for the homotrimer and 1a9a for the heterotrimer.

RESULTS

Expression and Characterization of HepV—We designed a fragment, referred to as HepV, which encompasses the complete NH$_2$-terminal part of the 30-kDa CNBr peptide defined by Yaoi et al. (15) and the sequence around the endoproteinase Glu-C cleavage site which was found to be determinant for heparin binding (Fig. 1). The resulting heparin binding site was thus narrowed down from the COOH-terminal end of the 30-kDa fragment to a 12-kDa polypeptide, HepV. An expression vector pT7-7, which encodes amino acids Ile$^{824}$ to Pro$^{950}$ of human \(\alpha_1(V)\) chain was constructed as described under “Experimental Procedures.” After induction with isopropyl-$\beta$-D-thiogalactopyranoside, SDS-PAGE analysis of the soluble and insoluble cellular extracts showed the presence of an additional protein band of 17 kDa in soluble extracts which was not found in transformed \(E.\ coli\) cells before induction. The difference between the apparent molecular mass and the predicted polypeptide mass of 12 kDa was attributed to the peculiar structure of collagen chains migrating slower than the globular standards, as well as the additional NH$_2$-terminal amino acid residues originating from the construct. Passage of the supernatant of a Mono S column separated the HepV domain from most of the contaminating bacterial proteins. Final purification was achieved by rerechromatography on a Mono Q column (Fig. 2A). Analysis of the NH$_2$-terminal amino acid sequence of the purified band indicated the sequence XRIPIKGD, which agreed with that of the HepV construct. The four first amino acid residues correspond to NH$_2$-terminal extension added for cloning purposes.

Binding of \(\alpha_1(V)\) Chain Fragments to Heparin—The binding interaction of heparin with collagen V fragments was studied using their affinities on a heparin-Sepharose column. We showed that HepV bound to the heparin-Sepharose column at physiological pH and ionic strength. Moreover, HepV was eluted from the column at 0.35 M NaCl (Fig. 2B), which is exactly the concentration required to elute isolated native \(\alpha_1(V)\) chains. In addition, binding to heparin is inhibited by incubation of HepV with free heparin or heparan sulfate but not with chondroitin sulfate before loading on the heparin-Sepharose column.

Furthermore, to assess the importance of the length of the sequence flanking the putative heparin binding site, we designed a peptide, HepP, containing the basic amino acid clusters KPGPRGQR but also including the basic residues close to the endoproteinase Glu-C cleavage site (at the carboxyl group of glutamate residue) (Fig. 1). Only 5% of the corresponding synthetic peptide was able to bind to a heparin-Sepharose column as indicated by the amino acid composition of the bound and unbound fractions (data not shown). Also, the peptide (added at concentrations up to 1 mg/ml; corresponding to a 200-fold molar excess) was unable to detach HepV bound to heparin-Sepharose (Fig. 3B) or to inhibit heparin binding to collagen V in a solid phase assay (not shown).

Binding of Heparin to the Different Collagen V Molecular Forms and Its Constitutive Chains—We showed that isolated \(\alpha_1(V)\), but not \(\alpha_2(V)\) and \(\alpha_3(V)\) chains, was able to bind heparin at physiological pH and ionic strength and was eluted from the column at exactly 0.35 M NaCl (Fig. 4A). To evaluate whether the binding site present in \(\alpha_1(V)\) is available when incorpo-
Mapping of the Heparin Binding Site on Collagen V Molecules—Because an active site might be available in small fragments or individual chains but masked in the context of the whole molecule, we then examined, by electron microscopy, the location of the heparin binding site on triple helices using heparin as a probe (according to Ref. 5). For this purpose, collagen V, and collagen I as control, were complexed with heparin-BSA-gold and prepared for rotary shadowing as mentioned under “Experimental Procedures.” Pepsinized \([\alpha_1(V)]_3\alpha_2(V)\) collagen V-heparin gold complexes were often observed as large intermolecular aggregates for which it was difficult to identify the precise location of heparin binding. However, selected areas contained a sufficient proportion of individual molecules to determine the position of the gold particles on collagen V molecules. The binding site was located at about 100 nm from one of the extremities of the molecules (Fig. 5A). This corresponds exactly to the \(\alpha_1(V)\) binding site position if we consider that the distance is measured from the NH2-terminal extremity of the collagen molecule. This binding site is specific to collagen V molecules since it was not observed on collagen I molecules labeled with heparin-gold (data not shown). Attempts to determine the polarity of collagen V ends using the recombinant \([\alpha_1(V)]_3\) homotrimer encompassing the entire N-propeptide were not successful. Indeed, the presence of the N-propeptide seemed to enhance the formation of intermolecular aggregates associated with heparin-gold, and individual labeled molecules were rarely observed. Therefore mapping experiments were undertaken with a 200-nm fragment product of the recombinant \([\alpha_1(V)]_3\) homotrimer (Fig. 5B). We have shown previously that this fragment resulted from a proteolytic cleavage occurring close to a flexible region present in the triple helical domain of the homotrimer (14). Interestingly, the NH2-terminal sequence of this fragment was found to start at residue Asp706, which is 65 amino acid residues upstream from the heparin binding site we have determined (Ile624 to Pro650) (14). This would correspond to about 20 nm from the NH2-terminal end of the fragment and allow the orientation of the molecules. As expected, when this fragment was applied to heparin-Sepharose it was retained on the column and was eluted in the same conditions as the entire recombinant homotrimer, thus confirming the presence of the heparin binding site (data not shown).

Mapping experiments on the fragments clearly showed a single location of heparin gold particles at a site very close to one end of the fragment, thus identified as the NH2-terminal extremity (Fig. 5). It is thus concluded that the heparin binding site we have determined on the \(\alpha_1(V)\) chain is available when incorporated in the triple helical structure of collagen V molecules.

CHO Cell Adhesion in Response to HepV Fragment—CHO cells were shown previously to interact with collagen V likely via a cell surface heparan sulfate proteoglycan (7). We show that not only intact collagen V but also recombinant HepV fragments were equally efficient in promoting cell attachment of EDTA-released CHO (Fig. 6). CHO cells inspected by inverted phase microscopy remained round in shape on a HepV substrate, whereas on a collagen V substrate, even though most of cells are round, a more flattened morphology was also observed (Fig. 6, upper panel). The glycosaminoglycan contribution to CHO adhesion was examined by plating the cells on collagen V and HepV substrates in the presence of heparin, heparan sulfate, and chondroitin sulfate. We show that heparin and heparan sulfate, but not chondroitin sulfate, are potent inhibitors of cell interaction with HepV and to a less extent with intact collagen V, whereas chondroitin sulfate and the irrelevant synthetic peptide RGDS have no effect (Fig. 6). Interestingly, binding of trypsin-released cells drastically affects cell adhesion to HepV, whereas adhesion to intact collagen V is only partially decreased. These results indicate that CHO cells possess at least two distinct cell surface receptors for collagen V: a trypsin-labile receptor that binds specifically to the hepa-
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Fig. 5. Electron microscopy images of pepsinized [α1(V)]_2α2(V) heterotrimer (panel A) and of truncated recombinant [α1(V)]_3 homotrimer (panel B) heparin-gold complexes observed by rotary shadowing. Panel C, mapping of the heparin-gold location on the 300-nm-long triple helical domain of collagen V molecules referred to as COL1 (upper panel) and on the truncated recombinant homotrimer (lower panel).

Fig. 6. CHO adhesion to HepV (left panel) and collagen V (right panel). Micrographs of CHO cells after a 40-min adhesion to HepV and collagen V (control) are shown. When wells were coated with HepV, the cells remained round in shape, whereas spread cells could be observed on collagen V substrate (upper panel). EDTA released cells adhesion in the absence (control) or in the presence of heparin (H), heparan sulfate (HS), chondroitin sulfate (CS), and irrelevant synthetic peptide (RGDS). Trypsin-released cells (Trypsin) result in a nearly total loss of cell adhesion to HepV, whereas adhesion to collagen V is only partially affected.

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arin binding site, HepV, and a trypsin-resistant receptor that binds to another region of the molecule. Altogether, the data support the conclusion that the interaction between CHO cells and the collagen V heparin binding site is mediated by a cell surface heparan sulfate proteoglycan receptor.

Representation of Heparin Binding Sequence Folded into Collagen V Triple Helices—We showed that HepV binds heparin as tightly as the parent α1(V) chains. However, even though the cluster of basic amino acids present in HepV contains conspicuously the sequence responsible for heparin binding activity, the synthetic peptide encompassing this sequence has no affinity for heparin. Furthermore, no amino acid sequence was found in HepV which fits one of the postulated consensus motifs identified in other heparin-binding proteins (XBBBXXB, where B designates a basic amino acid and X any other amino acid). This probably means that the secondary structure of collagen holds the sequence of interest in a particular spatial pattern that fits more to heparin or that residues flanking this sequence contribute to the charge density of the binding site. The spatial pattern of the putative binding site was assayed by analyzing the amino acid distribution of residues 905–921 (KPGPRGQRGPTGPRGER) in a helical wheel representation. The representation of a collagen helix known as a polyproline II helix has been shown to differ from a true α-helix in the sense that proline residues induce a more extended structure, and thus the helical wheel contains 17 residues instead of 18 for an α-helix (21). Interestingly, the representation of the peptide sequence segregates the basic residues to one side of the helical face and forms a cluster of 5 basic residues within a stretch of 9 amino acids. It is clear that the spatial distribution of the basic amino acids promotes the formation of a high positive charge region. The helical wheel representations for peptides flanking this sequence, even if they do correspond to basic amino acid-rich regions, do not allow the observation of positive charge clusters (data not shown). Because HepP encompasses this region, this indicates that the designed peptide, although it did not bind to a heparin column, corresponds to the sequence that fits the best the postulated criteria for heparin recognition.

A similar representation for heterotrimer and homotrimer collagen molecules is also proposed considering that a molecule is constituted by three individual α chains twisted to form the triple helix with glycol residues, from the Gly-Xaa-Yaa triplets, strictly located at the center of the helix (Fig. 7). Alternatively, to gain insight into the three-dimensional arrangement of the basic amino acid residues, a molecular model was generated for the triple helix structures, which was based on the three-dimensional structure of a collagenous triple helix recently solved from x-ray crystal diffraction for the collagen-like peptide (PP*G)4PP*A(PP*G)5 (22). The coordinates of the three NH2-terminal GPP* triplets of each chain were used to calcu-
late the corresponding distance constraints and dihedral angles to generate the triple helical conformation as described under “Experimental Procedures.” All of the generated structures satisfied these constraints (Fig. 7). They are no violations of distances or dihedral constraint (respectively no distances deviation >0.5 Å and no dihedral deviation >5°). Examination of calculated structures showed that the interchain hydrogen bonds between amide protons of glycine residues and carboxyl groups of proline residues were established according to the molecular model proposed from x-ray diffraction (22). The validity of these structures was confirmed by the rather low energy found for the calculated molecules (−280.5 and −248.7 kcal mol⁻¹ for homo- and heterotrimer, respectively) and the low deviations from ideal covalent geometry (data not shown).

The two-dimensional representation of the heterotrimer segregates the two arginines, Arg₅₅₉ and Arg₅₆₉ from the opposite sequence of α₂(V) chain on the molecules surface. Apparently, from our experimental data this is not sufficient to allow the α₂(V) chain binding to heparin. The molecular model of the two helices suggests that even if these two arginines can contribute to the heparin site according to their distribution in close vicinity to α₁(V) basic amino acids Lys⁹⁰⁵ and Arg⁹¹₂, the lack of positively charged residues in the regions between (Lys⁹⁰⁵–Arg⁹¹₂ and Arg⁹¹₂–Arg⁹₂⁴ from the α₁(V) chain), can affect heparin binding by decreasing the net positive charge of the heterotrimer compared with the homotrimer.

DISCUSSION

Collagen V was shown to bind heparan sulfate proteoglycans through its heparin binding site (7, 15). The same site might mediate interaction with syndecan-1 (4, 5). A first approach to study these interactions in terms of function and possible involvement in physiological events is to characterize better the heparin binding site on individual chains and also on collagen V triple helix molecules. In the present study we have narrowed the sequence involved in the interaction on the individual α₁(V) chains and identified the heparin binding site on collagen V molecules. Indeed, experiments with individual chains or small fragments cannot solve the question of whether the same specific domain is required for heparin binding to triple helical molecules. As a matter of fact collagen I binds to heparin, but it was shown that the triple helical structure is strictly required for heparin binding (4, 25, 26). This suggests that triple helical formation can generate new combinations of basic amino acid residues from the association of the two distinct constitutive chains creating a heparin binding site. Therefore, it appears essential to determine the level of structural organization of collagen V required for binding to heparin.

The results presented here strongly suggest that the recombinant 12-kDa (HepV) fragment is necessary and sufficient for heparin/heparan sulfate binding activity, whereas a more restricted sequence (HepP) including a cluster of basic amino acid residues is not sufficient to mediate binding to heparin. The lack of heparin binding activity of this peptide might be the result of the need for neighboring amino acid residues for the interaction to occur or the failure of the peptide to assume a correct secondary structure consistent with its configuration in the folded domain.

It is now clear that specific structural motifs rather than just ionic interactions are required for protein binding to heparin (20, 27). This is supported by studies where the use of heparin-binding synthetic peptides led to a reduction of affinity (28, 29).
As a matter of fact, HepV was shown to fold into the characteristic collagenous structure, the polyproline II helix, by circular dichroism, whereas the peptide tested in the same experimental condition was not (data not shown). A large range of extracellular proteins interacts specifically with heparin, and a common motif emerged from comparison of the various heparin binding sequences (20). Such sequences were not found in the entire \( \alpha(V) \) chain. However, from the research on the identification of a common motif in heparin binding sequences, Marzilli et al. (27) defined the motif KPGPRQGR as the sequence responsible for heparin recognition. Even though this sequence was considered to fold into a \( \beta \) strand, which appears to be unlikely since this sequence is included within the triple helical domain known as a polyproline II helix, analysis of the basic residue distribution in the helical wheel representation adapted to this peculiar structure ensures the segregation of the basic amino acid residues on one side of the helix. This representation allowed us to observe that all of the basic residues contained in the synthetic peptide sequence (HepP) form a cluster of positive charge which likely interacts with heparin. Because the formation of this cluster is dependent on the correct folding of the peptide, we suggest that the peptide does contain the information to bind heparin but fails to adopt the correct conformation.

Thus it appears that heparin binding motif of collagenous structure exhibits the same characteristics already described for other proteins (folded into a helix or \( \beta \) strand): a spatial distribution of the basic amino acid forming an amphipathic structure (20, 27). Although it is not possible to generalize to the other collagens containing heparin binding sites within the triple helical domain, this is at least confirmed for the heparin binding sites present on the acetylcholinesterase collagenous tail (21).

A preferred site of attachment of heparin gold on collagen triple helices was positioned at about 100 nm from the NH\(_2\)-terminal extremity of the collagenous domain, indicating that the heparin binding site occurs at the same position on individual chains and on triple helical molecules. Nevertheless the level of affinity for heparin depends on the chain composition constituting the collagen molecules. Collagen V can associate to at least three different molecules: \( \alpha(V)_2\beta(V) \), found in most tissues; \( \alpha(V)_2\beta(V)\delta(V) \), described only in human placenta; and the homotrimer \( \alpha(V)_3 \), produced by hamster lung cells and present in some embryonic tissues (11–13). Indeed, a different apparent affinity for the two heterotrimers for heparin has been described recently by others as a way to purify the \( \alpha(V)_3 \) fragment and particularly on the recombinant \( \alpha(V)_3 \) chain. However, this approach described here is an essential step in the elucidation of the function of the collagen V heparin binding site in cell and matrix interactions. Moreover, the basic amino acid residue(s) crucial for heparin binding activity can be investigated further by mutagenesis experiments on the recombinant \( \alpha(V) \) fragment and particularly on the recombinant homotrimer. As we have shown that collagen V binds to cell surface proteoglycans and integrins (9, 10), it is tempting to speculate that these sites can act cooperatively as described already for other extracellular matrix proteins such as laminin (32, 33) and fibronecetin (34).

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