Unraveling the Amino Acid Sequence Crucial for Heparin Binding to Collagen V*

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We have previously shown that a recombinant 12-kDa fragment of the collagen α1(V) chain (Ile824–Pro950), referred to as HepV, binds to heparin and heparan sulfate (Delacoux, F., Fichard, A., Geourjon, C., Garrone, R., and Ruggiero, F. (1998) *J. Biol. Chem.* 273, 15069–15076). No consensus sequence was found in the α1(V) primary sequence, but a cluster of 7 basic amino acids (in the Arg900–Arg924 region) was postulated to contain the heparin-binding site. The contribution of individual basic amino acids within this sequence was examined by site-directed mutagenesis. Further evidence for the precise localization of the heparin-binding site was provided by experiments based on the fact that heparin can protect the α1(V) chain heparin-binding site from trypsin digestion. The results parallel the alanine scanning mutagenesis data, i.e. heparin binding to the α1(V) chain involved Arg912, Arg918, and Arg921 and two additional neighboring basic residues, Lys905 and Arg909. Our data suggest that this extended sequence functions as a heparin-binding site in both collagens V and XI, indicating that these collagens use a novel sequence motif to interact with heparin.

Collagen V is a minor component of connective tissues that plays a fundamental role in matrix organization. Indeed, direct evidence was obtained from collagen V gene mutations that provoke obvious alteration of fibril aggregates (1–4). Aside from its role in collagen fibril formation, collagen V interacts specifically with a variety of macromolecules in the extracellular matrix (5) and with several cell-surface receptors such as integrins (6, 7), tyrosine kinase receptors (8, 9), and proteoglycans (10, 11). Such interactions are important in regulating cell behavior and fibril formation during development and physiological events. Although collagen V is involved in a plethora of specific interactions and thus might possess various domain-specific functions, binding sites have not yet been mapped except for the heparin-binding site. Heparin is abundant in animal tissues in the form of heparan sulfate proteoglycans both on the cell surface and in the extracellular matrix. A 30-kDa heparin-binding fragment of the α1(V) chain has been isolated and was shown to bind heparin with the same affinity as the complete parental chain (12). The binding site is exclusive to this chain since the two other chains, viz. α2(V) and α3(V), which can also be part of collagen V molecules, have no affinity for heparin (11–13). Using a recombinant approach, we have narrowed this region down to a 12-kDa fragment referred to as HepV. Interestingly, the recombinant fragment HepV expressed in *Escherichia coli* was shown to support heparin-dependent cell adhesion (11).

Heparin-binding sites are found in a wide range of proteins including collagens and in a broad repertoire of extracellular matrix proteins, viz. fibroectin, tenascin, and laminin. They are all characterized by an overall positive charge, and common structural motifs have been proposed from the analysis of the different heparin-binding site primary sequences. The consensus sequences BBXB, XBBXB, and XBBBXXBX (where B designates a basic amino acid and X designates any other residues) have been identified (14). A thorough inspection of the α1(V) chain primary sequence indicates that no sequence matches any of the proposed motifs above. HepV does contain a stretch of cationic amino acids (Arg900–Arg924), but a synthetic peptide that was designed to encompass this sequence was shown to have negligible affinity for immobilized heparin under physiological conditions. Also, the synthetic peptide failed to compete with HepV or with the complete α1(V) chain in a heparin binding assay. This result led to the hypothesis that the peptide conformation or/and the flanking residues might be crucial for efficient binding to heparin (11). The characteristics of the collagen V heparin-binding site might be somehow more subtle than previously thought. Based on the fact that recombinant HepV binds to heparin with the same relative affinity as the parental α1(V) chain, all 7 basic residues contained in the region Arg900–Arg924 were individually mutated, and the resulting expression products were tested for their capacity to bind heparin. In addition, trypsin digestion of HepV bound to heparin was used to map the heparin-binding site owing to the fact that heparin protects this site from trypsin cleavage. The results indicate that collagen V binds to heparin through an extended binding site in which the different basic residues involved do not have the same contribution.

**EXPERIMENTAL PROCEDURES**

*Construction and Expression of HepV and Mutants*—The overexpression of the wild-type recombinant fragment has been previously described (11). Briefly, the HepV module cDNA was generated by PCR using clone 302 of the human α1(V) chain cDNA (15) as a template. The oligonucleotides corresponding to the 5′-end of the module (5′-WT primer, 5′-TATGAATTCATTCAAGGGTGATCGGGGGAGA-3′) and to the 3′-end of the module (3′-WT primer, 5′-TATCTGCAAGATTAGGCTCCCGCTTCACCAAGGGCGGCCAGCTG-3′) (see Fig. 1) contained exogenous restriction sites: EcoRI on the 5′-end and PstI on the 3′-end for convenient subcloning downstream of the *E. coli* phage T7 promoter of a pT7-7 expression vector (16). The recombinant plasmid pHePv was transformed in an *E. coli* BL21(DE3) strain carrying the T7 RNA polymerase gene under the control of the lac promoter/operator.

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1 The abbreviations used are: PCR, polymerase chain reaction; WT, wild-type; CAPS, 3-(cyclohexylamino)propanesulfonic acid; HPLC, high pressure liquid chromatography.
**Collagen V Heparin-binding Site**

**Fig. 1.** Alanine scanning mutagenesis of basic residues from Arg<sup>900</sup> to Arg<sup>924</sup> of the human α1(V) chain. The recombinant HepV fragment (Ile<sup>824</sup>–Pro<sup>950</sup>) has been previously obtained (11) using two degenerate oligonucleotide primers (5'-WT and 3'-WT) for convenient subcloning in the E. coli expression vector. The boxed amino acid residues (aa) located within the region Glu<sup>896</sup>–Gly<sup>928</sup> of the α1(V) chain have been singly mutated. The sequences of oligonucleotides designed for basic amino acid-to-alanine substitution are listed; the mutated nucleotides are underlined, and the resulting amino acid substitutions are in boldface.

The recombinant protein was collected 6 h after the induction of the promoter by 0.4 mM isopropyl-β-D-thiogalactopyranoside.

Site-directed mutagenesis was carried out according to the procedure of Kamnan et al. (16). It is based on a two-round PCR amplification, with the first round leading to the generation of a mega-mutagenic primer, which is then used in a second PCR round, which allows the synthesis of the whole mutated fragment. The HepV plasmid previously obtained was used as a template for the PCR. During the first PCR, the sequence between one of the different mutagenic primers described in Fig. 1 that contains the targeted mismatched bases in the center and the 3'-WT primer was amplified. Mutations were designed to substitute alanine for any of the basic amino acids. A control using a non-basic amino acid was made by substitution of Thr<sup>915</sup> with alanine.

The fragment obtained was then used as a primer together with the 5'-WT primer to generate the 399-base pair PCR fragment. The PCR product was subcloned in the expression vector. The recombinant fragment referred to as HepV that encompasses the complete N-terminal part of the 30-kDa CNBr peptide defined by Yaoi et al. (12) and the sequence around the endoproteinase Glu-C cleavage site that was found to be determinant for heparin binding (Fig. 1) (11). This recombinant fragment, which spans Ile<sup>824</sup>–Pro<sup>950</sup> of the human α1(V) chain, has been incubated in batch on a 1-ml heparin-Sepharose CL-6B column (Amersham Pharmacia Biotech) in 50 mM Tris-HCl (pH 7.5) for 15 min at room temperature, and the unbound material was removed with 50 volumes of the above buffer. The bound material was then digested with trypsin (sequencing grade modified trypsin, Promega) for 10 min at room temperature (enzyme/substrate ratio of 1:10). The digestion was stopped by addition of soybean trypsin inhibitor (Sigma), and the gel was washed with 20 volumes of buffer containing soybean trypsin inhibitor to remove unbound trypsin-digested HepV fragments. Elution of bound fragments was achieved by adding 0.35 M NaCl to the starting buffer. The HepV heparin-binding tryptic fragments were separated by reverse-phase chromatography on a C<sub>18</sub> column (Delta-Pak, Waters), and the complete sequences of the purified tryptic fragments were determined by N-terminal sequencing.

**RESULTS**

**Expression and Characterization of Recombinant HepV and Mutants**—We have previously designed and produced the recombinant fragment referred to as HepV that encompasses the complete N-terminal part of the 30-kDa CNBr peptide defined by Yaoi et al. (12) and the sequence around the endoproteinase Glu-C cleavage site that was found to be determinant for heparin binding (Fig. 1) (11). This recombinant fragment, which spans Ile<sup>824</sup>–Pro<sup>950</sup> of the human α1(V) chain, was found to bind to heparin with the same affinity as the complete human α1(V) chain and thus was identified as the collagen V heparin-binding site (11). To further characterize this binding site, we undertook to analyze the Arg<sup>900</sup>–Arg<sup>924</sup> region, which contains a stretch of 7 basic residues, by alanine scanning mutagenesis of the basic residues. With this aim, the different arginine and lysine residues spanning this sequence were singly converted to alanine, and the irrelevant Thr<sup>915</sup> residue was also mutated to alanine as a control (Fig. 1). The mutated sequences of the
recombinant cDNA were all checked thoroughly. Wild-type and mutant recombinant HepV fragments were expressed in bacteria and passed through a cation-exchange chromatography column to remove most of the bacterial protein contaminants from samples before determination of heparin affinities. All the mutated fragments were identified by their migration pattern by SDS-polyacrylamide gel electrophoresis analysis and, when necessary, by N-terminal sequencing after electrotransfer of the band of interest (Fig. 2). For R921A, a band migrating at the position of HepV and two additional bands migrating faster were observed on the gel. These three bands showed identical N-terminal sequences, indicating that this mutant underwent, for some reason, C-terminal trimming. To determine its heparin affinity, only the upper band was considered.

Binding of Recombinant HepV and Mutants to Heparin—The relative affinities of the recombinant proteins for heparin were assessed by sodium chloride elution from a heparin-Sepharose column. The wild-type fragment was eluted from the column at 0.35 M NaCl (Fig. 3A), which is identical to the concentration obtained with the complete natural α1(V) chain (11). Alanine scanning showed that all mutants retained the heparin-binding activity, but their shift in elution position was compared with the wild-type HepV elution. A considerable difference in the degree of affinities was observed. As expected, the T915A mutation did not affect the fragment affinity for heparin. Mutants R909A and R924A behaved roughly the same as the wild-type HepV fragment. The affinities of mutants K905A and R909A for immobilized heparin were only slightly reduced (0.29 and 0.26 M, respectively), whereas mutations of Arg912, Arg918, and Arg921 reduced heparin binding drastically. Actually, these three mutations demonstrated the weakest binding. They all eluted at 0.2 M, which is close to the physiologic ionic strength (0.15 M NaCl). Overall, a limited number of basic residues that form a continuous patch of positively charged residues are critical for heparin binding, viz. Arg912, Arg918, and Arg921. Interestingly, when concentrations of NaCl required for elution were plotted as a function of the position in the primary sequence of any mutated basic residue (Fig. 3B), the heparin affinity gradually decreased at the vicinity of the Arg912–Arg918–Arg921 cluster, which showed the lowest heparin affinities, and then increased with the next mutated residue, i.e. Arg924. This indicates that heparin binding of the α1(V) chain involves at least 3 basic residues within the HepV fragment and that additional neighboring basic residues (viz. Lys905 and, to a lesser extent, Arg909) might also contribute to heparin binding.

Mapping of the Heparin-binding Site by Trypsin Digestion—Site-directed mutagenesis provides a unique way to determine the residues directly involved in molecular interactions. However, inherent to this method is the possibility that the amino acid substitution is not conservative and thus can modify the overall charge of the polypeptide or its conformation. Therefore, we looked for other evidence to localize the heparin-binding domain based on the fact that heparin might be able to protect the binding site of the complete recombinant HepV polypeptide from proteolytic digestion.

With this aim, HepV was bound to a heparin-Sepharose column and subsequently digested with trypsin. The trypsin cleavage sites are Arg-X and Lys-X. These 2 residues are crucial to heparin binding and, as expected, are frequent in the HepV sequence. We speculated that when involved in heparin binding, such residues are protected from trypsin attack. Thus, the use of trypsin in this case might favor the determination of the minimal α1(V) sequence directly involved in heparin binding.

After trypsin digestion, the bound fragments were eluted with 0.5 M NaCl and separated by reverse-phase chromatography.
phy (Fig. 4A). Several different peaks were obtained, and the elution positions of undigested HepV and trypsin were easily identified in comparison with profiles obtained with the different proteins loaded separately (data not shown). Besides these two identified peaks, five peaks numbered from 1 to 5 on the chromatogram (Fig. 4A) were selected for protein sequencing. The profile clearly indicates that one of the proteolytic fragments, peak 2, is predominant. The complete sequence of this major eluted fragment, determined by Edman degradation, reveals that it spans the sequence Gly901–Arg924 (Fig. 4B). This sequence encompasses the 3 basic residues Arg912, Arg918, and Arg921. Thus, this corroborates our site-directed mutagenesis experiments and likewise demonstrates the crucial role of these 3 Arg residues in heparin binding. The amino acid sequences of the fragments contained in the other peaks were also determined. The analysis of peak 1 revealed the presence of two fragments named 1A and 1B. The sequence data showed a major sequence (Gly901–Arg912, peak 1B) and a minor one (Gly901–Arg909, peak 1A) (Fig. 4B). Interestingly, these two sequences encompass Lys905 and Arg909, which were shown to contribute to heparin binding; thus, the results are once again in excellent agreement with our point mutation results.

Likewise, the larger proteolytic fragment obtained (peak 3) was identified as the sequence Gly901–Arg924, which exactly overlapped the two shorter sequences determined for peaks 1 and 2, viz. Gly901–Arg912 and Gly910–Arg924 (Fig. 4B). This observation argues for a larger contribution of Lys905 and Arg909 to heparin binding than expected from the mutagenesis experiments.

The N-terminal sequences of peaks 4 and 5 indicated that the fragments started at Gly910 and at the first amino acid residue of the HepV construct, respectively. As expected, these two minor peaks, which showed a longer retention time, likely correspond to incomplete trypsin digestion of HepV, leading to the persistence of uncleaved C-terminal (peak 4) or N-terminal (peak 5) regions.

**DISCUSSION**

Collagen V occurs in tissues in different chain associations, viz. α1(V), α2(V), α1(V) α2(V), α3(V), and α1(V)3. The α1(V) chain was shown to be responsible for heparin binding, whereas the two other chains failed to bind heparin under physiological conditions. This can explain the increased affinities for heparin of the different molecules related to the number of α1(V) chains they contain (11, 13). As a general rule, strong heparin affinity generally predicts the potential for a given protein to bind matrix- or membrane-bound heparan sulfate proteoglycans. The binding is mostly dominated by electrostatic interactions between clusters of basic amino acids and the anionic heparin. Such a cluster actually exists in the α1(V) chain. We have previously mapped the binding site within the third N-terminal part of the helicoid domain referred to as the Coll domain (11). This region encompasses a linear protein sequence from Arg900 to Arg924 containing an obvious cluster of 7 basic residues that were proposed to include part of the heparin-binding site. The molecular model we have previously proposed (11) predicts that the positively charged cluster Lys905–Arg921, which would be expected to form a positive ring all around the surface of the molecule, might function as a heparin-binding site. However, if electrostatic interactions are predominant, the spatial organization of the basic residues can greatly contribute to binding and confer a certain specificity to the interaction (18). A common spatial pattern was shown to be required for heparin binding, in which basic residues form an amphipathic structure and the basic amino acids at the extremities of the motif are located −20 Å apart. This holds true for α-helix and β-strand conformations, but no data are available for the collagogenous domain that corresponds to a polyproline type II structure. The molecular modeling of the putative collagen V heparin-binding site did not reveal any obvious spatial arrangement of the basic residues when included into the triple helical collagen V molecule, and inspection of the molecular model did not help us to predict which basic residues within this region are part of the binding site. Moreover, the primary sequence does not fit a consensus sequence already described for other proteins (14, 18). Numerous collagens contain heparin-binding sites, but they are generally located within the noncollagogenous domain, such as is the case for collagen XIV (19) and collagen XVIII (20), or within a short interruption of the collagenous domain, as in collagen IV (21). Collagen I possesses a heparin-binding site that has been mapped within the collagenous domain (22); but so far, the precise site has not been identified.

In this report, we identified, for the first time, the basic residues that confer affinity for heparin to collagen V. The contribution of individual basic residues spanning the region from Arg900 to Arg924 has been examined by site-directed mutagenesis of the recombinant HepV fragment. To precisely determine the relative importance of charged residues in this sequence to heparin-mediated binding, we prepared a series of recombinant HepV mutants containing alanine substitutions for the amino acid of interest and tested them by heparin-Sepharose chromatography. The results presented here indicate that binding of the α1(V) chain to heparin involves at least 3 neighboring arginines: Arg912, Arg918, and Arg921. Mutations of any one of these residues to alanine strongly decreased the affinity of the HepV fragment for heparin. A lesser but still significant decrease in the concentration of NaCl required for elution from a heparin-Sepharose column was observed for Lys905 and Arg909. Mutations of Arg905 and Arg924 had little (if any) effect on binding, indicating that reduction of the net positive charge cannot account for the observed effects of the other mutations. The results are in agreement with the crucial
role of Arg921 in heparin interactions previously suggested by the biochemical data of Yaoi et al. (12), i.e., they located the heparin-binding site on a 30-kDa CNBr fragment of the α1(V) chain, and they showed that its further cleavage at the Glu920–Arg921 bond by proteolytic digestion greatly reduced heparin binding to the resulting peptides.

From the mutagenesis data, the role of Lys905 and Arg909 in heparin binding was not clear. The contributions of the specific basic residues to heparin affinity are not equal, and the nature of these residues can be important. Fromm et al. (23) showed that arginine-containing peptides bind stronger to heparin than the analogous lysine peptides. This could explain why point mutation of Lys905 only slightly affected heparin affinity. Since both residues have an identical charge of +1, it was suggested that the difference could be due to the intrinsic properties of the side chains. Although the frequency of arginine residues in known heparin-binding sites is greater than that of lysine residues, the latter were shown to be crucial for various heparin-binding sites (24–26). Thus, despite the low affinity observed between lysine residues and heparin, their role is likely not negligible and might be related to a more flexible side chain that can pair the anionic heparin more readily.

To gain more insights into the collagen V heparin-binding site, we sought to determine the exact sequence that fits heparin. With this aim, the ability of heparin to protect the collagen V heparin-binding site against trypsin digestion was assessed. We showed that a good correlation exists between the mutagenesis experiments and the biochemical data. The two major peaks contained fragments that spanned the 3 arginine residues Arg912, Arg918, and Arg921 and the residues Lys905 and Arg909, respectively. More important, from the third most important peak, we sequenced a peptide that covered the extended sequence from Lys905 to Arg921. This result strengthens the crucial role of Lys905 in binding.

Heparin is a negatively charged polymer of a regular disaccharide repeat sequence. The long chain of heparin can interact with extended protein sequence. Heparin-binding sites have been structurally mapped on fibronectin, revealing that both type III repeats 12 and 14 are required for complex heparin binding and that the heparin polymer is supposed to span the FN13 and FN14 domains, located 60 Å away in the three-dimensional structure (27). The maximum distance from Lys905 to Arg921 in the collagen V molecule is ~45 Å, indicating that 12–16 saccharide units are required for optimal binding.

Collagens V and XI share common structural and biological features, although their tissue distribution is different (5). The most common chain association for collagen XI is the heterotrimer α1(XI) α2(XI) α1(II). However, likely due to their highly identity, the α1(V) and α1(XI) chains can substitute for each other to form heterotopic molecules. Indeed, collagen V chains can assemble with the α1(XI) chains and form a stable molecule (α1(XI) α2V) described in bone (28) and vitreous (29) tissues and in the A204 rhabdomyosarcoma cell line (30). One interesting characteristic common to α1(V), α1(XI), and α2(XI) chains, but not to α2(V) and α3(V) chains, is the capacity to bind heparin under physiological conditions (11, 13, 31). However, the binding sites on collagen XI chains have not been identified so far. Owing to the high homology among the three α1(V), α1(XI), and α2(XI) chains, alignment of the heparin-binding site was previously proposed and showed that the basic residues from Arg909 to Arg924 are well conserved in the human protein sequences (5). This alignment thus could not reveal more information regarding the residues directly involved in heparin binding, and our data were really determinant in unraveling them. Inspection of the corresponding sequences of the α2(V) and α3(V) chains that do not bind heparin was more informative. Indeed, on one hand, alignment of the α2(V) chain with the α1(V) chain showed that the net positive charge of the α2(V)-facing sequence is greatly reduced (~2 instead of +7 for α1(V)) and that all basic residues shown in this report to be involved in binding are not conserved (Fig. 5). This corroborates our experimental data. On the other hand, the corresponding region of α3(V), now available (32), was aligned with the α1(V) chain heparin-binding domain (Fig. 5). This chain, supposed to be closely related to the α1(V) chain, has no heparin affinity, as mentioned above. Indeed, the alignment shows a notable reduction in the net positive charge of α3(V) versus α1(V) sequences (e.g., +5/7). However, Arg912, Arg918, and Arg921 are surprisingly perfectly conserved in the human α3(V) chain. The main difference consists of substitutions of Lys905 and Arg909 by glutamine and glutamate, respectively, which highlighted their crucial role in binding (Fig. 5). The replacement of the lysine residue by glutamine is not drastic since this residue is uncharged, but still has polar character. This suggests that the role of Lys905 in heparin binding is perhaps more important than predicted from the alanine mutagenesis data. Basic residues are present in the α3(V) sequence next to the position of the substituted lysine, increasing the net positive charge of the sequence, but apparently without any effect on heparin binding. This is consistent with the idea that electrostatic interactions by themselves are probably not sufficient for specific heparin binding. Overall, our data fit a model whereby a tight and specific binding is more dependent on strategically positioned basic residues than on electrostatic interaction between a positively charged cluster and the anionic heparin.

A novel sequence motif is thus described for heparin binding. Based on the data, we propose that residues including Lys905 might form a primary recognition site for heparin that allows the heparin molecule to subsequently interact with additional basic residues such as Arg912, Arg918, and Arg921 to reinforce the initial binding. The results provide a satisfying explanation for the capacity of the collagen V chains to bind heparin that is consistent with the different affinities observed for the three known collagen V chain associations. It becomes evident that through combinatorial chain associations, the affinity of heparin/heparan sulfate for a given collagen V molecule can be suited to its physiological role in tissues. The identification of the heparin-binding site in this study will clearly help future investigation on molecular mechanisms whereby heparan sulfate proteoglycans interact with collagen V molecules.

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