A Novel Strategy for Defining Critical Amino Acid Residues Involved in Protein/Glycosaminoglycan Interactions*

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The binding of proteins to glycosaminoglycans (GAGs) is the prerequisite for a large number of cellular processes and regulatory events and is associated to many pathologies. However, progress in the understanding of these mechanisms has been hampered by the lack of simple and comprehensive analytical tools for the identification of the structural attributes involved in protein/saccharide interaction. Characterization of GAG binding motifs on proteins has so far relied on site-directed mutagenesis studies, protein sequence mapping using synthetic peptides, molecular modeling, or structural analysis. Here, we report the development of a novel approach for identifying protein residues involved in the binding to heparin, the archetypal member of the GAG family. This method, which uses native proteins, is based on the formation of cross-linked complexes of the protein of interest with heparin beads, the proteolytic digestion of these complexes, and the subsequent identification of the heparin binding containing peptides by N terminus sequencing. Analysis of the CC chemokine regulated on activation, normal T-cell expressed, and secreted (RANTES), the envelope glycoprotein gC from pseudorabies virus and the laminin-5 ζ3LG4/5 domain validated the techniques and provided novel information on the heparin binding motifs present within these proteins. Our results highlighted this method as a fast and valuable alternative to existing approaches. Application of this technique should greatly contribute to facilitate the structural study of protein/GAG interactions and the understanding of their biological functions.

Glycosaminoglycans (GAGs) are complex polysaccharides abundantly found on cell surfaces and in extracellular matrices. Over the last 15 years, GAGs have emerged as critical regulators of most events involving cell response to its external environment. Through their ability to bind a wide range of proteins, they participate to biological processes as diverse as cell proliferation, cell-matrix interactions, migration and chemotaxis, inflammation, angiogenesis, embryo development and patterning, or viral attachment (1–5). GAG chains consist in a linear succession of disaccharide units comprising alternating uronic acid, either β-iduronic (IdoUA) or β-glucuronic (GlcA), and hexosamine, either β-glucosamine (GlcN) or β-galactosamine (GalN). With the exception of hyaluronic acid, GAGs can be sulfated to a variable extent, leading to a high degree of sequence heterogeneity. For heparin and heparan sulfate (HS), the most sulfated and complex GAGs, both in term of structural variability and biological activities, sulfation occurs at C-2, C-6, and occasionally C-3 of glucosamines and C-2 of uronic acids (mostly IdoUA). This process is finely tuned by the concerted activities of multiple biosynthesis enzyme families and controls the generation of specific motifs with appropriate sulfation pattern that will constitute the binding sites for their protein ligands (6–8).

Binding of proteins to HS can influence their activity through many ways. HS is involved in storage functions, protection against proteolysis, and formation of chemotactic gradients (9, 10). It can activate proteins such as antithrombin III (11), plays a role of coreceptor for a number of growth factors including fibroblast growth factors (12, 13), inhibits binding of interferon γ to its cell surface receptor (14), and can direct entry of herpes simplex virus-1 into the cell (15). These multiple activities are intimately related to the structural features of the polysaccharide. A well documented example is that of basic fibroblast growth factor, for which subtle sulfation variations can lead HS modulatory properties toward inhibition or stimulation of the growth factor signaling activity (16, 17). Understanding the functional role of HS/protein binding thus requires the identification of the structural determinants involved in the interaction, both on the saccharide and on the protein side.

On proteins and in absence of structural data, the characterization of heparan sulfate binding sites remains a complex issue. Among studies based on heparin-binding protein sequence comparison, an early work led to the proposition of two HS binding consensus sequences, the XBBXXB and XBBBXXB motifs (where B stands for a basic and X for a neutral/hydrophobic amino acid) (18). However, analysis of more HS ligands has seriously challenged the universality of this paradigm. Site-directed mutagenesis (19) or structural characterization of protein-heparin complexes by x-ray crystallography (20), NMR (21), or molecular modeling (22) clearly

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§The abbreviations used are: GAG, glycosaminoglycan; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; HBD, heparin binding domain; Hp-bd, heparin bead; HS, heparan sulfate; NHS, N-hydroxysuccinimide; PRV, pseudorabies virus; RANTES, regulated on activation, normal T-cell expressed, and secreted; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline.
indicate that HS binding sites are not exclusively composed of linear sequences. They can also include conformational epitopes comprising distant amino acids organized in a precise spatial orientation through the folding of the protein. Therefore, identification of putative HS binding sites solely on the basis of sequence analysis remains highly speculative.

In this study, we have developed an approach, which relies on the proteolytic digestion of protein-heparin stabilized complexes and subsequent analysis of the polysaccharide-bound peptides to identify HS binding motifs. This technique has been used to cartograph HS binding sites on three proteins: the pseudorabies virus (PRV) envelope glycoprotein gC participating to the virus-cell attachment process, the CC chemokine RANTES, a small and soluble signaling protein, and the C-terminal fragment of the laminin-5 α3 chain corresponding to the globular domains 4 and 5 (α3LG4/5), which is part of the complex network of structural proteins forming basement membranes. The results obtained confirmed data from earlier studies and provided additional information on the interaction of these proteins with HS and heparin.

**EXPERIMENTAL PROCEDURES**

*Materials and Methods—Automated Edman degradation was performed using a model 492 gas-phase sequencer, a model 140C HPLC system, and the model 610A data analysis software package (version 2.1) for protein sequencing from Applied Biosystems. Heparin beads (TSK gel heparin 5PW) were from Tosoh Bioscience. EDC/NHS reagents were supplied by BIACore. Thermolysin was from Sigma.*

*Production of the α3LG4/5 Fragment—The α3LG4/5 fragment was produced as previously described (23). Briefly, cDNA corresponding to the α3LG4/5 fragment (C135 to the end) was inserted in-frame with the BM-40 signal peptide in the mammalian expression vector pCEP-Pu. Recombinant plasmid was introduced by electroporation into the human embryonic kidney cell line 293-EBNA (Invitrogen). Transfected cells were selected with 0.5 mM G418 and grown to confluence in Dulbecco’s modified Eagle’s medium. The secreted recombinant LG4/5 fragment was purified from the culture medium by affinity chromatography on a HiTrap heparin column (Amersham Biosciences), and elution was achieved with a linear NaCl gradient from 0.1 to 1.2 M. Protein concentration was determined by microprotein assay using bicinchoninic acid.*

*Coupling of Proteins to Heparin Beads—Heparin Beads (Hp-bds, 25 μl) were first resuspended in PBS, and the carbohydrate groups were activated with 3 mM EDC and 7.5 mM NHS for 15 min, at room temperature, under agitation. Reactants in excess were inactivated by addition of β-mercaptoethanol (15 mM final) and removed by centrifugation and washing with PBS. RANTES (9–68), PRV gC, and laminin-5 α3LG4/5 fragment (9–10 μM) were incubated in the activated Hp-bds in 200 μl of PBS for 2 h at room temperature, under agitation. The reaction was then quenched by addition of 20 mM of 1 M Tris, pH 7.5, and beads were washed with PBS, 2 mM NaCl to remove non-covalently bound proteins. Prior to proteolytic digestion and analysis, beads were stored in PBS at 4 °C.*

**Proteolytic Digestion and N-terminal Sequencing of Proteins on Heparin Beads—Proteins on Hp-bds were denatured by heating at 60 °C for 15 min in PBS, 2 μl resuspended in 50 μl of PBS, and digested overnight with 53 μIU of thermolysin, at 50 °C under constant agitation. Released peptides were removed by extensive washing with PBS, 2 mM NaCl, 15 mM β-mercaptoethanol, 1% Triton. Hp-bds were rinsed in water and packed on a Biobrene-treated filter. Within the filter holder, a 5-μm Durapore membrane was fitted underneath the filter to prevent beads being washed throughout the system. Peptides cross-linked to Hp-bds were sequenced using an Edman degradation automated protocol. Phenylthiohydantoin amino acid derivatives removed at each sequencing cycle were identified and quantified on-line by reverse phase HPLC. Retention times and integration values of peaks were compared with chromatograms obtained for a standard mixture of derivatized amino acids. For clarity, irrelevant residues that could not be detected by sequencing for technical reasons (cysteine that cannot be identified or low yield amino acids at the end of the sequenced peptides) were shown as “detected” in the results. All procedures and reagents were used as recommended by the manufacturer.*

Results:

**Principle of the Beads Approach—**The technique developed in this study is designed to identify heparin binding domains (HBDs) on proteins. The principle of the beads approach (Fig. 1) is based on the capture of the protein of interest on heparin immobilized beads, using a cross-linking reaction between amine-bearing amino acids of the protein and EDC/NHS-activated carbonyl groups of the polysaccharide. The protein/polysaccharide conjugate formed can be submitted to proteolytic digestion. Finally, peptides remaining attached to the Hp-bds, and thus comprising the HBD, can be identified by N terminus protein sequencing. Contrary to site-directed mutagenesis, this technique does not require any modifications of the protein before the formation of the complex with heparin chains, which thus constitutes a native structure. The cross-linking step used to stabilize the complex was designed to occur specifically within the HBD and to avoid unwanted protein-protein interactions. Finally, proteolytic digestion conditions were setup to apply to all kinds of proteins.

**Analysis of gC from Pseudorabies Virus—**PRV is an α herpes virus that causes Aujesky’s disease in swine. Similarly to the prototypical herpes simplex virus type 1 (HSV-1), the PRV cell entry process involves multiple viral envelope glycoproteins. These include the gC protein, which participates in the virus initial attachment by interacting with HS (24–26). Within the 457-amino acid sequence of mature gC, up to seven exact or close matches to the XBBX8 or XBBXXBXB heparin binding consensus sequences can be found (Fig. 2A). Among these, sequences HB1D (457SRKPPR352), HB2 (458AHGRKR3101), and HB2D (458YRRGRFR141), located within the N-terminal...
first third of the protein, have been identified as three independent heparin binding sites with distinct saccharide requirements (27, 28). Interestingly, gC mutants for two of any of the three HBDs could still efficiently bind to heparin (27).

The glycoprotein gC (∼600 nM) was incubated with EDC/NHS-activated Hp-bds. After removal of unbound protein, the gC-heparin conjugate was submitted to proteolytic digestion, using thermolysin. Released peptides were discarded, while fragments cross-linked to the Hp-bds were analyzed by N-terminal sequencing. Results systematically led to the simultaneous identification of two distinct sequences corresponding to domains HBD1 and HBD2 (Fig. 2B, i and ii). Amino acids (marked in red) involved in cross-linking, i.e. undetected Lys (K) and Arg (R) residues, are in red. Lys and Arg residues shared by multiple sequences are in pink, as coupling to the polysaccharide could not be determined. Identified heparin binding motifs are framed and pasted back onto the whole gC sequence (in pale gray).

Analysis of RANTES-(9–68)—RANTES is a 68-amino acid proinflammatory chemokine displaying chemotactic, leukocyte activating, and anti-human immunodeficiency virus activities (29). As most chemokines, RANTES binds to GAGs and particularly to HS (30, 31). This interaction may play multiple roles in RANTES biological activity, including protection and storage of the chemokine, formation of chemotactic gradients, or induction of dimerization, which is thought to be a critical step for eliciting chemokine receptor signaling (9, 10). RANTES amino acid sequence (Fig. 3A) features two clusters of basic residues, 44RKNR47 and 55KKWVR59, the first one having been identified by site-directed mutagenesis as the primary heparin binding site. However, mutations of all basic residues within the 44RKNR47 cluster did not completely abrogate interaction with heparin, suggesting the existence of complementary binding motifs (32).

To tackle this issue, we decided to further characterize RANTES HBD, using the beads approach. One of the major hurdles in the study of RANTES is its very potent tendency to self-aggregate. To circumvent this difficulty, all analyses were performed on RANTES-(9–68), an N-terminal eight-amino acid truncated form of the chemokine, which remains monomeric in solution (33). RANTES-(9–68) affinity for heparin is similar to that of wild type RANTES, which is in agreement with the presence of the heparin binding residues toward the C terminus of the chemokine (22, 31, 32). RANTES-(9–68) was incubated with EDC/NHS-activated Hp-bds at a concentration of 1011 nM, which is in the range of the chemokine affinity for the polysaccharide (31). Typical results yielded a single sequence, 42VTR-NRQ48, which corresponded to the reported HBD (Fig. 3B). Amino acid Lys45 could not be detected, indicating that this residue was the one involved in the cross-linking with the polysaccharide (see above). Incomplete digestions led occasionally to the detection of sequences corresponding to partly degraded or intact material. For instance, in Fig. 3B, ii, peptides PCCFAYI and VTR-NRQ were simultaneously sequenced.
While the latter matched RANTES HBD (with an undetected Lys involved in cross-linking), the former arose from the N terminus of the undigested protein. However, a participation of such unrelated peptide to heparin binding was easily ruled out, as no cross-linked residues could be identified within this sequence. Our data thus further support the primary role of the 4KRKNR motif in heparin binding. Increasing the concentration of RANTES-(9–68) incubated with the Hp-bds to the micromolar range (1–10 μM) led to the detection of additional sequences, with cross-linking occurring at the level of a second cluster of basic amino acids: 55KKWVR (Fig. 3C, i–iii). This second domain could correspond to an accessory, low affinity binding site that may account for the residual heparin binding activity detected, even at the highest concentration tested (1 μM). Interestingly, Arg83 and Lys85 are located within a highly basic peptide, K80KLRIKSKEK89, whose sequence corresponds to a HBD consensus motif. In contrast, both Lys144 and Lys260 are relatively isolated within the sequence (Fig. 3D). As mentioned before, heparin binding motifs are often partly composed of linear epitopes corresponding to clusters of basic amino acids and remote residues that are added to the motif through folding of the protein. Accordingly, it is likely that the whole K80KLRIKSKEK89 sequence would constitute a heparin binding site, along with Lys144, possibly Lys142, and Lys260.

**DISCUSSION**

While increasing interest arises from the biological functions of protein/GAG interactions, the lack of simple analytical tools for the structural characterization of these interactions remains one of the major hurdles to their study. Here, we have developed a technique enabling a mapping of protein sequences to locate HBDs. This approach is based on the capture of the protein of interest on heparin immobilized beads, followed by proteolytic digestion of the protein. Peptides retained, i.e. containing amino acids involved in covalent linkages with the polysaccharide, can then be identified by solid phase N-terminal protein sequencing performed directly on the heparin beads (Fig. 1).
The first step of the technique corresponds to the immobilization of the protein of interest on the heparin chains. This was achieved, using an adapted protocol of a zero length, two-step cross-linking procedure (38). Heparin carboxyl groups were activated by incubation with EDC in the presence of NHS to form succinimide esters. Excess of cross-linking reagents were easily removed by both neutralization of free EDC with H9252-mercaptoethanol and washing of the beads with PBS. The protein was incubated with the activated heparin and was captured upon binding, through formation of covalent linkages between the succinimide esters on the polysaccharide and amine groups present within the HBD of the protein. The use of such a procedure has two major advantages. First, the direct reaction of succinimide esters with amine groups does not introduce any spacer. Cross-linking should thus only occur at the level of the protein HBD. Second, as heparin activation is performed prior to incubation with the protein, the latter is never let in contact with the cross-linking reagents, thus preventing putative protein/protein cross-linking side reactions. Such a technique has been successfully used in the past to study the stoichiometry of protein/GAG complexes (31, 38, 39).

Once cross-linked to the Hp-bds, proteins were submitted to proteolytic digestions. Results showed that this step was critical for the quality of the sequencing data. Incomplete digestions, or the use of enzymes with high cleavage specificities, resulted in the generation of large fragments, with a N terminus remote from the interaction domain. In such cases, sequencing, which is only achievable for a limited number of residues, would not be able to reach and identify heparin cross-linked residues. Moreover, partial digestions generated multiple intermediate fragments with distinct N terminus but overlapping sequences. The presence of these peptides devoid of information dramatically increased the complexity of the amino acid attribution and sequence analysis. In particular, saccharide cross-linking on Lys or Arg residues shared by multiple sequences could not be determined. To prevent this, digestions were routinely performed using thermolysin, a proteolytic enzyme with a very broad specificity (cleavage upstream Leu, Phe, Ile, Val, Met, Ala, Tyr, Gly, Thr, and Ser), and were facilitated by addition of a denaturing step (heat/urea/β-mercaptoethanol treatment) beforehand.

Finally, fragments conjugated to heparin were identified by sequencing, using an automated protocol of Edman degradation. The system was adapted for direct sequencing on beads by insertion in the reaction chamber of a filter (5 μm pore size) underneath the beads to prevent their wash out. Analysis of the reverse phase HPLC profiles corresponding to each sequencing cycle revealed the presence of several non-attributed peaks. These most likely corresponded to peptide-heparin fragments conjugates released from the progressive degradation of the polysaccharide under the very stringent conditions of Edman degradation. In agreement with this observation, yields of material recovered calculated at the end of each cycle were found to be lower than those of standard analyses (65 versus 95%). However, although this loss of material limited the number of sequenceable residues, it did not affect the readability and the significance of the results obtained. For the beads approach, a major advantage of N-terminal sequencing analysis was that cross-linking events could be easily visualized, as saccharide conjugated amino acids were not eluted, which resulted in “gaps” within the reverse phase HPLC profiles. Another critical observation was that cross-linking was also observed at the level of arginine residues. NHS esters react with primary amine groups, which should restrict cross-linking on

**Fig. 4.** Mapping of laminin α3LG4/5 heparin binding domain. A, amino acid sequence of laminin-5 α3LG4/5 fragment. Lysine and arginine amino acids are in bold. B, sequences analysis of α3LG4/5 after immobilization on Hp-bds (incubation at 300 nM) and digestion with thermolysin. i and ii are the results from two different experiments. As in Fig. 2, saccharide-conjugated residues are in red, and the results are summarized (in black) on the whole protein sequence (in pale gray).
Mapping of Heparin Binding Sites within Proteins

the proteins to the α-amino group of the N terminus and ε-amino groups found on lysine residues. To our knowledge, coupling of NHS-esters to arginines has never been reported. However, a major difference to typical protein-protein complexes is the presence of heparin negatively charged sulfate groups, which could allow the cross-linking reaction to proceed.

The analysis of two proteins with well-characterized HBDS was used to validate this approach. Results on both PRV gC and RANTES (9–68) were in agreement with previously published data (28, 32). The reported sequences 72RKKPRR92 and 95AHGKRRI101 for gC and 44RKNR47 for RANTES, were found to be the main HBDS of the proteins (Figs. 2 and 3). Surprisingly, gC reported HBDS, 139YRGRFR147, was never found (Fig. 2B). As for RANTES, high protein concentrations were incubated with Hp-bds but did not allow to detect low affinity binding activity. The site-directed mutagenesis study of gC that led to the identification of all three HBDS indicated that HBD3 on its own was able to efficiently sustain heparin binding (27, 28). However, it could not assess its actual participation when both HBD1 and HBD2 were present and the protein was in its natural conformation. One hypothesis would be that the structural organization of gC could not support the simultaneous binding of all three HBDS to a single heparin chain and that a synergetic effect of both HBD1 and HBD2 makes up for a much higher affinity binding site than HBD3. The previously observed heparin binding activity of HBD3 may also be the result of mutagenesis induced conformational changes of the protein that would expose normally hidden epitopes. In any case, results obtained with the beads approach question the actual role of HBD3 within the wild type protein. Our data also suggested the contribution of additional residues to heparin binding. Results strongly supported a role of gC Lys92, as this residue was consistently found cross-linked to the polysaccharide (Fig. 2B). A participation of this residue located between HBD1 and HBD2 would further support a synergetic role of these two domains to form a large, contiguous binding interface. For RANTES, a possible involvement of Lys83 was proposed, although this result was not absolutely definitive, as coupling to this lysine was only observed at times when high concentrations of the chemokine were incubated with the Hp-bds. Another interesting observation was that modulation of the protein concentration incubated with the polysaccharide beads could affect the nature of the sequences detected. This was seen with RANTES (9–68) and led to the identification of a low affinity binding site within the chemokine, the sequence 58KKWVR59. The presence of this domain could account for the residual binding activity observed for RANTES mutated in the 44RKNR47 primary binding site (32). Moreover, this would be in agreement with an earlier study, which showed that a monoclonal antibody recognizing an epitope overlapping this region was inhibited by heparin (40). In the last part of this study, we have used the beads approach to characterize the HBD of laminin-5 α3LG4/5 fragment, for which few data were yet available. Our results consistently pointed out four residues, the first twos, Arg83 and Lys85, being located within a cluster of basic residues (Fig. 4). These data enabled us to propose a HBD for this protein, composed by a linear sequence K80KLRIKSEEK59 supplemented by residues Lys144, possibly Lys142, and Lys260. In support to our results, site-directed mutagenesis analyses of other laminin chains (α1 and α4) identified critical heparin binding residues within the regions corresponding to the α3 chain K80KLRIKSEEK59 domain (19, 41). In contrast, our data did not support a previous study, which suggested a role of the N55SFMALYLSKGRR70 peptide within the α3 chain (42). However, this motif was identified by competition assays with synthetic peptides, thus in a system where both surface exposition and structural conformation of the epitope within the whole protein were not taken into account.

Yet, application of the beads approach is subject to a number of technical limitations. First, the procedure relies on the formation of artificially stabilized complexes. However, it has to be noted that no nonspecific cross-linking was observed in any of the experiments, despite the presence of a large number of putative coupling residues in the proteins tested. This clearly demonstrates the specificity of the EDC/NHS based cross-linking for heparin/protein interfaces and emphasizes the relevance of our observations. Another limitation is that cross-linking would only occur on lysine and arginine residues. The participation of other amino acids, such as asparagine, serine, glutamine, or tyrosine, which are commonly found in HS binding sites (43), could thus not be clearly established. In this case, our approach would only provide indications and potentially draw attention to such residues present at the vicinity or within clusters of identified lysines and arginines. Nevertheless, results obtained provided new and important data on the molecular determinants involved in the interaction of the proteins studied with heparin and have highlighted the beads approach as a powerful methodology, fast and very easy to implement. This is particularly well illustrated with our work on the laminin α3LG4/5 fragment. As a comparison, Suzuki et al. (44) synthesized and screened over 110 laminin α1 chain synthetic peptides and Yamashita et al. (19) mutated each of the 27 basic residues within α4LG4 domain to identify heparin binding residues on these proteins. Contrary to site-directed mutagenesis, the beads approach is prospective; it does not require the preselection of amino acid targets and thus is more adapted to the identification of isolated residues. The analysis is performed on native proteins, while the study of mutants can be distorted by conformational alterations, and the use of synthetic peptides cannot reproduce the structural context within the whole protein. Another interesting feature of this method is that a number of technical developments can be envisaged. First, beads could be functionalized with other GAGs, heparin/HS from different sources and structure. A wealth of studies reported the formation of conjugates through the saccharide reducing ends that could be used for such purpose (45, 46). The immobilization of oligosaccharides with defined sizes could also be achieved by insertion of a spacer. Another strategy, which is presently investigated by others, would be to perform the protein/GAG cross-linking reaction in solution. Despite practical handling complications due to the absence of solid phase support, this alternative approach would allow the use of small structurally defined oligosaccharides without the risk of bead steric hindrance. Other detection techniques could be coupled to the beads approach. For instance, characterization of the proteolysis-released fragments could be performed by mass spectrometry for identifying the missing peptides involved in the cross-linking with the polysaccharide. However, this indirect approach would not allow visualization of the cross-linked residues, this being a critical parameter to precisely locate heparin binding motifs within large peptide sequences. The use of alternative solid supports to immobilize GAG chains could also be considered. An attractive perspective would be the adaptation of the technique to magnetic beads and the 96-well plate format that would allow automated medium throughput analyses. With regards to the number and the functional diversity of proteins that bind to GAGs, we believe that this technique is of general interest and should greatly contribute to progress in the study of protein/GAG interactions.

2 M. Lyon, personal communication.
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