Communication

Characterization of Glycosaminoglycan-binding Domains Present in Insulin-like Growth Factor-binding Protein-3*

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Matrix metalloproteinase 3 cleaves insulin-like growth factor-binding protein-3 (IGFBP-3) into six fragments, four of which bind heparin-Sepharose (Fowlkes, J. L., Enghild, J. J., Suzuki, K., and Nagase, H. (1994) J. Biol. Chem. 269, 25742–25746). Sequence analysis of IGFBP-3 heparin-binding fragments shows that all fragments contain at least one of two highly basic, putative heparin-binding consensus sequences present in IGFBP-3. Epitope-specific antibodies generated against synthetic peptides containing these domains recognized IGFBP-3, yet were significantly inhibited from binding in the presence of heparin, demonstrating that these regions of IGFBP-3 contain functional heparin-binding domains. IGFBP-3 peptides containing one of the two heparin-binding consensus sequences bound heparin in a solid phase binding assay in a dose-dependent and saturable manner. However, the IGFBP-3 peptide containing the heparin-binding consensus sequence 146KKKQCRP216 bound heparin with 4-fold less affinity than the IGFBP-3 peptide containing the longer heparin-binding consensus sequence 219GYKKQCRPR256. Examination of several well characterized glycosaminoglycans to inhibit the binding of heparin to both heparin-binding IGFBP-3 peptides revealed that the most potent inhibitors were heparin, heparan sulfate, and dermatan sulfate; chondroitin sulfate A and hyaluronic acid were intermediate in their inhibitory activities; and chondroitin sulfate C caused no inhibition. These studies identify and characterize the glycosaminoglycan-binding domains in IGFBP-3, providing a basis for the better understanding of IGFBP-3-glycosaminoglycan interactions at the cellular and extracellular interface.

Insulin-like growth factor (IGF)3-binding proteins (IGFBPs)

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The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; GAG, glycosaminoglycan; rh, recombinant human; bHep, bird heparin; HS, heparan sulfate; are a group of six homologous, high affinity carrier proteins for IGF-I and IGF-II, which are produced by a wide variety of cells and tissues as soluble proteins (for recent reviews see Refs. 1–4). However, at least two IGFBPs, IGFBP-3 and IGFBP-5, have been shown to associate with cell surfaces and/or extracellular matrix (1–4). This association may facilitate the localization of IGF-IGFBP complexes into close proximity of IGF receptors, thereby enhancing IGF bioavailability. The mechanisms by which IGFBP-3, the major carrier of serum IGFs, interacts with cell surfaces and/or extracellular matrix remain unclear. Oh et al. (5) have provided evidence that IGFBP-3 may interact with a specific cell-surface receptor, yet other data suggest that IGFBP-3 may interact with glycosaminoglycan (GAG)-containing molecules (i.e. proteoglycans) present at the cell surface and/or in extracellular matrix (6, 7). IGFBP-3 binds avidly to heparin-Sepharose (8–10), and we have recently reported that proteolysis of recombinant human (rh) IGFBP-3 by matrix metalloproteinase 3, an IGFBP-3-degrading protease (11, 12), produces six IGFBP-3 fragments, four of which bind heparin-agarose (13). Sequence analysis reveals that the non-heparin-binding fragments comprise the first 100–110 amino acids of IGFBP-3, demonstrating that no heparin-binding domains reside in this segment of the binding protein. However, amino acid analysis of the heparin-binding fragments shows that each contains at least one of two heparin-binding consensus sequences present in IGFBP-3 (11, 13, 14). Herein, we examine whether these putative heparin-binding domains are involved in IGFBP-3-GAG interactions and define their affinities for heparin, as well as for other GAGs.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGFBP-3 produced in Escherichia coli (rhIGFBP-3313) and glycosylated rhIGFBP-3 produced in Chinese hamster ovary cells (rhIGFBP-3313) were kindly provided by Dr. Christopher Maack, Celtrix Pharmaceuticals, Santa Clara, CA (15). Low molecular mass heparin, heparan sulfate (HS), chondroitin sulfate A (CS-A), dermatan sulfate (DS), chondroitin sulfate C (CS-C), and hyaluronic acid (HA) were purchased from Sigma. Biotin-hydrazide, MES, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), and SulfoLinkTM resin were purchased from Pierce. Reagents used for SDS-polyacrylamide gel electrophoresis were purchased from Bio-Rad. Molecular weight markers and Hyperfilm-ECL were obtained from Amersham Corp. Synthetic peptides based on internal sequences contained in IGFBP-3 were prepared as described elsewhere (13). The sequences are as follows: 5SRILRAYLPPAPART107 (peptide I), 146KKKQCRP216 (peptide II), 219GYKKQCRPR256 (peptide III), and 249KDGKGYKKQCRPR256 (peptide IV). All peptides were synthesized with an additional N-terminal cysteine for use in coupling reactions (see below), each was shown to be >95% pure by high pressure liquid chromatography, and sequence verification was performed by electrospray mass spectrometry.

Production, Purification, and Characterization of Epitope-specific Antibodies—Three mg of each peptide was conjugated to keyhole limpet hemocyanin using m-maleimidobenzoyl-N-hydroxysuccinimide, utilizing the terminal -SH group of each peptide for conjugation. The peptide-conjugate was mixed with an equal volume of complete Freund’s adjuvant and injected in a pair of New Zealand White rabbits. Repeat injections were performed on five separate occasions over ~105 days. Crude antisera from immunized rabbits was applied to each epitope and compared with preimmune serum as assessed by an enzyme-linked immunosorbent assay using BSA-conjugated peptide in the solid phase.

CS-A, chondroitin sulfate A; DS, dermatan sulfate; CS-C, chondroitin sulfate C; HA, hyaluronic acid; BSA, bovine serum albumin; PBS, phosphate-buffered saline; MES, N-morpholinoethanesulfonic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; IgG, 50% inhibitory concentration; EC50, 50% effective concentration.

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Epitope-specific antibodies were purified from crude antisera using affinity columns prepared from each peptide covalently linked via its terminal cysteine to SulfoLink™ according to the manufacturer’s instructions. Three ml of peptide-affinity matrix was incubated with 20 ml of crude antisera and 20 ml of PBS and mixed in a 50-ml conical tube at room temperature for 2–3 h. Antibodies were eluted from the column by 0.1 M glycine buffer, pH 2.5, and neutralized with 1 M Tris-HCl, pH 9.5. Antibodies were dialyzed into 5 mMphosphate buffer, pH 7.4, and then lyophilized and stored at −20 °C. Little or no cross-reactivity was observed among the three antibody preparations when analyzed by dot blotting using all three peptides (data not shown).

### Immunoblotting—rhIGFBP-3 CHO (500 ng/lane) and rhIGFBP-3 CHO (data not shown) were prepared based on a method used by Yu and Toda (17) for biotinylating hyaluronan. Briefly, 4 mg of heparin was dissolved in 1 ml of 0.1 M MES, pH 5.5 (MES buffer), and then reacted with 20 μl of a 50 mM solution of biotin-hydrazide dissolved in dimethyl sulfoxide. To the mixture was added 13 μl of EDC buffer (100 mg/ml EDC dissolved in MES buffer). The mixture was mixed overnight at 22 °C in an end-over-end fashion. The solution was then desalted over a Quick Spin™ column (Boehringer Mannheim, Indianapolis, IN) and exchanged into PBS, pH 7.4, 0.05% NaN₃.

The solid-phase peptide binding assay was performed using a modification of a method reported by Kost et al. (18). Fifty-microliter aliquots of various concentrations of synthetic peptides dissolved in carbonate buffer, pH 9.6, were absorbed onto 96-well tissue culture plates overnight at 4 °C. The wells were then saturated for 1 h at room temperature with 100 μl/well PBS, pH 7.4, containing 3% BSA, which had been denatured at 60 °C for 30 min. The plate was washed with PBS, pH 7.4, containing 0.1% Tween 20 (PBST) and then incubated for 3 h at room temperature with biHep diluted in PBST, 0.2% BSA (final concentration, 5 μg/ml) with or without various concentrations of unlabeled heparin, HS, CS-A, CS-C, DS, or HA. After washing the plate, 50 μl of streptavidin-conjugated horseradish peroxidase (Amersham Corp.) diluted 1:1000 in PBST, 0.2% BSA was added to each well and incubated for 1 h at room temperature. After a final wash, the peroxidase substrate 3,3'-5,5'-tetracarbethoxydihydrochloride (Sigma) was added, and the reaction was terminated with the addition of 2 M H₂SO₄. The plate was read in an automated plate reader at A₄₅₀.

### Binding of epitope-specific antibodies to rhIGFBP-3 CHO

![Table 1](image-url)

**Table 1.** Effect of heparin on the binding of epitope-specific antibodies to rhIGFBP-3 CHO and rhIGFBP-3 CHO

| Antipeptide antibody | Inhibition of antibody binding by heparin (S.E.) % |
|----------------------|-----------------------------------------------|
| I                    | 5.62 ± 5.9                                    |
| IV                   | 91.7 ± 1.0                                    |
| VI                   | 77.9 ± 1.2                                    |
| rhIGFBP-3 CHO        | 15.8 ± 7.9                                    |
| IV                   | 67.9 ± 6.8                                    |
| VI                   | 65.3 ± 3.3                                    |

* p < 0.005.
* p < 0.05.

(Fig. 1) or rhIGFBP-3 CHO (data not shown). These data were examined by densitometry, and the results are presented in Table I. Because heparin markedly inhibited the binding of antibodies to both peptide IV and peptide VI, these findings suggested that within these regions of IGFBP-3 reside functional heparin-binding domains.

### Characterization of Heparin Binding to IGFBP-3 Heparin-Binding Domains—A solid-phase binding assay using immobilized peptides IV and VI and biHep as ligand was used to characterize the relative affinities of these IGFBP-3 domains for heparin. As shown in Fig. 2A, both peptides bound biHep in a dose-dependent fashion. Peptide IV bound biHep (5 μg/ml) with an EC₅₀ of 15 μg/ml peptide (750 ng/well), while peptide VI bound biHep with an EC₅₀ of 3.6 μg/ml peptide (180 ng/well), demonstrating that peptide VI bound biHep with a 4-fold higher affinity than peptide IV. Furthermore, binding of biHep to both peptides was saturable at concentrations of 30 μg/ml (1.5 μg/well) for peptide IV and 10 μg/ml (500 ng/well) for peptide VI. As shown in Fig. 2B, binding of biHep to both peptides was specific. When peptides were coated onto plates at a maximal concentration (1.5 μg/well for peptide IV and 500 ng/well for peptide VI), heparin displaced biHep in a dose-dependent manner with an IC₅₀ of ~3 μg/ml for both heparin peptides. Together, these data demonstrated that heparin bound both peptides, yet it bound peptide VI more avidly than peptide IV.

### Binding of GAGs to Heparin-binding IGFBP-3 Peptides—Since GAGs constitute a diverse group of complex macromolecules, we examined the ability of several well-characterized GAGs to inhibit the binding of biHep to both heparin-binding
IGFBP-3 contains at least two GAG-binding domains, it is unclear if both domains participate in binding GAGs under physiologic conditions. While both domains demonstrate specific binding of heparin, the heparin-binding domain present in the C terminus of the molecule demonstrates the highest affinity for heparin. Interestingly, IGFBP-5 and IGFBP-6 also contain C-terminal sequences, which are homologous to the C-terminal heparin-binding consensus sequence present in IGFBP-3 (1–4, 5, 10, 27), suggesting that the C-terminal heparin-binding sequence of all three IGFBPs may be important in GAG interactions. This is supported by the findings that synthetic peptides containing the C-terminal heparin-binding domains from IGFBP-3, -5, and -6 inhibit IGFBP-3 and IGFBP-5 binding to endothelial cell monolayers (27, 28). Consistent with these observations, Andress (29) has recently shown heparin prevents the association of intact IGFBP-5 with mouse osteoblasts, yet heparin does not interfere with the binding of a C-terminally truncated form of IGFBP-5 lacking the heparin-binding domain. Furthermore, Arai et al. (30) have demonstrated that point mutations of basic amino acids present in the C-terminal heparin-binding domain of IGFBP-5 can significantly reduce its affinity for heparin. Data from our laboratory suggest that the C-terminal heparin-binding sequence from IGFBP-5 has a similar affinity for heparin as does the homologous heparin-binding domain from IGFBP-3, while the homologous IGFBP-6 consensus sequence binds heparin much less...
of these GAGs for IGFBP-3 heparin-binding domains may be avidly,\(^2\) possibly explaining why IGFBP-6 has not been shown to associate with cell monolayers (27, 28). Further studies using epitope-specific antibodies as described herein and site-directed mutagenesis of IGFBPs should provide further insights into the specificity of IGFBP-GAG interactions.

The specificity involved in GAG-protein interactions has only recently been appreciated. For instance, both antithrombin III and heparin cofactor II bind heparin, but only heparin cofactor II binds DS (21). Our data suggest that both heparin-binding domains identified in IGFBP-3 bind several different GAGs but with different affinities. Both IGFBP-3 peptides containing heparin-binding domains demonstrated the greatest overall binding to heparin, HS, and DS, suggesting that common features shared among these three GAGs might provide clues as to the specificity involved in this interaction. A major structural similarity among heparin, HS, and DS is that each contains \(\alpha\)-L-iduronic acid residues, suggesting that the higher affinity of these GAGs for IGFBP-3 heparin-binding domains may be dictated, at least in part, by the disaccharide backbone of the GAG. Consistent with our data, Arai et al. (31) demonstrated that heparin, HS, and DS were the most potent GAGs in inhibiting IGFBP-5-IGF interactions. Although these authors did not examine directly the binding of GAGs to IGFBP-5, their data suggested that GAG interactions which inhibited the formation of the IGFBP-5-IGF complex contained primarily O-sulfate groups in either the 2- or 3-carbon positions. While sulfation of GAGs may affect protein binding, it is unclear from our studies to what extent the degree of sulfation or the position of the sulfate group modulates GAG binding to IGFBP-3. For instance, CS-A and CS-C contain the same disaccharide unit and both are sulfated GAGs. Nevertheless, CS-A is principally sulfated at the 4-carbon position, while CS-C is sulfated primarily at the 6-carbon position, suggesting that sulfation at the 4-carbon position promotes binding to IGFBP-3 heparin-binding domains, especially the short heparin-binding domain (see Fig. 3). In contrast, HA, which is not sulfated, bound both peptides with similar affinities to CS-A. Taken together, these data would suggest that sulfation at the 6-carbon position may inhibit IGFBP-3-GAG binding. Because GAGs are commonly covalently attached to protein cores (i.e. proteoglycans), it is possible that protein-protein interactions may also modulate IGFBP-3-GAG binding. For instance, several proteoglycans including decorin, fibromodulin, and biglycan have core proteins containing leucine-rich repeats, which are homologous to sequences found in the acid-labile subunit that binds IGFBP-3-IGF complexes in serum (32).

In conclusion, while other reports have established that IGFBP-3 binds heparin-Sepharose, the studies herein are the first to localize and characterize the specific domains within the IGFBP-3 molecule that bind heparin. Furthermore, they suggest that IGFBP-3 may interact in a selective way with certain GAG moieties. Thus, these studies should provide essential information for the better understanding of IGFBP-3-GAG interactions and how these interactions mediate the effects of IGFBP-3 at the cellular and extracellular interface.

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\(^3\)E. coli and rhIGFBP-3CHO from Dr. Christopher Maack (Celtrix Pharmaceuticals, Santa Clara, CA).

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