Substitution of Specific Amino Acids in Insulin-like Growth Factor (IGF) Binding Protein 5 Alters Heparin Binding and Its Change in Affinity for IGF-I in Response to Heparin*

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Heparin binding to insulin-like growth factor (IGF)-binding protein 5 (IGFBP-5) leads to a 17-fold decrease in its affinity for IGF-I, and a region that contains several basic amino acids (Arg[201]–Arg[218]) may be involved in this affinity shift. In the present study, mutagenesis was used to analyze the effect of substitutions for basic amino acids in the Arg[201]–Arg[218] region of IGFBP-5 on heparin-binding and the heparin-induced affinity shift. Nine mutant forms were prepared. Their association constants (K_a) for IGF-I were similar to native IGFBP-5. When 10 μg/ml of heparin was added, the K_a of native IGFBP-5 decreased 17-fold, and the K_a of the K134A/R136A mutant decreased 16-fold. In contrast, substitutions for specific basic amino acids in the Arg[201]–Arg[218] region decrease the affinity shift to 1.1–3.2-fold. Lys[211] was especially important. When a mutant containing that single substitution was tested, heparin caused only a 2.5-fold reduction in IGF-I affinity. Affinity cross-linking studies showed that heparin was equipotent in inhibiting the formation of ^{125}I-IGF-I·K134A/R136A mutant complexes compared to native IGFBP-5. In contrast, heparin had minimal effects on the formation of complexes between ^{125}I-IGF-I and the other mutants. The heparin-binding activity of each mutant was determined. Four mutants, R201A/K202N, K202A/K206A/R207A, R201A/K202N/K206N/K208N, and K211N/R214A/K217A/R218A, had reduced heparin binding compared to native IGFBP-5. The other five mutants, including the K211N mutant, showed no change in heparin binding. The four mutants with reduced heparin binding could be dissociated from heparin-Sepharose with much lower NaCl concentrations, indicating that they had reduced affinity. These findings suggest that Arg[201], Lys[202], Lys[206], and Arg[214] are important for heparin binding. In contrast, Lys[211] is not important for the binding of IGFBP-5 to heparin, but substitution for it reduced the heparin-induced affinity shift.

Insulin-like growth factors (IGFs)[1] in extracellular fluids are bound to insulin-like growth factor-binding proteins (IGFBPs), and IGFBPs are important regulators of IGF's biological ac-
tions (1). When IGFBPs are present in a soluble, high affinity state they reduce the amount of IGF-I or -II that is available for receptor interaction and inhibit IGF bioactivity (2–4). However, IGFBP-5, unlike IGFBP-1, -2, and -4, binds to both cell surfaces and extracellular matrix (ECM). IGFBP-5 binding to ECM results in a reduction in its affinity for IGF-I and enhancement of IGF-I's biologic actions (5, 6). Therefore, it is important to determine the specific amino acids in IGFBP-5 that account for ECM binding and for the reduction in its affinity. Glycosaminoglycans are abundant components in ECM that can modulate cell and protein attachment. That IGFBP-5 may bind to glycosaminoglycans, such as heparin and heparan sulfate, is suggested by the observation that incubation of IGFBP-5 with glycosaminoglycans results in a 17-fold decrease in the affinity of IGFBP-5 for IGF-I (7). Peptide competition studies have suggested that a basic amino acid-rich region (Arg[201]–Arg[218]) of IGFBP-5 contains the amino acids that are necessary for this reduction to occur. The reduction of the affinity has been proposed to be due to a conformational change of IGFBP-5, which is induced by heparin binding, since the heparin and IGF-I binding sites of IGFBP-5 are distinct (7).

The Arg[201]–Arg[218] region of IGFBP-5 contains 10 basic amino acids including a putative heparin binding domain containing a BBB*XX*B motif where B is a basic amino acid and *X* is a neutral one. Since heparin and heparan sulfate are composed of repeating disaccharides and are highly sulfated (8), they are strongly anionic. These groups are believed to align with the basic residues in heparin-binding proteins. The heparin-induced affinity shift of IGFBP-5 for IGF-I has been proposed to be a two-step process, heparin-binding followed by a conformational change of IGFBP-5 that results in a decrease in its affinity. The purpose of this study was to determine the effect of substitutions for basic amino acids on the binding of IGFBP-5 to heparin and on the heparin-induced reduction in affinity for IGF-I. We prepared nine mutants of IGFBP-5 in which basic amino acids were substituted by neutral ones. Their heparin-binding activities and affinity shifts in response to heparin were compared.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGFBP-5 was synthesized in transfected Chinese hamster ovary cells and purified as described previously (3). Recombinant IGF-I was obtained from Bachem, Inc. (Torrance, CA). ^{125}I-IGF-I was a gift from Dr. Louis E. Underwood (University of North Carolina, Chapel Hill). Heparin (187 USP units/mg) was purchased from Sigma. Heparin-Sepharose and Sepharose were purchased from Pharmacia Biotech Inc. Dithiothreitol was purchased from Sigma. Disuccinimidyl suberate was purchased from Pierce. Dimethyl sulfoxide was purchased from Mallinckrodt Chemical Co. (Paris, KY). Eagle's minimum essential medium (EMEM) was purchased from Hazelton (Denver, PA). Tween 20 and polyethylene glycol (M_r 8,000–12,000) were obtained from Sigma. EDTA was obtained from Fisher. Heparin, heparan sulfate, chondroitin sulfate A, and chondroitin sulfate C were purchased from Sigma. Two peptides that contain sequences of IGFBP-5

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[1] The abbreviations used are: IGF-I, insulin-like growth factor-I; IGFBP-5, insulin-like growth factor binding protein-5; EMEM, Eagle's minimum essential medium; ECM, extracellular matrix; AT-III, antithrombin III; PAI, plasminogen activator inhibitor.

were synthesized and purified (9). They were designated peptide A (residues R[201]KGFYKRKQCKPSRGRKR[218]) and peptide B (residues A[131]VKKDRRKKLT[141]) (7).

Mutagenesis—A full-length human IGFBP-5 cDNA was cloned into the *Hin*dIII and *Not*I sites of a mammalian expression plasmid pRcRS-VhPB-5 which had been prepared from the plasmid pRcCMV (Invitrogen, La Jolla, CA). The pRcRSVhPBP-5 contains a bacteriophage origin of replication (f1) that allows production of plasmid DNA in a single-stranded form suitable for site-directed mutagenesis. Mutants from pRcRSVhPB-5 were generated by using site-directed mutagenesis. Two micrograms of plasmid DNA were transfected into *Escherichia coli* strain CJ236. A fresh colony of CJ236 was used to inoculate a 60-ml culture and grown to an OD of 0.25–0.30 (600 nM) before infection with helper phage R408 in a multiplicity of infection of 10 to 1. The culture was grown for 5 h, and the bacteria were pelleted. The secreted phagemid particles were precipitated in 16% polyethylene glycol, 2.8 M ammonium sulfate, and the single-stranded phagemid DNA was isolated by adherence to glassmilk according to a protocol provided by the manufacturer (Bio 101, Inc., La Jolla, CA). Complementary oligonucleotides containing mutagenic mismatches were synthesized by the Lineberger Cancer Research Center, Nucleic Acids Core facility. The following sequences were used: acgggaaggttgcactgcttc, Lys[211] to Asn; cttctttctggcgtcgttcttcactgc, Lys[134] and Arg[136] to Ala; gcactgctttgccgcgtagaatccattgcggtcac, Lys[202], Lys[206], and Arg[207] to Ala; gcagatgccagccgcgcggccagcggaagggtt, Lys[211] to Asn, Arg[214], Lys[217], and Arg[218] to Ala; gggaagggttgcactgctttgccttgtacaa, Lys[134] and Arg[136] to Ala, Lys[211] to Asn; gggaactgggttgcactgctttgccttgtagaa, Arg[207] to Ala, Lys[211] to Asn; tttgcactgatttctattgtagaatcc, Arg[201] to Ala, Lys[202], Lys[206], and Lys[208]; attggcgtcacaatt, to Asn. Synthetic oligonucleotides were phosphorylated with 15 units of T4 polynucleotide kinase for 1 h at 37 °C. Ten μl of synthesis mixture were used to transform *E. coli* strain DH5 αF′, and ampicillin-resistant colonies were selected. DNA from resulting colonies were amplified and used for sequencing. Sequencing of double-stranded DNA was performed by using the Sequenase (U. S. Biochemical Corp.) protocol followed by a 6% polyacrylamide, Tris, borate, EDTA, urea gel electrophoresis and autoradiography (10). The clones containing the correct sequences were amplified and plasmid DNA prepared using silica gel anion exchange resin chromatography as recommended by Qiagen (Chatsworth, MA).

Transfection of Mammalian Cells—Chinese hamster ovary K-1 cells were obtained from the Lineberger Comprehensive Cancer Tissue Culture facility. The cells were maintained in α-minimal essential medium, 10% fetal calf serum, supplemented with penicillin and streptomycin. Twenty four hours before transfection, the cells were seeded into six-well tissue culture plates at approximately 15% confluency. DNA was introduced into the cells by a standard calcium phosphate precipitation procedure (11). A DNA-calcium phosphate precipitate was formed by mixing 0.5 ml of 0.25 M calcium chloride with 10 μg of plasmid DNA, and 2 μg of the calcium chloride-DNA complex were added to the wells containing 3 ml of medium. The plates were then incubated at 37 °C for 5 h. Calcium-containing medium was removed, and medium containing 10% glycerol was applied for 3 min. After rinsing, the medium was replaced, and the cells were incubated for 48 h. The treated cells were then trypsinized and plated in medium containing 800 μg/ml neomycin analog G418. Fresh G418 was applied every 3 to 4 days for 10–12 days when stable colonies of transfected cells began to appear. The colonies were isolated by cloning rings, trypsinized, and transferred into individual wells of a 24-well plate. Medium was analyzed by immunoblotting for secretion of IGFBP-5 after reaching confluency. The positive clones were maintained in a long term culture in 400 μg/ml G418.

Conditioned medium containing the IGFBP-5 mutants was collected and centrifuged at $10,000 \times g$ for 20 min to remove cellular debris. The mutants were purified as described previously (12). The amount of each mutant IGFBP-5 was quantified by comparing their high performance liquid chromatography peak areas to an IGFBP-5 standard. The protein concentration of the standard was determined by amino acid composition analysis. To further ensure that a heparin-induced change in affinity could be validly estimated for each mutant, Scatchard analysis was used to calculate the affinity of each mutant for IGF-I, and the results were compared to native IGFBP-5 (see Table I).

Scatchard Analysis—To determine the affinity of the IGFBP-5 mutants for IGF-I, ^{125}I-IGF-I (20,000 cpm/tube) was incubated with native or mutant IGFBP-5 (0.35 nM) in 0.1 M HEPES, 0.1% bovine serum albumin, pH 6.0. Duplicate tubes received increasing concentrations of unlabeled IGF-I (0.053-l.33 nM), and some tubes also received heparin (10 μg/ml). The bound and free ^{125}I-IGF-I were separated by precipitation using 12.5% polyethylene glycol (M_r 8,000–12,000) as described previously (3). The data were analyzed according to the method of Scatchard.

Affinity Cross-linking Studies—Affinity cross-linking was performed as described previously (7). ^{125}I-IGF-I (30,000 cpm/tube) was added into 100 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, and incubated with native IGFBP-5 (4 nM) or each mutant in the presence of various concentrations of heparin (0, 0.1, 1, 10, and 100 μg/ml) at room temperature. After 1 h, the samples were cross-linked by addition of 10 μl of 5 mM disuccinimidyl suberate and further incubated for 20 min. The reaction was stopped by the addition of 10 μl of 0.5 M Tris, pH 7.4. The samples for SDS-polyacrylamide gel electrophoresis were exposed to 0.1 M dithiothreitol in Laemmli (13) sample buffer, then electrophoresed through a 12.5% gel. The gel was fixed with 25% isopropanol containing 10% acetic acid and 2.5% glycerol for 30 min, then dried and autoradiographed using Kodak X-Omat film. The autoradiographic intensities of radiolabeled bands were determined by scanning densitometry using a Hoffer scanning densitometer, model GS-300.

Binding of IGFBP-5 to Heparin-Sepharose Beads—The heparin-binding activity of native IGFBP-5 and each mutant was determined by comparing their binding to heparin-Sepharose beads. The methods were similar to ones described previously (7). IGFBP-5 (80 nM) was added in 50 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, 0.1% Tween 20, and 20 mM EDTA. 40 μl of heparin-Sepharose beads were diluted with 960 μl of Sepharose beads. This mixture of diluted heparin-Sepharose beads is hereafter termed stock heparin-Sepharose beads. The heparin concentration of the beads was 50 μg of heparin/μl. The stock heparin-Sepharose beads were further diluted with Sepharose beads such that the final mixtures contained 0.1, 0.05, 0.025, and 0.01 μl of heparin-Sepharose beads in 5 μl of total bead volume. To correct for nonspecific binding, duplicate tubes containing Sepharose beads only were used for each test condition. In other experiments, increasing concentrations of native IGFBP-5 or each mutant (3.33–26.6 nM) were added to duplicate tubes with 0.025 μl of heparin-Sepharose beads (containing 1.25 μg of heparin). After an overnight incubation at 4 °C, the samples were centrifuged at $16,000 \times g$ for 1 min. The IGFBP-5 that remained in the supernatants (20 μl) was analyzed directly by ligand blotting. The pellets of the heparin-Sepharose and the Sepharose beads were rinsed twice with the same buffer, incubated with 50 μl of Laemmli sample buffer for 10 min at 60 °C, and then centrifuged. The IGFBP-5 in these supernatants (20 μl) was also analyzed by SDS-PAGE. Band intensities were quantified by scanning densitometry. The results are expressed as the percentage of each mutant form that bound to heparin-Sepharose. To determine the affinity of each mutant for heparin, 0.025 μl of heparin-Sepharose beads was incubated with 80 nM of each form of IGFBP-5 and NaCl concentrations that varied from 150 to 500 mM (increasing in 50 mM increments (14). The amount of IGFBP-5 that remained and was analyzed by SDS-PAGE and quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA). To examine the specificity of heparin binding to native IGFBP-5, increasing concentrations of soluble heparin (0.01–0.5 mg/ml), heparan sulfate (0.5 mg/ml), chondroitin sulfate A, or dermatan sulfate (0.5 mg/ml) were added to additional tubes.

To determine the region of IGFBP-5 that contained the heparin binding site, competition studies were carried out as described previously (7). IGFBP-5 (80 nM) was incubated with 0.025 μl of heparin-Sepharose beads in the presence of various concentrations (0, 0.27, 2.7, or 27 μM) of peptide A or B. After an overnight incubation, the samples were centrifuged as described previously, and both the IGFBP-5 that bound to heparin-Sepharose beads and that remained in the supernatant were analyzed by immunoblotting.

Cell Culture and Preparation of Extracellular Matrix—Normal skin fibroblasts (GM-10) were obtained from Coriell Institute (Camden, NJ) and grown to confluency as described previously (12). The extracellular matrix was prepared from confluent quiescent cultures using a previously described method (6). Tenascin was a gift from Dr. Harold Erickson, Duke University. Tenascin appeared to be a heparan sulfate proteoglycan, since exposure to heparinase followed by immunoblotting showed that it underwent a gel shift to a lower molecular weight. Two μg of protein were layered onto a plastic tissue culture plate. The ECM or tenascin was exposed to heparinase (Sigma), 0.1 unit/ml, in PBS containing 2 mM CaCl2, pH 7.4 for 4 h at 37 °C. The ECM proteins and tenascin were incubated with IGFBP-5 (80 ng/ml) for 14 h at 4 °C, washed three times in phosphate-buffered saline, then extracted in Laemmli sample buffer, and the bound IGFBP-5 was determined by immunoblotting.

Immunoblotting and Ligand Blotting—Samples were electrophoresed on 12.5% SDS-polyacrylamide gels and then transferred to polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA). The membranes were probed with ^{125}I-IGF-I as described previ-

The basic amino acids (Lys and Arg) were substituted with neutral ones (Ala and Aln) and their positions are indicated. The association constant (K_a) of IGFBP-5 for IGF-I was determined using Scatchard analysis. The results are expressed as the ratio of K_a of each IGFBP-5 mutant to native IGFBP-5 (the left column). The K_a of IGFBP-5 for IGF-I in the absence or presence of heparin (10 μg/ml) was also determined. The ratios of K_a in the absence of heparin to K_a in the presence of heparin are shown (the right column).

Form of IGFBP-5	K_a of mutant/K_a of native IGFBP-5	K_a without/K_a with heparin
Native IGFBP-5	N/A[a]	17
K134A/R136A	1.47	16
K134A/R136A/K211N	1.12	2.1
K211N	1.11	2.5
R201A/K202N	1.34	1.7
R207A/K211N	1.16	3.2
K217A/R218A	1.27	2.8
K202A/K206A/R207A	1.12	1.5
R201A/K202N/K206N/K208N	1.15	1.3
K211N/R214A/K217A/R218A	1.20	1.1

[a] Not applicable.

ously (12). In other experiments, the filters were probed using 1:1,000 dilution of a polyclonal rabbit to human IGFBP-5 serum (12). After an overnight incubation at room temperature, the immunoblots were developed by 3-h incubation with goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate (Sigma) in a final dilution of 1:2,000 in Tris-buffered saline plus 1% bovine serum albumin, followed by three washes with Tris-buffered saline containing 0.01% Tween 20. Bands were visualized using the Proto Blot system immunoblotting reagents following the technique recommended by the manufacture (Promega, Madison, WI).

RESULTS

Effect of Mutagenesis on Affinity for IGF-I—Nine mutants of IGFBP-5 were prepared (Table I). We previously have shown that the basic amino acid-rich region (Arg[201]–Arg[218]) in IGFBP-5 is involved in the affinity shift of IGFBP-5 for IGF-I in response to heparin, but another basic amino acid-rich region (Ala[131]–Thr[141]) is not (7). Therefore the K134A/R136A mutant was used as a control, since its two substitutions for Lys[134] and Arg[136] are located in the Ala[131]–Thr[141] region. In contrast, the other eight mutants each contained basic amino acid substitutions in the Arg[201]–Arg[218] region. The association constants (K_a) of native and mutant forms of IGFBP-5 for IGF-I were determined using Scatchard analysis. No major change in K_a for IGF-I was detectable in any of the mutants (Table I). The K_a of each mutant was comparable to native IGFBP-5, and the ratio of K_a of each mutant to K_a of native IGFBP-5 was between 1.1 and 1.3 except for the K134A/R136A mutant which was 1.5 (Table I). These results show that substitutions for these basic amino acids do not alter their affinities for IGF-I. This result is consistent with our previous observations which showed that a peptide containing the Arg[201]–Arg[218] sequence of IGFBP-5 did not alter ^{125}I-IGF-I binding to native IGFBP-5 (7).

IGFBP-5 Binding to Heparin and Other Glycosaminoglycans—When native IGFBP-5 (80 nM) was incubated with 0.025 μl of heparin-Sepharose beads (1.25 μg of heparin) (Fig. 1A, *lane 2*), most of it bound, and only minimal amounts could be detected in the supernatant. Coincubation with soluble heparin (0.02–0.5 mg/ml or 1–25 μg/tube) inhibited native IGFBP-5 binding to heparin-Sepharose beads in a concentration-dependent manner (Fig. 1A, *lanes 3–6*). These results show that low concentrations of soluble heparin compete with native IGFBP-5 for binding to heparin-Sepharose beads. In addition, coincubation with 0.5 mg/ml heparan sulfate (Fig. 1B, *lane 4*) also inhibited native IGFBP-5 binding to heparin-Sepharose beads. In contrast, chondroitin sulfate A (Fig. 1B, *lane 5*) did not affect
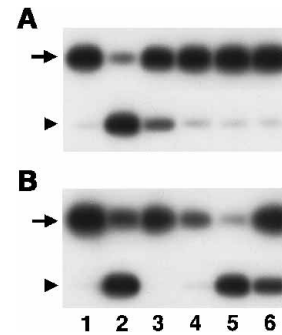

FIG. 1. *A*, effect of soluble heparin on IGFBP-5 binding to heparin-Sepharose beads. IGFBP-5 (80 nM) was incubated with 5 μl of Sepharose or heparin-Sepharose beads in 50 μl of EMEM, supplemented with 20 mM, HEPES, 0.1% Tween 20, and 20 mM EDTA. Some tubes received the indicated concentrations of soluble heparin in the buffer. After an overnight incubation, the samples were centrifuged. The IGFBP-5 in both the pellets and the supernatants of Sepharose beads or heparin-Sepharose beads was analyzed by ligand blotting as described under "Experimental Procedures." The *arrow* denotes the position of the unbound IGFBP-5 in the supernatant, and the *arrowhead* denotes the bound IGFBP-5 in the pellet. *Lane 1*, Sepharose beads; *lanes 2–6*, heparin-Sepharose beads. *Lanes 1* and *2*, no heparin; *lane 3*, heparin 0.02 mg/ml; *lane 4*, 0.05 mg/ml; *lane 5*, 0.25 mg/ml; *lane 6*, 0.5 mg/ml. *B*, specificity of IGFBP-5 binding to heparin. The experiment was performed as described in *A* except that the incubation buffer included: *lanes 1* and *2*, no glycosaminoglycan; *lane 3*, heparin, 0.2 mg/ml; *lane 4*, heparan sulfate, 0.5 mg/ml; *lane 5*, chondroitin sulfate A, 0.5 mg/ml; *lane 6*, dermatan sulfate, 0.5 mg/ml. *Lane 1*, Sepharose beads, *lanes 2–6*, 0.025 μl of heparin-Sepharose beads.

native IGFBP-5 binding. Dermatan sulfate had an intermediate effect (Fig. 1B, *lane 6*). These results show that native IGFBP-5 binding to heparin or heparan sulfate is specific.

Native IGFBP-5 binding increased when increasing amounts of heparin-Sepharose beads were used. 0.01 μl of heparin-Sepharose beads bound nearly 50% of the native IGFBP-5 (Fig. 2, *lane 2*), and 99% of the material was pelleted when 0.025 μl was used (Fig. 2, *lane 3*). Therefore 0.025 μl of heparin-Sepharose beads was selected as the minimum volume to be used in any experiment.

Region of IGFBP-5 That Mediates Heparin Binding—Our previous result showed that the region Arg[201]–Arg[218] is responsible for the reduction in the affinity of IGFBP-5 for IGF-I that occurs in response to heparin, suggesting that this region may contain heparin-binding site of IGFBP-5. To determine if this sequence was important for heparin binding, competitive binding studies were carried out using these test conditions. Coincubation with peptide A (Arg[201]–Arg[218]) inhibited native IGFBP-5 binding to heparin-Sepharose beads (Fig. 3, *lanes 3–5*). In contrast, the effect of peptide B, which contains a similar charge to mass ratio, was minimal (Fig. 3, *lanes 6–8*). To verify that proteoglycans in the ECM could bind to IGFBP-5 through glycosaminoglycan side chains, fibroblast ECM and purified tenascin were exposed to heparinase, and IGFBP-5 binding was determined. IGFBP-5 binding to both ECM and purified tenascin was reduced by heparinase exposure (Fig. 4).

To identify the basic amino acids in the Arg[201]–Arg[218] region that are involved in heparin binding, we compared the amounts of native and of each IGFBP-5 mutant that bound to 0.025 μl of heparin-Sepharose beads. Native IGFBP-5 (Fig. 5A, *lanes 4–7*) and the K211N mutant (Fig. 5A, *lanes 11–14*) bound to heparin-Sepharose beads dose dependently. Scanning densitometry showed that the heparin binding activity of the K211N mutant was equal to native IGFBP-5. The binding ratio defined as a percentage of IGFBP-5 that binds the heparin-Sepharose beads divided by the total detectible IGFBP-5 (the amount bound in the pellet plus the supernatant) was calculated. The binding
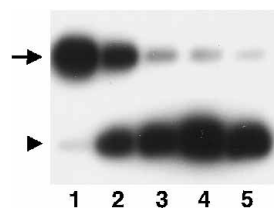
FIG. 2. **IGFBP-5 binding to heparin-Sepharose beads.** Native IGFBP-5 (80 nM) was incubated with Sepharose beads or the indicated volume of heparin-Sepharose beads in 50 μl of EMEM, supplemented with 20 mM HEPES, 0.1% Tween 20, and 20 mM EDTA. After an overnight incubation, the samples were centrifuged. The IGFBP-5 in both the pellets and the supernatants was analyzed by ligand blotting as described under "Experimental Procedures." The *arrow* denotes unbound IGFBP-5 in the supernatant, and the *arrowhead* denotes bound IGFBP-5 in the pellet. *Lane 1*, Sepharose beads; *lane 2*, heparin-Sepharose beads, 0.01 μl; *lane 3*, 0.025 μl; *lane 4*, 0.05 μl; and *lane 5*, 0.1 μl.
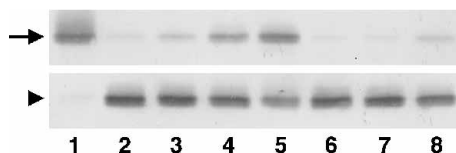

FIG. 3. **Competition binding of IGFBP-5 to heparin-Sepharose beads.** Native IGFBP-5 (80 nM) was added in 50 μl of EMEM supplemented with 20 mM HEPES, 0.1% Tween 20, and 20 mM EDTA and incubated with 0.025 μl of heparin-Sepharose beads or Sepharose beads in the presence of the indicated concentrations of Arg[201]–Arg[218] peptide, or Ala[131]–Thr[141] peptide. After an overnight incubation, the samples were centrifuged. Both the IGFBP-5 that bound to heparin-Sepharose beads and remained in the supernatant were analyzed by immunoblotting as described under "Experimental Procedures." The *arrow* denotes unbound IGFBP-5 in the supernatants, and the *arrowhead* denotes bound IGFBP-5 in the pellets. *Lane 1*, Sepharose beads; *lanes 2–8*, heparin-Sepharose beads. *Lanes 1* and *2*, no peptide; *lanes 3*, *4*, and *5*, Arg[201]–Arg[218] peptide, 0.27, 2.7, and 27 uM, respectively; *lanes 6*, *7*, and *8*, Ala[131]–Thr[141] peptide, 0.27, 2.7, and 27 μM, respectively.
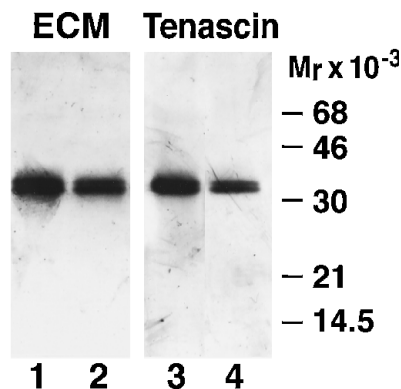

FIG. 4. **Effect of heparinase on ECM and tenascin binding of IGFBP-5.** ECM was prepared or purified tenascin was layered on to 35-mm plastic tissue culture plates. The ECM and tenascin were exposed to heparinase (0.1 unit/ml) for 2 h at 37 °C. IGFBP-5 (3.4 nM) was incubated with the ECM or tenascin, and the amount of bound material was determined by immunoblotting. *Lane 1*, ECM control; *lane 2*, ECM after heparinase; *lane 3*, tenascin control; *lane 4*, tenascin after heparinase.

ratios of 1.67, 3.33, and 6.66 pmol of native IGFBP-5 to heparin-Sepharose beads were 96, 93, and 88%, respectively (Table II), and for the K211N mutant they were 97, 99, and 96%, respectively (Table II). Similarly, the K134A/R136A/K211N mutant (Fig. 5*C*), the R207A/K211N mutant (Fig. 5*E*), and the K217A/R218A mutant (Fig. 5*G*) bound as well to heparin-Sepharose beads as native IGFBP-5 (Table II). In contrast, heparin-binding activity of the K202A/K206A/R207A mutant
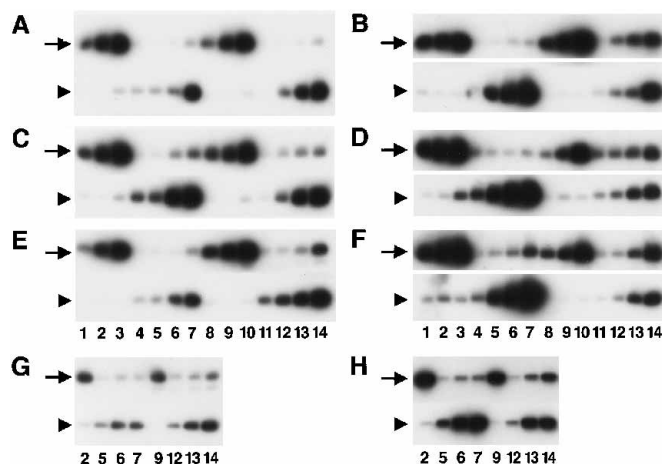

FIG. 5. *A–H*, heparin binding activity of IGFBP-5. Native IGFBP-5 or each mutant was incubated with Sepharose beads or heparin-Sepharose beads (0.025 μl) in 50 μl of EMEM, supplemented with 20 mM HEPES, 0.1% Tween 20, and 20 mM EDTA. After an overnight incubation, the samples were centrifuged. The amount of each form of IGFBP-5 in both the pellet and the supernatant was determined by ligand blotting and scanning densitometry as described under "Experimental Procedures." The results were confirmed by immunoblotting (data not shown). *Lanes 1–7* of each panel contain native IGFBP-5, and *lanes 8–14* contain each mutant; these include *A*, K211N; *C*, K134A/R136A/K211N; *E*, R207A/K211N; *B*, K202A/K206A/R207A; *D*, R201A/K202N/K206N/K208N; *F*, K211N/R214A/K217A/R218A; *G*, K217A/R218A; *H*, R201A/K202N. *Lanes 1–3* and *8–10*, Sepharose beads; *lanes 4–7* and *11–14*, heparin-Sepharose beads. *Lanes 4* and *11*, IGFBP-5 (3.33 nM); *lanes 1*, *5*, *8*, and *12*, IGFBP-5 (6.66 nM); *lanes 2*, *6*, *9*, and *13*, IGFBP-5 (13.3 nM); and *lanes 3*, *7*, *10*, and *14*, IGFBP-5 (26.6 nM). The *arrows* denote the position of unbound IGFBP-5 that remained in the supernatant, and the *arrowheads* denote the bound IGFBP-5 that remained in the pellet.

(Fig. 5*B*), the R201A/K202N/K206N/K208N mutant (Fig. 5*D*), the K211N/R214A/K217A/R218A mutant (Fig. 5*F*), and the R201A/K202N (Fig. 5*H*) mutant were decreased. When 1.6 pmol of each of these mutants were added, only 37, 41, 46, and 62% of each of these mutants, respectively, bound to the heparin-Sepharose beads, compared to 96% for native IGFBP-5 (Table II). Nonspecific binding was very low, since native IGFBP-5 and each mutant bound only minimally (<7%) to Sepharose beads. Since the NaCl concentration that is necessary to inhibit the binding of proteins to heparin is inversely proportional to the K_d value, we quantified the binding of native IGFBP-5 and the mutants to heparin-Sepharose using NaCl concentrations between 150 and 500 mM. As shown in Table III, the maximal decrease in binding of native IGFBP-5 binding to heparin occurred when NaCl concentrations between 300 and 350 mM were added. Similarly the K134A/R136A, K134A/R136A/K211N, K211N, R207A/K211N, and K217A/R218A mutants showed maximal decreases between 300 and 350 mM. In contrast, the R201A/K202N mutant showed the greatest change between 200 and 250 mM NaCl, and the K202A/K206A/R207A, R201A/K202N/K206N/K208N, and K211N/R214A/K217A/R218A mutants had maximum reductions between 150 and 200 mM NaCl. This indicates that they have an affinity for heparin that is considerably less than native IGFBP-5.

Taken together the results show that five basic amino acids are potentially required to maintain the heparin binding activity of IGFBP-5. These include positions 201, 202, 206, 208, and 214. Although mutants containing single amino acid substitutions will be required to determine the necessity of each of these residues, some preliminary conclusions can be inferred. The K211N/R214A/K217A/R218A mutant had markedly reduced heparin binding. Since the K211N, K217A, and R218A

TABLE II
Heparin binding activity of IGFBP-5

The values represent the percentage of IGFBP-5 that bound to Sepharose beads or heparin-sepharose beads compared to the total detectable IGFBP-5 (bound plus unbound). The values for each point were determined by scanning densitometry. Counting the band density of *lanes 4, 8,* and *11* in Fig. 4 was technically impossible, since the IGFBP-5 bands in *lanes 3,* and *7,* and *10* in Fig. 4 were so intense that their signals interfered with the quantitation in adjacent lanes.

Form of IGFBP-5	IGFBP-5 added (pmol)				
	Sepharose		Heparin-Sepharose		
	3.33	6.67	1.67	3.33	6.67
	%				
Native IGFBP-5	1.6	3.8	96	93	88
K134A/R136A	0	0	93	84	91
K134A/R136A/K211N	0	1.8	83	86	86
K211N	0	1.1	97	99	96
R207A/K211N	0	0.5	91	84	70
K217A/R218A	ND[a]	0	92	89	80
R201A/K202N	ND[a]	1.2	70	66	65
K202A/K206A/R207A	0.9	0.4	37	42	56
R201A/K202N/K206N/K208N	6.9	1.5	41	59	54
K211N/R214A/K217A/R218A	0	0	46	51	42

[a] ND, not determined.

TABLE III
Inhibition of IGFBP-5 binding to heparin-Sepharose by NaCl

The values are the percentage of IGFBP-5 that remained bound to heparin-Sepharose beads at each NaCl concentration compared to control tubes containing 150 mM NaCl. The amount of IGFBP-5 that remained bound to the beads at each NaCl concentration was determined by PhosphorImager analysis.

Form of IGFBP-5	NaCl concentration (mM)				
	200	250	300	350	400
	%				
Native IGFBP-5	94	82	68	31	19
K134A/R136A	89	80	59	23	17
K134A/R136A/K211N	86	67	45	12	6
K211N	87	70	53	25	13
R207A/K211N	99	66	49	20	14
K217A/R218A	99	67	47	10	3
R201A/K202N	68	33	13	5	2
K202A/K206A/R207A	44	11	4	1	<1
R201A/K202N/K206N/K208N	27	10	1	<1	<1
K211N/R214A/K217A/R218A	9	4	1	<1	<1

substitutions had no effect, this suggests that Arg[214] may be a critical determinant of heparin binding or that some combination of Arg[214] with the other three basic amino acids may be necessary. We also noted a substantial reduction in binding of the K202A/K206A/R207A mutant to heparin. Arg[207] is probably not important, since the R207A/K211N mutant bound heparin normally, although the effect of altering Arg[207] alone was not determined.

Amino Acid Substitutions That Alter the IGFBP-5 Affinity Change in Response to Heparin—We examined effect of heparin (10 μg/ml) on the K_a of native IGFBP-5 and the IGFBP-5 mutants for IGF-I using Scatchard analysis (Table I). Heparin decreased the K_a of native IGFBP-5 and the K134A/R136A mutant by 17- and 16-fold, respectively. In contrast, the change in affinity in the other eight mutants in response to heparin was much less (*e.g.* 1.1–3.2-fold). The K134A/R136A/K211N mutant and the K211N mutant had similar reductions in K_a in response to heparin (2.1- and 2.5-fold, respectively). These results suggest that only basic amino acids in the Arg[201]–Arg[218] region are responsible for the heparin-induced affinity shift of IGFBP-5 for IGF-I and that Lys[134] and Arg[136] are not important. The results show that the Lys[211] residue contributes greatly to the heparin-induced affinity shift and that alteration of Lys[134] and Arg[136] in the Ala[131]–Thr[141] region has no additional effect. The greatest reduction in K_a in response to heparin was found in the K211N/R214A/K217A/R218A mutant followed by the R201A/K202N/K206N/K208N mutant and the

K202A/K206A/R207A mutant, respectively. This suggests that the reduced binding of these mutants to heparin contributes to this reduction. However, the magnitude of the reductions in K_a of the K211N mutant (2.5-fold) and for the K217A/R218A mutant (2.8-fold) were much less than for native IGFBP-5, suggesting that Lys[211] and Lys[217] or Arg[218] are important basic amino acids for inducing the change in affinity in native IGFBP-5 when it is bound to heparin. Substitution for Arg[207] with Lys[211] did not cause a further reduction in K_a in response to heparin. However, since the K202A/K206A/R207A and R201A/K202N mutants also had significant reductions in the heparin-induced affinity shift, the Lys[211], Lys[217], and Arg[218] substitutions are not absolutely required. Substitutions for Lys[211] or for Lys[217] and Arg[218] result in nearly complete loss of the reduction in affinity in response to heparin but have no effect on heparin binding. This suggests that heparin binding to IGFBP-5 leads to a conformational change which contributes to reduction in affinity of IGFBP-5 for IGF-I and that Lys[211] and Lys[217] or Lys[218] are important for heparin to induce this conformational change. This conformational change may be conferred by several amino acids, but our data do not identify single amino acids, other than the Lys[211], that alter the conformational change without altering heparin binding. Additional mutations at positions 134, 136, or 207 combined with the K211N substitution resulted in no additional effect on the IGFBP-5 response to heparin binding. Each of the four other mutants that contained substitutions that resulted in a change

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in the heparin-induced affinity shift had reduced heparin binding; therefore, the contribution of their substituted amino acids to the change in affinity in response to heparin could not be determined.

The degree of change in affinity of each mutant for IGF-I in response to heparin was also determined using cross-linking studies. Coincubation with heparin inhibited ^{125}I-IGF-I-native IGFBP-5 complex formation in a dose-dependent manner (Fig. 6*A* and Table IV). The ^{125}I-IGF-I·K134A/R136A mutant complex formation was inhibited by heparin, and the inhibition was comparable to its effect on the ^{125}I-IGF-I-native IGFBP-5 complexes (Fig. 6*B*) (Table IV). In contrast, the responsiveness of the other eight IGFBP-5 mutants to heparin was decreased compared to native IGFBP-5. When ^{125}I-IGF-I was cross-linked

to the other eight mutants (Fig. 6, *C–J*) in the presence of heparin, the band intensities of the complexes were greater at all heparin concentrations tested compared to the ^{125}I-IGF-I-native IGFBP-5 or to the ^{125}I-IGF-I·K134A/R136A mutant complex. These results confirm that the basic amino acids in the Arg^{201}–Arg^{218} region are responsible for the heparin-induced affinity shift. Similar responsiveness to heparin was found between the K134A/R136A/K211N mutant (Fig. 6*C*) and the K211N mutant (Fig. 6*D*), further suggesting that Lys^{134} and Arg^{136} do not contribute to the affinity shift in response to heparin.

DISCUSSION

In this study we extended our previous observations (7) to report that site-directed mutagenesis of specific basic residues in IGFBP-5 results in a reduction of the capacity of this protein to associate with heparin. Since we had shown previously (7) that a peptide containing residues in the basic region Arg^{201}–Arg^{218} could nullify the effect of heparin on the change in IGFBP-5 affinity for IGF-I, we reasoned that basic amino acids in this region might be involved in heparin binding. In the present study the possibility that this region contained amino acids that formed the heparin binding site of IGFBP-5 was confirmed. Coincubation of native IGFBP-5 with a peptide that contained the Arg^{201}–Arg^{218} sequence inhibited native IGFBP-5 binding to heparin-Sepharose beads. In contrast, a peptide containing the Ala^{131}–Thr^{141} sequence in IGFBP-5 that has a similar charge to mass ratio had no effect. We next evaluated the contribution of specific basic amino acids in this region to heparin binding using the IGFBP-5 mutants. Four of the mutants showed a significant reduction in heparin binding, and binding of these mutants to heparin was inhibited by lower NaCl concentrations than were required to inhibit the binding of native IGFBP-5 to heparin. In contrast, four other mutants that also contained substitutions or basic amino acids within the Arg^{201}–Arg^{218} region had no reduction in heparin binding, suggesting that the specific positional locations of the basic residues may be important.

The degree of reduction in the affinity of the IGFBP-5 mutants for heparin is similar to that reported for the effects of specific amino substitutions on the affinity of plasminogen activator inhibitor I (PAI-1) binding to heparin (14). In that study, the investigators reported that the wild type protein required 293–318 mM NaCl to disassociate PAI-1 from heparin-Sepharose whereas the PAI-1 mutants were dissociated with NaCl concentrations between 175 and 238 mM. Native IGFBP-5 required somewhat higher salt concentration for significant inhibition of heparin binding (*e.g.* 300–350 mM) but the binding

FIG. 6. *A–J*, inhibitory effect of heparin on forming IGF-I·IGFBP-5 complexes. ^{125}I-IGF-I (30,000 cpm/tube) was incubated with each form of IGFBP-5 (4 nM) in 100 μl of EMEM supplemented with 20 mM HEPES, pH 7.3, in the presence of the indicated concentrations of heparin. After a 1-h incubation at room temperature, the samples were cross-linked using 0.5 mM disuccinimidyl suberate and the reaction was stopped by addition of 10 μl of 0.5 M Tris, pH 7.4. The samples were subjected to SDS-PAGE under reducing conditions (0.1 M dithiothreitol). A gel was fixed, dried, and autoradiographed as described under "Experimental Procedures." *Lanes 1–6*, IGFBP-5 (4 nM). *Lanes 1* and *6*, no heparin; *lane 2*, 0.1 μg/ml heparin; *lane 3*, 1 μg/ml heparin; *lane 4*, 10 μg/ml heparin; *lane 5*, 100 μg/ml heparin. *Lane 6*, IGF-I (13.3 nM). *A*, native IGFBP-5; *B*, K134A/R136A; *C*, K134A/R136A/K211N; *D*, K211N; *E*, R207A/K211N; *F*, K202A/K206A/R207A; *G*, R201A/K202N/K206N/K208N; *H*, K211N/R214A/K217A/R218A; *I*, R201A/R202N; *J*, R217A/R218A.

TABLE IV

Inhibitory effect of heparin on the formation of IGF-I · IGFBP-5 complexes

Affinity cross-linking studies were performed as described under "Experimental Procedures." The band intensities of the ^{125}I-IGF-I · IGFBP-5 complexes in Fig. 5, *A–J* were determined by scanning densitometry. The percentages were obtained by dividing the band intensities of the samples that included the indicated concentration of heparin in the incubation buffer by the band intensities of samples that did not include heparin. The results are the mean of three separate experiments.

Form of IGFBP-5	Heparin (μg/ml)				
	0	0.1	1	10	100
			%		
Native IGFBP-5	100	65	33	25	19
K134A/R136A	100	87	57	42	30
K134A/R136A/K211N	100	106	77	64	50
K211N	100	98	61	61	62
R217A/R218A	100	94	86	75	66
R201A/K202N	100	96	84	78	70
R207A/K211N	100	97	75	59	47
K202A/K206A/R207A	100	97	85	69	67
R201A/K202N/K206N/K208N	100	98	94	89	81
K211N/R214A/K217A/R218A	100	75	68	63	62

of our mutants was inhibited using NaCl concentrations that were similar to those used to inhibit mutant PAI-1 binding (*e.g.* 150–200 mM). These results indicate that these substitutions for basic residues in IGFBP-5 had a significant effect on its affinity for heparin-Sepharose.

Substitution for two residues within the linear BBB*XX*B motif (positions 207 and 211) (15) did not alter heparin binding. In contrast the three-dimensional structure of antithrombin III (AT-III) a heparin-binding protein, suggests that the basic amino acids in the BBB*XX*B motif (positions 131–136) (15) are located in or near the heparin binding region (16, 17). No natural or site-directed AT-III mutant that has a substitution for the basic amino acids in positions 131–136 has been analyzed (18, 19). In contrast, mutation of basic amino acids outside the motif can result in major reduction in heparin binding (18, 19). Chemical modification of Lys^{136} in AT-III suggests that it contributes to low affinity heparin binding (17, 20, 21), but Lys^{125}, which is outside the BBB*XX*B motif, is an important residue for high affinity heparin binding. The positions of Arg^{132} and Lys^{133} in AT-III correspond to Arg^{192} and Arg^{193} in heparin cofactor II, and mutagenesis of these residues results in decreased dermatan sulfate binding (22). Therefore it is possible that Lys^{132} and Arg^{133} in AT-III are important, but this has not been determined. In summary, several basic amino acids in AT-III and IGFBP-5 that are responsible for heparin binding are located outside the proposed heparin binding BBB*XX*B motif, suggesting that for both proteins the determinants of heparin binding in IGFBP-5 may be more complex.

A reduction in heparin binding is not required to induce the affinity shift since the K211N or Lys^{217} plus Ala^{218} substitutions alter the response to heparin extensively. AT-III mutants that alter its function have been analyzed extensively. However, studies that show mutations that have no effect on heparin binding but alter the conformational change in AT-III that occurs with heparin binding have not been reported. The AT-III position that corresponds to the Lys^{211} position within IGFBP-5, *e.g.* lysine 136, has not been analyzed in this manner. Therefore a direct comparison is not possible. It is possible that the conformational change that occurs in IGFBP-5 that alters its affinity for IGF-I in response to heparin is based on a more simplified model than AT-III or other serpins, and therefore its conformational change in response to heparin binding may be altered more extensively by single amino acid substitutions.

Our previous report (7) showing that IGFBP-1, IGFBP-2, and IGFBP-4 do not contain the Arg^{201}–Arg^{218} sequence and do not undergo the heparin-induced affinity shift further suggests that this sequence is important for either heparin binding and conformational changes in affinity for IGF-I that are induced by heparin. IGFBP-3, like IGFBP-5, contains 10 of 18 amino acids in the region corresponding to Arg^{201}–Arg^{218} that are basic, and all of these positions have been conserved (23). However, we do not note as great an affinity shift after heparin binding with IGFBP-3, suggesting that, even though its affinity for heparin appears to be similar to IGFBP-5 (7, 24), IGFBP-3 has other structural determinants that limit its change in affinity in response to heparin binding.

Mutagenesis did not induce significant changes in the affinity of any of the IGFBP-5 mutants for IGF-I. Slight increases in the affinity were detected, but all were less than 1.5-fold. These results suggest that these basic amino acids play a minimal role in the binding of IGFBP-5 to IGF-I. This conclusion is consistent with our previous results (7) showing that the Arg^{201}–Arg^{218} region does not directly compete with IGFBP-5 binding to IGF-I and excludes the possibility that both the affinity shift and heparin binding changes noted herein are simply due to changes in the affinity of each mutant for IGF-I.

Recent evidence has been presented that the binding of IGFBP-3 and IGFBP-5 to proteoglycans or glycosaminoglycans may play a significant role in the regulation of cellular responses to IGF/IGFBP combinations. Smith *et al.* (25) reported that IGFBP-3 is associated with Leydig cell surface proteoglycans, and this association influences IGFBP-3 clearance from conditioned medium. Martin *et al.* (26) reported that IGFBP-3 associated with the fibroblast cell surface is displaced by the addition of heparin in conditioned medium, suggesting that IGFBP-3 binds to cell surface proteoglycans. We recently have shown that heparin binding to IGFBP-5 or IGFBP-3 leads to a decrease in the binding affinity of IGFBP-5 or IGFBP-3 for IGF-I (7). Importantly IGFBP-3 contains a sequence that is identical to the Arg^{201}–Arg^{218} region of IGFBP-5, and this region in IGFBP-3 has been proposed to mediate glycosaminoglycan binding (22). These findings have led ourselves and others to hypothesize that IGF-I·IGFBP-5 or IGF-I·IGFBP-3 complexes adhere to heparan sulfate proteoglycans on cell surfaces or in ECM. Such adherence results in a shift in the IGFBP affinity for IGF-I, allowing release from the complex and thus making free IGF-I available to bind to receptors. This hypothesis is supported by our previous reports (6, 27) showing the affinity of IGFBP-3 for IGF-I in conditioned medium is 12-fold higher than the affinity of IGFBP-3 associated with cell surface and that the affinity of IGFBP-5 in the conditioned medium is 8-fold higher than for IGFBP-5 that is associated with ECM. More importantly the ability of IGFBP-3 or IGFBP-5 to potentiate IGF-I action appears to require the affinity shifts, since when these forms are present in solution they usually inhibit IGF-I actions, whereas when they are associated with either ECM or cell surface, they have been shown to potentiate IGF-I actions (4–6).

We previously reported that human fibroblasts secrete a serine protease that cleaves IGFBP-5 (28). Heparin binds to this protease and multiple glycosaminoglycans inhibit its activity (29). Furthermore, the effect of heparin on this protease can be enhanced by AT-III or heparin cofactor II, suggesting that heparin binding may function to regulate IGFBP-5 abundance as well as its affinity for IGF-I (28). Since extracellular matrix contains multiple proteoglycans, these proteoglycans in ECM and on cell surfaces may also serve to modulate the activity of this protease and therefore indirectly alter cellular responsiveness to the IGFs.

Proteoglycans in ECM represent an important potential reservoir for binding IGFBP-5 and thereby modulate its activity. They may provide an important means for controlling its affinity for IGF-I (6) and its cleavage by serine proteases (29). The effect of these mutations on susceptibility to proteolysis and the responsiveness of fibroblasts to IGF-I deserves further analysis.

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