Molecular Biology of the Cell
Vol. 9, 2383–2392, September 1998

Binding of Insulin-like Growth Factor (IGF)–Binding Protein-5 to Smooth-Muscle Cell Extracellular Matrix Is a Major Determinant of the Cellular Response to IGF-I

Alex Parker, Catherine Rees, Jane Clarke, Walker H. Busby, Jr., and David R. Clemmons*

Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599

Submitted May 7, 1998; Accepted July 2, 1998
Monitoring Editor: Carl-Henrik Heldin

Insulin-like growth factor–binding protein-5 (IGFBP-5) has been shown to bind to fibroblast extracellular matrix (ECM). Extracellular matrix binding of IGFBP-5 leads to a decrease in its affinity for insulin-like growth factor-I (IGF-I), which allows IGF-I to better equilibrate with IGF receptors. When the amount of IGFBP-5 that is bound to ECM is increased by exogenous addition, IGF-I's effect on fibroblast growth is enhanced. In this study we identified the specific basic residues in IGFBP-5 that mediate its binding to porcine smooth-muscle cell (pSMC) ECM. An IGFBP-5 mutant containing alterations of basic residues at positions 211, 214, 217, and 218 had the greatest reduction in ECM binding, although three other mutants, R214A, R207A/K211N, and K202A/R206N/R207A, also had major decreases. In contrast, three other mutants, R201A/K202N/R206N/R208A, and K217N/R218A and K211N, had only minimal reductions in ECM binding. This suggested that residues R207 and R214 were the most important for binding, whereas alterations in K211 and R218, which align near them, had minimal effects. To determine the effect of a reduction in ECM binding on the cellular replication response to IGF-I, pSMCs were transfected with the mutant cDNAs that encoded the forms of IGFBPs with the greatest changes in ECM binding. The ECM content of IGFBP-5 from cultures expressing the K211N, R214A, R217A/R218A, and K202A/R206N/R207A mutants was reduced by 79.6 and 71.7%, respectively, compared with cells expressing the wild-type protein. In contrast, abundance of the R201A/K202N/R206N/R208A mutant was reduced by only 14%. Cells expressing the two mutants with reduced ECM binding had decreased DNA synthesis responses to IGF-I, but the cells expressing the R201A/K202N/R206N/R208A mutant responded well to IGF-I. The findings suggest that specific basic amino acids at positions 207 and 214 mediate the binding of IGFBP-5 to pSMC/ECM. Smooth-muscle cells that constitutively express the mutants that bind weakly to ECM are less responsive to IGF-I, suggesting that ECM binding of IGFBP-5 is an important variable that determines cellular responsiveness.

INTRODUCTION

The abundance of the insulin-like growth factors (IGFs) in extracellular fluids and their capacity to bind to cell surface receptors is determined by insulin-like growth factor–binding proteins (IGFBPs) (Rechler, 1993; Jones and Clemmons, 1995). All extracellular fluids that have been analyzed appear to have an excess of IGF-binding capacity (McCusker *et al.*, 1988). These proteins each have a higher affinity for IGF-I

* Corresponding author. E-mail address: dpm@med.unc.edu.

© 1998 by The American Society for Cell Biology

and -II than the IGF-I receptor; therefore, their ability to modulate cellular responses to the IGFs is dependent on which specific forms of IGFBPs are present and their M concentrations. The affinity of some forms of IGFBPs for IGF-I and -II has been shown to be lowered by either binding to extracellular matrix (ECM) or by proteolytic cleavage (Guidice *et al.*, 1990; Jones *et al.*, 1993a; Blat *et al.*, 1994). Human fibroblasts synthesize IGFBP-5, which is the predominant form of IGFBP in their ECM (Camacho-Hubner *et al.*, 1992; Jones *et al.*, 1993a). In contrast, other forms of IGFBPs adhere weakly or not at all to ECM. Fibroblasts and smooth-muscle cells (SMCs) release a protease that cleaves IGFBP-5 into non-IGF–binding fragments, thus allowing release of IGF-I to receptors (Nam *et al.*, 1994; Duan *et al.*, 1996). In contrast, when IGFBP-5 is associated with the ECM, its affinity for IGF-I and -II is lowered 8- to 15-fold, and it is protected from proteolysis; therefore, ECM-associated IGFBP-5 can act as a reservoir for the IGFs and can slow their clearance from the pericellular microenvironment (Jones *et al.*, 1993a). Similarly, because of the reduction in IGFBP-5 affinity, the IGF-I that is bound to IGFBP-5 within the ECM is in better equilibrium with receptors, and an increase in ECM-associated IGFBP-5 has been shown to result in potentiation of the fibroblast growth response to IGF-I (Jones *et al.*, 1993a).

In vitro mutagenesis has been used previously to determine the amino acids within IGFBP-5 that are required for it to bind to fibroblast ECM (Parker *et al.*, 1996). However, whether mutant forms of IGFBP-5 that are being synthesized constituitively will have reduced ECM binding and whether this results in an altered replication response of cells to IGF-I has not been determined. We have recently reported that porcine aortic smooth-muscle cells (pSMCs) release a protease that cleaves IGFBP-5 and that its activity is so abundant that no intact IGFBP-5 is detected in the medium unless the activity of the protease is inhibited. Because intact IGFBP-5, but not its 22-kDa fragment, inhibits the cellular replication response to IGF-I (Imai *et al.*, 1997), we used this model system, which does not have the confounding variable of intact IGFBP-5 in interstitial fluid, to more clearly determine the effect of lowering the amount of constituitively synthesized IGFBP-5 in the ECM on the cellular replication response to IGF-I. To test the hypothesis we initially determined the residues in IGFBP-5 that were the most important for binding to ECM then transfected pSMC using two mutant cDNAs that encoded the forms of IGFBP-5 with the greatest reduction in ECM binding. We then compared the DNA synthesis responses of pSMC expressing those mutants to pSMC expressing wild-type IGFBP-5 and a control mutant form that had an equal number of amino acid substitutions but no reduction in ECM binding.

MATERIALS AND METHODS

Cell Culture

Smooth-muscle cells were isolated from porcine aortas using a previously described method (Ross, 1971). Cells that migrated from explants were cultured in DMEM (Hazelton Systems, Danver, PA) supplemented with 10% FBS (Life Technologies, Grand Island, NY). The medium was also supplemented with penicillin, 100 U/ml, and streptomycin, 100 μg/ml (Life Technologies). Cells were grown in 10-cm dishes (3001; Falcon, Becton Dickinson, Rutherford, NJ). For experiments to test binding to the ECM, the cells were grown in 35-mm tissue culture dishes (Falcon 3010), and for DNA synthesis experiments, they were grown in microtest plates (Falcon 3004). Cells were seeded at a density of 5000 cells/cm^2 and grown for 7–10 d until confluency was reached. The medium was changed every third day.

Preparation of ECM and Measurement of IGFBP-5 Binding to ECM

In previous studies we had directly measured IGFBP-5 binding to fibroblast ECM that was adherent to tissue culture plastic surfaces. However, when this method was attempted using pSMC/ECM, the nonspecific binding of IGFBP-5 to the plastic dishes was high (e.g., ~50% of the total binding). Therefore, an extraction method was devised to lower the nonspecific binding. Although this method results in disruption of the ECM in order to focally concentrate it on membranes, the known proteins that are present in the ECM that bind to IGFBP-5, such as vitronectin and plasminogen activator inhibitor-1, are completely extracted with SDS and rebind to the membrane; therefore, assessment of binding to specific substituents of the ECM is possible.

ECM was prepared as follows. Cells were grown to confluency for at least 7 d and then washed three times with serum-free DMEM. The cell monolayer was removed by exposure to 2.0 M urea for 10 min. This resulted in complete removal of nuclei and cytoskeletal elements. The ECM was removed by adding 1.0 ml of 0.3 M Tris (pH 7.2) containing 3% SDS to each 10-cm dish. This extract (0.4 ml) was added to a slot blotter apparatus (model 2643; Bethesda Research Laboratories, Gaithersburg, MD) and concentrated on a polyvinylidine diflouride (PVDF) membrane (Immobilon; Millipore, Bedford, MA).

For competitive binding experiments, the ECM that was bound to the PVDF filter was incubated with ^{125}I-IGFBP-5 (specific activity, 63 μCi/μg; 80,000 cpm/ml) alone or with increasing concentrations (10–10000 ng/ml) of unlabeled IGFBP-5 or the IGFBP-5 mutants for 14 h at 4°C. The incubation buffer was 0.02 M Na_2PO_4 containing 0.2% Triton X-100 (pH 7.2). Before adding the ^{125}I-IGFBP-5, the filters were blocked by incubating them with TBS (pH 7.2) and 3% BSA for 5 h. After incubation, the filters were washed extensively with 0.2 M Na_2PO_4 (pH 7.2), and then the bound ^{125}I-IGFBP-5 was counted directly using a gamma spectrometer. Nonspecific binding was determined by subtracting the counts per minute bound in the presence of 50 μg/ml unlabeled IGFBP-5. This was consistently $<$15% of total binding. ^{125}I-IGFBP-5 was prepared by adding 0.5 mCi of NaI and one iodobead (Pierce, Rockford, IL) for 10 min in 0.2 M Na_2PO_4 (pH 7.0). After 10 min at 22°C, the bead was removed, and the mixture was purified by Sephadex G-100 chromatography.

To validate this assay, two types of experiments were performed. To determine that similar amounts of ECM were loaded, duplicate filters were immunoblotted for vitronectin. Anti-vitronectin antiserum (1:1500 dilution; Sigma, St. Louis, MO) was incubated with the filters for 4 h, and the immune complexes were detected by chemiluminescence using goat anti-rabbit–conjugated alkaline phosphatase and the supersignal CL-H substrate system (Pierce) as described previously (Parker *et al.*, 1995). The signal intensities were analyzed by PhosphorImager analysis using model 455 (Molecular Dynamics, Sunnyvale, CA). Total ECM protein was determined by the BCA assay (Pierce). When increasing concentrations of ECM

extract were added over the range of 0.5–10 mg of total protein, there was a linear increase in vitronectin band intensity. Furthermore, when reproducibility was assessed in four separate experiments, the scanning units ranged from 56,000 to 63,000 units/mg of matrix protein. The technique had an interexperimental variation of ±10.1%, and the intraassay variability was ±3.7%. To further validate that the IGFBP-5 binding to the ECM on the filter was reproducible, increasing amounts of ECM protein (0.5–10 mg) were added to the filters, and the ECM-containing filters were incubated with ^{125}I-IGFBP-5 (specific activity, 84 μCi/μg; 200,000 cpm/ml) for 14 h at 4°C, and then the filters were washed, as previously described. The filters were analyzed by autoradiography, which showed no signal intensity outside the area that contained the ECM proteins. The results were also quantified by PhosphorImager analysis. The increase in signal intensity was proportional to the amount of ECM protein that was added to the incubation mixture. When this experiment was repeated four times, the scanning units ranged from 141,000 to 166,000 units/mg of ECM protein for an interexperimental variation of ±9.6%. The intraexperimental variability was ±4.1%.

For Western ligand blotting and immunoblotting, the ECM proteins were extracted in Laemmli sample buffer (250 μl/35-mm dish), and the extract was heated to 60°C for 10 min. The proteins were resolved on SDS-PAGE using a 12.5% gel and then transferred to PVDF membranes. For Western ligand blotting, the filters were probed using ^{125}I-IGF-I (specific activity, 125 μCi/μg; 500,000 cpm/ml). The membranes were washed as described previously (Hossenlopp *et al.*, 1986). Signal intensity was determined by autoradiography and PhosphorImager analysis. For immunoblotting, the filters were probed with an anti-IGFBP-5 polyclonal antiserum using a 1:500 dilution (Camacho-Hubner *et al.*, 1992). The antiserum was incubated overnight at room temperature, as described previously (Camacho-Hubner *et al.*, 1992), and the immunoblots were developed using a sheep anti-guinea pig immunoglobulin G alkaline phosphatase conjugate (Boehringer Mannheim, Indianapolis, IN), following the manufacturer's recommended procedure. This antibody is specific for IGFBP-5 and has <0.5% cross-reactivity with other forms of IGFBPs.

Preparation of Human IGFBP-5

Human IGFBP-5 was purified to homogeneity from conditioned media that was obtained from Chinese hamster ovary (CHO) cells (American Type Culture Collection, Rockville, MD) that had been stably transfected (Camacho-Hubner *et al.*, 1992). Before transfection, the IGFBP-5 cDNA was inserted into the expression plasmid pNUT obtained from Richard Palmiter (University of Washington, Seattle, WA) as previously described (Jones *et al.*, 1993b). Human IGFBP-5 was purified using a previously described method (Camacho-Hubner *et al.*, 1992). The pure material was indistinguishable from the native protein that had been purified from conditioned medium from a human glioblastoma line (T98G, American Type Culture Collection) as determined by size estimates by SDS-PAGE with silver staining and by determination of its affinity for IGF-I (Camacho-Hubner *et al.*, 1992).

Preparation of IGFBP-5 Mutants

In vitro mutagenesis was conducted using the pRcRSV vector (Invitrogen, La Jolla, CA). The human IGFBP-5 cDNA had been ligated into this vector using a previously described method (Arai *et al.*, 1996b). The method that was used for mutagenesis and the base substitutions that were used to prepare each mutant have been previously published (Arai *et al.*, 1996b; Parker *et al.*, 1996). The mutant cDNAs were transfected into CHO K-1 cells obtained from the Lineberger Cancer Center (Chapel Hill, NC) tissue culture facility. Transfection was conducted as previously described, using calcium phosphate precipitation (Sambrook *et al.* 1989). The cells were maintained in α-Minimum Essential Media (Life Technologies) supplemented with G-418 (500 μg/ml) and 10% FBS. After clonal selection, the clones were maintained in the same medium. Serum-free conditioned medium containing the mutants was collected for 48 h and centrifuged to remove cellular debris, and the mutants were then purified to homogeneity as previously described (Camacho-Hubner *et al.*, 1992). The amount of each mutant was quantified by comparing its HPLC peak area to the peak area of a known amount of wild-type IGFBP-5 that had been quantified by amino acid composition analysis (Arai *et al.*, 1996b). The affinity of each mutant for ^{125}I-IGF-I was determined using a solution binding assay as previously described (Parker *et al.*, 1995), and the results were analyzed by the method of Scatchard.

Immunoprecipitation of IGFBP-5

Confluent quiescent cultures (35-mm dishes) were washed with serum-free DMEM and then exposed to low-methionine (10^{-7} M) DMEM containing 50 μCi/ml [^{35}S]methionine (56 Ci/mmol; Amersham, Arlington Heights, IL) for 6 h. The medium also contained heparin (100 U/ml) to prevent proteolysis. After 6 h, 1.0 cc of medium was collected and incubated with a 1:500 dilution of IGFBP-5 antiserum, and the complexes were precipitated with protein A-Sepharose (Duan *et al.*, 1996). The precipitates were analyzed by SDS-PAGE with flourography and autoradiography as described previously (Duan *et al.*, 1996).

Preparation of Transfected pSMC Cell Lysates and ECM

The cDNAs encoding three of the IGFBP-5 mutants and the nonmutated IGFBP-5 were ligated into the pMEP vector (Stratagene, La Jolla, CA). This vector is episomal and contains a region of DNA that permits extrachromosomal replication (Kingston, 1994). It also contains a *trans*-activating factor encoding a DNA-binding protein that permits stable plasmid expression, thereby allowing transfected cells to stably retain 10–200 copies of plasmid DNA per cell. The plasmid also contains a metallothionine promoter and hygromycin resistance genes. The cDNAs were excised from the pRc plasmid with xba-1 and filled in with T_4 DNA polymerase. The resulting DNAs were then digested with KPH, and the excised fragments were separated by agarose gel purification. The pMEP vector was prepared in a similar manner so that it contained a 5′-KPH overhanging end and a 3′ blunt end. The ligation was accomplished by adding 50 ng of pMEP DNA, 1.5 U of T4 DNA ligase, and 50 ng of each IGFBP-5 insert. This yielded a vector-to-insert ratio of 1:5. The reaction proceeded overnight, and then competent XLT Blue bacteria (Stratagene) were transformed. After selection, a large-scale plasmid preparation was isolated and purified (Sambrook *et al.*, 1989). Transfection was accomplished by growing the SMC cultures (third or fourth passage) to 70–80% of confluent density. The cells were washed with serum-free DMEM, and then 300 μl of media containing 5 μg/ml DNA, 1% FBS, and 10 ng/ml poly-L-ornithine (Dong *et al.*, 1993) (Sigma) were added to six-well plates (Falcon 3036). The cells were incubated in this mixture for 6 h at 37°C. DMSO (25%) was added for 4 min and then removed, and the cultures were washed three times with 5 ml of serum-free DMEM. The cultures were allowed to recover for 72 h in DMEM with 10% FBS. At that time, they were trypsined and replated in selective media containing 100 μg/ml hygromycin and then maintained until discrete colonies appeared (usually 8–10 d).

To prepare cell lysates, the transfected, confluent cultures were washed three times with serum-free DMEM and then exposed to 2 M urea (1.0 ml/35-mm dish) for 10 min at room temperature. The remaining ECM was extracted with Laemmli sample buffer as described previously. The cellular extract was centrifuged at 400 × *g* for 10 min to remove the nuclei, and then the supernatant was centrifuged at 40,000 × *g* for 30 min. The resulting supernatant was concentrated by placing 1 ml of extract in a filtration apparatus (UFU 28 C10, Millipore) and centrifuged at 5000 × *g* for 1 h, which

concentrated it to 100 μl. The membrane has a molecular mass cutoff of 10,000 Da. Forty microliters of the concentrate were analyzed by Western ligand blotting (Hossenlopp *et al.*, 1986) and immunoblotting (Camacho-Hubner *et al.*, 1992) for IGFBP-5. The relative abundance of IGFBP-5 was determined using the PhosphorImager. Image analysis was performed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). ECM was prepared from confluent cultures as described above. To determine the amount of IGFBP-5 that was present, between 15 and 32 μl of each extract were analyzed by SDS-PAGE followed by Western ligand blotting (Hossenlopp *et al.*, 1986) and immunoblotting (Camacho-Hubner *et al.*, 1992). The amount of ECM that was loaded was determined by the relative abundance of each protein in the cell lysate to correct for differences in transfection efficiency and protein expression.

Cell Replication Assays

To determine the effect of ECM-associated IGFBP-5 on pSMC replication, transfected pSMCs were used. The cells were plated into 96-well microtest plates (Falcon 3004) at a density of 5000 cells per well in DMEM supplemented with 10% FBS and hygromycin (400 μg/l). After 5 d, the medium was aspirated from the confluent cultures, and increasing concentrations of IGF-I were added to triplicate cultures in DMEM containing 0.2% human platelet-poor plasma (PPP) (Clemmons *et al.*, 1990) and 0.5 μCi of [^{3}H]thymidine. After 30 h, the amount of [^{3}H]thymidine that had been incorporated into DNA was quantified (Clemmons and Van Wyk, 1985). To assess changes in cell number, the cells were plated at 3000 cells/cm^2 in 24-well plates (Falcon 3047) in DMEM with 0.2% FCS for 48 h to induce quiescence. At that time fresh DMEM and 0.1% PPP were added with 50 ng/ml IGF-I. After 48 h cell number was determined using a particle data counter (model ZBI; Coulter Electronics, Hialeah, FL).

RESULTS

The selection of specific residues for alteration by mutagenesis was based on several observations. We had reported previously that the region of IGFBP-5 between residues 201 and 218 was very important for binding to fibroblast ECM (Parker *et al.*, 1995). Within that region 10 of 18 residues are basic; therefore, all of the mutants that we selected contained substitutions for charged residues in that region. Several of the mutants that were prepared also contained multiple substitutions. These were chosen if mutants containing single substitutions had no effect on ECM binding. To determine that mutagenesis did not alter the affinity of each mutant for IGF-I, Scatchard analysis was performed. The affinity of some of the mutants for IGF-I had been reported previously (Parker *et al.*, 1995). None of the mutants had a significant alteration in its affinity for IGF-I (Table 1). The results indicate that the region of IGFBP-5 between residues 201 and 218 is not involved in IGF-I binding.

To determine the relative affinity of each of the IGFBP-5 mutants for ECM, increasing concentrations of each mutant were incubated with the ECM-containing filters and ^{125}I-IGFBP-5, and specific binding was determined. As shown in Figure 1, wild-type IGFBP-5 inhibited the binding of the ^{125}I-IGFBP-5, and half-maximal inhibition occurred at 425 ng/ml. The two mutant forms of IGFBP-5, K202A/K206A/R207A and K211N/R214A/K217A/R218A, did not compete for binding with the wild-type protein. The R207A/K211N, and R214A mutants had major reductions in their ability to compete for binding. The R201A/K202N and K217A/R218A, and R201A/K202N/K206N/K208N mutants competed for binding, but their activities were reduced compared with native

Table 1. Affinity of IGFBP-5 mutants for IGF-I

Mutant	K_a of mutant/K_a of native IGFBP-5
R214A	1.22
K211N	1.11
R201A/K202N	1.34
R207A/K211N	1.16
K217A/R218N	1.27
K202A/K206A/R207A	1.12
R201A/K202N/K206N/K208N	1.15
K211N/R214A/K217N/R218A	1.2

The association constant (K_a) of IGFBP-5 for IGF-I was determined using Scatchard analysis. A single high-affinity binding site was detected.

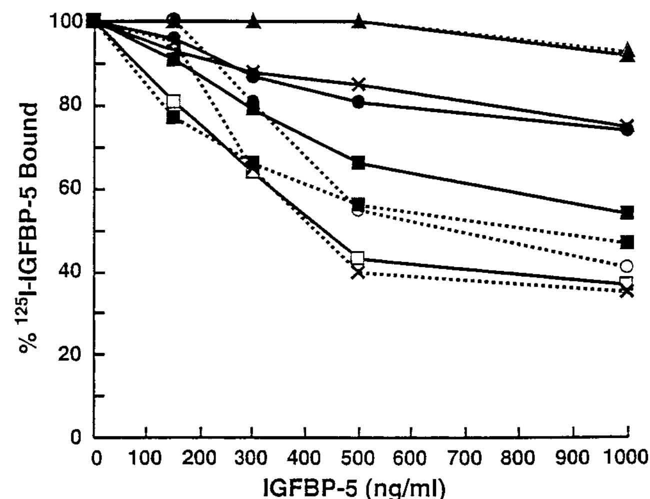

Figure 1. Competition for binding to the ECM between native IGFBP-5 and IGFBP-5 mutants. The IGFBP-5 mutants listed in Table 1 were added in increasing concentrations with ^{125}I-IGFBP-5 and incubated with Immobilon membranes that contained the ECM extracts. The binding reaction was carried out as described in MATERIALS AND METHODS. Specific binding was determined by subtracting the counts bound in the presence of 50 μg/ml IGFBP-5 from the total counts per minute bound. This was consistently <15% of total binding. Each point represents the mean of quadruplicate determinations from three independent experiments. The individual mutants that were added were as follows: K211N/R214A/K217/R218A (▲- - -▲); K202A/K206A/R207N (▲—▲); K201A/K202A/R206A/K208N (○- - -○); R217A/R218A (■—■); R214A (×—×); native IGFBP-5 (×- - -×); K211N (□—□); R207A/K211N (●—●); R201A/K202N (■- - -■).

Table 2. Affinity of IGFBP-5 mutants for ECM

Mutant	K_a (M^{-1})
Non-mutated IGFBP-5	1.8×10^9
K211N	1.8×10^9
R201A/K202N	1.0×10^9
R201A/K202N/K206N/K208N	8.4×10^8
K217A/R218A	6.1×10^8
R207A/K211N	1.7×10^8
R214A	1.6×10^8
K202A/K206A/R207A	1.1×10^7
K211N/R214A/K217N/R218A	1.4×10^7

The affinity constant (K_a) of IGFBP-5 for ECM was determined using Scatchard analysis. Scatchard analysis showed two sites, a low-affinity site (K_a = 2.3×10^6 for intact IGFBP-5) and a high-affinity site were present. The data are shown only for the high-affinity site. Concentrations between 100 ng and 10 μg/ml of each form of IGFBP-5 were used and the binding assays were performed as described in MATERIALS AND METHODS. Nonspecific binding was the counts per minute bound in the presence of 50 μg/ml nonmutated IGFBP-5, and this value was substracted from each experimental determination to calculate specific binding.

IGFBP-5. To determine the affinity of each mutant, Scatchard analysis was performed. Competitive binding assays were repeated using unlabeled IGFBP-5 concentrations as high as 50 μg/ml. As can be seen from the data in Table 2, the K211N and R201A/K202N/K206N/K208N mutants had an affinity for ECM that was similar to native IGFBP-5, and the R201A/K202N and K217A/R218A mutants had two-fold reductions. In contrast, R214A and R207A/K211N mutants had 10-fold reductions, and the K211N/R214A/K217N/R218A and K202A/R206A/R207A mutants had >100 fold reductions in their affinities.

To determine whether changing these basic residues would alter the abundance of the IGFBP-5 mutants within the ECM following ECM synthesis and assembly, the wild type and three of the mutant cDNAs were transfected into pSMC, and the abundance of the expressed proteins in pSMC/ECM was determined. Two mutants were selected because they had the greatest reduction in ECM binding (Table 2). The control mutant was selected because it had substitutions for four charged amino acids but no reduction in ECM affinity. To adjust for differences in protein expression, two types of analysis were performed, including ligand blotting of the cell lysates and [^{35}S]methionine labeling followed by immunoprecipitation. The cells that were transfected with the wild-type IGFBP-5 and the K202A/K206A/R207A mutant cDNAs had the highest IGFBP-5 concentrations in their cellular lysates (Figure 2A). PhosphorImager analysis of the cell lysate signal intensities showed that there was no more than a 2.1-fold difference between the lowest and highest producing cultures. To confirm the accuracy of that conclusion, we measured IGFBP-5 synthesis using metabolic labeling and immunoprecipitation. As shown in Figure 2B, the media samples from the transfected cultures contained predominantly intact IGFBP-5 and a 22-kDa fragment. We have previously reported that these cells synthesize and secrete IGFBP-5 in the intact form, but that after secretion that it is nearly completely cleaved to a 22-kDa fragment by a serine protease that is present in the culture medium (Duan *et al.*, 1996; Imai *et al.*, 1997). To inhibit the protease, heparin (100 U/ml) was included in the medium. The amounts of IGFBP-5 that were detected were similar to the results of the cell lysate analysis, indicating that there were no major differences in the amount of IGFBP-5 that was being synthesized and secreted (Figure 2B).

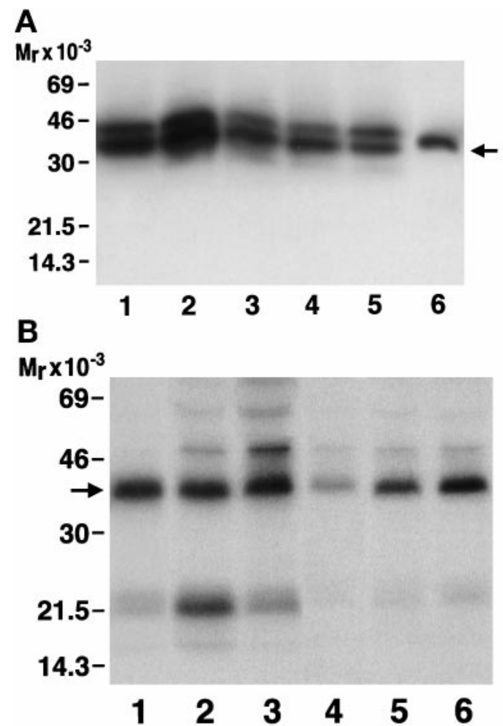

Figure 2. Relative abundance of IGFBP-5 in the cell lysates (A) and conditioned medium (B) of pSMCs that are constitutively expressing IGFBP-5 or IGFBP-5 mutants. (A) pSMC cell lysates (50 μl) from each cell line were electrophoresed and transferred to filters that were analyzed by ligand blotting using ^{125}I-IGF-I. Lane 1, R202A/R206A/R207A; lane 2, wild-type IGFBP-5; lane 3, R201A/K202N/R206A/R208A; lane 4, K211N/R214A/K217A/R218A; lane 5, mock-transfected cells; lane 6, pure IGFBP-5 standard (10 ng). The arrow denotes the position of intact, purified IGFBP-5. (B) Radiolabeled conditioned medium was collected from the cultures expressing mutants shown in A for 6 h. One milliliter was analyzed by immunoprecipitation using anti-IGFBP-5 antiserum. The lane positions are as follows: lane 1, K202A/R206A/R207A; lane 2, native IGFBP-5; lane 3, K211N/R214A/K217A/R218A; lane 4, nontransfected; lane 5, R201A/K202N/R206A/R208A; lane 6, mock transfected. The arrow denotes the position of intact IGFBP-5. The 22-kDa band represents an IGFBP-5 fragment.

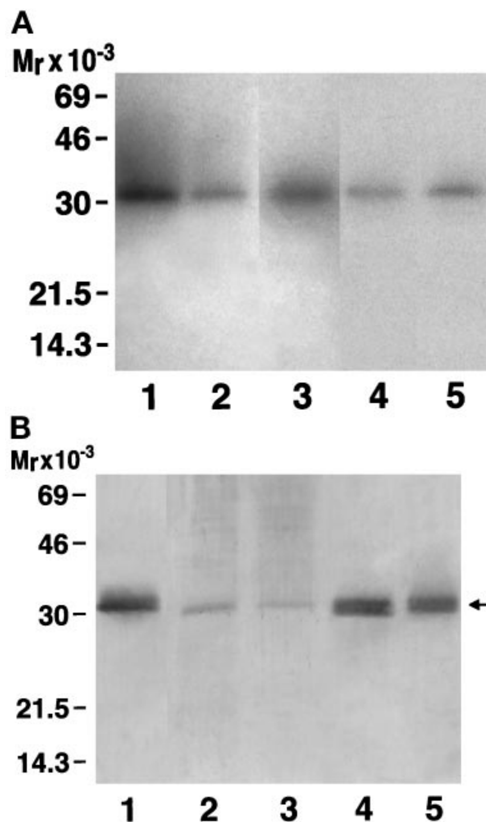

Figure 3. (A) Ligand blot of IGFBPs in the ECM. The cultures that were secreting the various forms of IGFBP-5 shown in Figure 2 were used. ECM extracts were prepared from confluent cultures as described previously and analyzed by SDS-PAGE followed by Western ligand blotting. The amount of ECM that was loaded in each lane was determined by the relative abundance of IGFBP-5 that was produced by each cell line shown in Figure 2A. Lane 1, wild-type IGFBP-5; lane 2, R201A/K202A/R207A; lane 3, R201A/K202A/R206N/R208A; lane 4, K211N/R214A/K217A/R218A; lane 5, mock transfected culture. The 31-kDa band that is shown represents intact IGFBP-5. No fragment is detected because IGFBP-5 within ECM is protected from proteolysis (Jones *et al.*, 1993a). The experiment was repeated three times with similar results. (B) Immunoblot of IGFBP-5 in the ECM. The cultures that were analyzed in A were also analyzed by immunoblotting as described in MATERIALS AND METHODS. Lane 1, wild-type IGFBP-5; lane 2, K211N/R214A/K217A/R218A; lane 3, R201A/K202A/R207A; lane 4, mock transfected; lane 5, R201A/K202A/R206N/R208A. The arrow denotes the position of IGFBP-5.

Using the results obtained in the experiments shown in Figure 2 to correct for differences in IGFBP-5 expression, we analyzed the abundance of IGFBP-5 in the ECM. Western ligand blotting showed that the abundance of the K202A/K206A/R207A (Figure 3A, lane 2) and K211N/R214A/K217A/R218A (Figure 3A, lane 4) mutants was markedly reduced compared with mock-transfected cells (Figure 3A, lane 5) or cells transfected with the native IGFBP-5 cDNA (Figure 3A, lane 1). In contrast, the R201A/K202N/K206N/R208N (Figure 3A, lane 3) mutant showed a minimal

Table 3. ECM abundance of constitutively expressed IGFBP-5 and mutants

Form of IGFBP-5	PhosphorImager units
IGFBP-5	162,623
K211N/R214A/K217N/R218A	33,086
K202N/R206A/R207N	45,987
R201A/K202N/R206A/R208A	147,886
Mock transfected	149,480

The results represent the mean of three separate experiments.

reduction in ECM binding compared with cells that were expressing native IGFBP-5. PhosphorImager analysis of the band intensities in the gel shown in Figure 3A showed that the abundance of the K211N/R214A/K217A/R218A mutant was reduced by 77% and the R201A/K202N/K207A mutant was reduced by 74% (Table 3). In contrast, the control mutant R201A/K202N/K206N/R208N was reduced only 14% compared with ECM from cultures that were expressing native IGFBP-5. To confirm that these reductions in IGFBP-5 as estimated by Western ligand blotting were indicative of a reduction in total ECM-associated IGFBP-5, the ECM extracts were also analyzed by immunoblotting (Figure 3B). The results were similar, indicating that the reduction in IGFBP-5 band intensity as determined by ligand blotting reflected a change in the amount of intact, immunoreactive protein.

To determine whether decreased abundance of IGFBP-5 mutants in the ECM resulted in an altered response to IGF-I, the capacity of each transfected cell line to synthesize DNA in response to IGF-I was analyzed. The cell lines expressing the mutants that had the greatest changes in the abundance of IGFBP-5 in the ECM were selected for analysis. Full IGF-I dose–response curves showed that the cells expressing the K211N/R214A/K217A/R218A mutant had marked attenuation in their ability to increase their [^{3}H]thymidine incorporation in response to this growth factor (Figure 4). Similarly, the cells that had been transfected with the R202A/K206N/R207A mutant had a substantially reduced response to IGF-I. In contrast, the cells expressing native IGFBP-5 showed the greatest response to IGF-I, and they responded better than the cells that had been transfected with the vector alone. Cultures expressing the R201A/K202N/K206N/R208N mutant and the mock-transfected cultures showed only a slight attenuation of IGF-I responsiveness compared with cells that had been transfected with the native IGFBP-5 cDNA. Analysis of the ECM after completion of the experiment showed that the cells expressing the K211N/R214A/K217A/R218A and R202A/K206N/R207A mutants had less IGFBP-5 in the ECM as compared with the

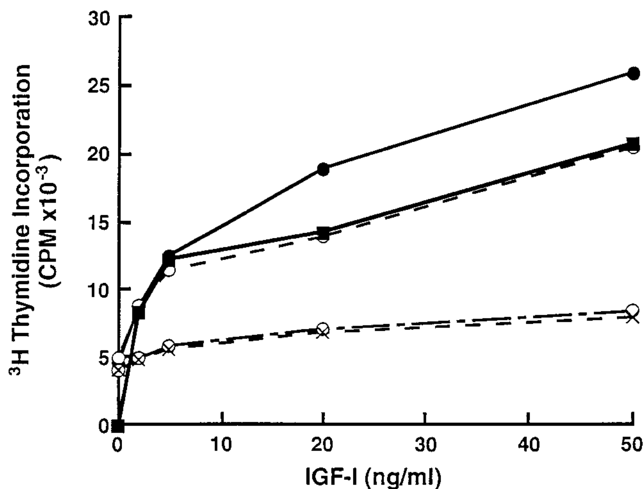

Figure 4. DNA synthesis response of cells expressing mutant forms of IGFBP-5. To determine whether the cells that were transfected with mutant forms of IGFBP-5 that resulted in less IGFBP-5 in their extracellular matrix would respond similarly to IGF-I, confluent, quiescent cultures were exposed to increasing concentrations of IGF-I for 36 h in DMEM containing 0.2% PPP. After 36 h, [^{3}H]thymidine incorporation into DNA was determined as described in MATERIALS AND METHODS. Each point represents the mean of triplicate determinations. The cultures expressing the wild-type IGFBP-5 (●—●) responded to IGF-I better than the mock-transfected cultures (■—■). Those synthesizing the R201A/K202N/K206N/K208N (○—○) mutant responded equally well. In contrast, the cultures synthesizing the K202A/K206A/K207A (○-··-○) or K211N/R214A/K217N/R218A (×---×) mutants had attenuated responses to IGF-I. The experiment was repeated three times with similar results.

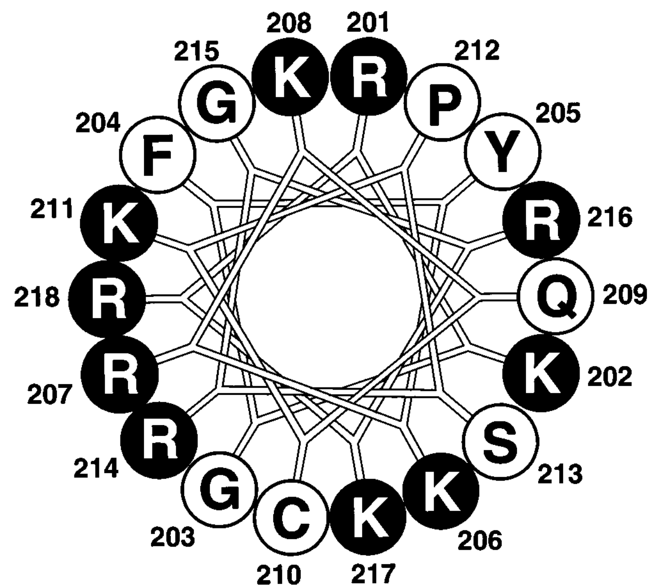

Figure 5. Helical wheel alignment of the region of IGFBP-5 between amino acids 201 and 218. The basic residues are shown in black.

control cultures (our unpublished results). The growth responses of two of the transfected cultures (those expressing the R201A/K202N/K206N/R208N and the K211N/R214A/K217A/R218A mutants) were also determined. IGF-I (50 ng/ml) increased cell number from 4908 ± 611 (n = 4) to 8166 ± 885 cells in the R201A/K202N/R206N/R208N-expressing cultures and from 4891 ± 869 (n = 4) 5641 ± 708 cells in the K211N/R214A/K217A/R218A-expressing cultures.

DISCUSSION

These findings show that IGFBP-5 binds to pSMC/ECM and that specific basic amino acids within the region between amino acid positions 201 and 218 mediate ECM binding. This region of IGFBP-5 contains 10 basic amino acids, and several of these have been shown to be important for its binding to fibroblast ECM and to heparan sulfate–containing proteoglycans (Arai *et al.*, 1996b; Parker *et al.*, 1996). Our previous studies had shown that residues 206, 207, 214, 217, and 218 appeared to be the most important for binding to fibroblast ECM. Therefore, these studies focused on the importance of those residues. The results show that the basic amino acids R207 and R214 appear to be very important for binding to pSMC ECM. Two mutants that each contained substitutions at position 207 and a mutant that contained a single substitution at 214 had a significant reduction in ECM binding. In contrast, mutants containing neutral substitutions at positions 201, 202, 206, 208, and 211 had no decrease in ECM binding, and a mutant with substitution for positions K217 and R218 showed a minimal reduction. Taken together, the findings suggest that R207 and R214 are the most important determinants of ECM binding and that the other basic residues are not important or contribute minimally.

Analysis of IGFBP-5 abundance in ECM prepared from transfected pSMCs that constitutively expressed the IGFBP-5 mutants confirmed the importance of the basic amino acids at positions 207 and 214. The two mutants containing alterations in these residues showed major reductions in IGFBP-5 content in the ECM, whereas a mutant containing four substitutions at positions 201, 202, 206, and 208 had only a 14% reduction. This suggests that the presence of these basic residues is important for IGFBP-5 incorporation into the ECM as it is synthesized de novo.

Helical wheel analysis of the 201–218 region of IGFBP-5 shows that the amino acids in positions 207, 211, 214, and 218 align asymmetrically on one side of the helical wheel (Cardin and Weintraub, 1989; Pratt *et al.*, 1992) (Figure 5). Of note is the observation that the amino acids in positions 207 and 214 are present in this cluster. In contrast, none of the basic amino acids in the four-point mutant with substitutions at posi-

tions 201, 202, 206, and 208 are within this cluster, and this mutant had a minimal alteration in ECM binding. This suggests that the helical wheel analysis of the spatial alignment of the basic residues that are required for binding may be predictive of the optimum charged amino acid alignment that mediates the binding of IGFBP-5 to ECM. Our results do not definitively prove that some residues outside this motif do not contribute to binding, although the contribution of residues such as K217 must be minimal.

Previously we showed that when IGFBP-5 is layered onto fibroblast ECM, its affinity for IGF-I is markedly reduced, and when fibroblasts are plated on a substratum that has been enriched in IGFBP-5, their cellular growth response to IGF-I is markedly enhanced (Jones *et al.*, 1993a). The results of this study extend that observation in two important ways. First, using transfected cultures that are constituitively synthesizing IGFBP-5, we show that cultures expressing the mutant forms that have reduced ECM affinity have less IGFBP-5 deposited in the ECM. Second, we show that these cultures have significantly reduced DNA synthesis responses to IGF-I. In contrast, the cultures expressing the R201A/K202N/K206N/R208N mutant have a minimal reduction in the amount of IGFBP-5 in their ECM and respond to IGF-I in a manner similar to the mock-transfected cells. The cultures that were expressing native IGFBP-5 constituitively had increased IGFBP-5 in their ECM and an enhanced DNA synthesis response to IGF-I. Therefore, there appears to be a relationship between the amount of IGFBP-5 within the ECM and the cellular DNA synthesis response to IGF-I. Because mutagenesis does not result in a change in the affinity in these mutants for IGF-I (Arai *et al.*, 1996b), we conclude that the reduction in IGFBP-5 binding to ECM results in a reduction in ECM-associated IGF-I (Parker *et al.*, 1996) and that this reduction in IGF-I and IGFBP-5 content leads to an attenuation of IGF-I actions.

Resistance or sensitivity of IGFBP-5 to proteolysis is also an important parameter of pSMC responsiveness (Imai *et al.*, 1997). IGFBP-5 in the ECM is resistant to proteolysis and has a major reduction in its affinity for IGF-I (Jones *et al.*, 1993a). In contrast, intact IGFBP-5 in extracellular fluids has a high affinity for IGF-I, and, if a 4:1 molar excess of intact IGFBP-5 to IGF-I is present, it can inhibit IGF-I interaction with its receptor and cellular responsiveness to IGF-I (Imai *et al.*, 1997). However, IGFBP-5 is cleaved in both pSMC and fibroblast culture media (Nam *et al.*, 1994; Duan *et al.*, 1996), and the fragments that are generated bind IGF-I with very low affinity. Therefore, IGFBP-5 may function to enhance IGF-I actions if there is a high concentration of intact, low-affinity IGFBP-5 in the ECM and a minimal amount of intact, high-affinity IGFBP-5 in the extracellular fluid. This suggests that proteolysis high-affinity IGFBP-5 in the interstitial fluid is also a major determinant of pSMC responsiveness.

IGFBP-5 is unique among members of the IGFBP family for its capacity to adhere to ECM. When IGFBP-1, -2, and -4 are added exogenously to fibroblast ECM, no binding can be detected (Jones *et al.*, 1993a). In the case of IGFBP-2, binding is detectable if an excess of IGF-I is added simultaneously (Arai *et al.*, 1996a). IGFBP-3 binds to fibroblast ECM but with at least 20-fold lower affinity compared with IGFBP-5 (Jones *et al.*, 1993a; Imai *et al.*, 1997). Because IGFBP-3 contains the same amino acid sequence that is present in the 201–218 region of IGFBP-5, the presumed explanation for this difference is that this region of IGFBP-3 is not surface exposed. IGFBP-3 also has a much lower affinity for heparan sulfate–containing glycosaminoglycans compared with IGFBP-5, and this may account for some of its reduced binding to ECM (Arai *et al.*, 1996b). Because ECM binding of IGFBP-5 appears to be an important component of the cellular response to IGF-I, and connective tissue cells, such as fibroblasts and osteoblasts, have abundant IGFBP-5 within their ECM, this may account for part of their IGF-I responsiveness compared with cell types that do not have this property.

The specific components of pSMC ECM that bind to IGFBP-5 have not been determined. For fibroblast ECM we have shown that tenascin (Imai *et al.*, 1997), type IV collagen (Jones *et al.*, 1993a), and plasminogen activator inhibitor-1 all bind with IGFBP-5 high affinity. Undoubtedly, other heparan sulfate–containing proteoglycans that are present in fibroblast ECM will be shown to bind this protein. Two specific components of pSMC ECM (thrombospondin and osteopontin) have been preliminarily reported to bind to IGFBP-5. These proteins are abundant components of the ECM within atherosclerotic lesions (Giachelli *et al.*, 1993; Borstein and Sage, 1994). Therefore, they have the potential to focally concentrate IGF-I and IGFBP-5 within the lesion ECM.

Several other growth factors have been shown to associate with ECM either by binding it directly or indirectly through binding to other ECM proteins (Gordon *et al.*, 1987; Yayon *et al.*, 1991; Gitay-Goren *et al.*, 1992; Nam *et al.*, 1997). In several cases, this association is required for growth factor action or for potentiating growth factor activity (Roberts *et al.*, 1988; Yayon *et al.*, 1991; Lopez *et al.*, 1993). Specific examples that meet these criteria, but are not identical to the IGF–IGFBP-5 system, include FGF association with heparin sulfate proteoglycans in ECM and TGF-β association with β-glycan, a cell surface–associated proteoglycan. In both cases, growth factor association with the proteoglycans facilitates receptor interaction (Gordon *et al.*, 1987; Yayon *et al.*, 1991). Growth factors that interact in this way with these types of extracellular molecules have been termed crinopectins (Feige

and Baird, 1995). Our data show that the IGF-I–IGFBP-5 interaction fulfills the criteria to be termed type I crinopectin interaction.

In summary, we have determined the specific amino acids in IGFBP-5 that are necessary for IGFBP-5 binding to pSMC/ECM. Synthesis of IGFBP-5 needs to remain high enough to maintain a critical level of low-affinity IGFBP-5 in the ECM to act as a reservoir for IGF-I, and a loss of ECM-associated IGFBP-5 results in reduced IGF-I response. In contrast, if a high concentration of intact IGFBP-5 is present in the media, it inhibits IGF-I response (Imai *et al.*, 1997). Therefore, the factors that determine ECM association and proteolysis of IGFBP-5 in interstitial fluids are important determinants of IGF-I actions.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Aaron Garmong. We also thank George Mosley for help in preparing the manuscript. This work was supported by grant HL 56580 from National Institutes of Health.

REFERENCES

Arai, A., Busby, W.H., and Clemmons, D.R. (1996a). Binding of insulin-like growth factor I or II to IGF binding protein 2 enables it to bind to heparin or extracellular matrix. Endocrinology *137*, 4571–4575.

Arai, T., Clarke, J., Parker, A., Busby, W.H., and Clemmons, D.R. (1996b). Effect of substitution of specific amino acids in insulin-like growth factor binding protein-5 on heparin binding and its change in affinity for IGF-I in response to heparin. J. Biol. Chem. *271*, 6099–6106.

Blat, C., Villaudy, J., and Binoux, M. (1994). In vivo proteolysis of serum insulin-like growth factor binding protein-3 results in minimal availability of IGF to target cells. J. Clin. Invest. *93*, 2286–2290.

Borstein, P., and Sage, E.H. (1994). Thrombospondins. Methods Enzymol. *245*, 62–85.

Camacho-Hubner, C., Busby, W.H., McCusker, R.H., Wright, G., and Clemmons, D.R. (1992). Identification of the forms of insulin-like growth factor binding proteins produced by human fibroblasts and the mechanisms that regulate their secretion. J. Biol. Chem. *267*, 11949–11956.

Cardin, A.D., and Weintraub, H.J.R. (1989). Molecular modeling of protein-glycosaminoglycan interactions. Arteriosclerosis *9*, 21–32.

Clemmons, D.R., and Gardner, L.I. (1990). A factor contained in plasma is required for IGF binding protein-1 to potentiate the effect of IGF-I on smooth muscle cell DNA synthesis. J. Cell Physiol. *145*, 129–135.

Clemmons, D.R., and Van Wyk, J.J. (1985). Evidence for a functional role of endogenously produced somatomedin-like peptides in the regulation of DNA synthesis in cultured human fibroblasts and porcine smooth muscle cells. J. Clin. Invest. *75*, 1914–1918.

Dong, Y., Skoulteki, A., and Pollard, J. (1993). Efficient DNA transfection of quiescent mammalian cells. Nucleic Acids Res. *21*, 171–1772.

Duan, C., Hawes, S., Prevette, T., and Clemmons, D.R. (1996). Insulin-like growth factor-I (IGF-I) stimulates IGF binding protein-5 synthesis through transcriptional activation of the gene in aortic smooth muscle cells. J. Biol. Chem. *271*, 4280–4288.

Feige, J.J., and Baird, A. (1995). Crinopexy: extracellular regulation of growth factor action. Kidney Int. *49*, s15–s18.

Giachelli, C.M., Bae, N., Almeida, M., Denhardt, D.T., Alpers, C.E., and Schwartz, S.M. (1993). Osteopontin is elevated during neointima formation in rat arteries and is a novel component of human atherosclerotic plaques. J. Clin. Invest. *92*, 1686–1696.

Gitay-Goren, H., Soker, S., Vlodavsky, I., and Nuefeld, G. (1992). The binding of vascular endothelial cell growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. J. Biol. Chem. *267*, 6093–6098.

Gordon, M.Y., Riley, G.P., Watt, S.M., and Graves, M.F. (1987). Compartmentalization of hematopoetic growth factor (GMCSF) by glycosaminoglycans in bone marrow microenvironment. Nature *326*, 403–405.

Guidice, L.C., Farrell, E.M., Pham, H., Lamson, F.G., and Rosenfeld, R.G. (1990). Insulin-like growth factor binding proteins in maternal serum throughout gestation and in the puerperium. J. Clin. Endocrinol Metab. *71*, 806–816.

Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S., and Binoux, M. (1986). Analysis of serum insulin-like growth factor binding proteins using Western blotting: use of the method for titration of the binding proteins and competitive binding studies. Anal. Biochem. *154*, 138–143.

Imai, Y., Busby, W.H., Smith, C.E., Clark, J.B., Horvitz, G.D., Rees, C., and Clemmons, D.R. (1997). Protease-resistant form of insulin-like growth factor-binding protein 5 is an inhibitor of insulin-like growth factor-I actions on porcine smooth muscle cells in culture. J. Clin. Invest. *100*, 2596–2605.

Jones, J.I., and Clemmons, D.R. (1995). Insulin like growth factor and their binding proteins: biologic actions. Endocr. Rev. *16*, 3–34.

Jones, J.I., Gockerman, A., Busby, W.H., Camacho-Hubner, C., and Clemmons, D.R. (1993a). Extracellular matrix contains insulin-like growth factor binding protein-5: potentiation of the effects of IGF-I. J. Cell Biol. *121*, 679–687.

Jones, J.I., Gockerman, A., Busby, W.H., Wright, G., and Clemmons, D.R. (1993b). Insulin-like growth factor binding protein-1 stimulates cell migration and binds to the $\alpha 5\beta 1$ integrin by means of its Arg-Gly-Asp sequence. Proc. Natl. Acad. Sci. USA *90*, 10553–10557.

Kingston, R. E. (1994). Introduction of DNA into mammalian cells. In: Current Protocols in Molecular Biology, ed. F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl, New York: Wiley-Interscience, 9.1.1–9.1.4.

Lopez, C.F., Wrena, J.L., and Massague, J. (1993). Betaglycan presents ligand to TGF-B signalling receptor. Cell *73*, 1345–1444.

McCusker, R.H., Campion, D.R., and Clemmons, D.R. (1988). The ontogeny and regulation of a 31,000 M_r insulin-like growth factor/somatomedin (IGF) binding protein in fetal porcine and plasma sera. Endocrinology *122*, 1–2079.

Nam, T.J., Busby, W.H., and Clemmons, D.R. (1994). Human fibroblasts secrete a serine protease that cleaves insulin-like growth factor binding protein-5. Endocrinology *135,* 1385–1391.

Nam, T.J., Busby, W.H., and Clemmons, D.R. (1997). Insulin-like growth factor binding protein-5 bound to plasminogen activator inhibitor I. Endocrinology *138*, 2972–2998.

Parker, A., Busby, W.H., and Clemmons, D.R. (1996). Identification of the extracellular matrix binding site for insulin-like growth factor binding protein-5. J. Biol. Chem. *271*, 13523–13529.

Parker, A., Gockerman, A., Busby, W.H., and Clemmons, D.R. (1995). Properties of an insulin-like growth factor binding protein-4 protease that is secreted by smooth muscle cells. Endocrinology *135*, 2470–2476.

Pratt, C.W., Whinna, H.C., and Church, F.C. (1992). A comparison of three heparin-binding serine proteinase inhibitors. J. Biol. Chem. *267*, 8795–8801.

Rechler, M.M. (1993). Insulin like growth factor binding proteins. Vitam. Horm. *47*, 1–114.

Roberts, R., Gallaher, J., Spooncer, E., Allen, T.D., Bloofield, F., and Dexter, T.M. (1988). Heparan sulfate bound growth factors: a mechanism or stromal cell mediated hematoapoesis. Nature *332*, 376–378.

Ross, R. (1971). The smooth muscle cell: growth of smooth muscle in cultures and formation of elastic fibers. J. Cell Biol. *50*, 172–186.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Sequencing by the Sanger dideoxy-mediated chain termination method. In: Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 13.42–13.53.

Yayon, A., Klagsbrun, M., Esko, J.D., Leder, P., and Ornitz, D.M. (1991). Cell surface heparin-like molecules are required for binding of basic fibroblast growth factor to its high affinity receptor. Cell *64*, 841–848.