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Identification of the Extracellular Matrix Binding Sites for Insulin-like Growth Factor-binding Protein 5*

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Fibroblast extracellular matrix (ECM) contains two forms of insulin-like growth factor-binding proteins (IGFBPs), IGFBP-3 and IGFBP-5. These studies were undertaken to identify the regions within IGFBP-5 that mediate its binding to fibroblast ECM. Synthetic peptides were prepared that were homologous with two regions of basic amino acids within IGFBP-5 (Arg^{201}-Arg^{218} and Ala^{131}-Thr^{141}). Increasing concentrations of both peptides competed with IGFBP-5 for binding to ECM but the Arg^{201}-Arg^{218} peptide was more potent. Mutagenesis was used to define the effect of substituting for these basic residues on ECM binding. Substitution for two peptide B residues K134A and R136A reduced binding by 40%. Substitution of a single basic residue within the peptide A region (K211N) reduced binding to ECM by 49%. Substitution for K211N, K134A, and R136A reduced binding by 52%. More extensive substitutions in the peptide A region, *e.g.* K211N,R214A,K217A,R218N, resulted in a greater (*e.g.* 88%) decrease. The positional location of basic residues appeared to be more important than the total number of substitutions since the mutant K202N,K206A,R207A had a 79% reduction in ECM binding. Two basic regions of IGFBP-5 contribute to its binding to ECM, but the region containing amino acids 201–218 has a greater contribution. ECM binding is mediated by charged residues and acts to stabilize IGFBP-5 by protecting it from proteolysis.

Connective tissue cells are embedded in a three-dimensional extracellular matrix (ECM).[1] Cellular attachment to the ECM results not only in anchorage dependence but also contributes to changes in cell shape, migration, and division (1, 2). Insulin-like growth factor binding proteins (IGFBPs) are soluble proteins that are present in extracellular fluids (3, 4). In recent studies, we have determined that two forms of IGFBPs, IGFBP-3 and IGFBP-5, are localized in fibroblast ECM and that IGFBP-1, -2, and -4 do not bind to fibroblast ECM (5). The association of IGFBP-5 with ECM results in a functional difference in fibroblast responsiveness to IGF-I. Specifically, when IGFBP-5 is ECM-associated, its affinity for IGF-I is lowered by approximately 8-fold (5). This lowering of its affinity may allow IGF-I to be more readily accessible to cell surface receptors since the addition of IGFBP-5 plus IGF-I results in a 60% increase in fibroblast growth in 48 h, whereas IGF-I alone results in a 31% increase. If type IV collagen is used in place of ECM, the fibroblast growth response to IGFBP-5 plus IGF-I is enhanced by 52%. Since binding of IGFBP-5 to ECM also protects it from degradation (5, 6), IGFBP-5 localization in ECM may provide a mechanism for concentrating IGF-I in discrete areas making it available to stimulate a proliferative response.

Increasing the salt concentration in the incubation buffer results in decreased binding of IGFBP-5 to fibroblast ECM (5). This suggests that the binding is ionic and not hydrophobic. Furthermore, IGFBP-5 binds to glycosaminoglycans, and glycosaminoglycan binding reduces the affinity of IGFBP-5 for IGF-I (7). Since glycosaminoglycans are present in ECM and protein binding to glycosaminoglycans is often ionic, this suggests that charged amino acids in IGFBP-5 are important for ECM binding. Unlike IGFBP-1, -2, -4, and -6, IGFBP-3 and -5 contain a region near the carboxyl terminus of the molecule in which 10 of 18 amino acids are basic (8). IGFBP-5 also contains another highly basic region in its central core region in which six of seven consecutive amino acids are basic (9). In order to determine if the NaCl-inhibitable binding of IGFBP-5 to ECM is mediated through these basic regions, we synthesized two peptides corresponding to these regions and used them to study the IGFBP-5 interaction with ECM. In addition, we used *in vitro* mutagenesis to convert several charged residues within these two regions of native IGFBP-5 to neutral ones in order to determine the effect of these substitutions on ECM binding.

EXPERIMENTAL PROCEDURES

Preparation of Extracellular Matrix—Human fetal dermal fibroblasts (GM-00010) were purchased from Coriell Institute, Camden, NJ and were maintained in Eagle's minimum essential media (Hazelton Systems, Denver, PA) supplemented with 10% calf serum (Colorado Serum Co., Denver), 100 μg/ml pyruvate, 30 μg/ml asparagine, 21 μg/ml serine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). Cells were grown on two types of culture dishes depending upon the purpose of the experiment. If the purpose was to compete with endogenously synthesized IGFBP-5, the cells were plated on 24-well plates, (Falcon No. 3047, Falcon Division, Becton Dickinson Laboratories, Rutherford, NJ). If the binding of exogenously added IGFBP-5 was to be tested, the cells were plated on positively charged 24-well plates (Primaria, Falcon) and grown for 7–10 days to confluence. Primaria plates were used in order to minimize nonspecific binding of IGFBP-5 to the dishes. The cells that were plated on Primaria plates were maintained in 10% fetal bovine serum (Hyclone Laboratories, Logan, UT) rather than calf serum because this was shown to limit the amount of endogenously synthesized IGFBP-5 that was associated with ECM (5). The ECM was prepared as described previously (5). The plates were kept at 4 °C during the preparation of ECM and binding assays. When the cultures reached confluency, the medium was aspirated and each well was rinsed twice with PBS. The cellular membranes were removed by incubation for 5 min in 0.5% Triton X-100 (Sigma) in PBS, pH 7.4. The adherent nuclei and cytoskeletal proteins were removed by incubation for 5 min in 25 mM ammonium acetate, pH 9.0 (10). The ECM which remained on the plates was rinsed twice in PBS and used as a substratum for binding experiments.

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[1] The abbreviations used are: ECM, extracellular matrix; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; PAGE, polyacrylamide gel electrophoresis; CHO, Chinese hamster ovary; 3,4-DCI, 3,4-dichloroisocoumarin; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; RSV, Rous sarcoma virus; FGF, fibroblast growth factor; TGF, transforming growth factor.

Western Ligand Blots and Immunoblots—Fibroblast ECM was prepared as above and scraped from the plates into Laemmli sample buffer (containing 3% SDS). Samples of the ECM preparations were heated to 60 °C for 10 min, and the ECM proteins were resolved by SDS-PAGE (using 12.5% gels) and transferred to polyvinylidene difluoride membranes (Immobilon, Millipore Corp.). For experiments done under reducing conditions, 0.1 M dithiothreitol (Sigma) was added and the solution was heated to 100 °C for 10 min. Following electrophoresis and Western blotting, the membranes were probed with ^{125}I-IGF-I (650,000 cpm in 5 ml). The probing and transfer buffers were as described previously (11). Autoradiographs were prepared by exposing the filters to hyperfilm MP, (Amersham Corp.). The autoradiographic signal intensities were determined by scanning densitometry using a Hoefer GS-300 scanner (Hoefer Scientific Instruments, San Francisco, CA). In some experiments, image intensity was also quantified using a PhosphorImager, model 455, Molecular Dynamics, Sunnyvale, CA. ^{125}I-IGF-I was iodinated to a specific activity of 125 μCi/μg and was a gift of Louis E. Underwood, University of North Carolina. For immunoblotting, a 1 to 500 dilution of polyclonal antiserum to human IGFBP-5 was incubated overnight at room temperature as described previously (5), and the immunoblots were developed for 3 h using a sheep anti-guinea pig IgG-AP conjugate (Boehringer Mannheim) using the manufacturer's recommended procedure. This antiserum is specific for IGFBP-5 and has <0.5% cross-reactivity with other forms of IGFBPs.

Preparation of Human IGFBP-5 and IGFBP-3—Human IGFBP-5 was purified to homogeneity from conditioned media from Chinese hamster ovary (CHO) cells (ATCC, Rockville, MD) that had been stably transfected (5). The IGFBP-5 cDNA was inserted into an expression plasmid (pNUT) obtained from Richard Palmiter, University of Washington, Seattle, as described previously (6). The human IGFBP-5 secreted by the transfected CHO cells was purified using a previously described method and was indistinguishable from IGFBP-5 that had been purified from conditioned medium obtained from a human glioblastoma cell line (ATCC, 1690) as determined by SDS-PAGE with silver staining, ligand blot, immunoblot, and the determination IGF binding affinity (6). Recombinant human IGFBP-3 that had been purified to homogeneity from conditioned medium obtained from transfected CHO cells was a gift from Genentech, Inc., South San Francisco, CA.

Binding of Purified IGFBP-5 or IGFBP-3 to ECM—The ECM was prepared from GM000-10 fibroblasts that had been grown to confluence on Primaria (positively charged) 24-well plates. The ECM was rinsed with PBS, then 0.5 ml of 0.03 M sodium phosphate, 0.1% bovine serum albumin, pH 7.4, was added. IGFBP-5 (40 ng–2.0 μg/ml) or IGFBP-3 (80 ng/ml) was added directly to the incubation buffer. After an overnight incubation at 4 °C, the wells were rinsed twice with PBS, and the ECM proteins were extracted with Laemmli sample buffer containing 3% SDS. The extracts were analyzed by SDS-PAGE followed by Western ligand blotting using ^{125}I-IGF-I (11) or immunoblotting (5). In some experiments, increasing concentrations of synthetic peptides (0.045 to 10 μg/ml) were coincubated with unlabeled IGFBP-5 or IGFBP-3 (80 ng/ml) as described previously (5). To determine if the peptides could compete for binding to ECM with endogenously synthesized IGFBP-5, ECM was prepared from GM000-10 fibroblasts that had been cultured in 10% calf serum on negatively charged 24-well plates (Falcon 3047). The binding assay was performed as described previously except that no IGFBP-5 was added to the incubation buffer. To determine if IGFBP-5 fragments that were detected in the ECM in the presence of peptide A were due to proteolysis, serine protease inhibitors were added to the incubation buffer with IGFBP-5. The protease inhibitors included 3,4-dichloroisocoumarin (3,4-DCI) (Sigma), a peptide containing the active site of α_1-antichymotrypsin (12), and a peptide containing the active site of antithrombin III, termed PB-145 (13). After overnight incubation, the ECM was extracted and the molecular sizes of the IGFBP-5 in the ECM and incubation buffer were estimated by immunoblotting (6).

Peptide Synthesis—Synthetic peptides were prepared by a previously described method (14). They were purified to >99% purity by reverse-phase HPLC. Two additional forms of one peptide containing amino acid substitutions for charged residues at positions 207 and 211 were also synthesized. A 14-amino acid synthetic peptide corresponding to the active site of the serine protease inhibitor, α_1-antichymotrypsin (12), was also prepared and purified. A synthetic peptide containing the active site of antithrombin III (13) was a gift from Charles Schastein, Monsanto Inc., St. Louis, MO.

In Vitro Mutagenesis—The full-length human IGFBP-5 cDNA was cloned into *Hin*dIII-*Not*I sites of the mammalian expression plasmid pRcRSVhBP-5 which had been prepared from the plasmid pRcCMV (Invitrogen). pRcCMV contains a bacteriophage origin of replication (f1) that allows production of plasmid DNA in a single-stranded form suitable for site-directed mutagenesis. The 655-base pair cytomegalovirus promoter was deleted from the pRcCMVhBP-5 plasmid by digestion with restriction enzymes *Nru*I and *Hin*dIII. The resulting 5-kilobase linear DNA was gel-purified, and the 636-base pair Rous sarcoma virus (RSV) long terminal repeat was generated from the plasmid pREP 9 (Invitrogen). After digestion with *Xba*I, the ends of the linear DNA were blunted with T4 DNA polymerase. Digestion with *Hin*dIII produced a fragment containing RSV long terminal repeat with ends compatible for directional cloning into *Nru*I-*Hin*dIII-digested pRchBP-5. The resulting fragment was gel-purified and ligated into pRchBP-5 using an insert to vector ratio of 6:1 and T4 DNA ligase. Following transformation of competent *Escherichia coli*, strain DH5α F′, DNA from the resulting colonies was screened for the presence of an additional 167-base pair *Eco*RI fragment in the RSV long terminal repeat containing clones.

Mutants of pRcRSVhBP-5 were generated by site-directed mutagenesis. Two nanograms of plasmid DNA was transfected into *E. coli* strain CJ236. A fresh colony of CJ236 was used to inoculate a 60-ml culture and grown to an optical density of 0.25–0.30 (600 nm) before infection with helper phage R408 at a multiplicity of infection of 10:1. The culture was grown for 5 h, and the bacteria were pelleted. Secreted phagemid particles were precipitated in 16% polyethylene glycol, 2.8 M ammonium acetate, and single-stranded phagemid DNA was isolated by adherence to glassmilk according to a protocol provided by the manufacturer (Bio 101, La Jolla, CA). Complimentary oligonucleotides containing mutagenic mismatches were synthesized by the Lineberger Comprehensive Cancer Research Center Nucleic Acids Core facility. The following sequences were used: acgggaaggttgcactgctttc, Lys^{211} → Asn; cttctttctggcgtcgttcttcactgc, Lys^{134}, Arg^{136} → Ala; gcactgctttgccgcgtagaatccattgcggtcac, Lys^{202} → Asn, Lys^{206}, and Arg^{207} → Ala; gcagatgccagccgcgcggccagcggaagggtt, Arg^{214} and Lys^{217} → Ala, Arg^{218} → Asn.

Synthetic oligonucleotides were phosphorylated with 15 units of T4 polynucleotide kinase for 1 h at 37 °C. Ten μl of the synthesis mixture was used to transform *E. coli* strain DH5αF′. Ampicillin-resistant colonies were selected, and their DNA was amplified. Sequencing of double-stranded DNA was performed by the Sequenase (U. S. Biochemical Corp.) protocol, followed by 6% polyacrylamide, Tris-borate-EDTA, urea gel electrophoresis, and autoradiography (15). The clones containing the correct sequences were then amplified, and plasmid DNA was prepared using silica gel anion exchange resin chromatography as recommended by Qiagen (Chatsworth, CA).

Transfection of Mammalian Cells—CHO K-1 cells were obtained from the Lineberger Comprehensive Cancer Center Tissue Culture Facility. The cells were maintained in α-minimum essential medium, 10% fetal calf serum, supplemented with penicillin and streptomycin. Twenty-four hours before transfection, the cells were plated into 6-well tissue culture plates at 12,000 cells/cm^2. DNA was introduced into the cells by a standard calcium phosphate precipitation procedure (16). DNA-calcium precipitate was formed by mixing 0.5 ml of 0.25 M $CaCl_2$ with 10 μg of plasmid DNA and 2 μg of $CaCl_2$ complexed DNA was added to wells containing 3 ml of medium. The plates were incubated at 37 °C for 5 h. Calcium-containing medium was removed, and medium containing 10% glycerol was applied for 3 min. The medium was replaced, and, after 48 h, the cells were trypsinized and plated in medium containing 800 μg/ml neomycin analogue, G418. Fresh G418 was reapplied every 3–4 days for 10–12 days when colonies of stably transfected cells began to appear. Colonies were isolated by cloning rings, trypsinized, and transferred to individual wells of a 24-well plate. Medium was analyzed by immunoblotting for production of IGFBP-5 after reaching confluency. Clones were maintained in 400 μg/ml G418.

Conditioned medium containing the mutants was collected and centrifuged at 10,000 × *g* for 20 min to remove cellular debris. The IGFBP-5 mutants were purified to homogeneity as described previously (6). The amount of each IGFBP-5 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. To ensure that Western ligand blotting was a valid method estimating the amount of each mutant that bound to ECM, the binding capacity and affinity of each mutant for ^{125}I-IGF-I was determined using Scatchard analysis (5). Since the affinity and binding capacity of each mutant were similar to nonmutated IGFBP-5 (Table I), an IGF binding capacity assay was used to obtain an independent estimate of the amount of each mutant and to confirm that the values obtained using the HPLC peak area method were correct. Increasing concentrations (0.1 to 20 ng/ml) of each mutant were coincubated with ^{125}I-IGF-I and the amount of bound ^{125}I-IGF-I determined as described previously (17). The concentration of each mutant was estimated by comparing the

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                                                  ↓
M V L L T A V L L L L A A Y A G P A Q S L   1
G S F V H C E P C D E K A L S M C P P S P   22
L G C E L V K E P G C G C C M T C A L A E   43
G Q S C G V Y T E R C A Q G L R C L P R Q   64
D E E K P L H A L L H G R G V C L N E K S   85
             (C)
Y R E Q V K I E R D S R E H E E P T T S E   106
M A E E T Y S P K I F R P K H T R I S E L   127
                      (D)
K A E A V K K D R R K K L T Q S K F V G G   148
  (B)
A E N T A H P R I I S A P E M R Q E S E Q   169
G P C R R H M E A S L Q E L K A S P R M V   190
P R A V Y L P N C D R K G F Y K R K Q C K   211
          (A)
P S R G R K R G I C W C V D K Y G M K L P   232
G M E Y V D G D F Q C H T F D S S N V E
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FIG. 1. **Amino acid sequence of IGFBP-5.** The amino acid sequence of human IGFBP-5 is shown beginning with the translation initiation residue and ending with the carboxyl terminus. The *arrow* denotes the beginning of the sequence of the secreted protein. The beginning of the sequences for each peptide termed A, B, C, and D are noted by the *letter in parentheses*. Seven of the eleven amino acids in peptide B are basic, and 10 of 18 are basic in peptide A whereas only 5 of 20 and 6 of 18 are basic in peptides C and D, respectively. The *numbers* indicate the amino acid position within the secreted protein. The peptide A sequence is also present in IGFBP-3.

amount of ^{125}I-IGF-I that bound to each mutant to the amount that bound to a known concentration of nonmutated IGFBP-5. For competitive binding experiments, ^{125}I-IGFBP-3 and -IGFBP-5 were prepared and purified as described previously (17). The specific activities were 27 and 16 μCi/μg, respectively. 2.5×10^5 cpm of IGFBP-3 and 1.7×10^5 cpm of IGFBP-5 plus increasing concentrations of each unlabeled protein (1.9–236 pM) or peptide A (250–16,000 pM) were added to 24-well plates containing fibroblast ECM in 0.5 ml of the binding buffer. After 14 h at 4 °C, the buffer was removed, the ECM was extracted with 0.3 N NaOH, and the bound ^{125}I-IGFBP-3 or -5 was determined. Nonspecific binding was determined by subtracting the counts/min bound in the presence of a 300 pM concentration of each protein.

RESULTS

To determine if the negatively charged regions of IGFBP-5 were ECM attachment sites, synthetic peptides containing the amino acid sequences shown in Fig. 1 (termed peptide A, B, C, or D) were incubated with IGFBP-5 and fibroblast ECM. Ligand blotting of the ECM extracts showed a dark M_r 31,000 band (Fig. 2*A*, *lane 2*) and a faint M_r 38,000 band. Immunoblotting (Fig. 3) indicated that the M_r 31,000 band that was detected was IGFBP-5 and that the M_r 38,000 band (M_r estimate corresponds to IGFBP-3 (6)) did not react with IGFBP-5 antisera. Addition of increasing concentrations of peptide A resulted in a decrease in the signal intensity of the M_r 31,000 band (Fig. 2*A*, *lanes 3–5*) and (Fig. 3, *lanes 2–4*). The addition of increasing concentrations of peptide B also resulted in competition (Fig. 2*A*, *lanes 6–8*, and Fig. 3, *lanes 5–7*). Scanning densitometry of autoradiographs obtained in 3 experiments showed that Peptide A (450 ng/ml) resulted in a 83 ± 7% reduction in band intensity and peptide B gave a 55 ± 12% decrease. Since the molar concentration of peptide B that was added was 40% greater than peptide A, this indicates that it was significantly less potent. Addition of peptide C or peptide D (4.5 μg/ml) (Fig. 2*B*) or a peptide containing the sequence of the active site of α_1-antichymotrypsin (5.0 μg/ml) (data not shown) resulted in no competition.

When increasing concentrations of either peptide A or B were incubated with fibroblast ECM that contained endogenously

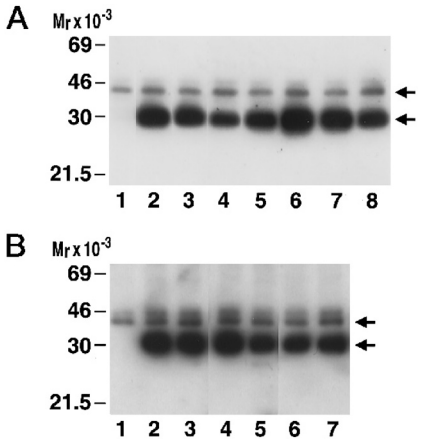

FIG. 2. **Ligand blot of IGFBP-5 in ECM in the presence of peptides A and B.** *A*, ECM was prepared from confluent fibroblast cultures as described under "Experimental Procedures." It was exposed to IGFBP-5 (80 ng/ml) and increasing concentrations of peptide A or peptide B. Following an overnight incubation, the ECM was extracted and the proteins in the extracts were separated by SDS-PAGE, transferred to Immobilon filters, and blotted with ^{125}I-IGF-I. The *arrows* denote the IGFBP-3 and IGFBP-5 bands. *Lane 1*, ECM alone; *lanes 2–8*, ECM plus IGFBP-5 (80 ng/ml); *lane 3*, peptide A (45 ng/ml); *lane 4*, peptide A (450 ng/ml); *lane 5*, peptide A (4.5 μg/ml); *lane 6*, peptide B (45 ng/ml); *lane 7*, peptide B (450 ng/ml); *lane 8*, peptide B (4.5 μg/ml). *B*, *lane 1*, ECM alone; *lanes 2–7*, ECM plus IGFBP-5 (80 ng/ml); *lane 3*, peptide C (450 ng/ml); *lane 4*, peptide C (4.5 μg/ml); *lane 5*, IGFBP-5 alone; *lane 6*, peptide D (450 ng/ml); *lane 7*, peptide D (4.5 μg/ml).

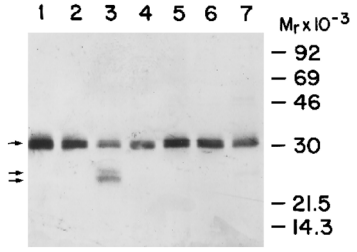

FIG. 3. **Immunoblot showing competition for binding of IGFBP-5 to ECM with peptides A and B.** ECM was prepared, and the binding assay was conducted as described in Fig. 2. Following SDS-PAGE, the proteins were transferred to Immobilon filters and immunoblotted as described under "Experimental Procedures" using a specific guinea pig anti-IGFBP-5 antiserum. *Lanes 1–7*, IGFBP-5 (80 ng/ml); *lane 2*, peptide A (45 ng/ml); *lane 3*, peptide A (450 ng/ml); *lane 4*, peptide A (4.5 μg/ml); *lane 5*, peptide B (45 ng/ml); *lane 6*, peptide B (450 ng/ml); *lane 7*, peptide B (4.5 μg/ml).

synthesized IGFBP-5, each peptide was capable of inhibiting IGFBP-5 binding to the ECM (Fig. 4). To determine if these peptides competed with IGFBP-3 for binding to ECM, human IGFBP-3 (80 ng/ml) was incubated with ECM and increasing concentrations of each peptide. As shown in Fig. 5, peptide A was significantly more potent than peptide B in competing with IGFBP-3 for binding to ECM. Scanning densitometry showed that peptide A was 20-fold more potent than peptide B. To confirm that peptide competition was specific, ^{125}I-IGFBP-5 or ^{125}I-IGFBP-3 was incubated with increasing concentrations of each unlabeled protein or peptide A. As shown in Fig. 6, the intact forms of each IGFBP were considerably more potent in competing with the iodinated ligand for binding to ECM as compared to peptide A. For IGFBP-5, a concentration of 7 pM resulted in half-maximal competition, whereas 1.5 nM peptide A was required. For IGFBP-3, half-maximal competition was obtained using 59 pM supporting the conclusion that IGFBP-5 binds to the ECM with higher affinity.

The addition of increasing concentrations of peptide A resulted in the appearance of IGFBP-5 fragments of 24 and 27 kDa in the ECM (Figs. 3 and 7*B*). In the absence of peptide A

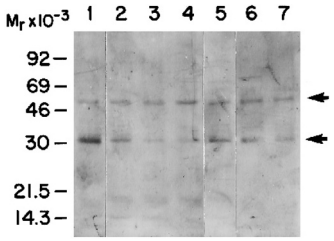

FIG. 4. **Inhibition of endogenously synthesized IGFBP-5 binding to ECM.** We have previously shown that endogenously synthesized IGFBP-5 can be detected in fibroblast ECM (5). ECM was prepared from fibroblasts that had been cultured on negatively charged plates in calf serum. The ECM was incubated with increasing concentrations of peptide A and B and the IGFBP-5 remaining in the ECM after an overnight incubation determined by immunoblotting. *Lane 1*, ECM alone; *lane 2*, peptide A (0.5 μg/ml); *lane 3*, peptide A (2.0 μg/ml); *lane 4*, peptide A (10 μg/ml); *lane 5*, peptide B (0.5 μg/ml); *lane 6*, peptide B (2.0 μg/ml); *lane 7*, peptide B (10 μg/ml).

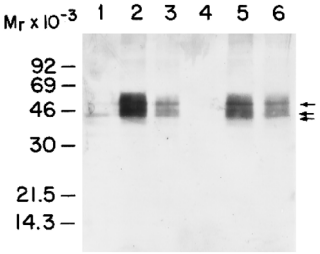

FIG. 5. **Competition for binding of IGFBP-3 to ECM.** IGFBP-3, 80 ng/ml, was incubated with ECM in the presence of increasing concentrations of peptides A and B and the ECM-bound IGFBP-3 determined by immunoblotting as described under "Experimental Procedures." *Lane 1*, ECM alone; *lanes 2–6*, IGFBP-3 (80 ng/ml); *lane 3*, peptide A (0.45 μg/ml); *lane 4*, peptide A (4.5 μg/ml); *lane 5*, peptide B (0.45 μg/ml); *lane 6*, peptide B (4.5 μg/ml).

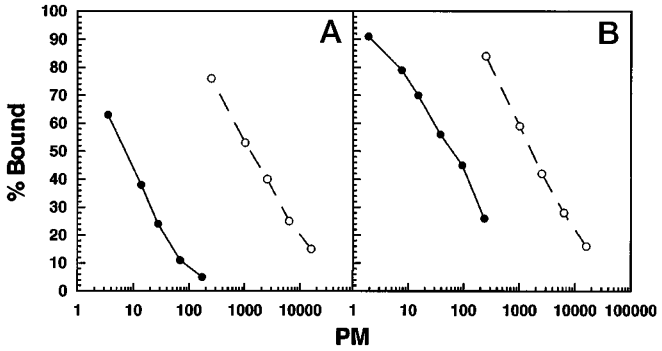

FIG. 6. *A* and *B*, competition for binding using radiolabeled IGFBP-3 and -5. Increasing concentrations of IGFBP-5 (*A*), IGFBP-3 (*B*), or peptide A (○– –○) were incubated with ^{125}I-IGFBP-3 or -5, and binding was determined as described under "Experimental Procedures." Specific binding was determined by subtracting the counts/min bound in the presence of a 300 pM concentration of each protein. The results are expressed as the percentage bound, and this was determined by dividing the counts/min bound using each concentration of unlabeled protein by the counts/min bound when ^{125}I-IGFBP-3 or -5 was added alone.

(Fig. 7, *A* and *B*, *lane 1*), these fragments could not be detected in the ECM, and minimal amounts were present in the assay buffer. Therefore, if peptide A was omitted, the binding of mutant and wild type IGFBP-5 binding to ECM did not appear to be altered significantly by proteolysis. In contrast, if peptide A was added, these fragments were detected (Fig. 7, *A* and *B*, *lane 2*). Since fibroblast-conditioned medium has been shown to contain a serine protease that cleaves IGFBP-5, we repeated the experiment with the serine protease inhibitor 3,4-DCI in the incubation buffer. 3,4-DCI inhibited the appearance of the 24- and 27-kDa fragments in ECM and in the assay buffer (Fig. 7, *A* and *B*, *lane 4*). Since the degree of reduction of intact

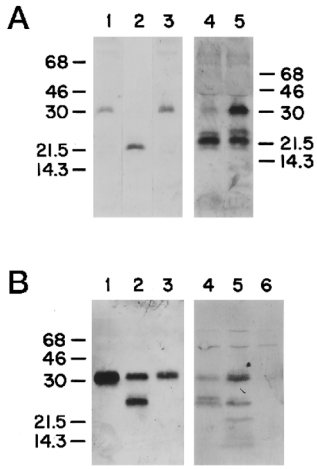

FIG. 7. *A*, proteolysis of IGFBP-5 in the binding assay buffer. The buffer was removed at the end of the binding assay, and IGFBP-5 content was determined by immunoblotting. *Lanes 1–5*, IGFBP-5 (80 ng/ml). *Lanes 2–5*, peptide A (0.45 μg/ml); *lane 3*, 3,4-DCI (5 mM); *lane 4*, α_1-antichymotrypsin (100 μg/ml); *lane 5*, PB-145 (165 μg/ml). The results show that IGFBP-5 remaining in the assay buffer is degraded. If peptide A is added, the fragments are more abundant, whereas if 3,4-DCI or PB-145 is added with peptide A, intact IGFBP-5 band intensity is increased. *B*, IGFBP-5 remaining in the ECM. Pure IGFBP-5 was incubated with ECM (*lanes 1–6*) and peptide A (*lanes 2–6*) (0.45 μg/ml). The IGFBP-5 remaining in the ECM was determined by immunoblotting. Serine protease inhibitors 3,4-DCI (5 mM) (*lane 3*), α_1-antichymotrypsin peptide (100 μg/ml) (*lane 4*), PB-145 (165 μg/ml) (*lane 5*), and heparin (10 μg/ml) (*lane 6*) were added to the incubation buffer. The ECM that was exposed to IGFBP-5 plus peptide A (*lane 2*) contains fragments of 24 and 27 kDa. The corresponding control extract of ECM exposed to IGFBP-5 only (*lane 1*) does not contain these bands. When 3,4-DCI and PB-145 but not α_1-antichymotrypsin are added with peptide A, the proteolytic fragment bands are decreased in intensity. Heparin inhibits IGFBP-5 binding to ECM.

IGFBP-5 band intensity was similar whether or not 3,4-DCI is added (Fig. 7*B*, compare *lane 2* with *lane 3*), the conclusion that peptide A competes with intact IGFBP-5 for binding and does not simply reduce the signal intensity by enhancing proteolysis appears to be valid. An antithrombin III analogue termed PB-145, partially inhibited the appearance of proteolytic fragments in the ECM and the assay buffer. A peptide containing the cleavage site of α_1-antichymotrypsin (Fig. 7, *A* and *B*) and EDTA, 5 mM (data not shown), had no effect on proteolysis. Since heparin totally inhibited IGFBP-5 binding to the ECM, its effect on proteolysis could not be determined. The findings suggest that in the presence of peptide A, a serine protease is released from the ECM into the assay buffer. IGFBP-5 is then cleaved into 24- and 27-kDa fragments which can rebind to ECM.

Mutagenesis was used to further analyze the importance of these basic residues in mediating ECM binding. Several mutants were constructed to alter charged residues within the peptide A and B regions (Table I). All of the mutants tested retained high affinity for IGF-I that was similar to wild type IGFBP-5. The effect of these mutations on ECM binding was analyzed by Western ligand blotting (Fig. 8). Immunoblotting was also used to confirm the Western ligand blotting results (Fig. 9). Substitution for two basic residues within the peptide B region K134A and R136A resulted in a 40 ± 11% decrease in ECM binding as determined by ligand blotting and scanning densitometry (Fig. 8). The coincubation of peptide B with the K134A,R136A mutant resulted in no further reduction in ECM binding (data not shown) indicating that further substitutions for basic residues within the peptide B region probably would have had little additional effect. A single substitution in the peptide A region, K211N, resulted in a 49 ± 13% decrease in

TABLE I

Binding affinity of each mutant for IGF-I and reduction in ECM binding

The affinity of each mutant for IGF-I was determined by Scatchard analysis. The fold change is the ratio of the affinity of the mutant divided by the affinity of nonmutated IGFBP-5. The stoichiometry of each mutant was determined to be 1:1. The percentage reduction in ECM binding was determined by dividing the band intensity on Western ligand blotting, as shown in Fig. 6, by the band intensity of control, nonmutated IGFBP-5. The affinity of nonmutated IGFBP-5 was 3.8×10^{10} liters/mol. Each value represents the mean ± S.D. of 3 separate experimental determinations.

Mutant forms of IGFBP-5	Fold change in IGF-I affinity	Reduction in ECM binding
		%
K211N	1.1	49 ± 13
K211N,K134A,R136A	1.1	53 ± 12
K134A,R136A	1.6	40 ± 11
K202N,K206A,R207A	1.1	79 ± 6
K211N,R214A,R217A,R218N	1.2	88 ± 7

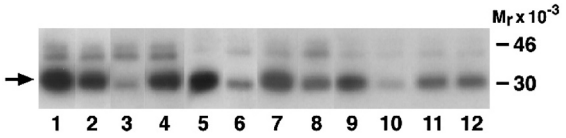

FIG. 8. **Ligand blot of binding of IGFBP-5 mutants to ECM.** Fibroblast ECM was prepared as described in Fig. 2. Each mutant (80 ng/ml) was incubated overnight with the ECM as described under "Experimental Procedures." The ECM was extracted, and IGFBP-5 binding was determined by SDS-PAGE followed by ligand blotting. The *arrow* denotes the position of the IGFBP-5 bands. *Lane 1*, IGFBP-5; *lane 2*, K211N; *lane 3*, K211N,R214A,K217A,R218N; *lane 4*, K134A, R136A; *lane 5*, IGFBP-5; *lane 6*, K202N,K206A,R207A; *lane 7*, IGFBP-5; *lane 8*, K134A,R136A,K211N; *lane 9*, IGFBP-5; *lane 10*, IGFBP-5 plus peptide A, 4.5 μg/ml; *lane 11*, plus K211A-peptide A; *lane 12*, plus K207A,K211A peptide A. Since the figure is a composite of 4 separate autoradiographs, the control lane for each separate autoradiograph that contained nonmutated IGFBP-5 is shown (*lanes 1*, *5*, *7*, and *9*).

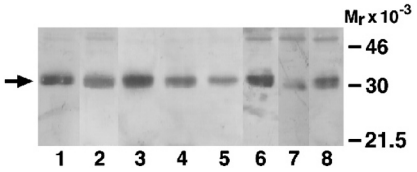

FIG. 9. **Immunoblot of binding of IGFBP-5 mutants binding to ECM.** Intact or mutant forms of IGFBP-5 (80 ng/ml) were incubated overnight with fibroblast ECM as in Fig. 6. Analysis of binding was performed by SDS-PAGE followed by immunoblotting. *Lane 1*, IGFBP-5; *lane 2*, K211N; *lane 3*, IGFBP-5; *lane 4*, K134A,R136A; *lane 5*, K202N,K206A,R207A; *lane 6*, IGFBP-5; *lane 7*, K211N,R214A, K217A,K218N; *lane 8*, K134A,R136A,K211N.

ECM binding (Fig. 7 and Table I). A mutant containing three substitutions K134A, R136A, and K211N had only a slightly greater reduction in binding (*e.g.* 53 ± 12%) than the K211N substitution. Further substitutions for basic residues in the peptide A region, *e.g.* K211N,R214A,K217A,R218N or K202N,K206A,R207A had greater effects (*e.g.* 88 ± 7% and 79 ± 6% respectively). To further analyze the specificity of the effect of peptide A, competition studies were performed using two peptides that were identical with peptide A except that they contained substitutions for $Lys^{211} \rightarrow Ala$ or Lys^{207} and $Lys^{211} \rightarrow Ala$. When compared to nonmutated peptide A, these two mutant peptides were much less effective in inhibiting the binding of IGFBP-5 to the ECM (Fig. 8). Peptide A reduced IGFBP-5 binding by 83%, but the altered peptides reduced binding by only 47 and 49%. This confirms that Lys^{211} is important for IGFBP-5 binding to ECM. To confirm these changes in ECM binding, immunoblotting was performed. The results show that the relative changes in the amount of each mutant that was associated with the ECM (Fig. 9) were similar to those obtained using ligand blotting (Fig. 8). For example, K211N,R214A,K217A,R218N-IGFBP-5 showed the greatest reduction in binding while the K211N and K134A,R136A mutants showed the least. Immunoblotting of the assay buffer for each experimental determination showed that the wild type and mutant forms of IGFBP-5 were minimally degraded (*e.g.* <10% of total), and no mutant was degraded preferentially compared to wild type IGFBP-5.

To verify these quantitative differences in ECM binding between the various mutants and nonmutated IGFBP-5, equilibrium binding assays were performed using increasing concentrations of each mutant and determining the amount of IGFBP-5 that was bound to ECM by phosphoimage analysis. As shown in Fig. 10 and Table II, when the mutants were tested over a wide range of concentrations, each had lower binding to ECM compared with wild type IGFBP-5. The mutants with substitution for 3 or 4 basic residues in the peptide A region had the greatest reduction. The reductions in ECM binding for 3 mutants K211N,K134A,R136A, K202N,K206A, R207A, and K211N,R214A,R217A,R218N as compared to nonmutated IGFBP-5 were similar to the decreases shown in the experiment in Fig. 7. The K134A,R136A mutant showed less reduction in binding to the ECM compared to the results shown in Fig. 7. When the assay buffer obtained at the completion of the incubation was analyzed by immunoblotting, there were minimal differences in the amount of fragment that was detected when the mutants and wild type IGFBP-5 were compared and the amount of fragment was consistently <10% of the total (data not shown).

DISCUSSION

These results show that two regions of IGFBP-5 (Arg^{201}-Arg^{218} and Ala^{131}-Thr^{141}) that contain several basic amino acids are important for its attachment to ECM. Peptide A was 4.1-fold more potent than peptide B in inhibiting IGFBP-5 binding to ECM and 20-fold more potent than peptide B in inhibiting IGFBP-3 binding. Since the peptide A sequence is the same within IGFBP-3 and IGFBP-5 and is not contained in the other forms of IGFBPs that do not bind to fibroblast ECM, this sequence has unique determinants for IGFBP-3 and IGFBP-5 ECM binding (5). This binding does not appear to be solely due to electrostatic interaction, since peptide B which has a charge to mass ratio that is similar to peptide A is less potent.

More definitive proof of the importance of the peptide A region in mediating attachment to ECM was provided by utilizing site-directed mutagenesis. Mutations in the peptide A region caused significant reductions in ECM binding. A single substitution K211N was effective, but a mutant containing four substitutions, K211N,R214A,K217A,R218N, had the greatest reduction. Substitution for basic residues at positions 202, 206, and 207 gave an intermediate result. These differences were noted over a wide range of IGFBP concentrations and therefore are unlikely to be due to differences in the estimation of protein concentration. Taken together, the findings strongly suggest that several basic residues within this 18-amino acid region are important for ECM binding and that the positional location of

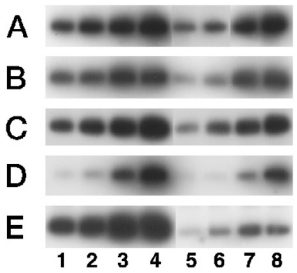

FIG. 10. **ECM binding activity of mutant forms of IGFBPs.** Increasing concentrations (80, 160, 320, and 800 ng/ml) of native IGFBP-5 (*lanes 1–4*) or mutant forms (*lanes 5–8*) were incubated with fibroblast ECM, and binding was determined as described under "Experimental Procedures." The figure shows the ligand blot band intensity for native IGFBP-5 and the form of each mutant that was used in that particular experiment. The mutants that were tested include: *A*, K134A,K136A; *B*, K211N; *C*, K134A,K136A,K211N; *D*, K202N,K206A, R207A; and *E*, K211N,R214A,K217A,R218N.

TABLE II
ECM binding of IGFBP-5 mutants

The values are expressed as the percentage of control nonmutated IGFBP-5 binding for that individual experiment. They were obtained by determining the amount of each mutant form of IGFBP-5 that bound to ECM and dividing it by the amount of nonmutated IGFBP-5 that bound in the same experiment. Binding was quantified by phosphoimage analysis, and each value represents the mean of 3 experiments. A representative experiment is shown in Fig. 10.

Mutant Form of IGFBP-5	IGFBP-5 added (ng/ml)			
	80	160	320	800
	% control IGFBP-5 binding			
K134A,R136A	76	82	76	73
K211N	55	42	55	65
K134A,R136A,K211N	41	41	47	54
K202N,K206A,R207A	38	32	29	31
K211N,R214A,K217A,R218N	5	5	10	7

these charged residues may be important.

Peptide B which contained six basic amino acids was an effective inhibitor of IGFBP-5 association with ECM but does not inhibit its binding to heparin (7). Mutagenesis of two basic residues, Lys^{134} and Arg^{136}, within the peptide B region resulted in a 25% reduction in ECM binding and competition studies using peptide B and this mutant showed no additional decrease in binding. This indicates that additional peptide B region substitutions probably would have had no additional effect. A mutant that combined the K134A,R136A substitutions with K211N showed only a slightly greater effect than the K211N substitution alone. These findings suggest the peptide B region is less important for binding to ECM.

The peptide A region of IGFBP-5 and IGFBP-3 contains the heparin binding domain *BBBXXB* where *B* represents basic amino acids and *X* represents neutral residues. This motif is present in several proteins that are known to bind to heparin (19), and it would be predicted that proteins that contain this sequence might bind to glycosaminoglycans that are present in the ECM. This conclusion is further supported by our observation that IGFBP-5 binds to heparan and heparan sulfate and that peptide A is a potent inhibitor of IGFBP-5 binding to heparin (7). In contrast, peptide D which contains the heparin binding motif *BBXB* had no effect on IGFBP-5 binding to ECM suggesting that the effect of the *BBBXXB* sequence is specific. The fact that both the K211N and the K202N,K206A,R207N substitutions resulted in decreased binding suggests that residues 206, 207, and 211 within this region are important for IGFBP-5 association with ECM. However, the K211N,R214A, R217A,R218N mutant had a greater loss of ECM binding than K211N indicating that basic residues other than those in this linear heparin binding sequence motif are also important. Further mutational analysis will be required to define the exact location of the amino acids that are required for ECM binding and whether these are the same residues that mediate glycosaminoglycan binding (7).

Previous studies have shown that the association of IGFBP-5 with ECM results in a reduction in its affinity for IGF-I and enhancement of IGF-I-stimulated cell growth. (5) Since the IGF-I binding site within IGFBP-5 is unknown, it was not clear whether mutagenesis of basic residues in peptide A or B regions of IGFBP-5 might alter its affinity for IGF-I. When we analyzed the affinity of each mutant for IGF-I, only the K134A,R136A mutant had any change and the increase (*e.g.* 1.6-fold) was minimal.

IGFBP-5 association with ECM also appears to alter its sensitivity to proteolysis. Immunoblotting shows no fragments of IGFBP-5 in fibroblast ECM, whereas a non-IGF-I-binding 22-kDa fragment is abundant in conditioned medium (6). Likewise, incubation of the 22-kDa purified IGFBP-5 fragment with ECM results in no detectable binding (6). This suggests that either that ECM-associated IGFBP-5 is resistant to proteolytic cleavage or that the fragment is completely released after proteolysis. However, competition with peptide A resulted in the appearance of larger fragments (*e.g.* 24 and 27 kDa) that were present in the binding buffer and in the ECM. Their appearance could be inhibited by the addition of a serine protease inhibitor to the incubation buffer (Fig. 7, *lane 3*) suggesting that they originate as the result of proteolytic cleavage. These findings suggest that a serine protease that can cleave IGFBP-5 is present in ECM; however, during usual assay conditions (*e.g.* 80 ng/ml IGFBP-5 added) there is not sufficient protease released to detect IGFBP-5 fragments in the incubation buffer. If more IGFBP-5 is added (*e.g.* 800 ng/ml), then the intensities of the fragment bands are increased but >90% of the IGFBP-5 is still intact and there are only minimal differences between the mutants and nonmutated IGFBP-5 in fragment abundance. This shows that the mutants are not degraded more rapidly and increased proteolysis does not account for their reduced ECM binding. This serine protease is distinct from the serine protease in fibroblast media (18) since its activity is not inhibited by α_1-antichymotrypsin or EDTA. The exact role of this ECM-associated protease in controlling IGFBP-5 abundance will require further study.

Other growth factors such as fibroblast growth factor (FGF) (20), transforming growth factor β (TGF-β) (21), interleukin 3, and granulocyte/macrophage colony stimulating factor have been shown to associate with ECM components. TGF beta binds to a glycoprotein, betaglycan (21), that is present in ECM, whereas FGF associates with heparan sulfate side chains of ECM proteins, such as heparan sulfate proteoglycan (22). Association of TGF-β, FGF, interleukin 3, and granulocyte/macrophage colony stimulating factor with ECM components has been shown to result in enhancement of the cellular responses to these growth factors (23–25). Similarly, proteases, such as tissue plasminogen activator, plasmin, or thrombin (26), as well as protease inhibitors, such as plasminogen activator inhibitor (27), have been shown to bind to specific ECM components. This suggests that systems exist for controlled release of these growth factors or their binding proteins from the ECM. Our findings suggest that the IGFBP-5/IGF-I system may be under the control of similar variables.

Our previous studies have shown that the activity of an IGFBP-5 protease that is secreted by fibroblasts is specifically inhibited by heparin and heparan sulfate (28). Since our results show that ECM proteoglycans also bind IGFBP-5, this binding may have dual functions, *e.g.* focal localization of IGFBP-5 in

ECM and protection from proteolysis. Both protection from proteolysis and conformational changes that reduce IGFBP-5 affinity are functions of ECM binding that would result in increased amounts of IGF-I being available to bind to receptors. Therefore, determining the matrix binding sites within IGFBP-5 may provide insights regarding the physiologic function of this protein in ECM and these mutants should be of help in discerning the mechanism by which ECM-sequestered IGF-I is presented to cell surface receptors.

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