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Substitutions for Hydrophobic Amino Acids in the N-terminal Domains of IGFBP-3 and -5 Markedly Reduce IGF-I Binding and Alter Their Biologic Actions*

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Insulin-like growth factor-binding protein-3 and -5 (IGFBP-3 and -5) have been shown to bind insulin-like growth factor-I and -II (IGF-I and -II) with high affinity. Previous studies have proposed that the N-terminal region of IGFBP-5 contains a hydrophobic patch between residues 49 and 74 that is required for high affinity binding. These studies were undertaken to determine if mutagenesis of several of these residues resulted in a reduction of the affinity of IGFBP-3 and -5 for IGF-I. Substitutions for residues 68, 69, 70, 73, and 74 in IGFBP-5 (changing one charged residue, Lys^{68}, to a neutral one and the four hydrophobic residues to nonhydrophobic residues) resulted in an ~1000-fold reduction in the affinity of IGFBP-5 for IGF-I. Substitutions for homologous residues in IGFBP-3 also resulted in a >1000-fold reduction in affinity. The physiologic consequence of this reduction was that IGFBP-3 and -5 became very weak inhibitors of IGF-I-stimulated cell migration and DNA synthesis. Likewise, the ability of IGFBP-5 to inhibit IGF-I-stimulated receptor phosphorylation was attenuated. These changes did not appear to be because of alterations in protein folding induced by mutagenesis, because the IGFBP-5 mutant was fully susceptible to proteolytic cleavage by a specific IGFBP-5 protease. In summary, residues 68, 69, 70, 73, and 74 in IGFBP-5 appear to be critical for high affinity binding to IGF-I. Homologous residues in IGFBP-3 are also required, suggesting that they form a similar binding pocket and that for both proteins these residues form an important component of the core binding site. The availability of these mutants will make it possible to determine if there are direct, non-IGF-I-dependent effects of IGFBP-3 and -5 on cellular physiologic processes in cell types that secrete IGF-I.

The insulin-like growth factor-binding proteins (IGFBPs)[1] are known to have high affinity for IGF-I and -II, and in most cases, the affinity of the intact proteins is greater than the IGF-I receptor (1). Therefore, IGFBPs are capable of regulating the equilibrium distribution of IGF-I and -II between that bound to the receptor and that bound to the binding proteins in solution or in extracellular matrix and on cell surfaces (2, 3). Because IGFBPs generally exist in a molar excess over IGF-I in extracellular fluids, they potentially control the amount of receptor stimulation that occurs under equilibrium conditions (4). Several investigators have been interested in the domains of the IGFBPs that mediate IGF-I and -II binding. In general, this has been analyzed by determining the affinities of various IGFBP fragments (5). Both N- and C-terminal fragments of IGFBP-3 and -5 have been shown to have detectable binding affinity for IGF-I and -II, although generally their affinities are reduced between 100- and 1000-fold compared with the native proteins (5–10). Affinities of the IGFBPs can also be modified by post-translational modifications other than proteolysis, including glycosylation and phosphorylation, but in general these studies have not led to hypotheses regarding the location of the IGF-I binding site (11–12). In addition, three-dimensional structural analysis of IGF-I and -II has enabled investigators to make predictions about the regions of IGFBPs that are likely to interact with hydrophobic and charged regions within the IGFs themselves (13–19).

The IGFBPs share a common domain organization. The highest conservation is found in the N terminus of the protein (*i.e.* the first 80 amino acids) in the C-terminal region. Twelve conserved cysteines are found in the N-terminal domain and six in the C-terminal domain. The central regions are much less well conserved and do not contain cysteines, with the exception of IGFBP-4.

It has been proposed, based on fragment analysis, that the high affinity binding site for IGFs is located in the N-terminal domain, although studies with IGFBP-2 and -3 have suggested that C-terminal domain binding components are also present (5, 20, 21). A recent study by Kalus *et al.* (6) using solution NMR demonstrated that the N-terminal domain of IGFBP-5 contained one high affinity site for IGF-I and -II binding. These investigators further determined that a hydrophobic patch (residues 49, 50, 69, 73, and 74) and a critical charged residue, lysine 68, existed within this motif that probably accounted for the binding of this fragment. Because the study by Kalus *et al.* (6) did not use the intact protein, these studies were conducted to determine if altering these residues by *in vitro* mutagenesis in the intact form of IGFBP-5 would result in reduced affinity for IGF-I and therefore whether the model of the binding pocket formulated using data obtained with the IGFBP-5 fragment was also valid for the intact protein. We further tested

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[1] The abbreviations used are: IGFBP, insulin-like growth factor-binding protein; IGF, insulin-like growth factor; PCR, polymerase chain reaction; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; pSMC, porcine aortic smooth muscle cells; DMEM, Dulbecco's modified Eagle's medium.

the importance of these specific residues and the applicability of the model by making homologous substitutions in IGFBP-3.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human IGF-I was a gift from Genentech (South San Francisco, CA). Des 1–3 IGF-I was a gift from Monsanto, Inc. (St. Louis, MO). Human dermal fibroblasts (GM-10A) were purchased from Coriell Institute (Camden, NJ). Chinese hamster ovary K1 cells were obtained from the Lineberger Comprehensive Cancer Tissue Culture Facility (Chapel Hill, NC). The mammalian expression vector pcDNA 3.1 and TOPO TA cloning kit were obtained from Invitrogen (Carlsbad, CA). Strataclean resin was purchased from Stratagene (La Jolla, CA). The antisera that had been prepared using human IGFBP-3 and IGFBP-5 have been described previously (22). The antiphosphotyrosine antibody (PY99) and an antibody against the β subunit of the IGF-I receptor (C-20) were obtained from Santa Cruz Biotechnology Co. (Santa Cruz, CA).

Construction of Plasmids That Express Native and Mutant Forms of IGFBPs—The preparation of expression vector pRcRSV-IGFBP-5 that contains a full-length human IGFBP-5 cDNA has been described previously (23). Mutant IGFBP-5 cDNA was prepared in pRcRSV-IGFBP-5 as described previously (23). Single-stranded phagemid-DNA was generated from pRcRSV-IGFBP-5, and mutations were introduced using synthetic oligonucleotides as substrates for antisense DNA synthesis. The following complementary oligonucleotide was used to mutate Lys^{68}, Pro^{69}, Leu^{70}, Leu^{73}, and Leu^{74} to Asn, Gln, Gln, Gln, and Gln: ccc gcg gcc gtg ctg ctg ggc gtg ctg ctg att ctc ctc gtc ctg.

A human IGFBP-3 cDNA was cloned into pcDNA 3.1 vector (pcDNA3-IGFBP-3) from pNUT-IGFBP-3 that contained a full-length human IGFBP-3 cDNA (22). Arg^{69}, Pro^{70}, Leu^{71}, Leu^{74}, and Leu^{75} of IGFBP-3 were mutated to Ser, Ala, Ser, Gln, and Gly using oligonucleotides that contained the desired substitutions and restriction enzyme cleavage sites as primers for PCR. The 5′-half of the IGFBP-3 sequence that included the start codon of IGFBP-3 to nucleotide 338 was amplified using a 5′-primer that had the wild type sequence and a 3′-primer that had the substituted sequence and an *Eco*RV site (primer A). The 3′-prime portion of the IGFBP-3 sequence that spans nucleotide 286 to the 3′-end of IGFBP-3 was amplified using a 5′-primer that contained the mutated sequence and a Msc site and a 3′-primer that contained the wild type sequence (primer B). PCR was performed using pcDNA3.1-IGFBP-3 as a template using an Advantage PCR kit (CLONTECH, Palo Alto, CA). The PCR products were then purified by adding 5 μl of Strataclean resin followed by centrifugation. The supernatant was digested with *Eco*RV (5′-fragment) or Msc (3′-fragment) for 3 h at 37 °C. The digests were run on 2% agarose gel (NuSieve), and the bands that corresponded to the expected molecular weights were excised, frozen, and thawed. The material that was recovered from the thawed gel was combined (5′-fragment and 3′-fragments) and ligated using T4 DNA ligase and then incubating at room temperature overnight. The ligation mixture was then amplified by PCR using 5′-primer A and 3′-primer B to create the full-length IGFBP-3 with the mutated sequences. This PCR product was cloned into pcDNA 3.1 using a TOPO TA cloning kit according to the manufacturer's protocol. After selection, the plasmids thus created were sequenced, and those containing the correct sequences were amplified and purified as described previously (24).

Preparation of Native IGFBP-3 and IGFBP-5 and the IGFBP Mutants from Chinese Hamster Ovary Cells That Were Expressing These IGFBPs—Chinese hamster ovary (K1) cells were transfected with pRcRSV that contained cDNA from either native or mutant IGFBP-5, pNUT-IGFBP-3 (22), or the pcDNA3.1 mutant IGFBP-3 using poly-L-ornithine (25). Positive clones were selected with 800 μg/ml G418 as described previously (23). The native forms of recombinant IGFBPs were purified from the conditioned medium of the transfected Chinese hamster ovary cells as described previously (3, 22). The mutant form of IGFBP-5 was purified by phenyl-Sepharose and IGF-I affinity chromatography (to remove IGFBP-4) as described previously (22). The material that was excluded from the column was equilibrated with 50 mM NaH_2PO_4, 2 mM EDTA, 0.1 M NaCl, pH 7.0, applied to an heparin-Sepharose affinity column and eluted in the same buffer containing 1.0 M NaCl. This fraction was purified to homogeneity by reverse phase HPLC as described previously (26). The IGFBP-3 mutant was purified by phenyl-Sepharose as described (22), then equilibrated with 50 mM NaH_2PO_4, 2 mM EDTA, 25 mM NaCl, applied to a heparin-Sepharose column, and eluted in the same buffer containing 1.0 M NaCl. The eluate was further purified by lectin affinity chromatography using wheat germ agglutinin. The active fractions were eluted using 0.5 M *N*-acetyl-D-glucosamine and purified further by HPLC (26). Purification was monitored by immunoblotting using specific, high affinity IGFBP-3 and -5 antisera. Purity was proven by SDS-polyacrylamide gel electrophoresis with silver staining, and the amount of each protein was determined.

^{125}I-IGF-I Binding Assay—The affinity of native and mutant IGFBPs for ^{125}I-IGF-I was determined as described previously (19). Briefly, 20,000 cpm of ^{125}I-IGF-I was incubated with 0–100 ng of native or mutant IGFBPs in 0.25 ml of 0.1 M HEPES, 44 mM sodium phosphate, 0.1% Triton X-100, 0.1% BSA, 0.2% sodium azide, pH 6.0, for 1 h at room temperature. Bound and free IGF-I were separated by precipitation using 12% polyethylene glycol (M_r 8000–12,000). Scatchard analysis was also performed. Duplicate tubes were incubated with increasing concentrations of IGF-I (0.053–1.33 M), and bound and free IGF-I were separated by precipitation using 12% polyethylene glycol (M_r 8000–12,000) as described previously (27). The data were then analyzed according to the method of Scatchard, and the results that were obtained with native IGFBP-3 or -5 were compared with those obtained using the mutants.

Ligand Blotting—Ten or forty nanograms of IGFBPs were mixed with Laemmli sample buffer in the absence of a reducing agent and incubated at 65 °C for 10 min. The samples were then separated on a 12.5% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride membrane as described previously (24). The membrane was blocked with 10 mM Tris, 150 mM NaCl, 0.5 mg/ml sodium azide, pH 7.4, plus 3% Nonidet P-40 for 15 min and then washed with this buffer containing 1% BSA for 2 h, followed by this buffer plus 0.1% Tween 20 for 10 min at 4 °C. The membrane was then incubated with 100,000 cpm/ml ^{125}I-IGF-I in this buffer (total volume 4 cc) containing 1% BSA and 0.1% Tween 20 for 20 h at 4 °C. The membrane was then washed three times with this buffer containing 0.1% Tween 20 and three times with this buffer alone for 15 min, each with gentle agitation at 4 °C. The membrane was dried and exposed to x-ray film (Kodak XAR) to visualize the binding of ^{125}I-IGF-I. The same membranes were rehydrated with methanol and probed with antisera against human IGFBP-3 or human IGFBP-5, as described below.

Proteolysis of Native/Mutant IGFBP-5 by the IGFBP-5-specific Protease—Serum-free conditioned medium was obtained from human dermal fibroblasts (GM10 cells). These cells have been shown to secrete an IGFBP-5-specific protease (28). Native or mutant IGFBP-5, 150 ng, was incubated with 50 μl of conditioned medium in 60 mM Tris and 4 mM $CaCl_2$ with or without 200 ng of IGF-I in a total volume of 60 μl overnight at 37 °C. The reaction mixture was then analyzed by immunoblotting as described below.

Immunoblotting—The samples were separated on SDS-polyacrylamide gel (8% for analysis of IGF-I receptor and 12.5% for analysis of IGFBP-3 and IGFBP-5) and transferred onto a polyvinylidene difluoride membrane, as described previously (24). For IGF-I receptor analysis, the membrane was probed with a 1:1000 dilution for PY99 or a 1:1000 dilution for C-20 and visualized by enhanced chemiluminescence, as described previously (29). IGFBP-3 and -5 were visualized by an alkaline phosphatase method using a 1:1000 dilution of IGFBP-3 antibody or a 1:1000 dilution of IGFBP-5 antibody, as described previously (24). The results were quantified by scanning densitometry (Hoeffer Scientific, San Francisco, CA). The signal intensities were analyzed using N.I.H. Image.

[^{3}H]Thymidine Incorporation into DNA—To determine the effect of IGFBP-3 and -5 and the mutants on [^{3}H]thymidine incorporation into DNA, increasing concentrations of mutant and wild type IGFBP-3 (25–700 ng/ml) or IGFBP-5 (250–7000 ng/ml) were added in the presence of 10 ng/ml of IGF-I. Quiescent, porcine aortic smooth muscle cells (pSMC) were isolated as described previously (23, 30). They were plated in 96-well plates at 5000 cells/cm^2 in DMEM supplemented with 10% fetal bovine serum. After 5 days the media were aspirated, and 0.2 ml of fresh media containing 0.2% human platelet-poor plasma and test concentrations of IGFBP-3 or -5 plus IGF-I and 0.5 μCi/well of [^{3}H]thymidine (specific activity 33 Ci/mmol) was added. After 36 h, the amount of [^{3}H]thymidine incorporated into DNA was determined after extracting the DNA as described previously (24).

Smooth Muscle Cell Migration—pSMC monolayers were grown to confluency in 6-well plates in DMEM supplemented with 10% fetal bovine serum. Confluent monolayers were wounded with a single-edged razor blade as described previously (30). Test concentrations of IGF-I and IGFBP-3 and -5 and their mutants were added in DMEM containing 0.2% fetal bovine serum. After 72 h, the number of cells migrating across the line of wounding were counted, as described previously (30). Cells were fixed and stained with methylene blue prior to counting. The

results are expressed as percent increase above a control containing 0.2% serum only.

Immunoprecipitation of the IGF-I Receptor—pSMC were grown to near confluency on 60-mm plates. The cultures were washed three times with serum-free DMEM and incubated with DMEM plus 0.01% BSA for 24 h. The medium was changed to DMEM with 0.01% BSA alone or this medium containing 1 μg/ml of each form of IGFBP. After 20 min at 37 °C, either vehicle or 100 ng/ml IGF-I was added for 10 min. The medium was removed, and the cultures placed on ice and then solubilized in 0.5 ml of lysis buffer (1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM EGTA, 150 mM NaCl, 50 mM HEPES, pH 7.5, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 0.3 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml leupeptin). The insoluble material was removed by centrifugation at 14,000 $\times$ *g* for 10 min, and the supernatant was incubated with a 1:250 dilution of C-20 antibody overnight at 4 °C. The immune complexes were precipitated by incubating with protein-A-Sepharose at 4 °C for 2 h, and then the immobilized protein A was sedimented by centrifugation at 7000 $\times$ *g* for 1 min and washed four times with the cell lysis buffer. The proteins were resuspended in Laemmli sample buffer with 0.1 M dithiothreitol. The immune complexes thus obtained were analyzed by immunoblotting.

RESULTS

Synthesis of Mutant Forms of IGFBP-3 and -5 That Have Reduced Affinity for IGF-I—Because the prior NMR study predicted that hydrophobic residues between Val^{49} and Leu^{75} of IGFBP-5 formed the core of the IGF binding site, five of the eight residues in that region that had been predicted to form the binding pocket (*i.e.* Lys^{68}, Pro^{69}, Leu^{70}, Leu^{73}, and Leu^{74}) were mutated to Asn, Gln, Gln, Gln, and Gln, respectively (13). Scatchard analysis, using the purified protein, showed that mutant IGFBP-5 had a K_d of ~1000 nM, whereas native IGFBP-5 had a K_d of 0.56 nM. A mutant form of IGFBP-3 that had substitutions for homologous residues was created by changing Arg^{69}, Pro^{70}, Leu^{71}, Leu^{74}, and Leu^{75} to Ser, Ala, Ser, Gln, and Gly, respectively. Native IGFBP-3 had a K_d of 0.69 nM, whereas the mutant had a K_d of 930 nM. Thus, for both mutants the affinity for IGF-I was reduced >1000-fold. When the ability of increasing concentrations of mutant IGFBP-3 to bind ^{125}I-IGF-I was studied, a significant increase in binding was not detected until 400 ng/ml was added. In contrast, a significant increase in ^{125}I-IGF-I binding was noted at 1.0 ng/ml when native IGFBP-3 was used (Fig. 1*A*). Similar results were obtained for mutant and wild type IGFBP-5, and concentrations of either mutant below 100 ng showed no significant increase in binding compared with controls that contained no IGFBP-5 (Fig. 1*B*).

The inability of both of the mutants to bind to ^{125}I-IGF-I was confirmed by Western ligand blotting. Neither 10 ng nor 40 ng of mutant IGFBP-3 and IGFBP-5 showed detectable binding. In contrast, both wild type proteins were easily visualized (Fig. 2*A*). When the same membrane was probed with antiserum against IGFBP-3 and IGFBP-5, it was shown that comparable amounts of native and mutant IGFBPs were adherent to the membrane (Fig. 2*B*).

IGF-I Failed to Protect the Mutant Form of IGFBP-5 from Proteolysis by the IGFBP-5 Protease—Native IGFBP-5 is cleaved by a serine protease that is released by cultured human fibroblasts (26). Previous studies have shown that incubation of IGF-I with IGFBP-5 partially protects it from degradation by this protease (20, 26). Because mutant IGFBP-5 is unable to bind IGF-I, we determined whether it was protected from proteolysis when incubated with the IGFBP-5-specific protease in the presence of IGF-I. After an overnight incubation, native IGFBP-5 was degraded and yielded a predominant 22-kDa fragment (Fig. 3, *lane 1*). Inclusion of 100 ng of IGF-I with native IGFBP-5 decreased proteolysis (Fig. 3, *lane 2*). When the experiment was repeated three times, scanning densitometry showed that the intensities of the intact native IGFBP-5 band was reduced by 55 ± 7% and in the presence of IGF-I by 24 ± 13% ($p < 0.05$). In contrast, the mutant IGFBP-5 band intensity was reduced by 48 ± 8% without IGF-I and by 57 ± 11% with IGF-I (*p*, N.S.) (Fig. 3, *lanes 5* and *6*). Therefore, binding of IGF-I to IGFBP-5 appears to be necessary for IGF-I to protect IGFBP-5 from proteolysis by this protease.

Native IGFBP-5 Inhibited IGF-I-stimulated Phosphorylation of IGF-I Receptor, whereas the Mutant IGFBP-5 Had Little Effect—The addition of intact IGFBP-5 to the culture medium has been shown to inhibit IGF-I-stimulated phosphorylation of the IGF-I receptor (6, 24). Therefore, we determined whether the mutant IGFBP-5 had any effect on IGF-I receptor phosphorylation. Incubation of pSMC with 100 ng/ml IGF-I resulted in the stimulation of tyrosine phosphorylation of the IGF-I receptor (Fig. 4). The addition of 1 μg/ml native IGFBP-5 to the IGF-I-stimulated cultures prevented phosphorylation of IGF-I receptor completely. On the other hand, the same amount of the mutant IGFBP-5 did not alter the IGF-I receptor phosphorylation stimulation by IGF-I. The stimulation of phosphorylation of the IGF-I receptor by des-1–3-IGF-I that binds IGFBP-5 with very low affinity was not affected significantly by the presence of native IGFBP-5 nor the mutant IGFBP-5 (data not shown). The incubation of cells with either native or mutant IGFBP-5 in the absence of IGF-I did not stimulate phosphorylation of IGF-I receptors (data not shown).

Migration—IGF-I is a potent simulator of cell migration (24, 30). This IGF-I action was also decreased by co-incubation of pSMC with native IGFBP-5, but not with the mutant IGFBP-5. 50 ng/ml IGF-I-stimulated pSMC migration 121 ± 33% over control and the addition of 2.0 μg/ml of native IGFBP-5 decreased this response to a 58 ± 16% increase over control (Table I). In contrast, in the presence of 2.0 μg/ml mutant IGFBP-5, migration was increased to 115 ± 22% over control, and this was not different than the cultures exposed to IGF-I alone. Native IGFBP-3 also inhibited the IGF-I-induced migration of pSMC, but the IGFBP-3 mutant had little effect. 50 ng/ml IGF-I increased the migration of pSMC to 114 ± 22% over control, and 500 ng/ml of native IGFBP-3 decreased the response to a 2 ± 11% increase. When the IGFBP-3 mutant was added with IGF-I, migration was increased 113 ± 18% over control (Table I).

[^{3}H]Thymidine Incorporation—A molar excess of IGFBP-3 or -5 can inhibit the ability of IGF-I to stimulate DNA synthesis (24). Therefore, when molar ratios of 1.12:1, 2.25:1, and 9:1 of wild type and mutant IGFBP-3 to IGF-I were added to quiescent pSMC cultures, the 4.3-fold increase in [^{3}H]thymidine incorporation that was stimulated by IGF-I was attenuated 84 ± 8, 77 ± 6, and 85 ± 8%, respectively ($p < 0.001$ compared with IGF-I alone, mean of three experiments) (Fig. 5*A*). In contrast, the IGFBP-3 mutant had no effect on the response to IGF-I. When IGFBP-5 was added using ratios of 2.9:1, 5.8:1, and 23:1, the [^{3}H]thymidine incorporation response to IGF-I was inhibited by 88 ± 5, 94 ± 4, and 98 ± 7%, respectively ($p <$ 0.001 compared with IGF-I alone) (Fig. 5*B*). The mutant IGFBP-5 had no effect.

DISCUSSION

These studies definitively demonstrate that a specific group of hydrophobic amino acids within the N-terminal one-third of IGFBP-3 and -5 is essential for IGF-I binding. Previous studies using solution NMR spectroscopy had determined that a fragment of IGFBP-5 containing this region of the protein folded in such a way that these residues formed a hydrophobic patch that was probably an important IGF-I binding site (6). Similarly, other investigators had reported that N-terminal fragments of IGFBP-2, -3, and -4 could bind to IGF-I (7–9, 25, 31, 32). Prior studies had to be conducted with IGFBP fragments,

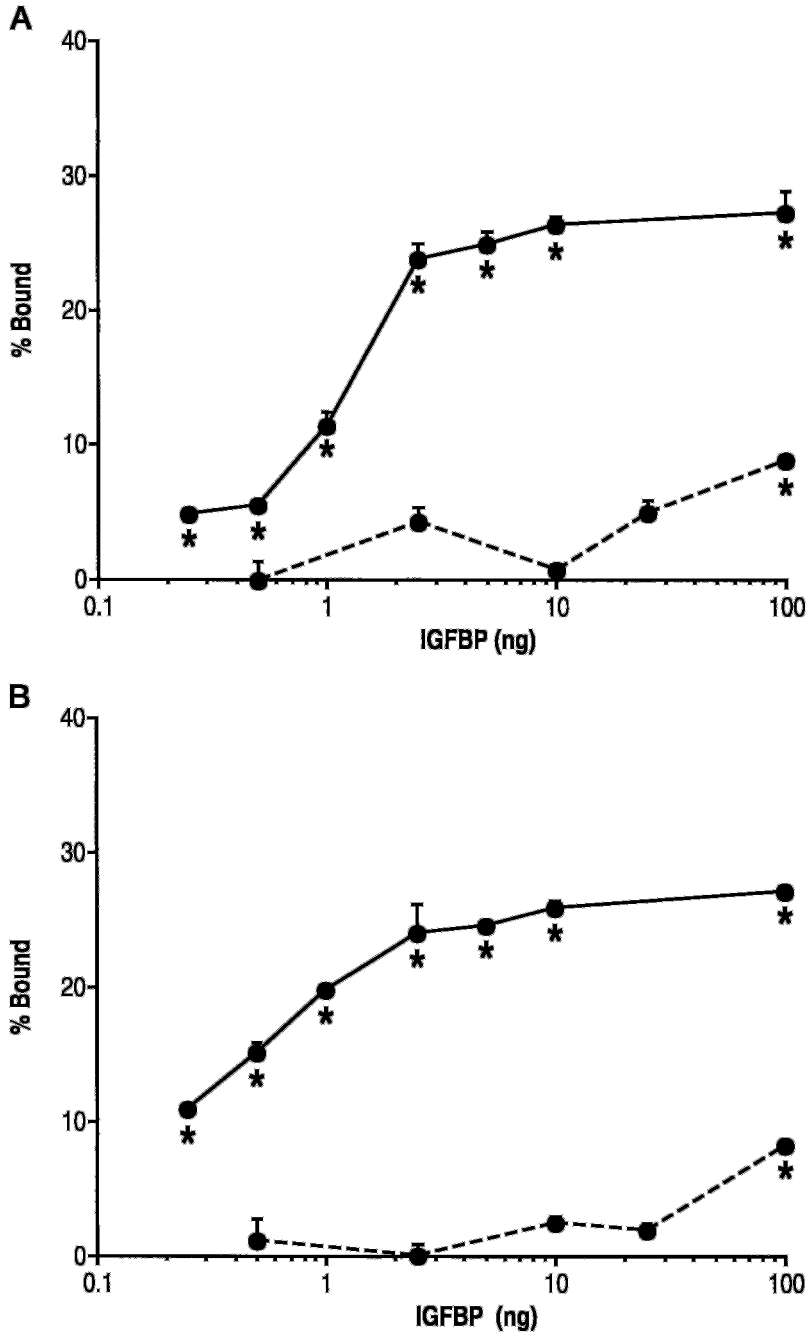

FIG. 1. **Binding of ^{125}I-IGF-I to native and mutant forms of IGFBP.** 0.25–100 ng of native (*solid line*) and mutant (*dashed line*) forms of IGFBP-3 (*A*) or IGFBP-5 (*B*) were incubated with ^{125}I-IGF-I, and the radioactivity that bound to IGFBPs was precipitated using polyethylene glycol, as described under "Experimental Procedures." The radioactivity precipitated in the absence of the IGFBPs was subtracted as background, and the data were expressed as a percentage of the total isotope added that bound. The result is the mean ± S.D. ($n = 4$) and is the result of two independent experiments. *, $p < 0.01$ compared with control tubes that contained no binding protein.

because when the intact proteins were used to make similar determinations, an unacceptable level of aggregation obscured the ability to make this determination by NMR spectroscopy. However, the IGFBP fragments that have been analyzed previously have a significantly reduced affinity for IGF-I. Therefore to determine if the putative solution structure of the binding epitope of the fragment would behave in a similar manner within the whole protein, we chose to selectively alter five of the eight residues of IGFBP-5 that were proposed to form this hydrophobic patch.

The strategy to use these five residues rather than all eight was dictated by the fact that the three other residues, at positions 49, 50, and 62, are separated from this pocket by several residues. Additionally, making a smaller number of substitutions would make it less likely that the effect of the substitutions was due solely to an alteration in the tertiary structure of the protein. This IGFBP-5 mutant had a >1000-fold reduction in affinity for IGF-I. Although the solution structure of IGFBP-3 was not determined in the prior study (6), homologous residues are present in IGFBP-3. Therefore we chose to mutate these homologous residues to either neutral or nonhydrophobic residues. The effect of these substitutions on the affinity of IGFBP-3 for IGF-I was similar to that observed with IGFBP-5 (*i.e.* greater than 1000-fold reduction in affinity).

Although these substitutions would have destroyed the hydrophobic patch as predicted from the NMR model, we cannot exclude the possibility that other changes in tertiary structure, such as alteration of the disulfide bonding pattern or important folding disruptions, occurred as a result of these substitutions. Because five substitutions were present, this is certainly a possibility. However, we favor the idea that this series of mutations disrupted the hydrophobic patch and that this is the principle reason that binding affinity is reduced. Several observations support this conclusion. First it is based on a rational protein folding model proposed by Kalus *et al.* (6). Second, no direct cysteine substitutions were performed, and therefore

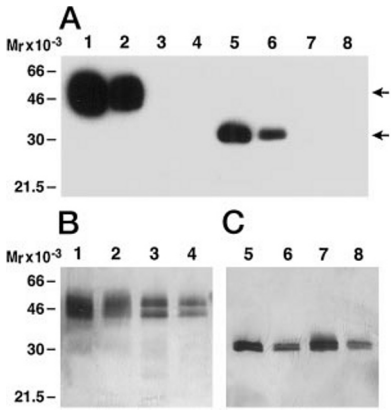

FIG. 2. **Ligand blot and immunoblot analysis of native and mutant IGFBP-3 and -5.** 10 or 40 ng of native and mutant IGFBP-3 and -5 were separated on 12.5% polyacrylamide gel and transferred onto polyvinylidene difluoride membranes. The membrane was first blotted with ^{125}I-IGF-I (*A*) and then probed with anti-IGFBP-3 antibody (*B*, *lanes 1–4*) or anti-IGFBP-5 antiserum (*C*, *lanes 5–8*), as described under "Experimental Procedures." *Lane 1,* 40 ng of native IGFBP-3; *lane 2,* 10 ng of native IGFBP-3; *lane 3,* 40 ng of mutant IGFBP-3; *lane 4,* 10 ng of mutant IGFBP-3; *lane 5,* 40 ng of native IGFBP-5; *lane 6,* 10 ng of native IGFBP-5; *lane 7,* 40 ng of mutant IGFBP-5; *lane 8,* 10 ng of mutant IGFBP-5. The *upper arrow* denotes the position of IGFBP-3, and the *lower arrow* indicates the position of IGFBP-5. The figure is the representative result of three experiments that gave similar results.

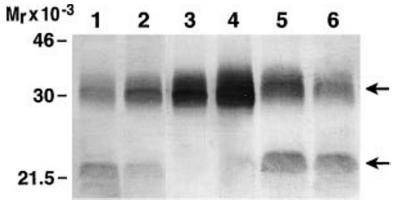

FIG. 3. **Proteolysis of native and mutant IGFBP-5 by the IGFBP-5-specific protease.** 150 ng of native (*lanes 1–3*) or mutant (*lanes 4–6*) IGFBP-5 was incubated for 14 h at 37 °C with fibroblast-conditioned medium that contained IGFBP-5 protease activity (*lanes 1, 2, 5*, and *6*). IGF-I (200 ng) was added to the samples shown in *lanes 2* and *6* and omitted from the samples shown in *lanes 1* and *5*. The samples shown in *lanes 3* and *4* were not exposed to the protease but were incubated in buffer alone. The reaction mixtures were separated on 12.5% SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting using an antiserum against IGFBP-5. The *upper arrow* denotes the position of intact IGFBP-5, and the *lower arrow* indicates the position of the major 22-kDa fragment. The figure is the representative result of three experiments that gave similar results.

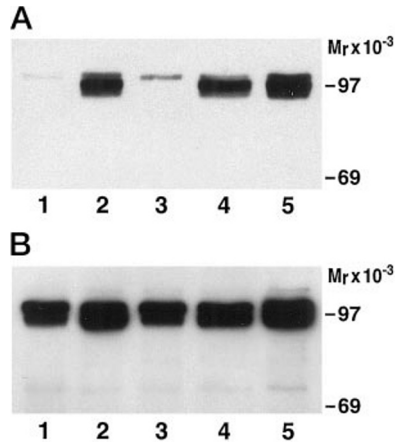

FIG. 4. **The effect of native and mutant IGFBP-5 on IGF-I-stimulated phosphorylation of the IGF receptor.** *A,* subconfluent pSMC were preincubated with 1 μg/ml of native IGFBP-5 or mutant IGFBP-5 for 20 min before the addition of 100 ng/ml of IGF-I. After a 10-min incubation with IGF-I, cells were lysed, and the IGF receptor was immunoprecipitated with anti-IGF receptor antibody. Precipitated protein was analyzed by immunoblotting (6% polyacrylamide gel) using an antibody against phosphotyrosine. *B,* when the membrane was blotted with anti-IGF receptor antibody (1:1000 dilution), the result confirmed that the amount of IGF receptor that was detected was similar for the different treatments. *Lane 1,* no treatment; *lane 2,* 100 ng/ml IGF-I; *lane 3,* 100 ng/ml IGF-I and 1 μg/ml native IGFBP-5; *lane 4,* 100 ng/ml IGF-I; *lane 5,* 100 ng/ml IGF-I and 1 μg/ml mutant IGFBP-5. Scanning densitometry values for the bands shown in *A* were: *lane 1*, 239; *lane 2*, 15,102; *lane 3*, 1663; *lane 4*, 16,060; and *lane 5*, 18,697. For *B* they were: *lane 1*, 19,265; *lane 2*, 21,431; *lane 3*, 18,940; *lane 4*, 19,801; and *lane 5*, 21,251.

TABLE I
Smooth muscle cell migration

The data are expressed as percent increase over control cultures that were exposed to 0.2% fetal bovine serum. *, $p < 0.001$ compared to IGF-I alone.

Treatment	% increase over control
IGF-I 50 ng/ml	114 ± 22
IGFBP-3 (wild type) 500 ng/ml	6 ± 15
IGF-I 50 ng/ml + IGFBP-3 500 ng/ml	2 ± 11*
IGFBP-3 (mutant) 500 ng/ml	19 ± 3
IGF-I 500 ng/ml + IGFBP-3 (mutant) 500 ng/ml	113 ± 18
IGF-I 50 ng/ml	121 ± 33
IGF-I 50 ng/ml + IGFBP-5 2.0 μg/ml	58 ± 16*
IGFBP-5 2.0 μg/ml	4 ± 12
IGF-I 50 ng/ml + IGFBP-5 (mutant) 2.0 μg/ml	115 ± 22
IGFBP-5 (mutant) 2.0 μg/ml	8 ± 10

there is no reason *a priori* to believe that the disulfide bonding pattern would be altered. Third, the identical substitutions in IGFBP-3 resulted in a similar attenuation in affinity, suggesting that if the effect were due solely to an alteration in tertiary structure that the identical change would have to occur in IGFBP-3, which has regions of sequence that are clearly distinct from IGFBP-5. Finally, the five substitutions are also in close physical proximity with one another, making it less likely that some other important structural determinant at a distant site in the molecule was altered. For all of these reasons, we believe that the model of Kalus *et al.* (6) is correct and is validated by these data as presented.

These data do not exclude the possibility of important binding determinants in the C terminus of the IGFBPs. Other laboratories have published data suggesting that fragments of IGFBP-3 or -5 containing the only C-terminal region have some affinity for IGF-I and -II (5, 21, 33). Other investigators have noted that the C-terminal region of IGFBP-2 contains an IGF-I binding site (20, 31, 34). Similarly, Bramani *et al.* (35) have presented data suggesting that substitutions for residues 205 and 207 in IGFBP-5 results in a major loss of affinity of this protein for IGF-I. The model of Kalus *et al.* (6) also proposed that the C-terminal region, although not containing a distinct high affinity site, could fold such that a critical C-terminal region interacted cooperatively with the hydrophobic binding pocket in the N terminus. This type of interaction has also been proposed to occur in IGFBP-4 (32). Our data show only that the N-terminal binding pocket is essential for high affinity binding but do not help in determining the essentiality of the C-terminal region binding domain. Similarly, they do not exclude its possible importance in determining the binding affinity of the whole protein.

Loss of binding resulted in major functional changes in IGFBP-3 and -5 actions. These proteins have been shown to be potent inhibitors of several physiologic processes that are stimulated by IGF-I. This was confirmed with the native forms of IGFBP-3 and -5 showing that they could attenuate the IGF-I response to cell migration and stimulation of [^{3}H]thymidine incorporation. Furthermore, we show that attenuation of IGF-I receptor-stimulated autophosphorylation, the first step in IGF-I signaling, is inhibited by wild type IGFBP-5, and the

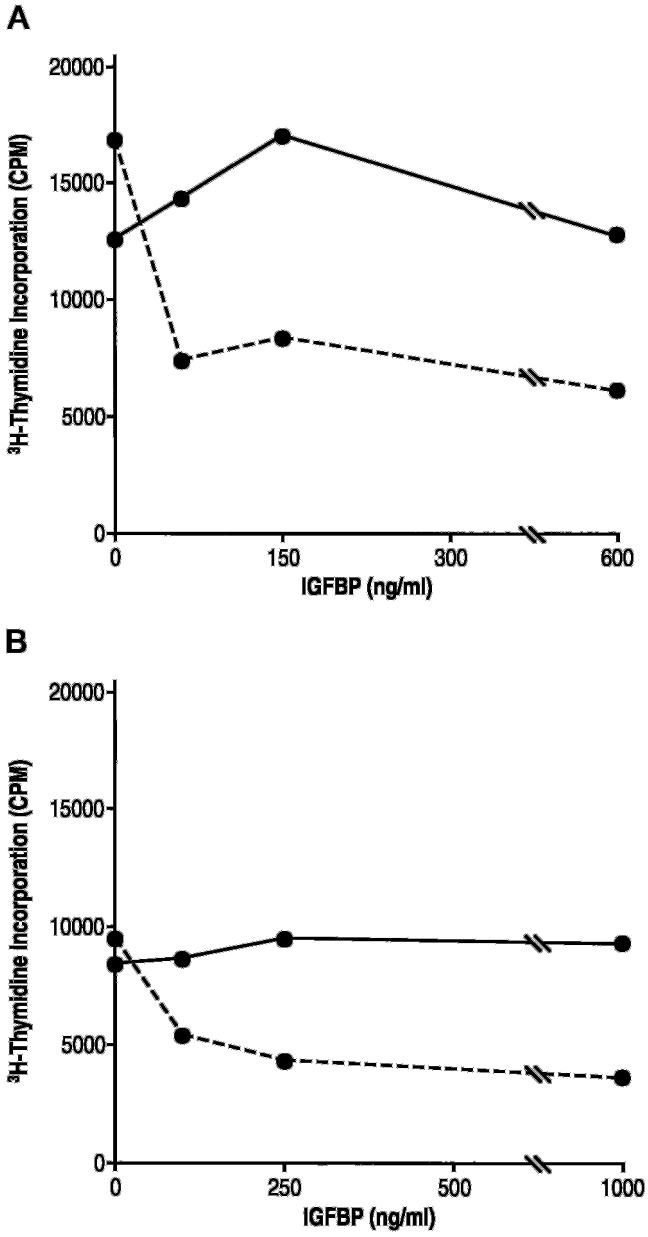

FIG. 5. **Absence of inhibition of IGF-I-stimulated DNA synthesis by IGFBP-3 and -5 mutants.** *A,* increasing concentrations of wild type (*dashed line*) or mutant (*solid line*) IGFBP-3 were incubated with a fixed amount (10 ng/ml) of IGF-I (*A*), and the DNA synthesis response to IGF-I was determined as described under "Experimental Procedures." *B,* the [^{3}H]thymidine incorporation response to IGF-I (10 ng/ml) in the presence of increasing concentrations of native (*dashed line*) or mutant (*solid line*) IGFBP-5. The experiments shown in *A* and *B* were repeated three times with similar results.

mutant form of IGFBP-5 had no effect in attenuating IGF-I signaling in this cell system. These data strongly suggest that the major effect of IGFBP-3 and -5 on IGF-I-stimulated actions is to prevent IGF-I receptor association. The data also show no direct effects of concentrations of 500 ng/ml or less of IGFBP-3 and -5 on [^{3}H]thymidine incorporation or cell migration in this cell type. This stands in contrast to breast tumor cells and other cancer cell lines, where IGFBP-3 has been shown to have direct growth attenuating effects (35–38). These data suggest that either nontransformed aortic smooth muscle cells are behaving differently in response to IGFBP-3 and -5 compared with these other cell types or that higher concentrations of these proteins are required to demonstrate direct effects. These mutants will be very useful in determining if IGFBP-3 and -5 have direct effects on cell types that secrete IGF-I, because they will allow investigators to be able to exclude the possibility that IGF-I produced in an autocrine or paracrine manner is modulating the IGFBP actions.

In an additional experiment, we were able to show that proteolytic cleavage of IGFBP-5 is partially inhibited by coincubation with IGF-I. This inhibition did not occur when IGF-I was incubated with the IGFBP-5 mutant. This result has two important implications. First, it is likely that the mutant IGFBP-5 is folded correctly to expose its proteolytic cleavage site because there was no difference in the amount of proteolytic cleavage of the wild type compared with the IGFBP-5 mutant. This further supports the conclusion that substitution for the hydrophobic patch residues did not result in a major alteration in conformation of the protein. Second, it has not been possible in prior studies to determine if IGF-I was inhibiting proteolysis by binding to the protease or by binding to IGFBP-5. These data strongly suggest that binding to IGFBP-5 is required for inhibition.

In summary, these studies demonstrate definitively that a 5-residue hydrophobic patch that has been postulated to be part of the primary binding site in IGFBPs for IGF-I is necessary for high affinity binding. Alteration of these hydrophobic residues to nonhydrophobic residues results in a greater than 1000-fold reduction in affinity of IGFBP-3 and -5 for this ligand. This strongly suggests that IGFBP-3 and -5 will not bind with high affinity without these residues. The minimum number of substitutions was not determined by this study, but the findings suggest that if a fewer number of substitutions allowed nearly equal reductions in affinity, it would be possible to exclude that the tertiary structure has been altered to any great extent.

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