Cumulative Mutagenesis of the Basic Residues in the 201–218 Region of Insulin-Like Growth Factor (IGF)-Binding Protein-5 Results in Progressive Loss of Both IGF-I Binding and Inhibition of IGF-I Biological Action

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We have reported previously that mutation of two conserved nonbasic amino acids (G203 and Q209) within the highly basic 201–218 region in the C-terminal domain of IGF-binding protein-5 (IGFBP-5) decreases binding to IGFs. This study reveals that cumulative mutagenesis of the 10 basic residues in this region, to create the C-Term series of mutants, ultimately results in a 15-fold decrease in the affinity for IGF-I and a major loss in heparin binding. We examined the ability of mutants to inhibit IGF-mediated survival of MCF-7 cells and were able to demonstrate that this depended not only upon the affinity for IGF-I, but also the kinetics of this interaction, because IGFBP-5 mutants with similar affinity constants (K_D) values, but with different association (K_a) and dissociation (K_d) rate values, had markedly different inhibitory properties. In con-

trast, the affinity for IGF-I provided no predictive value in terms of the ability of these mutants to enhance IGF action when bound to the substratum. Instead, these C-Term mutants appeared to enhance the actions of IGF-I by a combination of increased dissociation of IGF-IGFBP complexes from the substratum, together with dissociation of IGF-I from IGFBP-5 bound to the substratum. These effects of the IGFBPs were dependent upon binding to IGF-I, because a non-IGF binding mutant (N-Term) was unable to inhibit or enhance the actions of IGF-I. These results emphasize the importance of the kinetics of association/dissociation in determining the enhancing or inhibiting effects of IGFBP-5 and demonstrate the ability to generate an IGFBP-5 mutant with exclusively IGF-enhancing activity. (*Endocrinology* 147: 338–349, 2006)

THE IGFs ARE important mediators of physiological growth, which exert their cellular actions by binding to and activating the type 1 IGF receptors. In turn, IGF receptor interactions are regulated by a family of six IGF-binding proteins (IGFBPs) that form high affinity complexes with both IGF-I and IGF-II (reviewed in Ref. 1). The IGFBP proteins all share a common protein structure that can conceptually be divided up into three domains (1) (see Fig. 1). There is a particularly high degree of conservation in the N- and C-terminal domains, with all IGFBPs containing six cysteine residues in the C-terminal domain of all species sequenced to date, whereas IGFBP-1 to -5 have 12 and IGFBP-6 has 10 cysteines in the N-terminal domain. A nonconserved region separating the N- and C-terminal domains contains most of the sites for proteolysis and posttranslational modifications of the proteins. IGFBP-5 is the most highly conserved of the IGFBP proteins; 95% conserved between human and mouse in the central domain and over 98% conserved in the N- and C-terminal domains. Both the N- and C-terminal cysteine-rich domains of IGFBPs have been demonstrated to contrib-

ute to the IGF-binding sites. This is supported by work in which fragments from the N-terminal cysteine-rich domains of IGFBP-1 (2), IGFBP-3 (3), IGFBP-4 (4), and IGFBP-5 (5, 6) and C-terminal cysteine-rich domains of IGFBP-2 (7, 8) and IGFBP-3 (3) were generated and shown to possess residual IGF-binding activity. Other groups have reported biosensor analysis of N- and C-terminal fragments of bovine IGFBP-2 and human IGFBP-3 (9–11), and all of these studies indicated that there were major contributions to the IGF-binding site provided by both the N- and C-terminal domains. Furthermore, others have demonstrated that isolated N- and C-terminal domains of IGFBP-3 cooperate in the presence of IGFs to form high affinity complexes (3). Our previous unpublished biosensor work has shown that the mouse and human IGFBP-5 proteins have identical affinities for binding IGFs.

A considerable body of work from several groups indicates that the major IGF-binding site is contained within the N-terminal domain (see Fig. 1), and an important nuclear magnetic resonance study localized this site to a hydrophobic patch on the surface of the IGFBP-5 protein (6). Subsequently, it was shown that substituting specific hydrophobic residues in this region of IGFBP-5 resulted in a large reduction in IGF-I binding (12), and more recently, our group was able to confirm by circular dichroism (CD) spectroscopy that these mutations do not cause a gross alteration in protein structure, and are, therefore, likely to be specific for IGF binding (13).
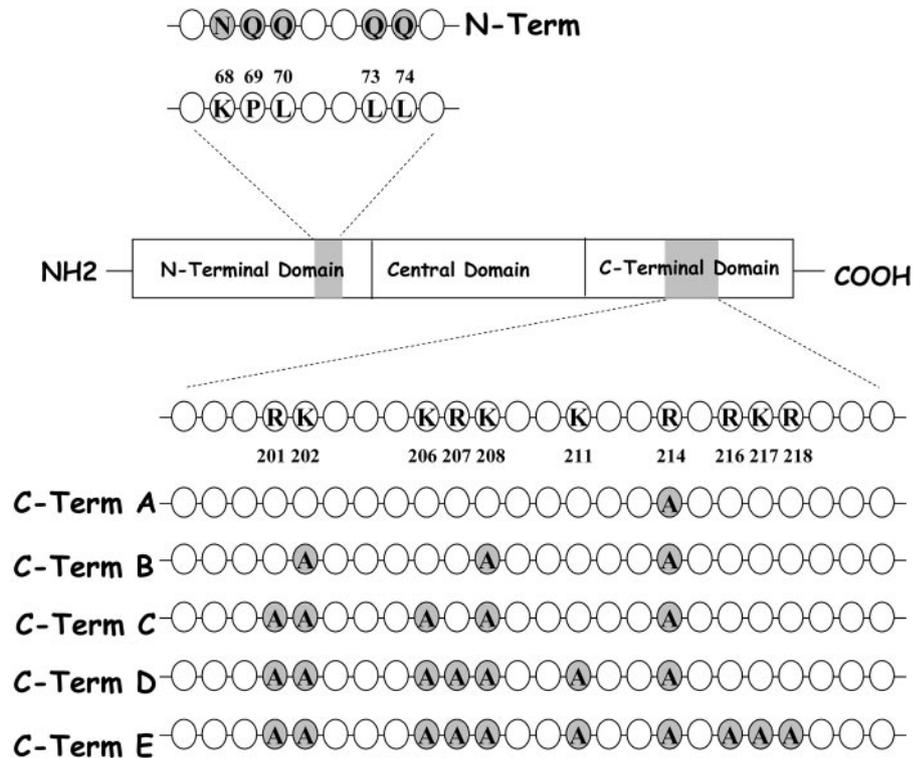
FIG. 1. Schematic representation of the amino acid substitutions made for the N-Term mutant and the C-Term series of mutants reported in this paper. The IGFBP-5 protein structure is divided up into the N-terminal, central, and C-terminal domains. The hydrophobic patch constituting the major IGF-binding site is shown in *gray* in the N-terminal domain, as is the 201–218 region in the C-terminal domain. Single-letter code and positions of the amino acids that were mutated are indicated, and the residues that these were substituted for are shown adjacent in *gray*.

Until recently, less attention had focused on identifying the C-terminal residues in IGFBPs that are involved in IGF interaction. There is a region common to the C-terminus of IGFBP-5 and -3 that is rich in basic amino acids (between 201–218 in IGFBP-5, where 10 of the 18 residues are basic Arg or Lys). Several of these basic amino acids have been shown to play a critical role in IGFBP-5 binding to a wide variety of different molecules, including heparin (14, 15), extracellular matrix (ECM) (16), the ECM proteins osteopontin and thrombospondin (17), acid-labile subunit (18), a putative IGFBP-5 receptor (19), and plasminogen activator inhibitor-I (20), in addition to acting as a nuclear localization signal (reviewed in Ref. 21). However, it has also been demonstrated that mutagenesis of up to four of the basic amino acids simultaneously had very little effect on IGF-I binding, as measured by solution phase binding assays, and it was concluded that these basic residues were not involved in the IGF/IGFBP interaction (22).

We have previously demonstrated that two nonbasic amino acids (G203 and Q209) in IGFBP-5, which lie within the basic 201–218 region and are completely conserved in all six binding proteins from those species studied, are involved in IGF binding (13, 23, 24). We also showed that the equivalent amino acids in IGFBP-2 were involved in IGF binding (13); more recently, others have demonstrated that this is also true for IGFBP-3 (25). We have argued that this demonstrates physical overlap of IGF- and heparin-binding sites in the C-terminal domain of both IGFBP-5 and -3.

In the present study we extended our mutagenesis strategy of the 201–218 region of IGFBP-5, by ultimately substituting all 10 basic Lys and Arg amino acids to Ala residues (creating the C-Term A through C-Term E series of mutants; see Fig. 1). We generated mutants that involved the basic residues K202 and K208, situated immediately adjacent to the residues involved in IGF binding, as well as a mutant in which a third basic residue, K206, which lies spatially as well as sequentially between the two IGF-binding residues, was mutated. We compared the IGF-I-binding abilities of these mutants with that of the wild-type (wt) protein by ligand blotting, solution phase IGF binding assays, and biosensor real-time analysis of binding kinetics. Furthermore, we carried out CD analysis on both wt IGFBP-5 and C-Term E mutant protein to establish whether the effects of these mutations are specific for IGF binding and not the result of a major conformational change in protein structure.

IGFBP-5 has been shown to have both inhibitory and enhancing effects on IGF actions (21). We hoped in this study to examine whether mutations that influence the ability of IGFBP-5 to interact with both IGF-I and the extracellular environment would help to provide mechanistic explanations for these apparently conflicting biological properties of IGFBP-5. To this end, we measured the survival of human breast cancer MCF-7 cells plated on the ECM components fibronectin, collagen, and laminin. Inhibitory effects of IGFBP-5 mutants on cell survival were determined by adding IGF-I and either wt IGFBP-5 or its various mutants after cell attachment, whereas enhancement of IGF-I action was observed when IGF-I and various IGFBP proteins were prebound to the substratum before cell attachment.

Materials and Methods

Site-directed mutagenesis

system (Stratagene, La Jolla, CA) following the protocol provided by the manufacturer. The rat and mouse protein sequences of IGFBP-5 differ by only a single amino acid at position 188 (Asp and Glu, respectively). Site-directed mutagenesis was employed to convert wt rat to wt mouse sequences (D188E) as described previously (13). This was then used as a template to make C-Term A (R214A) using the oligonucleotides 5'-GCA GTG CAA GCC TTC TGC AGG CCG CAA ACG TGG CAT-3' and 5'-GAT GCC ACG TTT GCG GCC TGC AGA AGG CTT GCA CTG C-3'. C-Term A was then used as a template to make C-Term B (K202A: K208A:R214A) using the oligonucleotides 5'-CTG TGA CCG CGC AGG ATT CTA CAA GAG AGC TCA GTG CAA GC-3' and 5'-GCT TGC ACT GAG CTC TCT TGT AGA ATC CTG CGC GGT CAC AG-3'. C-Term B was then used as a template to make C-Term C (R201A:K202A:K206A: K208A:R214A) using the oligonucleotides 5'-CCC AAC TGT GAC GCC GCA GGA TTC TAC GCG AGA GCT CAG TG-3' and 5'-CAC TGA GCT CTC GCG TAG AAT CCT GCG GCG TCA CAG TTG GG-3'. C-Term C was then used as a template to make C-Term D (R201A:K202A:K206A: R207A:K208A:K211A:R214A) using the oligonucleotides 5'-GAT TCT ACG CGG CAG CTC AGT GCG CGC CTT CTG CAG G-3' and 5'-CCT GCA GAA GGC GCG CAC TGA GCT GCC GCG TAG AAT C-3'. C-Term D was then used as a template to make C-Term E (R201A: K202A:K206A:R207A:K208A:K211A:R214A:R216A:K217A:R218A) using the oligonucleotides 5'-CGC GCC TTC TGC AGG CGC CGC AGC TGG CAT CTG CTG GTG TGT GG-3' and 5'-CCA CAC ACC AGC AGA TGC CAG CTG CGG CGC CTG CAG AAG GCG CG-3'. N-Term (K68N: P69Q:L70Q:L73Q:L74Q) was made as described previously (13). All oligos were synthesized by MWG BioTech (Ebersberg, Germany). After site-directed mutagenesis, automated DNA sequencing (Lark Technologies, Takeley, UK) was carried out on all clones to confirm that the correct mutations had been made.

Bacterial expression and purification of recombinant proteins

The expression of recombinant IGFBP-5 proteins was carried out using conditions identical with those described previously (13). After cell lysis, the fusion proteins were extracted from the soluble fraction only and subsequently subjected to two rounds of purification. Cell lysates were loaded onto glutathione-Sepharose columns (Amersham Biosciences, Little Chalfont, UK), and the purified IGFBPs were then eluted after removal of the glutathione-*S*-transferase tag with PreScission protease (Amersham Biosciences). Subsequently, the untagged IG-FBPs were purified by reverse phase HPLC (RP-HPLC) using a polymeric column (PLRP-S; 300 Å; 8 μm; diameter, 4.6 × 150 mm; Polymer Laboratories Ltd., Church Stretton, UK) equilibrated at 30 C with a mobile phase consisting of 3 parts solution A (0.1% trifluoroacetic acid in water) and 1 part solution B (0.1% trifluoroacetic acid in acetonitrile). Aliquots (1–2 ml) of affinity-purified protein [2–3 mg/ml in 50 mM Tris HCl (pH 7), 150 mM NaCl, and 1 mM EDTA] were injected onto the column, which was run at 1 ml/min throughout. After 5 min of isocratic elution, a linear gradient from 25% solution B to 40% solution B was applied over the next 50 min. Absorbance of column effluent was monitored at 220 nm; IGFBP proteins were collected, typically between 25 and 30 min after sample injection, and assessed for homogeneity by SDS-PAGE (Fig. 2). Gels were stained with 0.3% (wt/vol) Coomassie Brilliant Blue R-250 (Sigma-Aldrich Corp., Poole, UK), 5% (vol/vol) methanol, and 7.5% (vol/vol) glacial acetic acid for 30 min and then destained in 30% (vol/vol) methanol and 10% (vol/vol) glacial acetic acid for 1 h with shaking, followed by fresh destaining, without shaking overnight. The gel was dried using the EasyBreeze system (Hoefer Scientific Instruments, San Francisco, CA) according to the manufacturer's instructions.

A single chromatography under these conditions usually yielded 200–600 μg pure protein, which was lyophilized to remove HPLC eluants then stored at −70 C. Proteins were redissolved, as required, in buffer appropriate for their subsequent experimental use.

CD spectroscopy and fluorescence

To compare the secondary structures of wt IGFBP-5 and C-Term E proteins, CD spectropolarimetry data were recorded using a Jasco J-810 spectropolarimeter (Easton, MD) in cells with a path length of 0.02 cm (far UV) using protein concentrations of 0.25 mg/ml for both wild-type
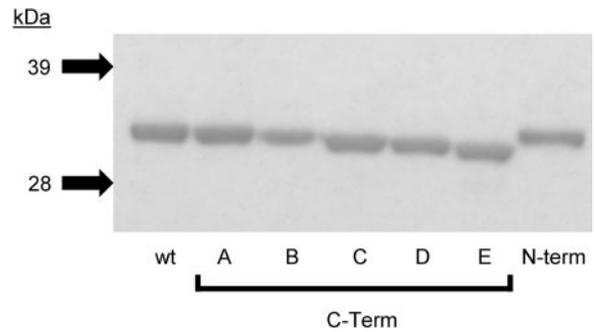

Fig. 2. Coomassie-stained gel of the wt and various mutant proteins after glutathione-*S*-transferase and HPLC purification. A single protein species was observed in each case. We also noted the size differences between the various proteins analyzed: the mature (minus signal peptide) wt mouse IGFBP-5 protein (28,461 Da) and the N-terminal pentapeptide extension (GPLGS) from pGEX-6P1 is 28,872 Da. The alanine substitutions in the 201–218 region of the C-Term mutant series result in a progressive reduction in size down to the C-Term E mutant at 28,112 Da, whereas the mutations in N-term lead to an increase in size to 28,934 Da.

and mutant IGFBP-5 samples (time constant, 0.2 sec; scan speed, 50 nm/min; eight scans). The secondary structure contents of wild-type and C-Term E were estimated using the CONTIN procedure (26), which was accessible from the DICHROWEB website (27). Secondary structure estimates were obtained using data values down to 185 nm in both the wt and C-Term E proteins. Fluorescence spectra were recorded using a PerkinElmer LS50B fluorometer (Wellesley, MA) in 1-cm path length cells using a protein concentration of 0.1 mg/ml for both wt and mutant samples (excitation, 290 nm; slits of 5 nm band pass).

Biosensor analysis

Reagents for biosensor analysis were purchased from BiaCore Ltd. (Stevenage, UK). Biosensor studies were performed with a Biacore 3000 instrument using protocols previously described, where IGF-I was the immobilized ligand present on the biosensor chip surface and the wt and mutant IGFBP-5 proteins were present in the mobile phase as analyte (13). Each protein was analyzed at five different doubling concentrations (0–50 nM), and IGFBP-5 proteins were injected randomly in duplicate across IGF-I surfaces. Analysis of data assumed a fully reversible 1:1 stoichiometry of binding between IGFBP and immobilized IGF, and the Langmuir model provided in the BiaEvaluation 3.1 software and the global data analysis option were used to fit data as described previously (13). Under the conditions described we observed no mass transport-associated effects. The methods used to investigate the interaction between wt and mutant IGFBP-5 proteins and heparin-coated biosensor surfaces have been described in detail previously (28). The wt and mutant proteins were analyzed for heparin binding at concentrations between 0–400 nM.

Solution phase IGF binding assay

Solution phase binding assays were performed as described previously (13), except that in this case, RP-HPLC-purified proteins were analyzed.

Assessment of biological activity of IGFBP-5 mutants

The human breast cancer line MCF-7 was routinely maintained in DMEM supplemented with 10% fetal calf serum, 0.29 mg/ml glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 C in 5% CO_2. Cell culture reagents were purchased from Invitrogen (Paisley, Scotland, UK).

Inhibition of the IGF-I response by IGFBP-5 mutants

Ninety-six-well tissue culture plates were coated with 1 μg/ml fibronectin, collagen, or laminin in PBS (pH 7.4). Fibronectin (F-0635),

laminin (L-2020), and collagen (C-7661) were obtained from Sigma-Aldrich Corp. Wells were rinsed twice, and approximately 1×10^4 MCF-7 cells/well were plated in DMEM/1% BSA and cultured overnight to allow attachment. Wells were rinsed again, and IGF-I (10 ng/ml) and various concentrations of wt or mutant IGFBP-5 (as indicated in the figures) were added in DMEM/1% BSA, for an additional 72 h of culture. Wells containing IGFBP-5 without IGF-I were included as controls. The cells were then fixed with 4% (wt/vol) paraformaldehyde and stained with 1 mg/ml crystal violet. The stain was solubilized in 0.1% Triton X-100, and the OD was read at 540 nm.

Enhancement of the IGF-I response by IGFBP-5 mutants

Ninety-six-well tissue culture plates were coated with 1 μg/ml fibronectin, collagen, or laminin in PBS, pH 7.4. Wells were rinsed and blocked with DMEM/1% BSA for 1 h at 37 C. IGF-I (1000 ng/ml) and wt or mutant IGFBP-5 (1000 ng/ml) were added either individually or in combination in DMEM/0.1% BSA and incubated overnight at 4 C. The wells were then rinsed to remove unbound IGF-I and IGFBP-5, and approximately 1×10^4 MCF-7 cells/well were plated in DMEM/0.1% BSA and cultured for 72 h. The cells were fixed with 4% (wt/vol) paraformaldehyde and stained with 1 mg/ml crystal violet. The stain was solubilized in 0.1% Triton X-100, and the OD was read at 540 nm.

Binding and dissociation studies of IGF-I/IGFBP-5 complexes

Determination of IGF-I release. Ninety-six-well tissue culture plates were coated with 1 μg/ml fibronectin, collagen, or laminin in PBS (pH 7.4). Wells were rinsed and blocked with DMEM/1% BSA for 1 h at 37 C. IGFBP-5 (1000 ng/ml) and 100,000 cpm [^{125}I]IGF-I were added in PBS/0.1% BSA and incubated overnight at 4 C. In experiments measuring initial binding, 1000 ng/ml unlabeled IGF-I was added to the tracer to achieve equivalent concentrations to those used in the enhancement studies described above. Total binding was determined by rinsing the wells with DMEM/1% BSA before eluting the bound material with 200 μl 200 mM HCl, incubated at room temperature for 30 min. To measure dissociation of [^{125}I]IGF-I, wells were rinsed, and 200 μl PBS/0.1% BSA (pH 7.4) was added. Incubation was continued at 37 C, and the medium containing dissociated IGF-I was sampled at intervals. Radioactivity was measured in a γ-counter (LKB Instruments, Gaithersburg, MD).

Determination of IGFBP-5 release. To determine binding and release of IGFBP-5, 96-well culture plates were coated with laminin, collagen, or fibronectin as described above, and each IGFBP-5 preparation (1000 ng/ml) was added to the well along with IGF-I (1000 ng/ml) and incubated overnight at 37 C. Unbound IGFBP-5 was removed by aspiration, the wells were rinsed, and the aspirates were combined for determination of unbound IGFBP-5. Fresh medium was added, and the wells were then incubated for an additional 24 h at 37 C, after which the medium was removed to determine dissociated IGFBP-5. The quantity of IGFBP-5 in all samples was determined by RIA as previously described (29), using each IGFBP-5 mutant as its own standard (although the antiserum recognized all the mutants with very similar avidity).

Results

After RP-HPLC purification, we obtained a single protein species for all the clones in this study, as indicated by SDS-PAGE followed by Coomassie Brilliant Blue staining (Fig. 2). Figure 3A and Table 1 show that the secondary structure estimates for wt IGFBP-5 and C-Term E determined by near and far UV CD spectroscopy were similar, exhibiting a strong negative band with a minimum of 202 nm in each case. The overall shapes of these spectra were very similar and indicated high proportions of random coil type structure in each case. Secondary structure estimates of these spectra gave low percentages of α-helical structure and suggested that the proteins contain a greater proportion of β-sheet, β-turns, and other structures (Table 1). To examine the conformational flexibility of IGFBP-5, both wt and C-Term E mutant proteins were analyzed in the presence of 50% trifluoroethanol (TFE). TFE has a lower dielectric constant than water, thus promoting intramolecular interactions by minimizing the interactions of the protein with the surrounding solvent molecules. In the presence of TFE, both proteins adopted very similar conformations that were more helical (Fig. 3A). The structural flexibility of IGFBP-5 may be important for its mode of interaction with the ECM or cell surfaces, thus providing a point of control in its ability to bind IGF. The fluorescence emission spectra showed that wt IGFBP-5 and C-Term E have very similar emission maxima around 385 nm, indicating that the tryptophan residues are fully exposed to solvent in each protein (Fig. 3B).
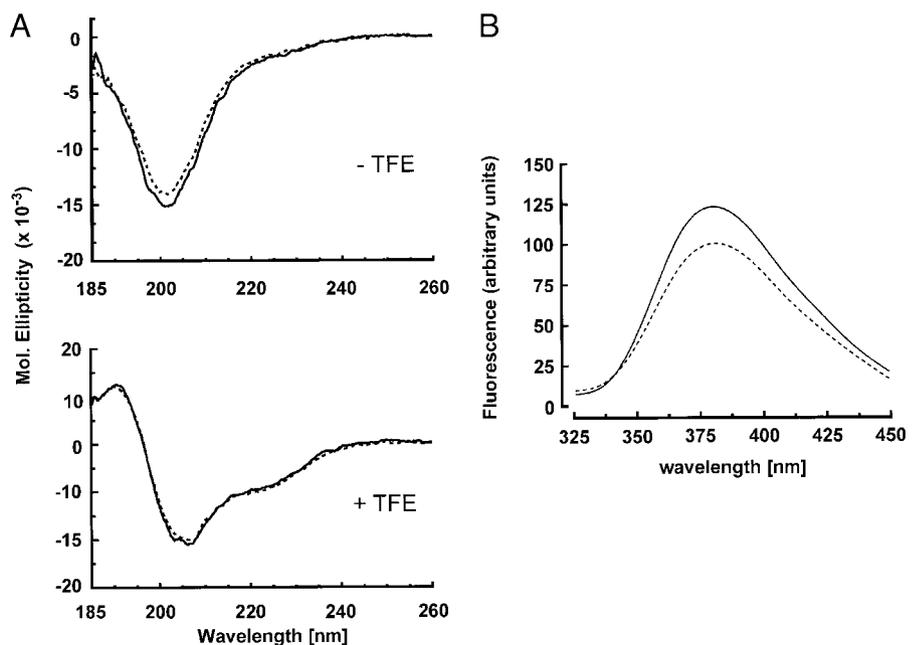

Fig. 3. A, Far UV CD spectra with and without TFE of wt IGFBP-5 (*solid line*) and C-Term E mutant (*dashed line*) proteins using values obtained by Bradford assay (wt, 0.5 mg/ml; C-Term, E 0.47 mg/ml). B, Fluorescence emission spectra of wt IGFBP-5 (*solid line*) and the C-Term E mutant (*dashed line*).

TABLE 1. Secondary structural estimations of wt and C-Term E mutant proteins

Contin	α-Helix	β-Sheet	β-Turn	Other	NMRSD
wt	6.4	20.3	15.2	58.0	0.070
+ 50% TFE	37.9	6.4	22.5	33.2	0.256
C-Term E	6.0	21.6	15.0	57.5	0.082
+ 50% TFE	38.2	5.8	23.8	32.1	0.229

NMRSD, Nuclear magnetic resonance study.

Detailed assessment of IGF/IGFBP binding affinities were obtained by biosensor analyses. Sensorgrams derived from biosensor analysis of wt and mutant IGFBP-5 binding to immobilized IGF-I sensor chips are presented in Fig. 4. The equilibrium constant obtained for the wt IGFBP-5 protein was almost identical with that reported previously (13) (0.2 nM). An examination of the derived equilibrium constants in Table 2 indicated an almost progressive decrease in affinity as mutations of basic amino acids within the C-terminal 201–218 region of IGFBP-5 accumulate, although we noted that C-Term D had a slightly higher affinity for IGF-I than C-Term B and C. This resulted in a mutant C-term D, which had a similar K_D to that of C-Term A and C-Term B, but had increases in both K_a and K_d rate constants. Additional mu-

TABLE 2. Biosensor-derived kinetic and equilibrium constants for interaction of wtIGFBP-5, C-Term-A to -E, and N-Term proteins binding to immobilized IGF-I

	K_a (M^{-1} s^{-1} × 10^5)	K_d (×10^{-4} s^{-1})	K_D (nM)	-Fold
wt IGFBP-5	21.3	5.1	0.24	1
C-Term A	6.3	4.3	0.68	2.8
C-Term B	4.9	5.6	1.1	4.6
C-Term C	5.1	6.1	1.2	5.0
C-Term D	7.6	6.9	0.91	3.8
C-Term E	2.3	8.3	3.5	14.6
N-Term	2.0	21.9	10.9	45.4

K_D is derived as K_d/K_a. The fold reduction in binding of mutant proteins relative to wt protein is indicated in the *right column*. For additional details see *Materials and Methods*.

tations produced a mutant, C-Term E, which had a K_D value of 3.5 nM, almost 15-fold lower than the wt protein. However, the affinity of the N-terminal mutant (13) was the most compromised, with a K_D value of 10.9 nM, 45-fold lower than that of wt IGFBP-5. This progressive loss in binding to immobilized IGF was also observed in IGF-II ligand blotting (data not shown).

We examined the affinity of these mutant proteins in a solution phase analysis of the IGF-I-IGFBP interaction (Fig. 5). It was clear from the saturation binding curves obtained that there was a progressive reduction in affinity for IGF-I from wt IGFBP-5 through the C-Term A–E series mutants to the N-term mutant, although we noted again that C-Term D had a slightly higher affinity for IGF-I than C-Term C (Fig. 5). A linear transformation, by means of a semilog plot, of the solution phase assay data allowed calculation of the quantity of each mutant required to bind an equivalent amount of [^{125}I]IGF-I (data not shown). The fold differences in IGF-I binding relative to the wt protein were very minor (< 2-fold) from C-Term A–D, whereas C-Term E and N-Term again showed major impairments in their binding to IGF-I (33- and 44-fold reductions in binding, respectively).

Biosensor analysis was also used to study the interaction of the wt IGFBP-5 protein and the various mutants with heparin immobilized to the sensor chip (Fig. 6). The affinity of interaction for the wt protein was 7.22 ± 2.22 nM (mean ± SE; n = 4), which is similar to that reported previously (28). For the C-Term A mutant, the value was 21.3 ± 0.6 (average ± range; n = 2), which indicates a slight reduction in heparin binding, whereas binding of the C-Term B-E mutants to heparin was so low that affinity constants could not be derived. For N-Term, there was a 2-fold reduction in the affinity (mean ± SE, 15.1 ± 4.0 nM; n = 4) relative to the wt protein, although this was not statistically significant ($P >$ 0.05). The loss in binding to immobilized heparin was also observed by ligand blotting (data not shown).

IGF-I exhibited a dose-dependent increase in MCF-7 cell survival independent of the substratum, achieving a maximal response at approximately 10 ng/ml (results not shown). However, the phenotype of the cells was substratum dependent, because cells grown on collagen or fibronectin exhibited an adherent phenotype, whereas those grown on laminin exhibited a much greater cell-cell interaction, with minimal cell-substratum interaction (Fig. 7).

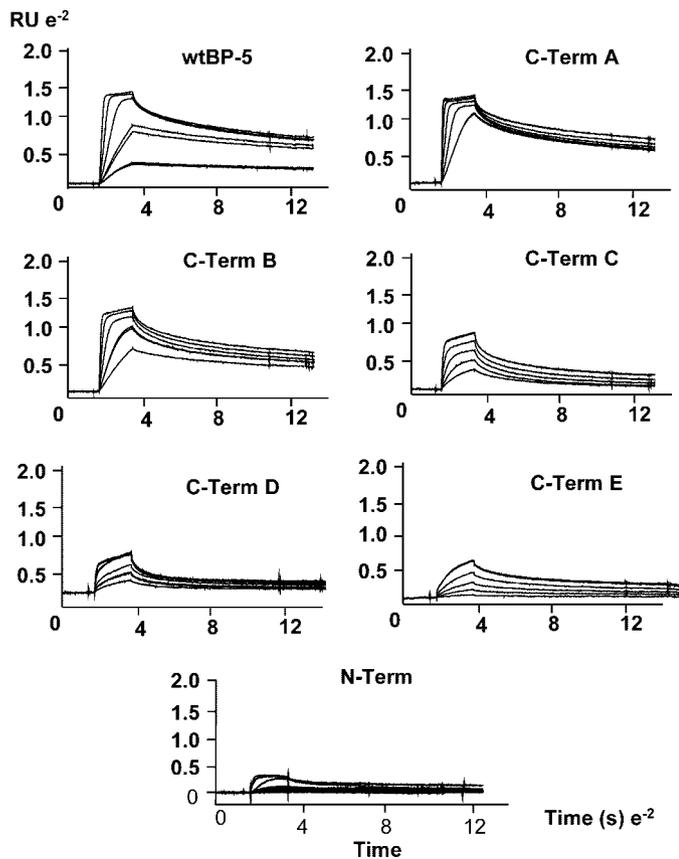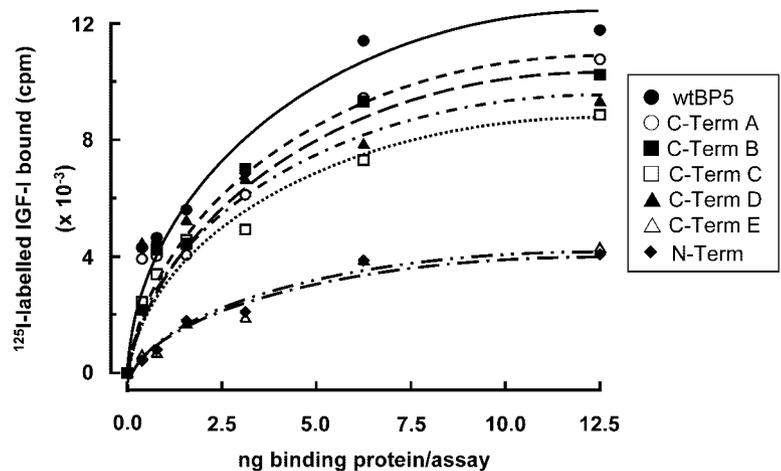IGFBP-5 was able to inhibit the effects of IGF-I in a dose-dependent manner regardless of substratum, whereas C-

RU e^{-2}

FIG. 4. Biosensor analysis of IGF-I binding. Individual sensograms for wt IGFBP-5, C-Term A–E, and N-Term binding to human IGF-I ligand, which was covalently captured by amine coupling chemistry to the carboxymethyl surface of a CM5 biosensor chip. See *Materials and Methods* for the details of the collection and analysis of data and the concentrations of IGFBP-5 proteins present as analyte in these experiments.

FIG. 5. Solution phase assay of IGF-I binding to wt and mutant IGFBP-5 proteins. The wt IGFBP-5 (●), C-Term A (○), C-Term B (■), C-Term C (□), C-Term D (▲), C-Term E (△), and N-Term (◆) were purified by HPLC (see above). Binding was assayed by incubation of proteins overnight with 25,000–30,000 cpm [^{125}I]human IGF-I and measurement of bound ligand by the method described previously (13). This experiment was repeated twice with similar results.

Term E and N-Term mutants were virtually ineffective (Fig. 8A). More conservative mutants (C-Term A and B) were also effective inhibitors of IGF-I, although there was a progressive diminution of this inhibitory activity such that C-Term D was largely ineffective (Fig. 8B). In the absence of IGF-I, IGFBP-5 had no effect on cell number (results not shown).

Figure 9A shows the ability of wt IGFBP-5 to enhance the effects of IGF-I on various substrata. Although IGF-I increased cell numbers by approximately 50% when added
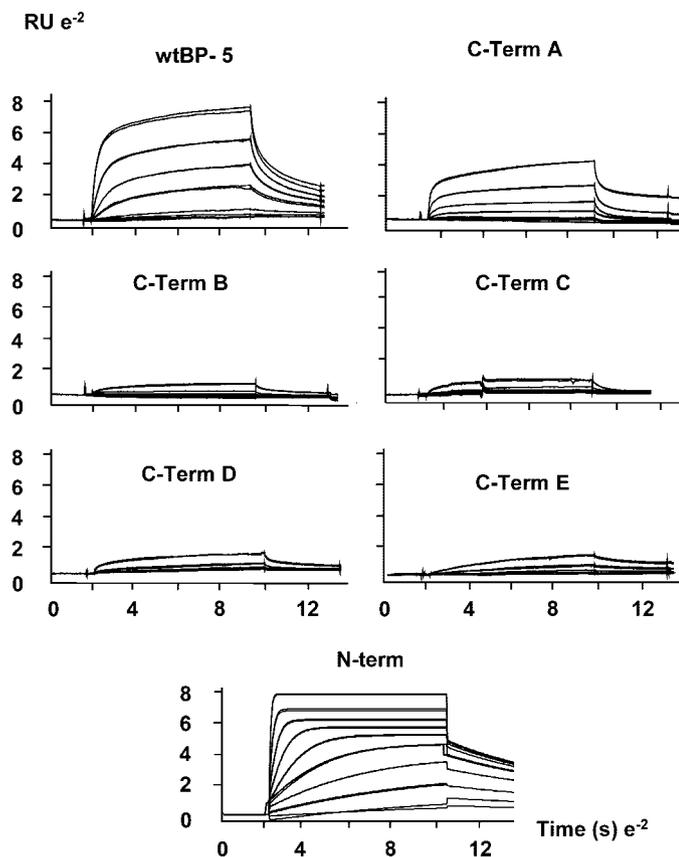

FIG. 6. Biosensor analysis of heparin binding. Individual sensograms for wt IGFBP-5, C-Term A–E, and N-Term binding to heparin coupled to the surface of a CM5 biosensor chip. Experimental procedures were exactly as described previously (28).

without IGFBP-5, in the presence of IGFBP-5, cell numbers were increased by approximately 100%. These effects of IGF-I and IGFBP-5 were independent of the substratum upon which the cells had attached. Furthermore, IGFBP-5, when added in the absence of IGF-I, failed to influence cell numbers. Figure 9B shows the enhancement effect for all mutants. Once again, C-Term A and B possessed activities similar to that of wt IGFBP-5. However, in contrast to their limited inhibitory potential, C-Term C–E were as effective as wt IGFBP-5 in augmenting the actions of IGF-I when captured on the substratum (Fig. 9B). However, N-Term was completely ineffective in this assay, adding additional support to the conclusion that the effects of IGFBP-5 in this system were dependent upon interaction with IGF-I. The discrepancy between the inhibitory and enhancing effects of the mutants was explained by determining the capture and release of IGF-I from the substratum. IGFBP-5 bound to the substratum was capable of binding approximately 30% of the IGF-I added, whereas in the absence of IGFBP-5, less than 1% of the IGF-I bound directly to the substratum. There was a progressive decrease in the amount of IGF-I bound to IGFBP-5 on the substratum with increasing number of mutations, although C-Term D bound more than anticipated (Fig. 10A). Despite the fact that all the C-Term mutants bound less IGF-I than wt IGFBP-5 during the culture period, some of the mutants released more IGF-I than did wt IGFBP-5 (Fig. 10B), and when expressed as a proportion of the amount bound, all the mutants released considerably more than wt IGFBP-5. Thus, although wt IGFBP-5 released approximately 5% of the total IGF-I bound during the first 24 h of culture, C-Term E released almost 20% (Fig. 10C). Once again, however, C-Term D behaved anomalously, releasing proportionately less IGF-I. These findings were again independent of the substratum. Because the release of bound IGF-I was dependent both upon its release from IGFBP-5 and upon the release of IGFBP-5 from the substratum, we examined IGFBP-5 release under identical conditions using an IGFBP-5 RIA. Seventy-five to 85% of wt IGFBP-5 added to the cultures was bound, independent of the substratum used (Fig. 11A). Similar amounts of the mutants were also bound, with the exception of C-Term E, in which this was approximately 50–70%. However, during the first 24 h in culture, progressively more
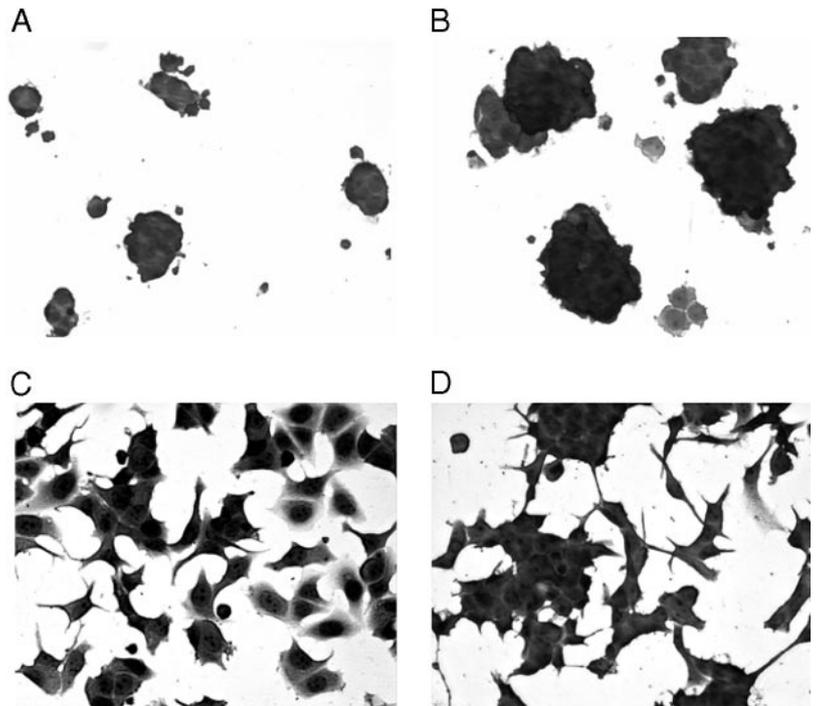
FIG. 7. MCF-7 cells grown in the absence (A and C) or presence (B and D) of IGF-I on laminin (A and B) or fibronectin (C and D). Cells on laminin demonstrate reduced cell-substratum interactions. Although cells on fibronectin demonstrate a flattened morphology indicative of cell-substratum contact, IGF-I induced a shift toward greater cell-cell contact, although these cells retained numerous cellular projections in contact with the substratum.

IGFBP-5 was released with increasing number of mutations (again with the exception of C-Term D; Fig. 11B) Thus, approximately 2% of wt IGFBP-5, N-Term and C-Term D, were released from the substratum, whereas this dissociation increased progressively for the other mutants, reaching approximately 10% for C-Term E (Fig. 11C). Note also that the proportion of IGF-I released (Fig. 10C) was consistently approximately twice the proportion of IGFBP-5 released (Fig. 11C).

Overall, these results suggest a faster dissociation of the IGFBP-5 mutants from the substratum as part of the explanation for the increased bioavailability of IGF-I despite their reduced ability to sequester IGF-I onto the substratum during the association phase.

Discussion

At the outset of this study it was considered essential to establish whether the effects on IGF-I binding of our amino acid substitutions in the IGFBP-5 sequence were specific or were simply the effect of a gross conformational change in protein structure. CD spectra were obtained to compare the secondary structures of wt IGFBP-5 and C-Term E, and these were virtually superimposable. Furthermore, the fluorescence spectra for both wt IGFBP-5 and C-Term E gave emission maxima of around 385 nm, indicating that the single tryptophan residue in both proteins was similarly orientated. Therefore, both fluorescence and CD structural analyses indicated that the overall secondary and tertiary structures of IGFBP-5 were not disrupted by the mutation, to alanine, of the 10 basic amino acids between 201 and 218.

Although we have previously reported that G203 and Q209 are important residues in the C-terminal domain of IGFBP-5 involved in IGF-I binding, we were unable to conclude that these were the only C-terminal residues involved

in this function (13). However, substitution of the two basic residues adjacent to G203 and Q209 had only a small (<2-fold) effect on the K_D. This adds additional support to the belief that residues G203 and Q209 are indeed critical for interaction with IGF-I, rather than reflecting a global change in the IGFBP-5 molecule as a result of mutagenesis. Furthermore, an additional mutation, K206A, which is predicted to lie on the same face of the molecule, juxtaposed between the two IGF-binding residues, had no effect on the K_D for IGF-I. However, we have demonstrated that mutation of all the basic Arg or Lys residues in the 201–218 region of IGFBP-5 ultimately produces a significant 15-fold reduction in affinity for ligand, as determined by biosensor analysis, although this was still less than that induced by mutation of the N terminus, which resulted in a 45-fold decrease in affinity for IGF-I.

At first sight, there would appear to be a discrepancy between our data and those of Parker *et al.* (22), who reported only very minor reductions in IGF-I binding when they substituted certain basic residues in this region of IGFBP-5. However, our solution phase data actually agree very well with their data when only a limited number of basic residues are mutated. The only mutant that is identical in the two studies is C-Term A (R214A), which their Scatchard analysis, derived from solution phase assays, demonstrated to have a 1.22-fold reduction in IGF-I binding relative to the wt IGFBP-5 protein, which compares well with the 1.35-fold reduction for this mutant in our study. Furthermore, the greatest number of simultaneous substitutions of basic residues made by Parker *et al.* (22) is four (R201A:K202N:K206N: K208N), and this mutant had a 1.15-fold reduction in IGF-I affinity. This is a very similar combination of mutations to our C-Term C mutant, which only has one extra residue mutated, (R201A:K202A:K206A:K208A:R214A) and has a 1.63-fold reduction in IGF-I binding in our solution phase
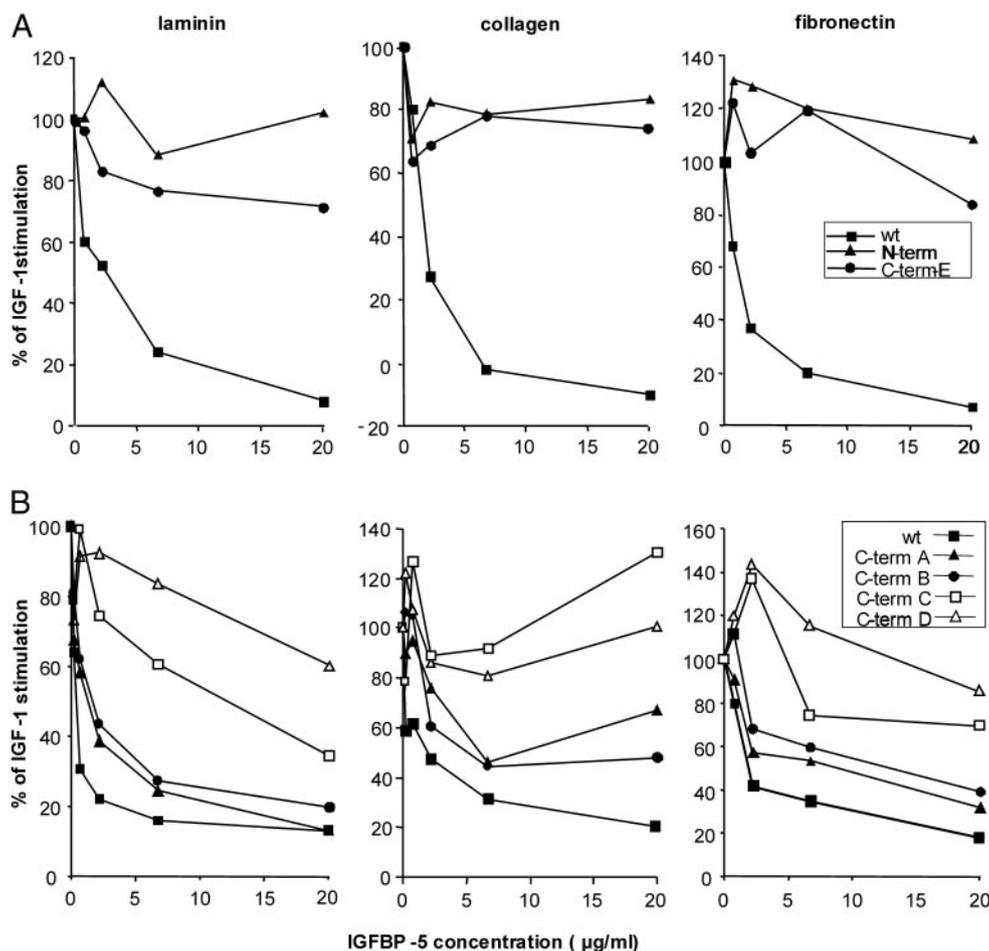
FIG. 8. Inhibition of IGF-I-mediated MCF-7 cell survival by wt IGFBP-5 (wt), C-Term E and N-Term (A), or wt and mutants C-Term A-D (B) cultured on laminin, collagen, or fibronectin. Values are the means of triplicate wells.

assays. Even C-Term D, with seven basic residues substituted, still demonstrated less than a 2-fold reduction in binding. The differences in affinity estimates thus seem to reside in the nature of the system used to determine it, rather than differences between laboratories. At present it is not possible to say which basic amino acids in the 201–218 region of IGFBP-5 contribute most quantitatively to IGF-I binding, and it may be that certain combinations of mutations are more effective than others in reducing affinity for ligand. Although our more sensitive biosensor analysis recorded slightly larger reductions in IGF-I binding for C-Term A–D, the most significant reduction in IGF-I binding (15-fold) only occurred when we mutated all 10 basic residues in C-Term E. Thus, the major effect on IGF binding in this study occurred with mutation of residues 216, 217, and 218 at the extreme C-terminal end of this region. Two of these residues (217 and 216) are in close proximity with residues 203 and 209, respectively, within a helical wheel alignment of the amino acids in this region, originally presented by Parker *et al.* (22). Definitive proof that the IGF-binding site primarily includes residues 203, 209, 216, and 217 will only be obtained once the crystal structure of the C-terminal domain of IGFBP-5 is determined, and its contact with IGF-I is resolved in detail.

Recently, others have examined the IGF-II-binding site in the C-terminal domain of IGFBP-6 by nuclear magnetic resonance (30). IGFBP-6 is unique among the IGFBP family in that it has a much higher affinity for IGF-II than IGF-I. Nevertheless, among the IGFBPs, IGFBP-5 has the highest binding affinity for both IGF-II and IGF-I. Although, IGFBP-6 shares seven of the 10 basic amino acids in the equivalent C-terminal region as 201–218 in IGFBP-5, the nuclear magnetic resonance study indicated that the main IGF-II-binding site involved three nonbasic residues (not including the equivalent G203 and Q209 residues in IGFBP-5). However, these researchers also showed that although mutating these three residues to alanines decreased binding to IGF-II, it had no effect on binding to IGF-I (30). Therefore, it may be that this particular site is only involved in binding IGF-II and confers on IGFBP-6 its unique preference for binding this growth factor. Certainly, in our present study mutation of the basic residues in the 201–218 region of IGFBP-5 led to progressive loss of binding to biotinylated IGF-II in ligand blot analysis (data not shown).

Although this present study clearly indicates a role for some or all of the basic residues in the 201–218 region of IGFBP-5 in binding IGF-I, it is crucial to remember that this region also contains a major heparin-binding site in both IGFBP-5 and -3, which has previously been shown to be
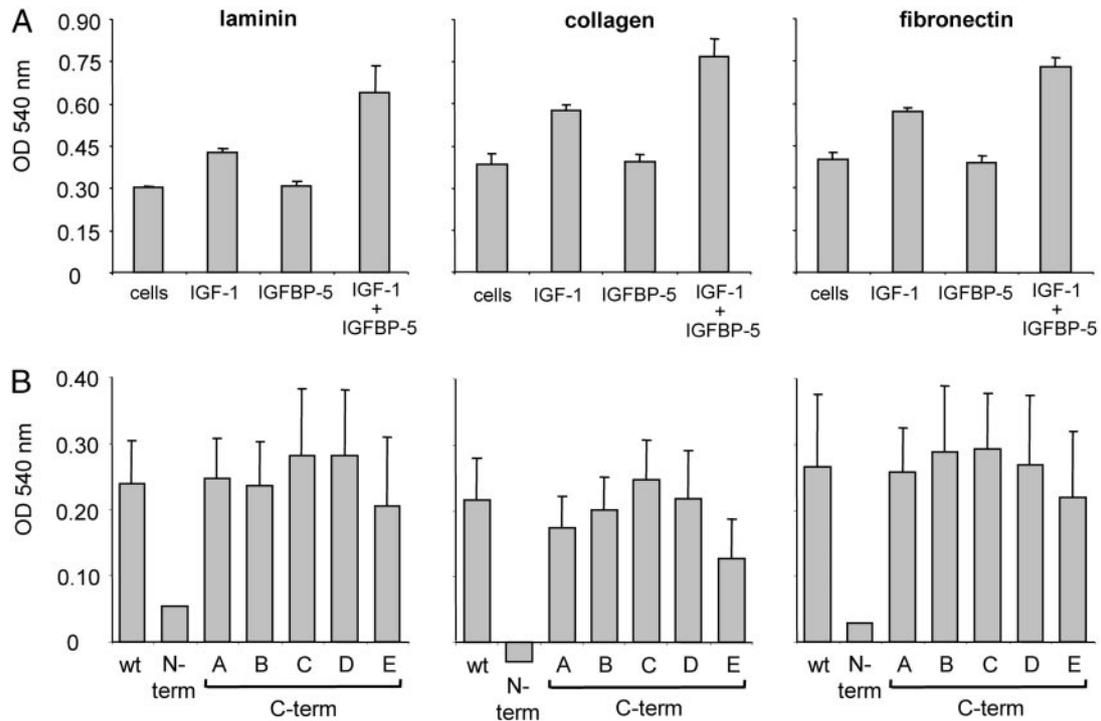
FIG. 9. Enhancement of the actions of IGF-I by IGFBP-5 when precomplexed and bound to the solid phase. A, Ability of wt IGFBP-5 to enhance the effect of IGF-I independent of the substratum. B, Enhancement of IGF-I action by wt IGFBP-5 and mutants. The enhancement is defined as the increase in cell number over and above that by IGF-I alone (calculated for wt IGFBP-5 by subtracting column 4 from column 2 in A). Values are the mean ± SEM of four experiments, except for N-Term (n = 2).

involved in binding to heparin and components of the ECM (14, 16). Although we demonstrated a minor contribution in heparin binding from R214, because C-Term A had only a slight reduction in binding to the heparin-coated biosensor chip, we found that C-Term B displayed a very sharp decrease in binding to levels below which affinity constants could be calculated. This is what we expected, because this mutant has one of the basic residues, K208, substituted in the putative classical heparin-binding motif (XBBBXXBX, where X is any amino acid, and B is basic) between YKRKQCKP (205–212). Taken together, these findings support the argument that within the C terminus of IGFBP-5, and probably also within IGFBP-3, there is overlap of residues involved in IGF-I and heparin binding, with IGF binding including 203, 209, 216, and 217, and heparin binding spanning 205–212. This has important implications for the biological actions of these two IGFBPs, because it implies that any of the numerous functions ascribed to the 210–218 region in IGFBP-5 may also impact upon IGF-I binding. Other functions ascribed to this region include binding to the acid-labile subunit, the putative IGFBP-5 receptor, and plasminogen activator inhibitor-I in addition to acting as a nuclear localization signal (21). In support of this hypothesis, others have clearly demonstrated that incubation of IGFBP-5 with heparin resulted in a 17-fold decrease in the affinity for IGF-I (31). These researchers argued that this lowering of the affinity of IGFBP-5 for IGF-I when 201–218 is bound by heparin may be critical in obtaining the correct balance between free and bound growth factors. Furthermore, recent biosensor analyses from our laboratory have shown that prebinding

IGFBP-5 with IGF-I inhibits the subsequent association of the binding protein with heparin, and conversely, that heparin prebound to IGFBP-5 inhibits the association of the binding protein with biosensor surfaces containing immobilized IGF-I (28). This led us to conclude that binding of IGF-I and heparin to IGFBP-5 is partly competitive in nature.

Although MCF-7 cells produce several IGFBPs, including IGFBP-5, the concentrations of IGFBP-5 used in this study were designed to mimic those occurring during involution of the mammary gland *in vivo*, which are typically 3 orders of magnitude greater than those in MCF-7 cultures. When we examined the biological activities of the C-Term mutants, we discovered that their ability to inhibit IGF-mediated cell survival was correlated with their affinity for IGF-I. However, C-Term D was an exception, because it had a K_D intermediate between that of C-Term A and B, but its ability to inhibit IGF-I was considerably reduced. This particular mutant had different kinetics in terms of its interaction with IGF-I, because it had increases in both K_a and K_d, suggesting a more kinetically dynamic binding complex. This, in turn, suggests that slower kinetic interactions favor inhibitory effects, presumably by reducing the likelihood of dissociation, which would decrease the prospect of binding to the IGF receptor. In contrast, the ability to enhance the actions of IGF-I was largely unaffected in any of the mutants, even C-Term E, which was incapable of inhibiting IGF-I in the liquid phase. The retention of this biological activity of the mutants appeared to be explained by a faster dissociation of IGF-I from the substratum (where it was bound via IGFBP-5) due to increases in K_d and is similar in concept to the interaction of
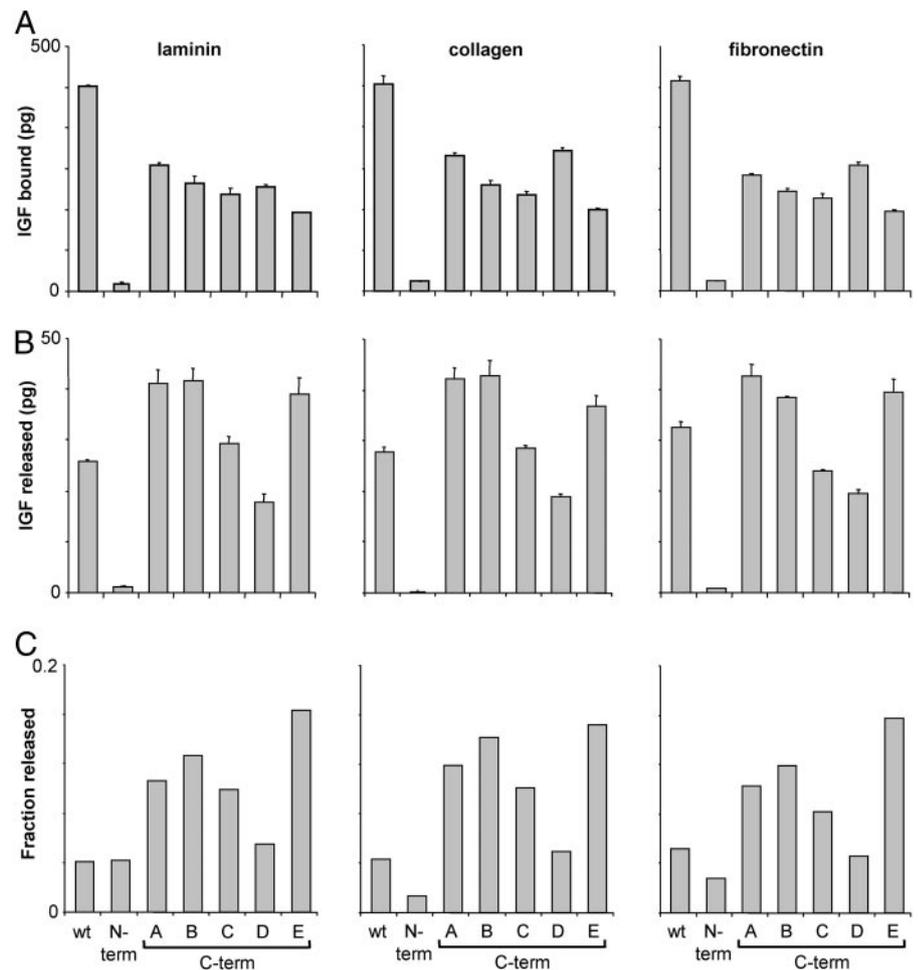
Fɪɢ. 10. Binding and dissociation of [^{125}I]IGF-I/IGFBP-5 complexes to laminin, collagen, or fibronectin. A, Quantity of IGF-I bound after 24 h of culture. After washes to remove unbound IGF-I, the quantity of radio-labeled IGF-I released from the substratum during the subsequent 24 h of culture was determined and is shown in B. The proportion of IGF-I released is shown in C. Note that the binding and release of IGF-I from N-Term IGFBP-5 were not significantly different from the binding and release of IGF-I in the absence of any IGFBP-5 (results not shown). Values are the mean ± ѕEM of three experiments.

IGFBP-5 with heparin, which reduces its affinity for IGF-I and results in enhancement of IGF actions. However, given that mutations in the C-terminal domain of IGFBP-5 led to reductions in both the affinity for IGF-I and that for the substrata, it was important to define which of these had the greatest influence on the release, and hence the bioavailability, of IGF-I. Examination of the dissociation of IGFBP-5 mutants from the substratum revealed that dissociation was progressively enhanced with increasing number of mutations (again with the exception of C-Term D), implying that increased release of IGF-I:IGFBP-5 complexes was able to at least partially explain the enhancing capabilities of the IGFBP-5 mutants despite their reduced ability to bind IGF-I. However, the proportion of IGF-I released from the substratum consistently exceeded (by ~2-fold) the proportion of IGFBP-5 released, suggesting that dissociation of IGF-I from the IGFBP-5 that remained bound to the substratum also made a contribution to the bioavailability of IGF-I. Thus, we conclude that the increased proportional bioavailability of IGF-I when bound to IGFBP-5 mutants in this model involves two processes, and these contribute approximately equal quantities of IGF-I: firstly, a more rapid release of IGFBP-5 mutants from the substratum (and presumably subsequent dissociation of IGF-I from that IGFBP-5), and secondly, dissociation of IGF-I from IGFBP-5, which remained bound to the substratum. These actions of IGFBP-5 mutants appeared

to be largely independent of whether cells exhibited a phenotype with extensive cell-substratum interactions (fibronectin and collagen) or a predominantly cell-cell contact (laminin). Because C-Term E was completely ineffective in terms of inhibition of IGF actions, but retained its ability to enhance IGF-I, this mutant represents an IGFBP-5 molecule with exclusively enhancing actions.

In summary, we have demonstrated that basic residues adjacent to residues in the region 201–218 important for IGF binding have little effect on affinity for IGF, supporting our previous conclusions that residues G203 and Q209 are important for the interaction with IGF-I. However, cumulative mutagenesis of the basic amino acids in the 201–218 region of IGFBP-5 led to a progressive loss of IGF-I binding. Ultimately, this produced a 15-fold reduction in binding when all 10 basic residues were mutated to alanines, an effect that was not due to gross conformational changes in protein structure. The ability of IGFBP-5 mutants to inhibit the actions of IGF-I on cell survival in MCF-7 cells showed a strong correlation with their relative affinities for IGF-I, determined by biosensor analysis, suggesting that affinity for IGF-I is an important factor in this biological property of IGFBP-5. However, we also identified that a mutant C-Term D, with a K_D similar to those of other mutants, but with faster kinetics, had a much reduced inhibitory capacity, indicating that the kinetics of the interaction with IGF-I are also important in
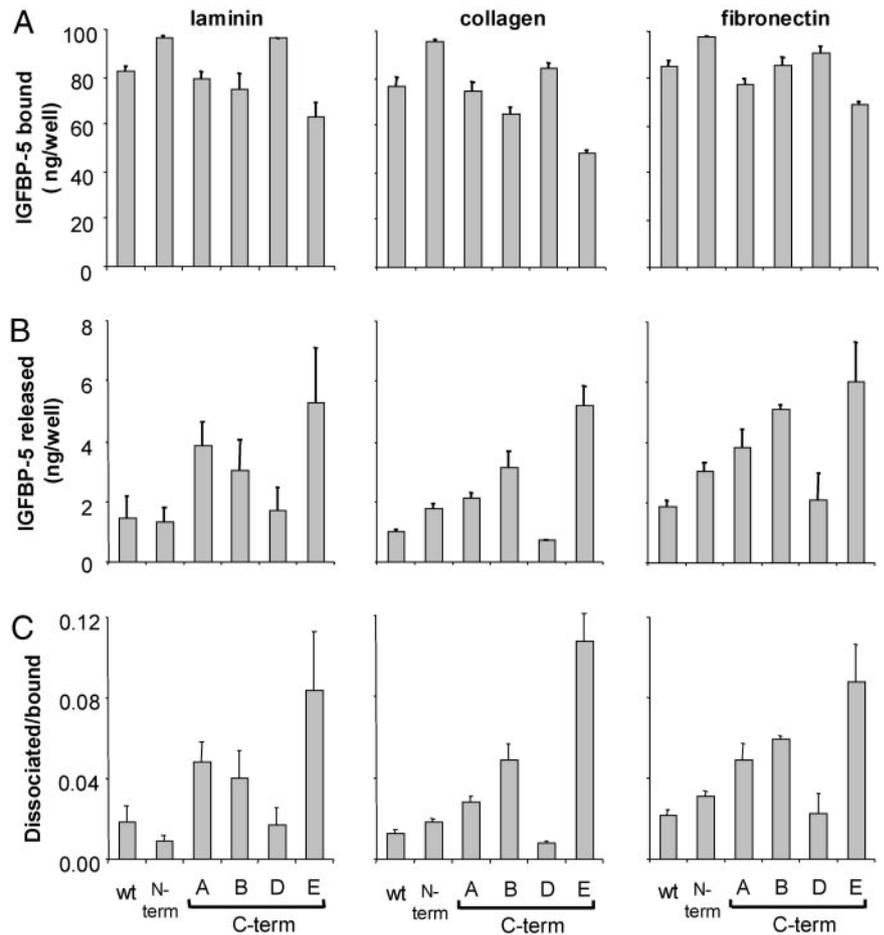
FIG. 11. Binding and dissociation of IGFBP-5 to laminin, collagen, and fibronectin. IGFBP-5 (1 μg/ml) was incubated with IGF-I (1 μg/ml) overnight at 37 C. The unbound material was removed, and the wells were washed before incubating for an additional 24 h to allow dissociation of the bound IGFBP-5. IGFBP-5 concentrations were determined by RIA using each mutant as its own standard. A, Bound IGFBP-5; B, dissociated IGFBP-5; C, the proportion of bound material that dissociated. Values are the mean ± SEM of three observations.

determining this property. In stark contrast to this, the affinity of IGFBP-5 mutants for IGF-I had no predictive value with respect to their ability to enhance the effects of IGF-I when bound to components of the ECM. Our results demonstrate that it is possible to engineer IGFBP-5 mutants (such as C-Term E) that have exclusively IGF-enhancing effects. Whether it is possible to generate IGFBP-5 mutants with exclusively inhibitory effects remains to be determined. This study does not take account of possible IGF-independent effects of such mutants, which are the subject of current studies.

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