Specific Amino Acid Substitutions Determine the Differential Contribution of the N- and C-terminal Domains of Insulin-like Growth Factor (IGF)-binding Protein-5 in Binding IGF-I*

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The actions of insulin-like growth factors (IGFs) are mediated by binding to and activating type 1 IGF receptors, which are found on the surface of most cell types. In turn, IGFs 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. 2). Because the affinity constants of the IGFBPs are between 2- and 50-fold greater for binding IGFs than that of the IGF type 1 receptor, it is thought that the IGFBPs are able to regulate the bioavailability of IGFs in different biological fluids.

IGFBPs are proteins of 216–289 residues, and all share a common protein structure that can be conceptually divided up into three domains, where a domain may be defined as a region of a protein that can fold into a tertiary structure independent of neighboring sequences (2) (Fig. 1). There is a particularly high degree of conservation in the N- and C-terminal domains, which contain 12 and 6 cysteine residues, respectively, in IGFBPs 1–6 of all species sequenced so far. A non-conserved region separating the N- and C-terminal domains contains most of the sites for proteolysis and post-translational modifications. Both the N- and C-terminal cysteine-rich domains of IGFBPs are believed to participate in IGF binding. This is supported by work where fragments from the N-terminal cysteine-rich domains of IGFBP-1 (3), IGFBP-3 (4), IGFBP-4 (5), and IGFBP-5 (6, 7) and the C-terminal cysteine-rich domains of IGFBP-2 (8, 9) and IGFBP-3 (10) were generated and shown to possess residual IGF binding activity. More recently, several groups have reported biosensor analysis of N- and C-terminal fragments of bovine IGFBP-2 and human IGFBP-3 (11–13), and all of these studies indicated that there were major IGF-binding sites in both the N- and C-terminal domains.

Work from several laboratories has already identified the specific amino acids in the N-terminal domain of the IGFBP molecule that contribute to IGF binding. Initially it was observed that Tyr60 was protected against iodination upon binding of bovine IGFBP-2 to IGF-II (14), and subsequently, the same group demonstrated that substitution of this amino acid leads to reduced affinities for IGF-I and -II (15). In addition, NMR was carried out on a bacterially expressed N-terminal fragment of IGFBP-5 (residues 40–92), and this clearly demonstrated that substitution of Tyr60 in IGFBP-5 led to a significant reduction in IGF-I binding, suggesting that the highly conserved Gly and Gln residues have a conserved IGF-I binding function in all six IGFBPs. Finally, although these residues lie within a major heparin-binding site in IGFBP-5 and -3, we also show that the mutations in C-Term have no effect on heparin binding.

The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; wt, wild type; GST, glutathione S-transferase.

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The NMR study described above also found that the N-terminal fragments of IGFBP-5 had a 10–200-fold lower affin-
IGF Binding of N- and C-terminal Domains of IGFBP-5

Figure 1. Conservation of the C-terminal glycine and glutamine residues in the IGFBP family. At the top is a schematic representation of the three structural domains of the IGFBPs. The box below shows a single letter amino acid line up for a region in the C-terminal domain for all six IGFBPs from several different species. The residue numbers at the start and end of each sequence are indicated in parentheses. The glycine and glutamine residues in question are shown in bold type, which clearly indicates the complete conservation of Gly\(^{203}\) and Gln\(^{209}\) in rat IGFBP-5 in all the other IGFBP sequences.

MATERIALS AND METHODS

Site-directed Mutagenesis—The full-length cDNAs for rat IGFBP-5 and -2 in pGEM\(^{\text{TM}}\)-7zF (Promega, Madison, WI), containing both initiator and signal peptide, was kindly provided by Dr. S. Guenette (John Wayne Cancer Institute, Los Angeles, CA). Site-directed mutagenesis was carried out using the QuikChange\(^{\text{TM}}\) system (Stratagene, La Jolla, CA), following the protocol provided by the manufacturer. The rat and mouse protein sequence of IGFBP-5 only differs by 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) using the oligonucleotides: 5'-GAC AGG AGT CTG AAC AAG GCC CCT GAC AAG CAT GCT CTG TAC AAC CTC AAA GCT-3'. The N- and C-term mutant was made by using the unique SacII restriction endonuclease site in the IGFBP-5 cDNA sequence to cut and ligate N-term and C-term composite fragments together. The wt rat IGFBP-2 cDNA was mutated to G203A/Q209A using the oligonucleotides 5'-AAC TGT GAC CGC AAA GCT TTC TAC AAG CAC GCA GCA CGC CCA CGC-3'. The N- and C-term mutant was made by using the unique SacII restriction endonuclease site in the IGFBP-5 cDNA sequence to cut and ligate N-term and C-term composite fragments together. The wt rat IGFBP-2 cDNA was mutated to G203A/Q209A using the oligonucleotides 5'-AAC TGT GAC CGC AAA GCT TTC TAC AAG CAC GCA GCA CGC CCA CGC-3' and 5'-CGG CCG CGG TCC TCG TGC TTC GAC ACC GCC CCT GCC-3' and 5'-GGG AGG GCC GGT ACG GTC AGT CTC TCC TCA TGA CAT CTT TCC TCT CTC ACG ACG CAG ACG ACG-3'. Our previously reported "heparin minus" (Hep-) mutant is defective in its ability to bind to heparin (22). All of the oligonucleotides were synthesized by MWG BioTech (Milton Keynes, UK). Following site-directed mutagenesis, automated DNA sequencing (MWG BioTech) was carried out on all clones to confirm that the correct mutations had been made.

Bacterial Expression—Expression of recombinant IGFBP-5 proteins was carried out using conditions identical to those described previously (24). Briefly, mouse wt IGFBP-5 and the various IGFBP-5 mutants were cloned without the signal peptide-encoding sequence into the pGEX 6P-1 vector (Amersham Biosciences), so that the proteins would have an N-terminal glutathione S-transferase (GST) tag. The proteins...
protein concentration of 0.1 mg/ml for both wild type and mutant samples; (excitation, 290 nm; slits of 5-nm band pass).

**IGF-I Ligand Blotting and Western Immunoblotting**—The proteins were electrophoresed on 12% acrylamide SDS-PAGE gels under nonreducing conditions and subsequently transferred to nitrocellulose membranes. The ligand blots were performed according to the method described previously (27), using IGF-I (ConPep Limited, Adelaide, Australia) radiolabeled to a specific activity of ~100 μCi/μg. Western blots with either an “in-house” sheep anti-rat IGFBP-5 antiserum or a commercial rabbit anti-bovine IGFBP-2 antibody (Upstate Biotechnology, Inc., Lake Placid, NY) were performed as described previously (23) and were used to monitor protein loading during ligand and heparin blotting experiments. The method used for heparin ligand blotting was also described previously (22).

**Solution Phase IGF Binding Assay**—Binding affinities for IGF-I were assayed in solution by the method described previously by Conover et al. (28). The assays were performed using the PreScission protease eluate fractions obtained after expression of wt IGFBP-5 and the C-Term mutant and the supernatant bacterial lysates containing recombinant wt IGFBP-2 and the C-Term 2 mutant. The optimal amount of each protein preparation was calculated following initial saturation binding assays containing 25,000–30,000 dpm (15–18 fmol) (125I)IGF-I. The affinities were then assessed by Scatchard analysis of binding curves obtained in the presence of increasing concentrations of unlabelled IGF-I. Only the affinities for the wt IGFBP-5 and C-Term are reported because binding to the N-Term and N+C-Term mutants was too low to be measured by this method.

**Biosensor Analysis**—Biosensor studies were performed using the Biacore 3000 instrument. Human IGF-I (10 μg/ml in 10 mM sodium acetate, pH 5) was immobilized into flow cell 2 of three separate CM5 biosensor chips at ligand densities of 50, 100, and 200 response units. To provide a control binding surface, insulin was immobilized in flow cell 1 of the three biosensor chips at the same level of ligand density. Both IGF-I and insulin were immobilized using amine-coupling chemistry according to protocols provided by the manufacturer. For kinetic studies, purified proteins were injected at five different concentrations and at a flow rate of 30 μl/min in 10 mM Hepes, pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20. All of the experiments were performed at ambient temperature. The protein concentrations were as follows: commercial mouse IGFBP-5 and wt mouse IGFBP-5 (in-house): 1.56, 3.125, 6.25, 12.5, and 25 nM; C-Term, 3.125, 6.25, 12.5, 25, and 50 nM; and N-Term and N+C-Term, 25, 50, 100, 200, and 400 nM. The association and dissociation phases of the binding curves were 3 and 15 min, respectively, for the commercial protein and 5 and 15 min, respectively, for all others. Following binding the surface of the chip was regenerated by two 30-s pulses of 50 mM NaOH, 1 mM NaCl. Protein samples and a buffer blank were injected in duplicate and in random order using each of the three biosensor chips. The data were analyzed assuming a 1:1 stoichiometry of interaction between IGF-I and IGFBP-5 using the Langmuir model provided in the BiaEvaluation 3.1 software and employing the global data analysis option.

**RESULTS**

N-terminal sequence analysis of the recombinant wt IGFBP-5 protein showed the expected sequence (GPLGLSGSLFVHCEPCDDEK) derived from pEX 6P-1 and the mature IGFBP-5 sequence minus signal peptide, indicating that there had been proper processing by PreScission protease.

It can be seen from the CD results (Fig. 2A) that despite the

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1. Davidson, personal communication.
apparently significant differences in amplitude, the secondary structure estimates (given in Table I) are broadly similar, exhibiting a strong negative band with a minimum close to 200 nm in each case. Secondary structure estimates of these spectra indicate a low percentage of α-helix and a greater proportion of β-sheet and β-turns for each protein. The fluorescence emission spectra (Fig. 2B) show that wt IGFBP-5, C-Term, and N-Term have very similar emission maxima (∼345 nm) with considerable differences in intensity. In view of the fact that intensity depends on the relative magnitudes of the rates of various processes that lead to the decay of the excited state, the emission maximum is generally regarded as a more reliable indicator of the polarity of the Trp environments in a protein (29). On this basis, the solvent exposure of the Trp side chains is very similar in the three proteins, indicating that little or no overall change in tertiary structure has occurred.

Following on from this, we compared [125I]IGF-I binding of the mutant IGFBP-5 proteins, N-Term, C-Term, and N+C-Term, to that of the wt protein using ligand blotting (Fig. 3, upper panel). The same filter was also subject to Western analysis with the appropriate antiserum to verify equivalent protein loading (Fig. 3, lower panel). Although all three mutant proteins displayed reduced IGF-I binding, we note that relative to wt IGFBP-5, the greatest effect on IGF-I binding was with N-Term and N+C-Term, which display no apparent binding on ligand blots (Fig. 3).

We also examined the affinity of these mutant proteins in a solution phase analysis of the IGF-I-IGFBP interaction (Fig. 4). Scatchard analysis could only be derived from binding curves for wt IGFBP-5 and C-Term (KD values of 0.13 and 3.54 nM, respectively), which indicated that C-Term had a 30-fold lower affinity than the wt protein for binding IGF-I. We also included a commercial mouse wt IGFBP-5 protein from R & D Systems to compare IGF-I binding of wt IGFBP-5 expressed in either mammalian cells or bacteria. Our data demonstrate that the commercial and in-house wt IGFBP-5 proteins have very similar affinities or KD values for binding to IGF-I, with 0.102 and 0.13 nM, respectively. However, in support of our ligand blotting data, binding of IGF-I to the N-Term and N+C-Term mutants was too low to be able to derive Scatchard data, although Imai et al. (1) were able to measure an ∼1000-fold reduction with the equivalent N-Term mutations. This apparent discrepancy could possibly be the result of differences in the solution phase assay techniques employed by the two groups. Nevertheless, both agree that amino acid substitutions in the N-terminal domain of IGFBP-5 result in very large reductions in IGF-I binding.

As a further confirmation of the IGF binding properties of these IGFBP-5 mutant proteins, we undertook a biosensor analysis of binding kinetics of the proteins to IGF-I immobilized to a biosensor surface (Fig. 5). In agreement with the solution phase data above, the commercial and in-house wt IGFBP-5 proteins had almost exactly the same KD values, with 0.22 and 0.21 nM, respectively. However, the KD values for N-Term, C-Term, and N+C-Term were 12.5, 2.1, and 25.9 nM, respectively. For N-Term and C-Term this demonstrates 60- and 10-fold reductions in affinity for binding IGF-I compared with the wt protein and a cumulative effect of the N+C-Term mutant, which displays a 126-fold reduction in binding. This type of analysis also gives details of on and off rate kinetic constants, from which equilibrium constants can be derived (Table II). Closer inspection of the biosensor data revealed that although the N- and C-Term mutants displayed similar changes to their K_on or rate of dissociation values, the overall larger reduction in IGF-I affinity observed for N-Term was primarily due to a substantially slower K_off or rate of association compared with C-Term. Finally, the highest reduction in IGF-I affinity observed for the N+C-Term mutant was largely the result of a cumulative effect on its K_off which was approximately twice as fast as that of either N- or C-Term. We also note that the absolute KD values for wt IGFBP-5 and C-Term varied by up to 2-fold between biosensor and solution phase assays and that C-Term displayed a greater reduction in binding relative to the wt protein in the latter system (30-fold versus 10-fold).

Because Gly203 and Gln209 lie within the heparin-binding site between amino acids 201 and 218 in the C-terminal domain of IGFBP-5, experiments were also carried out to investigate...
the heparin binding properties of the C-Term mutant. Using a heparin blotting methodology we have previously reported that wt IGFBP-5 and the two single mutants, G203A and Q209A, bind to heparin in this system, whereas the Hep/H11002 mutant, which contains mutations at four basic residues in this region (R201L, K202E, K206Q, and R214A) binds heparin very poorly (22). We have now used this system to compare heparin binding between wt IGFBP-5, C-Term (G203A/Q209A combined) and Hep- (Fig. 6, upper panel). Although wt IGFBP-5 and C-Term display comparable binding to heparin in this system, there is no evidence of binding for Hep-. As with the IGF ligand blotting, corresponding Western blots were carried out to verify equivalent protein loading (Fig. 6, lower panel). Therefore, the residues Gly203 and Gln209 within the 201–218 region are in-

![Heparin blotting methodology](image)

**TABLE II**

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{on}$</th>
<th>$K_{off}$</th>
<th>$K_D$</th>
<th>Fold decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>$37.5 \pm 23.1 \times 10^5$</td>
<td>$0.72 \pm 0.54 \times 10^{-3}$</td>
<td>$0.206 \pm 0.089$</td>
<td>-</td>
</tr>
<tr>
<td>C-Term</td>
<td>$14.5 \pm 3.2$</td>
<td>$2.47 \pm 1.67$</td>
<td>$2.08 \pm 1.87$</td>
<td>10</td>
</tr>
<tr>
<td>N-Term</td>
<td>$2.18 \pm 0.35$</td>
<td>$2.70 \pm 0.4$</td>
<td>$12.45 \pm 0.15$</td>
<td>60</td>
</tr>
<tr>
<td>N + C-Term</td>
<td>$2.21 \pm 0.65$</td>
<td>$5.14 \pm 0.26$</td>
<td>$25.9 \pm 8.8$</td>
<td>126</td>
</tr>
</tbody>
</table>

**FIG. 5. Biosensor analysis of IGF-I binding.** Individual sensograms for commercial mouse IGFBP-5, wt IGFBP-5 (in-house), C-Term, N-Term, and N + C-Term binding to human IGF-I ligand immobilized on the surface of a biosensor chip. The sensograms show $K_D$ values calculated as the ratio of the rate constants $K_{off}/K_{on}$. See “Materials and Methods” for concentrations of IGFBP-5 proteins present as analyte in these experiments.
volved in IGFBP-5 binding to IGF-I but appear to play no part in binding to heparin.

Finally, because Gly\textsuperscript{203} and Gln\textsuperscript{209} in IGFBP-5 are completely conserved in all six IGFBPs from all species sequenced to date, we investigated whether these residues were also involved in IGF-I binding in a different IGFBP species, IGFBP-2. Ligand Western analysis and solution phase IGF binding assays were carried out on wt IGFBP-2 and the C-Term 2 mutant, which contains the corresponding amino acid substitutions (G211A/Q217A) in the rat IGFBP-2 sequence (Fig. 7). IGF ligand blotting demonstrated that there was a very significant reduction in C-Term 2 binding to \[^{125}\text{I}]\text{IGF-I} relative to wt IGFBP-2, where only a faint band was detectable for C-Term 2 (Fig. 7A, upper panel). We note that Western blot analysis of the same filter using an anti-IGFBP-2 antiserum demonstrated equivalent protein loading of wt IGFBP-2 and C-Term 2 (Fig. 7A, lower panel). Further support of this conclusion comes from the observation that the solution phase binding curves also demonstrate compromised IGF-I binding for C-Term 2 relative to the wt protein (Fig. 7B). Scatchard analysis derived from binding curves for wt IGFBP-2 and C-Term 2 (K\textsubscript{D} values of 0.14 and 0.61 nM, respectively) indicated that C-Term 2 had a 4.5-fold lower affinity than the wt protein for binding IGF-I. Taken together, these results clearly demonstrate that Gly\textsuperscript{211} and Gln\textsuperscript{217} in the IGFBP-2 sequence are involved in binding to IGF-I.

**DISCUSSION**

At the outset, we considered it essential to establish whether the effects of our amino acid substitutions in the IGFBP-5 sequence on IGF-I binding 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, C-Term, and N-Term, and it was observed that the overall shapes of the spectra were similar for all three proteins. Interestingly, similar CD spectra have also been shown for other proteins, including oxidized ribonuclease and a subclass of all-ß proteins (termed ß-II proteins) such as ß-chymotrypsin, elastase, and soybean trypsin inhibitor protein. X-ray diffraction data have shown that these ß-sheet proteins are either highly distorted or made up of short irregular ß strands (30, 31), and this could also hold true for IGFBP-5. The fluorescence spectra show that wt IGFBP-2, C-Term, and N-Term have very similar emission maxima, which indicates that the orientation of the Trp side chains is very similar in the three proteins. However, subtle changes in the microenvironments of the respective side chains could effect the efficiency of quenching by moieties such as disulfide bonds, and it is possible that this could account for the significant differences observed in the intensity of fluorescence spectra for the three proteins. Nevertheless, these spectra would argue that little or no overall change in tertiary structure has occurred between the wt protein and the two mutants. Therefore, taken together our structural analyses confirm that the amino acid substitutions that we have made in both N-Term and C-Term have not led to gross changes in protein structure.

Bearing this in mind, our experiments confirm the previous work of Imai et al. (1), which showed that these N-Term mutations led to the disruption of a major IGF-I-binding site in IGFBP-5, because both groups report very large decreases in binding with the same amino acid substitutions. However, although we could not detect any binding to N-Term and N+C-Term using solution phase IGF binding assays, Imai et al. (1) reported an \(-1000\)-fold reduction in IGF-I binding for their corresponding N-Term mutant. This apparent discrepancy is likely to be due to minor variations in the solution phase IGF binding protocols employed by the two labs. Another difference is that our analyses were with bacterially expressed proteins, whereas Imai et al. (1) expressed their recombinant proteins in Chinese hamster ovary cells (1). However, we also demonstrate that a commercial mouse wt IGFBP-5 protein, which was expressed in mammalian cells and is therefore likely to be properly post-translationally modified (phosphorylated and glyco-
sylated), has almost exactly the same affinity for binding IGF-I in solution phase assays and biosensor analysis as our bacteriaially expressed wt protein. This is supported by other work where it was shown that mutagenesis to remove the glycosylation sites or expression in a prokaryotic nonglycosylating system had no effect on the binding of IGFBP-3 to IGF-I (32, 33). Therefore, post-translational modifications of either IGFBP-5 or -3 do not appear to affect IGF-I binding.

In addition to confirming that there is a major IGF-binding site in the N-terminal domain of IGFBP-5, the major findings presented here identify the important residues in the C-terminal domain that are involved in IGF-I binding: glycine 203 and glutamine 209. Mutagenesis of both of these amino acids in the C-Term mutant has led to a cumulative effect over the single mutations reported previously (23), with either a 30- or 10-fold reduction in the affinity of C-Term for IGF-I when measured by either solution phase assays or biosensor analysis respectively. It is possible, of course, that other residues in the C-terminal domain of IGFBP-5 may contribute to IGF-I binding. However, it is interesting to compare our findings with the biosensor analyses of Carrick et al. (11), where they measured the IGF-I binding affinity of recombinant fragments of bovine IGFBP-2. Relative to full-length IGFBP-2, they observed a 5-fold reduction in IGF-I binding with their 1–185 fragment, which contains the entire N-terminal and central domains. This is very close to the 4.5-fold reduction in affinity for IGF-I that we observe for our C-Term mutant relative to wt IGFBP-2, as derived from Scatchard analysis. Therefore, relative to the native proteins, the removal of the entire C-terminal domain in IGFBP-2 leads to a reduction in IGF-I binding very similar to that observed for both our C-Term and C-Term 2 mutants, which only carry substitutions of the C-terminal residues Gly203 and Gln209 or residues Gly211 and Gln217 in IGFBP-5 or -2, respectively. This would imply that these two amino acids are the major, if not the only, residues in the C terminus of both IGFBP-5 and -2 that are involved in IGF-I binding. The fact that they are also highly conserved throughout the IGFBP family (Fig. 1) would also suggest that these residues are specifically involved in binding IGF-I in all six IGFBP proteins.

In this respect, it is important to remember that Gly203 and Gln209 lie within a major heparin-binding site in both IGFBP-5 and -3, which has previously been shown to be involved in binding to heparin and to components of the extracellular matrix (18, 19). Although the work described above clearly indicates a role for Gly203 and Gln209 in binding IGF-I, we also demonstrate that these two amino acids play no part in heparin binding because heparin-ligand analysis demonstrates similar activities for wt IGFBP-5 and C-Term. Therefore, it would appear that within the C terminus of IGFBP-5, and likely also within IGFBP-3, there is an overlap of residues involved in IGF-I and heparin binding. Furthermore, because Gly203 and Gln209 are conserved in all six binding proteins, whereas the heparin-binding site is only common to IGFBP-5 and -3, this must mean that the IGF-I binding function of this region pre-dates heparin binding during evolution. Taken together, this has important implications for the biological actions of these two IGFBPs, because it implies that any of the numerous functions ascribed to this region (amino acids 201–218 in IGFBP-5) may be mutually exclusive to effective IGF-I binding. Other functions ascribed to this region in IGFBP-5 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 (reviewed recently in Ref. 34). 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 (35). This lowering of IGF-I affinity when the 201–218 region is otherwise occupied may be critical in obtaining the correct balance between free and bound growth factor.

Our site-directed mutagenesis strategy has also enabled us to determine the differential contributions of the N- and C-terminal domains of IGFBP-5 in binding IGF-I. Independent of the technique used to measure IGF-I binding, it is clear that the amino acid substitutions in the N-terminal domain have led to a significantly greater reduction in IGF-I binding, which would argue that the major IGF-binding site is in this domain. Nevertheless, we note that Gly203 and Gln209 in the C terminus also make a significant contribution to IGF-I binding. Another advantage of our site-directed mutagenesis approach over analysis of N- and C-terminal IGFBP fragments is that we were able to combine the mutations in both termini by making the N+C-Term mutant. This clearly demonstrates that the disruption of IGF-I-binding sequences in both the N and C termini simultaneously has a cumulative effect and leads to a far greater (126-fold) reduction in IGF-I binding as measured by biosensor analysis.

Study of the individual \( K_a \) and \( K_d \) values from the biosensor analysis allows us to observe that the N-Term has a considerably slower association rate than C-Term (17- and 2.6-fold slower than wt IGFBP-5, respectively). However, the N- and C-Term mutants have comparable increases in dissociation rates, (both have \(-3.5\)-fold faster dissociation rates than wt IGFBP-5). The effect of combining the mutations, as is the case with the N+C-Term mutant, appears to be more pronounced on the dissociation rate (which is now 7-fold faster relative to wt IGFBP-5). Thus, there would appear to be an additive effect of the N- and C-term mutations on dissociation. Taken together, these biosensor results would suggest that although the N-terminal sequences play a major role in the association of IGF-I with IGFBP-5, the C-terminal sequences are more involved in stabilization of the IGFBP/IGF complex, because mutating these sequences increases the speed of dissociation.

In conclusion, we have identified Gly and Gln as the important residues in the C-terminal domain of IGFBP-5 that are involved in IGF-I binding. Although their substitution leads to a significant loss of ligand binding, it is less than that observed when several residues in the major IGF-binding site in the N-terminal domain are mutated. Further advances in this area will require the determination of the three-dimensional structure of the IGF-IGFBP complex by x-ray crystallography.

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REFERENCES

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