BIAcore Analysis of Bovine Insulin-like Growth Factor (IGF)-binding Protein-2 Identifies Major IGF Binding Site Determinants in Both the Amino- and Carboxyl-terminal Domains*

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In the absence of a complete tertiary structure to define the molecular basis of the high affinity binding interaction between insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs), we have investigated binding of IGFs by discrete amino-terminal domains (amino acid residues 1–93, 1–104, 1–132, and 1–185) and carboxyl-terminal domains (amino acid residues 96–279, 136–279, and 182–284) of bovine IGFBP-2 (bIGFBP-2). Both halves of bIGFBP-2 bound IGF-I and IGF-II in BIAcore studies, albeit with different affinities (1–132IGFBP-2, \( K_D = 36.3 \) and 51.8 nM; 136–279IGFBP-2HIS, \( K_D = 23.8 \) and 16.3 nM, respectively). The amino-terminal half appears to contain components responsible for fast association. In contrast, IGF binding by the carboxyl-terminal fragment results in a more stable complex as reflected by its \( K_D \). Furthermore, des(1–3)IGF-I and des(1–6)IGF-II exhibited reduced binding affinity to 1–279IGFBP-2HIS, 1–132IGFBP-2, and 136–279IGFBP-2HIS biosensor surfaces compared with wild-type IGF. A change reversal at positions 3 and 6 of IGF-I and IGF-II, respectively, affects binding interactions with the amino-terminal fragment and full-length bIGFBP-2 but not the carboxyl-terminal fragment.

IGFs are polypeptide hormones that elicit mitogenic and metabolic effects upon binding the IGF and insulin receptors (reviewed by Jones and Clemmons (1)). A family of at least six IGF-binding proteins (IGFBPs) modulates the bioavailability of IGF-I and IGF-II. IGFBPs play an important role in regulating IGF actions by increasing their circulating half-life and by affecting their tissue distribution and localization. IGFBPs have been shown to inhibit IGF action by preventing interactions between IGFs and IGF receptors. IGFBPs also potentiate IGF action in vitro by co-localizing the IGF-IGFBP complex with cell surface receptors and promoting the subsequent release of IGF through a number of mechanisms, including proteolysis (2). Tissue expression and serum levels of IGFBPs are under a variety of influences, such as developmental, hormonal, and physiological conditions including pregnancy, and several disease states including tumorigenesis (reviewed by Rajaram et al. (3)).

IGFBPs 1–6 are generally thought to consist of three structural domains of approximately equal length (4, 5). All six IGFBPs are characterized by highly conserved cysteine-rich amino- and carboxyl-terminal domains, joined by a linker domain unique to each IGFBP species. Although the role of the IGFBPs in modulating IGF activity is widely accepted, the exact mechanism of interaction and the IGF binding sites on the IGFBPs have yet to be elucidated. The proposed IGF binding site on the IGFBPs is believed to be located within the highly conserved amino- and carboxyl-terminal domains, as studies have shown both contain residues important for IGF binding (reviewed by Hwa et al. (4) and Baxter (2)).

The earliest insights into IGF binding regions of IGFBPs arose from observations that proteolysed fragments often retained residual affinity for IGFs. IGF binding activity has been reported for amino-terminal fragments of IGFBP-1, -3, -4, and -5 (6, 8–12). Although these fragments demonstrate IGF binding properties, there is a distinct loss of affinity, suggesting a requirement for additional components. Interestingly, IGFBP-related proteins, which share sequence similarities with the amino-terminal domain of the high affinity IGFBPs, have also been reported to bind IGF-I, IGF-II, and insulin (4, 13–15).

Naturally occurring carboxyl-terminal fragments of IGFBP-2, -3, and -5 have also been shown to possess IGF binding activity; however, they have significantly lower affinities than native IGFBPs (7, 16–18). Ho and Baxter (17) recently isolated a carboxyl-terminal fragment of IGFBP-2 (169–289hIGFBP-2) from human milk, which demonstrated less than 10% binding to \(^{125}\)I-IGF-I and 25% binding to \(^{125}\)I-IGF-II compared with wild-type hIGFBP-2. Furthermore, failure of des(1–6)IGF-II to displace IGF-II tracer from this carboxyl-terminal fragment highlighted the importance the amino-hexapeptide of IGF-II in the binding interaction (17). Horney et al. (19) have complemented these studies using photoaffinity-labeled hIGFBP-2 to confirm that carboxyl-terminal residues 212–227 and 266–287 are involved in IGF-I interactions.

Deletion and chemical modification studies have also contributed significantly to the elucidation of critical residues involved in IGF binding. Removal of either amino- or carboxyl-terminal residues from IGFBP-1 severely disrupted IGF binding (20, 21), whereas deletion of amino acid residues 222–284 of bIGFBP-2 significantly altered the IGF binding capabilities of bIGFBP-2 (22). However, chemical iodination and site-directed mutagenesis of bIGFBP-2 by Hobba et al. (23, 24) showed the amino-terminal residue Tyr-60 contributed to IGF binding. NMR analysis and site-directed mutagenesis confirmed the involvement of the equivalent residue of hIGFBP-5 (10, 25), whereas recombinant amino-terminal fragments of rIGFBP-3 (26) and hIGFBP-4 (27) have also been shown to retain residual binding affinity.

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The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; bIGFBP-2, bovine IGFBP-2; HIS, HEPEIS-buffered saline; rpHPLC, reverse phase high performance liquid chromatography; PCR, polymerase chain reaction.

27120 This paper is available on line at http://www.jbc.org

Received for publication, February 12, 2001, and in revised form, April 27, 2001

Published, JBC Papers in Press, May 16, 2001, DOI 10.1074/jbc.M101317200

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**Construction of bigFBP-2 Truncations**

cDNA encoding amino acid residues 280–284 of the wild-type bigFBP-2 was replaced with cDNA encoding histidine residues using the primer 5′-TTG AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ to incorporate a hexa-histidine tag in bigFBP-2 expressed using the vector pXMT2 (pGF8) (22) and resulting in the clone pGF8HIS. cDNA encoding 1–279IGFBP-2HIS was also cloned into the pET32a (+) expression vector (Novagen, Madison, WI) for E. coli expression (pET32a-2HIS) using the Ncol-Smal fragment of pGF14 (29) and the Smal-EcoRI fragment of pGF8HIS.

**Amino-terminal Expression Constructs**

Amino-terminal constructs were generated by the introduction of stop codons in the wild-type bigFBP-2 cDNA by PCR, using the common forward primer 5′-TTG AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ and the reverse primers 5′-TTT AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (1–132IGFBP-2), 5′-TTT AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (1–279IGFBP-2HIS), 5′-TTT AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (1–369IGFBP-2HIS), 5′-TTT AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (1–185IGFBP-2HIS), or 5′-TTT AAG CTT GTA GTG GTG GTG GTG CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (1–136IGFBP-2HIS), which introduced stop codons after the cDNA sequences encoding His-93, Gln-104, Gly-132, and His-135, respectively. The reverse primers also introduced an EcoRI restriction site (underlined) at the 3′ end of the PCR product to facilitate its religation into the parent vector pXM72. These PCR products were also cloned into the pET32a (+) expression vector (pET1–132IGFBP-2 and pET1–185IGFBP-2) using the convenient Ncol and EcoRI restriction sites.

**Carboxyl-terminal Expression Constructs**

To enable the secretion of carboxyl-terminal domains expressed in mammalian cells, an ApaI restriction site was introduced at the 3′ end of the sequence encoding the bigFBP-2 leader sequence of pGF8. This ApaI site allowed subsequent introduction of cDNAs encoding carboxyl-terminal domains into the expression vector. The ApaI restriction site was generated using the Bio-Rad in vitro mutagenesis kit and mutagenic oligonucleotide 5′-CAC TCT GCC GGG CCC CCC GGC GCG GGT CAC CCC TCG AGC CCC CTG CTG CTC GTC GTA-3′ (23) to introduce the carboxyl-terminal expression constructs into the expression vector. The carboxyl-terminal expression construct (pET32a (+) expression vector (pET1–132IGFBP-2 and pET1–185IGFBP-2) using the convenient Ncol and EcoRI restriction sites.

**EXPERIMENTAL PROCEDURES**

**Materials**

Recombinant bigFBP-2 and truncated mutants were expressed in COS-1 (ATCC: CRL 1650) monkey kidney cells or Escherichia coli BL21 cells (E. coli B F’ damT hsdS (Rb mB) gal (DE3)). Receptor grade IGF-I and IGF-II were purchased from Genentech (San Francisco, CA) and Lambda (5–10 μM) from Collaborative (Lexington, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Molecular biology and biochemical reagents were purchased from Life Technologies (Grand Island, NY) and NEN Research Products (Boston, MA).

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E. coli Protein Expression

The pET constructs were transformed into E. coli BL21 cells for large scale expression. Two-liter cultures were grown in Luria-Bertani broth containing carbenicillin (100 μg/ml) at 37 °C with shaking and induced with 1 mM isopropyl-β-D-thiogalactopyranoside at an A600nm of ~0.6. Protein was expressed for a minimum of 4 h prior to pelleting the cells (5000 × g for 5 min at 4 °C). The cells were then resuspended to one-tenth of the original volume in 2.5 mM EDTA, 20 mM Tris, pH 8.0, with lysozyme (100 μg/ml) and sonicated (Sonifier cell disruptor B-30) three times for 30 s each on ice. The soluble fraction was collected (10000 × g for 10 min at 4 °C) and filtered.

Purification

The expressed products of wild-type and amino-terminal bIGFBP-2 constructs were purified from transfected COS-1 cell conditioned medium as described previously (22). The carboxyl-terminal histidine-tagged proteins were purified by Ni-IDA chromatography (Bioserve). Briefly, nonspecific interacting proteins were removed with 60 mM imidazole, and specifically bound protein was eluted with 1 M imidazole. Wild-type and truncated bIGFBP-2 preparations were further purified by rpHPLC as described by Hobba et al. (23). The eluted protein was detected by absorbance at 215 nm. The amino-terminal fusion protein was cleaved from the E. coli expressed proteins by enterokinase (0.5 units/mg of protein, Invitrogen EkMax), and the products were separated by rpHPLC. Purified protein samples were analyzed by separation on 12.5% polyacrylamide gels under nonreducing conditions and stained with Coomassie Blue R250. Molecular weight markers were derived 1–279IGFBP-2HIS and 136–279IGFBP-2HIS (Fig. 2). Despite predicted molecular masses of 13,604, 19,439, 21,002, and 17193 Da, respectively, all appeared by SDS-polyacrylamide gel electrophoresis analysis to be greater than 21 kDa. IGFBP fragments consisting of amino acid residues 1–93, 1–104, and 182–284 of bIGFBP-2 were not detected in either the soluble secreted material or in COS-1 cell lysates. Amino-terminal fragments 1–132IGFBP-2 and 1–185IGFBP-2 bound IGF-II by Western ligand blot, and 1–185IGFBP-2 exhibited at least a 79-fold lower affinity than wild-type IGFBP-2 for IGFBP-2 in solution binding assays. SDS-polyacrylamide gel electrophoresis analysis of the carboxyl-terminal mutant 96–284IGFBP-2 indicated high levels of secreted protein in the conditioned medium; however, it possessed little IGF-II binding capability by Western ligand blot or standard IGF-II affinity chromatography. Consequently, a carboxyl-terminal hexahistidine tag was introduced (96–279IGFBP-2HIS).

RESULTS

Expression and Purification of Recombinant bIGFBP-2 Truncations

SDS-polyacrylamide gel electrophoresis analysis indicated successful expression of 1–132IGFBP-2, 1–185IGFBP-2, 96–279IGFBP-2HIS, and 136–279IGFBP-2HIS (Fig. 2). Despite predicted molecular masses of 13,604, 19,439, 21,002, and 17193 Da, respectively, all appeared by SDS-polyacrylamide gel electrophoresis analysis to be greater than 21 kDa. IGFBP fragments consisting of amino acid residues 1–93, 1–104, and 182–284 of bIGFBP-2 were not detected in either the soluble secreted material or in COS-1 cell lysates. Amino-terminal fragments 1–132IGFBP-2 and 1–185IGFBP-2 bound IGF-II by Western ligand blot, and 1–185IGFBP-2 exhibited at least a 79-fold lower affinity than wild-type IGFBP-2 for IGF-II in solution binding assays. SDS-polyacrylamide gel electrophoresis analysis of the carboxyl-terminal mutant 96–284IGFBP-2 indicated high levels of secreted protein in the conditioned medium; however, it possessed little IGF-II binding capability by Western ligand blot or standard IGF-II affinity chromatography. Consequently, a carboxyl-terminal hexahistidine tag was introduced (96–279IGFBP-2HIS).

Characterization of bIGFBP-2 Mutants

Amino-terminal sequencing of the COS-1-derived material confirmed the correct amino termini for 1–132IGFBP-2 (EVLF–), 1–185IGFBP-2 (EVLF–), 96–279IGFBP-2HIS (AYEYA–), and 136–279IGFBP-2HIS (GAGKR–). An alternatively processed form of the COS-1 protein preparations with the amino-terminal sequence GARAE–, was co-purified as previously described (22). Electrospray mass spectroscopy was consistent with the amino-terminal sequencing data for COS-1-derived material, identifying both native and GARAE forms for 1–185IGFBP-2 (19,429 and 19,784 Da) and 96–279IGFBP-2HIS (21,002 and 21,355 Da) preparations. As a consequence of the cloning strategy, material derived from E. coli expression incorporated an additional three amino-terminal amino acid residues (AMA), which were reflected in the molecular masses: 1–179IGFBP-2HIS (31,177 Da), 1–132IGFBP-2 (13,866 Da), and 136–279IGFBP-2HIS (17,374 Da). Kinetic analysis of IGF binding by bIGFBP-2 and 1–279IGFBP-2HIS showed that they were essentially identical (Table I). In addition, a comparison using BIAcore analysis of the IGF binding affinities of E. coli-derived and mammalian-derived 1–279IGFBP-2HIS and 136–279IGFBP-2HIS revealed that protein from both sources behaved identically. Furthermore, the change in intrinsic fluorescence of Trp-243 of
bIGFBP-2 was the same for both 1–279IGFBP-2HIS and 136–279IGFBP-2HIS upon IGF binding, indicating a similar change in environment of Trp-243 in both full-length and truncated bIGFBP-2.2

**BIACore Analysis**

**Kinetic Study of Wild-type bIGFBP-2 and the Truncated Constructs—**BIACore analysis provided insight into the association and dissociation kinetics of the interaction between either wild-type or the bIGFBP-2 fragments and IGF-I or IGF-II. Kinetic analyses were performed in both orientations of protein immobilized to the CM5 gold chip; i.e., the CM5 chips were derivatized with either bIGFBP-2/truncated bIGFBP-2 or IGF, with the analyte therefore being IGF or bIGFBP-2/truncated bIGFBP-2, respectively. Bovine IGFBP-2, 1–279IGFBP-2HIS, 1–132IGFBP-2, 1–185IGFBP-2, 136–279IGFBP-2HIS, and 136–279IGFBP-2HIS bound IGF-I and IGF-II (Fig. 3) but did not bind either insulin or protein G, indicating that interactions with IGF were specific. Using a 1:1 Langmuir binding model to fit binding curves, 1–279IGFBP-2HIS exhibited a fast association rate with both IGF-I ($k_a = 1.14 \times 10^5$ Ms$^{-1}$) and IGF-II ($k_a = 0.87 \times 10^5$ Ms$^{-1}$) biosensor surfaces. The resultant complexes between 1–279IGFBP-2HIS and the IGF peptides were stable as illustrated by slow dissociation rates ($k_d = 0.23 \times 10^{-2}$ 1/s, $k_d = 0.25 \times 10^{-3}$ 1/s) (Fig. 3, a and c). Kinetic analysis of the biosensorgram curves suggests that 1–279IGFBP-2HIS exhibits a 1.2-fold preference for IGF-I ($K_D = 2.2$ nM) over IGF-II surfaces ($K_D = 2.7$ nM) (Table I).

The interactions of the amino-terminal mutants 1–132IGFBP-2 and 1–185IGFBP-2 with IGF-II and IGF-I biosensor surfaces are shown in Fig. 3 (panels b and f and panels c and g, respectively). The kinetic analysis shows faster association rates and significantly increased dissociation rates compared with 1–279IGFBP-2HIS (Table I). 1–185IGFBP-2 exhibited association rates with IGF-I ($k_a = 7.94 \times 10^5$ Ms$^{-1}$) and IGF-II ($k_a = 1.35 \times 10^5$ Ms$^{-1}$) that were seven- and 1.6-fold, respectively, more rapid than 1–279IGFBP-2HIS (IGF-I, $k_a = 1.14 \times 10^5$ Ms$^{-1}$; IGF-II, $k_a = 0.87 \times 10^5$ Ms$^{-1}$) (Table I). Similarly, 1–132IGFBP-2 associated with IGF-I ($k_a = 6.36 \times 10^5$ Ms$^{-1}$) and IGF-II ($k_a = 2.89 \times 10^5$ Ms$^{-1}$) biosensor surfaces 5.6- and 3.3-fold more rapidly than 1–279IGFBP-2HIS. Both of the amino-terminal fragments associated more rapidly with IGF-I than IGF-II. The dissociation rates of 1–132IGFBP-2 and 1–185IGFBP-2 from both IGF-I and IGF-II surfaces were also increased compared with 1–279IGFBP-2HIS (1–132IGFBP-2, 92- and 65-fold, and 1–185IGFBP-2, 24.8- and 73-fold for IGF-I and IGF-II, respectively) (Table I). The amino-terminal fragments mimic the preference of 1–279IGFBP-2HIS for IGF-I ligated over IGF-II (Table I). 136–279IGFBP-2HIS exhibits a slower association with both IGF-I ($k_a = 6.5 \times 10^4$ M$^{-1}$ s$^{-1}$) and IGF-II ($k_a = 6.8 \times 10^4$ M$^{-1}$ s$^{-1}$) biosensor surfaces compared with 1–279IGFBP-2HIS (0.57- and 0.78-fold, respectively), and these rates are essentially identical (Table I). The dissociation rate of 136–279IGFBP-2HIS from IGF is much slower than that of the amino-terminal fragment, but more rapid, however, than the wild-type protein (6.2- and 4.9-fold from IGF-I and IGF-II surfaces, respectively) (Table I). Interestingly, unlike the amino-terminal fragment 1–132IGFBP-2, which exhibits a slight preference for IGF-I ($K_D = 36.3$ nM) over IGF-II ($K_D = 51.8$ nM), 136–279IGFBP-2HIS has a 1.8-fold preference for IGF-I ($K_D = 16.3$ nM) over IGF-II ($K_D = 23.8$ nM), attributable to its dissociation component (Table I). The carboxyl-terminal fragment 96–279IGFBP-2HIS binds both IGF-I and IGF-II biosensor surfaces so poorly that association and dissociation kinetics could not be quantitated.

**Reconstitution of the High Affinity IGF/IGFBP Complex on IGF Biosensor Surfaces—**Reconstitution of the wild-type binding interaction between bIGFBP-2 and IGF ligands was investigated by two mechanisms. Initially, the amino- and carboxyl-terminal fragments of bIGFBP-2 were preincubated in HBS running buffer prior to injection across IGF biosensor surfaces. Secondly, IGF-II and the truncated IGFBP fragments were sequentially injected across IGF biosensor surfaces. The 1–185IGFBP-2 and 96–279IGFBP-2HIS fragments could not be used concurrently in the same BIACore experiment due to nonspecific interactions with each other, presumably through the common hydrophobic central region (amino acid residues 96–185). Interestingly, the carboxyl-terminal fragment 96–279IGFBP-2HIS bound IGF-I and IGF-II biosensor surfaces 5.6- and 65-fold, and 1–185IGFBP-2, 24.8- and 73-fold for IGF-I and IGF-II, respectively (Table I). The amino-terminal fragments mimic the preference of 1–279IGFBP-2HIS for IGF-I ligated over IGF-II (Table I). 136–279IGFBP-2HIS exhibits a slower association with both IGF-I ($k_a = 6.5 \times 10^4$ M$^{-1}$ s$^{-1}$) and IGF-II ($k_a = 6.8 \times 10^4$ M$^{-1}$ s$^{-1}$) biosensor surfaces compared with 1–279IGFBP-2HIS (0.57- and 0.78-fold, respectively), and these rates are essentially identical (Table I). The dissociation rate of 136–279IGFBP-2HIS from IGF is much slower than that of the amino-terminal fragment, but more rapid, however, than the wild-type protein (6.2- and 4.9-fold from IGF-I and IGF-II surfaces, respectively) (Table I). Interestingly, unlike the amino-terminal fragment 1–132IGFBP-2, which exhibits a slight preference for IGF-I ($K_D = 36.3$ nM) over IGF-II ($K_D = 51.8$ nM), 136–279IGFBP-2HIS has a 1.8-fold preference for IGF-I ($K_D = 16.3$ nM) over IGF-II ($K_D = 23.8$ nM), attributable to its dissociation component (Table I). The carboxyl-terminal fragment 96–279IGFBP-2HIS binds both IGF-I and IGF-II biosensor surfaces so poorly that association and dissociation kinetics could not be quantitated.

### Table I

| $k_a$ Relative to $k_a$ | $k_d$ Relative to $k_d$ | $K_D$ Relative to $K_D$ | Relative to IGF-I | Relative to IGF-II |
|----------------------|-----------------------|------------------------|------------------|-------------------|
| $10^5$ Ms$^{-1}$ | $10^2$ 1/s | $10^9$ M$^{-1}$ |
| IGF-I | 1–279IGFBP-2HIS | 1.14 | 1.00 | 1.00 | 0.83 |
| bIGFBP-2 | 1–132IGFBP-2 | 1.48 | 1.37 | 0.96 | 1.63 | 0.73 | 0.51 |
| IGF-II | 1–185IGFBP-2 | 6.36 | 5.58 | 23.1 | 92.4 | 36.3 | 16.4 | 0.7 | 0.62 |
| 136–279IGFBP-2HIS | 7.94 | 6.96 | 6.2 | 24.8 | 78.1 | 35.2 | 0.62 |
| IGF-I | 1–279IGFBP-2HIS | 0.65 | 0.57 | 1.55 | 6.2 | 23.8 | 10.7 | 1.77 |
| bIGFBP-2 | 1–132IGFBP-2 | 0.87 | 1.00 | 1.00 | 0.71 |
| IGF-II | 1–185IGFBP-2 | 0.81 | 0.93 | 0.26 | 1.13 | 3.2 | 1.2 |
| 136–279IGFBP-2HIS | 2.29 | 3.32 | 15 | 65.2 | 51.8 | 19.5 |
| IGF-I | 1–185IGFBP-2 | 1.94 | 1.55 | 16.8 | 73 | 127 | 47 |
| 136–279IGFBP-2HIS | 0.68 | 0.78 | 1.1 | 4.9 | 16.3 | 6.1 |

**References**

2 B. Forbes, unpublished data.
IGFBP-2HIS was identical whether HBS, 1–132IGFBP-2, or 136–279IGFBP-2HIS was present in the dissociation phase (Fig. 5a). When 136–279IGFBP-2HIS was passed over the IGF-II-complexed 1–132IGFBP-2 biosensor surface, a slower dissociation rate was observed compared with HBS or buffer containing 1–132IGFBP-2 (Fig. 5b). This effect could be attributed to a combination of 136–279IGFBP-2HIS binding to the IGF-II-complexed 1–132IGFBP-2 biosensor surface and competition by the higher affinity 136–279IGFBP-2HIS for IGF-II. Similarly, the increased response upon addition of 1–132IGFBP-2 across the IGF-II-complexed 136–279IGFBP-2HIS biosensor surface suggests that 1–132IGFBP-2 binds to the complex (Fig. 5c).

Orientation of the IGF/IGFBP Molecules in the High Affinity State Complex—The kinetic data obtained from binding of IGF to immobilized bIGFBP-2 mutant biosensor surfaces were evaluated using both the 1:1 Langmuir model and the two state conformational change model. Binding kinetics derived from both models were similar. Interestingly, the two state conformational change model was a better fit than the 1:1 Langmuir binding model when comparing a single concentration of IGF over IGFBP biosensor surfaces, a slower dissociation rate was observed compared with HBS or buffer containing 1–132IGFBP-2 (Fig. 5a). This effect could be attributed to a combination of 136–279IGFBP-2HIS binding to the IGF-II-complexed 1–132IGFBP-2 biosensor surface and competition by the higher affinity 136–279IGFBP-2HIS for IGF-II. Similarly, the increased response upon addition of 1–132IGFBP-2 across the IGF-II-complexed 136–279IGFBP-2HIS biosensor surface suggests that 1–132IGFBP-2 binds to the complex (Fig. 5c).

Native IGF-I and IGF-II biosensorgrams have been presented showing binding to each of the immobilized bIGFBP-2 analogue biosensor surfaces (Fig. 6). R$_3$IGF-I and R$_6$IGF-II bind 1–279IGFBP-2HIS, 1–132IGFBP-2, and 136–279IGFBP-2HIS with higher affinity than the truncated analogues, consistent with previously reported solution binding assays (34, 35). Relative to native IGF-II, R$_6$IGF-II exhibits 3.4- and 4.0-fold decreased equilibrium affinities for 1–279IGFBP-2HIS and 1–132IGFBP-2 biosensor surfaces, respectively (Table II). In contrast, R$_3$IGF-I and R$_6$IGF-II bind 136–279IGFBP-2HIS biosensor surfaces as well as do their respective native IGFs (Fig. 6, c and f). These results suggest that the glutamate residue is specifically involved in binding the amino-terminal domain of bIGFBP-2. In contrast, des[1–3]IGF-I and des[1–6]IGF-II exhibited reduced binding to 1–279IGFBP-2HIS, 1–132IGFBP-2, and 136–279IGFBP-2HIS biosensor surfaces. The amino-tripeptide of IGF-I and hexapeptide of IGF-II, therefore, influenced binding to both the amino and carboxyl termini of bIGFBP-2. It is unclear whether the low affinity interaction between des-IGFs and bIGFBP-2 is due to structural perturbations in the IGF molecule (to date, no structure analysis has been performed, although receptor binding is not affected (34, 36)) or is due specifically to loss of the amino-terminal residues of IGF.
DISCUSSION

We have shown that both the amino- and carboxyl-terminal domains of bIGFBP-2 bind IGF-I and IGF-II. The amino-terminal fragments 1–132IGFBP-2 and 1–185IGFBP-2 exhibit rapid association but lack the components essential for maintaining a stable complex. In contrast, 136–279IGFBP-2HIS binds IGFs less rapidly than either amino-terminal fragments or 1–279IGFBP-2HIS but retains the ligand in a more stable complex than 1–132IGFBP-2. These findings suggest that both the highly conserved amino- and carboxyl-terminal domains of bIGFBP-2 are required for sequestering IGF ligands. Indeed, they appear to act in a coordinated fashion in the high affinity interaction. Furthermore, we have shown that the aminoterminal residues of IGF are critical binding determinants for both 1–132IGFBP-2 and 136–279IGFBP-2HIS, whereas the glutamate at positions 3 and 6 of IGF-I and IGF-II, respectively, is important for the interaction with the amino-terminal domain of bIGFBP-2.

The present investigation shows in real time the high affinity association of the amino-terminal fragments 1–132IGFBP-2 and 1–185IGFBP-2 exhibit rapid association but lack the components essential for maintaining a stable complex. In contrast, 136–279IGFBP-2HIS binds IGFs less rapidly than either amino-terminal fragments or 1–279IGFBP-2HIS but retains the ligand in a more stable complex than 1–132IGFBP-2. These findings suggest that both the highly conserved amino- and carboxyl-terminal domains of bIGFBP-2 are required for sequestering IGF ligands. Indeed, they appear to act in a coordinated fashion in the high affinity interaction. Furthermore, we have shown that the aminoterminal residues of IGF are critical binding determinants for both 1–132IGFBP-2 and 136–279IGFBP-2HIS, whereas the glutamate at positions 3 and 6 of IGF-I and IGF-II, respectively, is important for the interaction with the amino-terminal domain of bIGFBP-2.

The present investigation shows in real time the high affinity association of the amino-terminal fragments 1–132IGFBP-2 and 1–185IGFBP-2 with IGF (8, 9, 26, 37–40). Interestingly, the central linker region appears to contribute to the structural integrity and hence the IGF binding potential of these amino-terminal fragments. Hashimoto et al. (26) highlighted the importance of inclusion of the linker region because a fragment of rat IGFBP-3 (amino acid residues 1–93) retained only 4.2% IGF binding, whereas addition of linker peptide residues (94–186) increased IGF binding to 12% of native rat IGFBP-3. A similar effect was seen in the present study. In fact, expression of 1–94IGFBP-2, 1–104IGFBP-2, and 182–284IGFBP-2 was not successful in the present investigation, presumably due to increased susceptibility to proteolysis resulting from a lack of structural integrity.

The present study has confirmed the ability of the carboxy-terminal half of bIGFBP-2 (amino acid residues 136–279) to bind IGF-I ($K_D = 23.8$ nM) and IGF-II ($K_D = 16.3$ nM). Previously, there was some debate about the ability of the carboxy-
terminal domain to bind IGF. Although carboxyl-terminal fragments of hIGFBP-4 (27) and hIGFBP-5 (10) had no demonstrable IGF binding, similar fragments, including a naturally occurring truncation of hIGFBP-2 (amino acid residues 169–289) (7, 16, 40), retained partial IGF binding. Further- more, carboxyl-terminal deletion studies of bIGFBP-2 clearly indicated the importance of the carboxyl-terminal domain in IGF binding (22). Removal of 14, 36, and 48 amino acid residues from the carboxyl terminus appeared to have no effect on IGF binding, whereas removal of a further 14 amino acid residues dramatically reduced IGF binding. Other studies have shown that removal of most or all of the carboxyl-terminal domains of IGFBP-3 (16, 41), IGFBP-4 (27), and IGFBP-5 (8) reduces IGF binding significantly.

It is clear that the amino- and carboxyl-terminal domains are required to work together to form the high affinity complex. However, whether the amino- or carboxyl-terminal domain of IGFBP is the major binding site is a matter of interpretation. The present investigation clearly shows the amino-terminal domain is required for rapid association of IGFBP-2 with IGF, and the carboxyl-terminal domain is required for the ability of IGFBP-2 to maintain IGF in a complexed form. Recently, Galanis et al. (42) reported that the recombinant expression of IGFBP-3 fragments encompassing amino- and carboxyl-terminal residues (1–88 and 165–264) bind IGF-I and IGF-II with reduced affinity. Consistent with the current findings, the amino-terminal fragment (N-88) dissociated 50- and 75-fold faster from IGF-I and IGF-II biosensor surfaces compared with the carboxyl-terminal fragment (C-165). In addition, Kalus et al. (10) purified two amino-terminal fragments of hIGFBP-5 (amino acid residues 1–94 and 40–92) that exhibited similar affinity for IGF, both with 200-fold reduced affinity compared with wild-type. Imai et al. (25) confirmed the importance of corresponding amino-terminal residues in IGFBP-3, and Qin et al. (27) did the same for IGFBP-4. Furthermore, as amino acid residues 1–39 of hIGFBP-5 did not contribute to maintaining the complex, it was concluded that additional components required for IGFBP-5 complex stability would be derived from the carboxyl-terminal residues of IGFBPs (10). It is feasible, therefore, that the IGF binding sites located in the ami-
IGF-II appeared to specifically affect IGF binding only represented schematically in Fig. 7. Furthermore, Glu-6 of minal domains of IGFBP-2. A summary of these findings is hexapeptide of IGF within both the amino- and carboxyl-ter-

no- and carboxyl-terminal domains of IGFBPs combine with each other to create one high affinity binding site. Indeed, Lys-222 to Asn-236 of bIGFBP-2 may be located in close enough proximity to the amino-terminal domain to allow both domains to interact with IGF (22). Site-directed mutagenesis of Gly-203 and Gln-209 in the equivalent region of rIGFBP-5 resulted in an 8- and 6-fold reduction in IGF-I binding affinity (43).

The amino-terminal residues of IGF are known to be essential for interaction with IGFBPs as IGF analogues des(1–3)IGF-I and des(1–6)IGF-II have significantly reduced binding affinity (34). Ho and Baxter (17) oriented the IGF molecule in complex with hIGFBP-2 using the analogue des(1–6)IGF-II. des(1–6)IGF-II was found to displace tracer from intact hIGFBP-2 with 10% the activity of native ligand; however, it was totally inactive in displacing IGF-II tracer from a carboxyl-
terminal fragment (amino acid residues 169–289). These results suggest that the amino terminus of IGF-II interacts with the carboxyl-terminal domain of rhIGFBP-2. Photoaffinity labeling experiments by Horney et al. (19) further supported this conclusion. However, the present study showed that the amino-

hexapeptide of IGF-II influenced binding not only to the carboxyl-terminal half of bIGFBP-2 but also to the amino-terminal half. This indicates the existence of binding sites for the hexapeptide of IGF within both the amino- and carboxyl-
terminal domains of IGFBP-2. A summary of these findings is represented schematically in Fig. 7. Furthermore, Glu-6 of IGF-II appeared to specifically affect IGF binding only to 1–132IGFBP-2 and did not alter binding to 136–279IGFBP-2HIS. It will be interesting to determine the ability of 136–279IGFBP-2 and 136–279IGFBP-2HIS to bind other IGF analogues known to influence interaction with IGFBPs (viz. IGF-I: Glu-3, Thr-4, Gln-15, Phe-16 (44); Thr-4, Glu-9, Phe-16 (45); Phe-49, Arg-50, and Ser-51 (46); IGF-II: Phe-26, Phe-48, Arg-49, and Ser-50 (47)). We are also identifying the specific residues of IGF-I and IGF-II involved in binding to either the amino- or carboxyl-
terminal domains by NMR.

Based on the present findings, we propose a model for the modulation of IGF activity (Fig. 7). Classical high affinity IGF binding only occurs when both the amino- and carboxyl-terminal domains of IGFBPs are linked together, as is evident also from partial proteolysis studies (reviewed by Conover (48)). Two binding sites exist, one in the amino-terminal domain that has a fast association component and one in the carboxy-
terminal domain that is essential for the stability of the com-
plex. These sites must be combined to form one high affinity binding site. Glu-3 of IGF-I or Glu-6 of IGF-II interacts directly with the amino-terminal domain of IGFBP-2, whereas the amino terminus of IGF is critical for binding of both the amino- and carboxyl-terminal fragments of IGFBP-2. Although the objective of this study was to elucidate the IGF binding site on IGFBP-2, it will be interesting to investigate the modulation of IGF activity by IGFBP fragments in biological assays.

The present experiments further emphasize the requirement for the two halves of the IGFBP to exist concomitantly in order to establish a high affinity binding complex with IGF, but we are not sure by which means the two domains come together to form the high affinity binding interaction. The IGF binding site of IGFBPs obviously requires further investigation. Determining the residues involved in the interaction between IGF and IGFBP will ultimately allow researchers to develop a comprehensive insight into the molecular dynamics of the binding interaction, making it possible to produce mutant binding proteins for therapeutic applications. Not until we can derive a three-dimensional structure of the complex, by NMR or x-ray crystallography, will we truly understand this interaction.

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