Localization of an Insulin-like Growth Factor (IGF) Binding Site of Bovine IGF Binding Protein-2 Using Disulfide Mapping and Deletion Mutation Analysis of the C-terminal Domain*

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We have investigated which region(s) of bovine insulin-like growth factor binding protein-2 (bIGFBP-2) interacts with insulin-like growth factors (IGFs) using C-terminally truncated forms of bIGFBP-2. Initially to aid in mutant design, we defined the disulfide bonding pattern of bIGFBP-2 C-terminal region using enzymatic digestion. The pattern is Cys^{186}-Cys^{220}, Cys^{231}-Cys^{235}*, and Cys^{242}-Cys^{255}*. In addition, cyanogen bromide cleavage of bIGFBP-2 revealed that the N- and C-terminal cysteine-rich domains were not linked by disulfide bonds. Taking the disulfide bonding pattern into consideration, C-terminal truncation mutants were designed and expressed in COS-1 mammalian cells. Following IGF binding assays, a region between residues 222 and 236 was identified as important in IGF binding. Specifically, mutants truncated by 14, 36, and 48 residues from the C terminus bound IGFs to the same extent as wild type (WT) bIGFBP-2. Removal of 63 residues resulted in a greatly reduced (up to 80-fold) ability to bind IGF compared with WT bIGFBP-2. Interestingly this mutant lacked the IGF-II binding preference of WT bIGFBP-2. Residues 236–270 also appeared to play a role in determining IGF binding specificity as their removal resulted in mutants with higher IGF-II binding affinity.

The mitogenic and metabolic effects of insulin-like growth factors (IGFs)§ are modified by a family of six or more insulin-like growth factor binding proteins (IGFBPs). IGF activity mediated via specific cell surface receptors can be inhibited or enhanced when bound to IGFBPs (1). In addition, IGFBPs are important considerations in design of our bIGFBP-2 mutants without the knowledge of the disulfide bonding pattern of IGFBPs, our first step in design of C-terminally truncated bIGFBP-2 mutants and analysis of their IGF binding abilities.

Comparisons of IGFBPs 1–6 indicate a high degree of conservation in the N- and C-terminal regions throughout all IGFBPs. Of particular note in those regions are the conserved cysteine residues, 14 of which are conserved throughout IGFBPs 1–6 of all species sequenced so far. All of the 18 cysteines in human IGFBP-3 (hIGFBP-3) and at least 16 in human IGFBP-1 (hIGFBP-1) are involved in disulfide bonds (3, 4). A non-conserved region separates the N- and C-terminal domains. It is likely that the regions of the IGFBPs which share sequence homology may confer similar tertiary structures leading to the formation of the IGF binding domain (5). However, relatively few studies have investigated which regions on the IGFBPs are important for binding IGFs.

Recently an additional low affinity IGFBP has been identified (human MAC25 (6)). Despite an overall low degree of sequence similarity to IGFBPs 1–6,11 or possibly 12 cysteines in the N-terminal domain remain conserved, indicating that conservation of the cysteines confers a structure that leads to a common IGF binding region.

Identification of fragmented IGFBPs and the analysis of their IGF binding has indicated that both the N-terminal (7–9) and the C-terminal (10) cysteine-rich domains can bind IGF independently. Interestingly, a monoclonal antibody directed to two sites in the last 47 C-terminal residues of hIGFBP-1 inhibited IGF binding (11). In addition, our recent chemical modification study has implicated Tyr^{60} in the N-terminal region of bovine IGFBP-2 (bIGFBP-2) as an important residue in IGF binding (12). Clearly further investigations to determine the specific residues of both the N- and C termini involved in IGF binding are required.

This study aims to further define the IGF binding site of bIGFBP-2 by recombinant production of C-terminally deleted bIGFBP-2 mutants and analysis of their IGF binding abilities. A similar approach of C-terminal deletion analysis was previously carried out by Brinkman et al. (13) using hIGFBP-1. Two important considerations in design of our bIGFBP-2 mutants were highlighted by the results obtained by Brinkman et al. (13). First, removal of 17 amino acids of hIGFBP-1 resulted in production of a mutant protein that formed aggregates. As the last cysteine had been removed this formation of aggregates suggested that an unpaired cysteine had been generated as a result of the deletion. Clearly, it is extremely difficult to design such mutants without the knowledge of the disulfide bonding pattern. Since there are currently no reports of the disulfide bonding pattern of IGFBPs, our first step in design of C-terminally truncated bIGFBP-2 mutants was to define the disulfide bonding pattern of the C terminus.

Second, despite identification of recombinant mutant protein, Brinkman et al. (13) failed to detect 125I IGF binding by Western ligand blotting. However, several reports (14, 15) have highlighted that fragmented IGFBPs are capable of binding IGFs in competitive binding assays and affinity labeling studies where binding is not detected by Western ligand blotting. Indeed recombinant C-terminally deleted human IGFBP-5
Deletion and Disulfide Pattern of IGFBP-2 C-terminal Domain

(hIGFBP-5) lacking the C-terminal domain but retaining all of the central non-conserved region was shown to bind IGF-I in charcoal binding assays (8). Therefore, in analysis of IGF binding by our C-terminal truncation bIGFBP-2 mutants, it was essential that purified mutants were subjected not only to Western ligand blotting but also to charcoal binding assays. The major outcome of this study has been the identification of a site in the C-terminal domain of bIGFBP-2 involved in IGF binding. In addition, in defining the disulfide bonding pattern of the C-terminal domain, we also demonstrated that the N- and C-terminal cysteine-rich regions of bIGFBP-2 are not linked by disulfide bonds. This indicated that bIGFBP-2 exists as two separate cysteine-rich domains separated by the non-conserved central region. The disulfide bonding pattern of the C-terminal domain was shown to be Cys186-Cys220 Cys231 Cys242 Cys244 Cys265.

EXPERIMENTAL PROCEDURES

Disulfide Bond Determination of the C-terminal Domain, Cyano gen Bromide (CNb) Cleavage of bIGFBP-2—Bovine IGF-II (5 μg), prepared as described below, was cleaved in 100 μl of 100 mM HCl using 100 μmol excess CNb (Sigma, Australia) over the number of methi onine in bIGFBP-2 at room temperature overnight. Analysis of cleavage involved separation on 12.5% SDS-polyacrylamide gels under non-reducing conditions (16) and Coomassie staining. The largest fragment was isolated for N-terminal amino acid sequencing using reverse phase high performance liquid chromatography (rpHPLC) as described by Hobbs et al. (12) on a C4 analytical column (Brownlee Aquapore B300, 7-μm particle size, 300-A pore size, 2.1 × 100 mm) at 40 °C with a linear acetonitrile gradient from 0 to 50% (v/v) in 0.1% trifluoroacetic acid over 50 min at 0.5 ml/min. N-terminal amino acid sequencing was performed using Edman degradation carried out automatically on an Applied Biosystems model 470A gas-phase sequencer with a 900A Control/Data Analysis Module. The other three sequences were performed following transfer to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) as described by Matsudaira (17).

Trypsin Cleavage of bIGFBP-2—Bovine IGF-II (100 μg) was dissolved in 100 μl of 10 mM acetic acid and diluted to 3.5 ml in 250 mM Tris, 20 mM CaCl2, pH 7.0. Trypsin (modified, sequencer grade, Boehringer, Mannheim, Germany) was added at a ratio of 1:10 (w/w, enzyme/substrate) for 5 h at 37 °C. A further 10 μg of trypsin was added for 18 h at 37 °C, and the reaction was stopped by aciddification with trifluoroacetic acid and stored at −20 °C.

Chymotrypsin Cleavage of bIGFBP-2—The peptide that resulted from the trypsin digest of bIGFBP-2 and eluted at 25.3 min on rpHPLC was lyophilized (10 μg) and reconstituted in 50 μl of 10 mM acetic acid. The peptide was incubated for 15 min at 37 °C in 6 μl urea, 100 mM Tris, 10 mM CaCl2, pH 7.4 (280 μl), to allow unfolding. Following dilution in 100 mM Tris, pH 7.4, and addition of 980 μl of a linear acetonitrile gradient from 0 to 50% (v/v) in 0.1% trifluoroacetic acid over 50 min at 0.5 ml/min, N-terminal amino acid sequencing was performed using Edman degradation carried out automatically on an Applied Biosystems model 470A gas-phase sequencer with a 900A Control/Data Analysis Module. The other three sequences were performed following transfer to polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA) as described by Matsudaira (17).

Mapping of Cleavage Products Using rpHPLC—Peptides generated following trypsin and chymotrypsin cleavages were separated by rpHPLC as described above. The absorbance of the peptide backbone bonds was monitored at 215 nm, Trp at 295 nm, and Tyr by specific methods described by Ross et al. (19). The fast and slow peaks were detected by the pXMT-2 expression vector (20), kindly provided by P. D. Rathjen (Department of Biochemistry, University of Adelaide) resulting in the plasmid pGF-8. Four C-terminal deletion mutants were created by polymerase chain reaction (PCR) mutagenesis as described by Clackson et al. (21) using pGF-8 template. The forward primer was 5' ATG GCC AAG GTC AAA CAC GAC G3' (corresponding to residues 61–69, Fig. 1), and the following were the reverse primers: mutant A, 5' TAC GAA TTC TAA GGT GTA GAA GAT AGC ACA CTC GGG 3' (residues 214–222); mutant B, 5' TAC GAA TTC TTA GGT AGG TAT CAC AGA CCA GCA CTA GAC GTA CCG CGC CAC GAG GGC CCG CCG CCG 3' (residues 223–236); mutant C, 5' TAC GAA TTC TTA GGT GAG ACA CTA GGT GAT CTT GGT CAT GCC ACC G 3' (residues 236–248); mutant D, 5' TAC GAA TTC TTA GGT ACA GGT GGG GTG TAG GGA 3' (residues 263–270). The PCR reaction conditions using Taq polymerase from Promega Corp., Australia, were as recommended by the manufacturer using 40 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C for 1 min, and extension at 72 °C for 1 min. PCR products were digested with NotI and EcoRI restriction enzymes and ligated to the pGF-8 NotI/EcoRI 9297 base pair fragment. Correct sequences of PCR inserts were confirmed by DNA sequencing (22).

Transfection of COS-1 Cells—COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies Inc.) containing 10% fetal calf serum. Large scale DNA preparations were purified by Superox 6 chromatography (Pharmacia Ltd., Australia) and transfected of 10 μg of DNA/5 × 106 cells was by electroporation at 0.27 V using a Bio-Rad gene pulser. Cells were cultured for 24 h in DMEM + 10% fetal calf serum and then washed and then transfected to phenol red free DMEM supplemented with insulin, transferrin, and sodium sialate (Boehringer, Mannheim, Germany), 0.1 mM β-mercaptoethanol, and glutamine. Medium was changed and collected every 24 h for up to 5 days.

Detection of bIGFBP-2 and Truncation Mutants—Bovine IGF-II and truncation mutants were separated by 12.5% SDS-polyacrylamide gel electrophoresis under non-reducing conditions (16). IGFs were detected by Western ligand blotting (23) with 125I IGF-II visualized using a Molecular Dynamics PhosphorImager and ImageQuant software, as well as by immunoblotting as described previously (24) with antibody raised by the same laboratory (Australia). Bound IGFs were eluted in 0.5 mM acetic acid and then applied directly to a cation exchange column (Resource S 1 ml column, Pharmacia Ltd., Australia). Using a gradient of 50 mM ammonium acetate, pH 6.0, to 1 mM ammonium acetate, pH 7.5, over 20 ml at 1 ml/min monkey IGF-II-3 was separated from IGF-II-2. Bovine IGF-II-2 or mutants were separated from monkey IGF-II-2 by rpHPLC using the same conditions described above for separation of CNBr-cleaved bIGFBP-2. To confirm the correct mass for each peptide electrospray mass spectroscopy was performed on a Perkin-Elmer Psi-ex triple quadrupole mass spectrometer by Yogi Hayasaka at the Australian Research Council Ems unit, Adelaide, 2018.

Activity Assays—IGF binding ability was determined by charcoal binding assays as described (23). IGF peptides were kindly provided by GruPep Pty. Ltd., Australia (25). Purification of bIGFBP-2 and Truncation Mutants from COS-1 Cell Medium—WT bIGFBP-2 and mutants were isolated from transfected COS-1 cell medium by IGF-II affinity chromatography (IGF-II purchased from GruPep Pty. Ltd., Australia, coupled to Affi-Fep-10 matrix as described by the same laboratory (Australia). Bound IGFs were eluted in 0.5 mM acetic acid and then applied directly to a cation exchange column (Resource S 1 ml column, Pharmacia Ltd., Australia). Using a gradient of 50 mM ammonium acetate, pH 6.0, to 1 mM ammonium acetate, pH 7.5, over 20 ml at 1 ml/min monkey IGF-II-3 was separated from IGF-II-2. Bovine IGF-II-2 or mutants were separated from monkey IGF-II-2 by rpHPLC using the same conditions described above for separation of CNBr-cleaved bIGFBP-2. To confirm the correct mass for each peptide electrospray mass spectroscopy was performed on a Perkin-Elmer Psi-ex triple quadrupole mass spectrometer by Yogi Hayasaka at the Australian Research Council Ems unit, Adelaide, 2018.

RESULTS

The bIGFBP-2 C-terminal Domain Disulfide Bonding Pattern—To solve the disulfide bonding pattern of the C-terminal region of bIGFBP-2 three separate cleavage steps were required. The first involved chemical cleavage with CNBr and showed that the N- and C-terminal regions were not linked by disulfide bonds. Subsequent cleavage with trypsin and chymotrypsin yielded the C-terminal disulfide bond pairing pattern. Cleavage sites for the three steps are shown in Fig. 2a.

CNBr Cleavage—Chemical cleavage of bIGFBP-2 with CNBr liberated four fragments separated by SDS-polyacrylamide gel electrophoresis (Fig. 2A). The largest product (fragment 1, Fig. 2A) was purified from the three other fragments by rpHPLC for N-terminal sequencing (chromatogram not shown). It had a single sequence corresponding to the N terminus of bIGFBP-2 (Fig. 2B). The other cleavage products were sequenced following transfer to polyvinylidene difluoride membrane (see “Ex-
The amino acid sequence of the secreted bIGFBP-2 is shown in the single letter code. Cys residues are boxed. CNBr (†) and chymotrypsin cleavage (❖) sites are indicated above the sequence.

Fig. 1. Cleavage sites in bIGFBP-2. The amino acid sequence of the secreted bIGFBP-2 is shown in the single letter code. Cys residues are boxed. CNBr (†), trypsin (●), and chymotrypsin cleavage (❖) sites are indicated above the sequence.

Fig. 2. CNBr cleavage of bIGFBP-2. A, CNBr cleavage products were separated on 12.5% SDS-polyacrylamide gels and Coomassie stained. Uncleaved bIGFBP-2, cleavage products (I–IV) and molecular weights are indicated. B, N-terminal sequences of the cleavage products are shown in the single letter code. Unassigned residues are indicated (?), and residues assumed to be cysteine are denoted (C).

Experimental Procedures). Fragment II had a single N-terminal sequence indicating cleavage after Met240. Separation of this C-terminal fragment from the N-terminal fragment under non-reducing conditions conclusively showed that the N- and C-terminal domains are not linked by disulfide bonds.

Further analysis of fragments III and IV confirmed this conclusion. Fragment III yielded three N-terminal sequences produced by cleavage after Met144, Met200, and Met232 (Fig. 2B). As they are present in a single fragment we can conclude that these cleavage products are linked by disulfide bonds. Similarly fragment IV yielded three N-terminal sequences due to cleavage at Met161, Met200, and Met232, again all products being present in the one fragment linked by disulfide bonds (Fig. 2B).

Trypsin Digestion—Trypsin was chosen to analyze the C-terminal disulfide bond pairing of bIGFBP-2 as four of the six cysteines within the C terminus are in separate tryptic fragments (Fig. 1). Tryptic products were separated by rpHPLC. To locate the tryptic fragments of interest tyrosine fluorescence and tryptophan absorbance at 295 nm were monitored. Cys242 and Cys244 are found within separate tryptic fragments which both contain a Tyr. In addition a single Trp243 is within a fragment encompassing Cys242 and Cys244 (Fig. 1).

Peak A (Fig. 3) was identified by fluorescence detection to contain Tyr (215 nm absorbance shown only). Following N-terminal sequencing and mass spectroscopy peak A was identified as a single peptide containing 2 N-terminal sequences of fragments encompassing Cys186 and Cys220 (Table I). We could conclude that Cys186 and Cys220 form a disulfide bond in bIGFBP-2.

Peak B (Fig. 3) not only contained Tyr fluorescence but also showed absorbance at 295 nm (not shown). This suggested that Cys265, within a Tyr-containing tryptic fragment, was linked to the fragment containing Cys242, Trp243, and Cys244. Indeed peak B when sequenced yielded three N termini (Table I). Further mass determination confirmed that this single product contained three peptides linked by disulfide bonds (Table I).

Chymotrypsin Digestion—Peak B of the trypsin digestion (Fig. 3) was cleaved by chymotrypsin to further delineate the disulfide bonding pattern of the C-terminal region of bIGFBP-2. Cleavage at Trp243 between Cys242 and Cys244 was achieved only in the presence of urea and was extremely inefficient. However, peak C (Fig. 3) was identified by N-terminal sequencing and mass spectroscopy (Table I) to contain Cys244 and Cys265 within a single peptide. We concluded therefore that they are disulfide linked. The peptide containing Cys231 and Cys242 was not located, but we could deduce that these must be disulfide bonded.

In summary the disulfide bonding pattern of bIGFBP-2 C-terminal region is as follows: Cys186-Cys220, Cys231-Cys242, and Cys244-Cys265.

Expression of bIGFBP-2 Truncation Mutants—A series of four bIGFBP-2 C-terminal truncation mutants were designed as shown in Fig. 4. Mutant A was truncated by 14 amino acids. Mutants B and C were truncated by 36 and 48 amino acids and had Cys244 → Ser and Cys265 → Ser substitutions, respectively, to eliminate the possibility of intermolecular disulfide bond formation. Mutant D was truncated by 62 amino acids.

Plasmids encoding the truncated forms of bIGFBP-2 were generated by PCR using the full-length bIGFBP-2 cDNA as a template (see “Experimental Procedures”). PCR products were digested with NotI and EcoRI restriction enzymes and ligated to the bIGFBP-2 pGF-8 NotI/EcoRI 5927-base pair fragment. The four mutant constructs were expressed in COS-1 cells, and the respective proteins were purified from endogenous monkey IGFBP-2 and -3 as described under “Experimental Procedures.”

Recombinant bovine IGFBP-2 and the four truncated mutants were of the expected size as determined by SDS-polyacrylamide gel electrophoresis (Fig. 5). Mass spectroscopy confirmed that bIGFBP-2, mutant A, mutant B, and mutant C were the correct mass within 2 mass units. Mutant D had an expected mass of 23,786, but its actual mass was measured as 23,658. N-terminal amino acid sequencing showed the correct sequence for five residues. Mutant D was digested with trypsin and the C-terminal fragment isolated and analyzed by N-terminal sequencing. This showed that the C-terminal sequence (Lys, mass = 128) was missing which was consistent with the observed mass difference. As the cDNA sequence of the clone isolated the C-terminal residue was Lys, mass = 128. As the DNA sequence of the clone encoding mutant D was correct, we can conclude that deletion of Lys225 occurred subsequent to secretion into the culture medium.

IGF Binding by WT bIGFBP-2 and Truncation Mutants—The ability of the mutants to bind IGF was analyzed initially by Western ligand blotting. Mutants A, B, and C bound 125I IGF-II to the same extent as bIGFBP-2 (WT, Fig. 5a). Mutant D, however, did not bind IGF-II despite equal amounts of protein being assayed as determined by immunoblotting the same filter (Fig. 5b).

IGF binding by bIGFBP-2 and mutants was further analyzed.
by charcoal binding assays. Initially titration of $^{125}$I IGF-I and $^{125}$I IGF-II with increasing amounts of binding protein revealed that mutants A, B, and C bound both radiolabeled ligands essentially to the same extent as WT bIGFBP-2. Mutant D, however, had a greatly reduced ability to bind both $^{125}$I IGF-I and $^{125}$I IGF-II (Fig. 6, a and b).

Competition binding assays (Fig. 6, c and d) revealed that mutant A had the same affinity as WT for $^{125}$I IGF-I and $^{125}$I IGF-II using competing IGF-I and IGF-II, respectively (summarized in Table II). Interestingly, mutants B and C demonstrated a higher affinity than WT for both IGF-I and IGF-II in competition assays. To assess the binding affinity of mutant D much higher amounts were used than WT bIGFBP-2 (1.26 versus 0.016 pmol). Under these conditions mutant D had a lower affinity for IGF-II tracer when competing with IGF-II and a slightly higher affinity for IGF-I tracer when competing with IGF-I.

Preliminary tryptic mapping of mutant D (as described under “Experimental Procedures”) suggests that alteration in binding of mutant D is not due to a gross structural malformation as a consequence of, for example, incorrect disulfide formation by the remaining cysteines but rather due to a lack of crucial residues. We isolated the peptide containing the fragments with Cys186 linked to Cys220 as confirmed by N-terminal sequencing. In addition the tryptic maps of unreduced mutant D and unreduced WT bIGFBP-2 appeared similar in that all Tyr-containing peaks eluted in the same positions, except the Tyr269-containing fragment which is not present in mutant D (data not included).

Relative binding affinities of WT bIGFBP-2 and mutants were deduced by comparing the $ED_{50}$ values in $^{125}$I IGF-I and $^{125}$I IGF-II competition assays (Table II). WT bIGFBP-2 had a 11.75-fold preference in IGF-II binding to $^{125}$I IGF-II over IGF-I binding to $^{125}$I IGF-I (Table II). The mutants A and B exhibited a similar preference for IGF-II over IGF-I. Mutant C, however, had a greater preference for IGF-II over IGF-I (21 fold) than WT bIGFBP-2 in competition for IGF-I and IGF-II tracers. Interestingly, mutant D exhibited an equal affinity for IGF-I and IGF-II in competition assays.

**DISCUSSION**

Our investigation involved the design of bIGFBP-2 C-terminal truncation mutants to be used as probes for identifying IGF binding sites. There is currently no information regarding disulfide mapping nor the three-dimensional structure of the IGFBPs, making the design of truncation mutants difficult. In this study our initial cleavage using CNBr showed that the N- and C-terminal cysteine-rich domains of bIGFBP-2 are not linked by disulfide bonds. In addition a combination of trypsin and chymotrypsin digestions allowed us to solve the disulfide

**Table I**

| N-terminal sequencing and masses of tryptic and chymotryptic digests |
|---|
| N-terminal sequences are shown in the single letter code. Fragments not sequenced completely are indicated (…). Estimated (Mass$_{est}$) and determined (Mass$_{det}$) masses are shown. |

| Peak A | Mass$_{est}$ | Mass$_{det}$ | N-terminal sequence(s) |
|---|---|---|---|
| Peak A | 3394 | 3393 | TP (C) QQELDQV... GPLEHLHSLH... Q (C) K |
| Peak B | 3546 | 3545 | GE (C) W (C) V... GDPE (C) H... (C) VNPPNTGK |
| Peak C | 1748 | 1748 | (C) VNPPNTGK GDPE (C) HLF |

**Fig. 3**. Reverse phase HPLC of tryp tic and chymotryptic cleavage products. Tryptic (a) and chymotryptic (b) products were separated as described under “Experimental Procedures.” Sequences of peptides corresponding to peaks indicated by arrows are shown below the chromatograms.
FIG. 6. Charcoal binding assays of hIGFBP-2 and mutants. Increasing amounts of WT hIGFBP-2 (●) and mutants A (▲), B (▲), C (▲), and D (■) were incubated with (a) 125I-IGF-I (11,600 cpm) and (b) 125I-IGF-II (2500 cpm). The bound IGFBPs were separated from the unbound IGF, and 125I radioactivity bound was quantified. The amount of tracer bound is expressed as % of total counts added. Using the same assay, competition of (c) IGF-I for 125I IGF-I (10,869 cpm) and (d) IGF-II for 125I IGF-II (2358) bound to labeled IGF was achieved are shown (ED 50). The fold difference in competition is indicated by the ratio of IGF-I:IGF-II.

### TABLE II

|            | IGF-I (ED50) | IGF-II (ED50) | IGF-I:IGF-II |
|------------|--------------|---------------|--------------|
| WT         | 0.94         | 0.08          | 11.75        |
| A          | 0.99         | 0.08          | 12.3         |
| B          | 0.37         | 0.03          | 12.3         |
| C          | 0.63         | 0.03          | 21.0         |
| D          | 0.52         | 0.54          | 0.96         |

The bonding pattern of the C-terminal region. All Cys residues in the C-terminal region are involved in disulfide bonds, and the pattern has been shown to be as follows: Cys214-Cys220, Cys231-Cys232, and Cys244-Cys256. Cleavages were carried out at pH 7.4 and below thereby reducing the probability of disulfide bond reshuffling, and indeed no alternate bonding patterns were identified following either the tryptic or chymotryptic digestions.

With the knowledge of the disulfide bonding pattern of the C-terminal domain, we proceeded to design four C-terminal deletion mutants. In those mutants with a disrupted Cys pair the remaining Cys was replaced by Ser, thereby eliminating the possibility of dimer formation or the formation of alternative Cys-Cys pairing. Indeed no dimers or aggregates were observed in production of any of the mutants.

Analysis of IGF binding ability of all mutants and particularly comparison of mutants C and D revealed that mutant D has lost residues between 222 and 236 crucial for IGF binding. Not only was mutant D unable to bind on a Western ligand blot but it bound IGFs poorly in charcoal binding assays. Its affinity for IGF-II was markedly reduced in 125I IGF-II competition assays. In addition it showed no preference for binding IGF-II over IGF-I, due to its greatly reduced affinity for IGF-II in comparison with WT hIGFBP-2.

In contrast, mutant A lacking 14 C-terminal amino acids behaved similarly to WT hIGFBP-2 in both activity assays (Western ligand blotting and charcoal binding assays). We conclude therefore that the last 14 amino acids do not play a major role in IGF binding by hIGFBP-2. Mutants B and C (lacking 24 and 48 C-terminal amino acids) also behaved similarly to WT hIGFBP-2 on Western ligand blots but displayed a greater binding affinity for IGF-II in 125I IGF-II competitive binding assays. Mutant B also showed a greater binding affinity for IGF-I in 125I IGF-I competition binding assays (Table II). However, the difference in relative IGF-I and IGF-II binding affinities for mutant B was similar to WT hIGFBP-2, whereas mutant C had a greater divergence in relative binding affinity for IGF-II over IGF-I. This indicates that in removing 24 or 48 amino acids these mutants were still able to bind IGFs to the same extent as WT hIGFBP-2, but there has been an alteration in the region of hIGFBP-2 which determines IGF binding specificity. Subsequent removal of the amino acids 222–236 in mutant D greatly altered the IGF binding site resulting in an IGFBP which binds IGFs poorly.

The fact that recombinantly produced C-terminally deleted hIGFBP-2 mutant D is a poorer IGF binder was not surprising. Proteolysed porcine IGFBP-2, identified as 25- and 16-kDa fragments, did not bind on Western ligand blots (27). Furthermore, fragments of various IGFBPs lacking some or all of the C-terminal region, generated following specific proteolysis or during purification procedures, have also been shown to have greatly reduced IGF binding capacity in competitive binding assays (8, 28). Also, recombinant production of a C-terminally deleted hIGFBP-5 lacking the C-terminal domain but retaining all of the central non-conserved region was shown to bind IGF-I in charcoal binding assays (29). However, like mutant D, its ability to bind IGF-I was greatly reduced. Indeed, N-terminally truncated rat IGFBP-2 encompassing half of the non-conserved region and all of the C-terminal domain bound IGF-I with lower affinity than native rat IGFBP-2 in competition assays (10). The same effect was seen with recombinantly expressed IGFBP-1 truncated from the N terminus by 60 residues. This mutant was unable to bind IGF on a Western ligand blot suggesting a greatly reduced ability to bind IGF (4). A similar result was noted with recombinantly expressed hIGFBP-3 frag-
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The results presented here and by others suggest that the individual domains of IGFBPs are unable to exhibit full IGF binding compared with native IGFBPs. It is likely that both domains are required for high affinity IGF binding. Indeed it is possible that the low affinity binding of IGFBP-3 identified by BIAcore analysis (31) results from binding to a single domain, while high affinity binding is a consequence of interaction of IGF with both domains.

Relating the deletion mutant information to the tertiary structure of bIGFBP-2 and IGFBPs in general is difficult without structural information. However, from our study it is possible to speculate that the C-terminal residues of bIGFBP-2, particularly residues 222–236, must lie in close proximity to the N-terminal domain to allow both domains to interact with IGF. In addition, we believe that the disulfide bonding pattern described for the bIGFBP-2 C-terminal domain in this paper could be the pattern for all IGFBPs as all six cysteines in the C-terminus are present in every IGFBP 1–6 sequenced so far. Therefore we would suggest that the C-terminal domains of all IGFBPs are likely to have a similar disulfide bonding pattern and hence adopt a similar structure. Thus, although the amino acid sequence in this region of bIGFBP-2, which is totally conserved across all species sequenced so far, has limited homology to other IGFBPs, residues corresponding to the crucial residues 222–236 of bIGFBP-2 could be important for IGF binding by other IGFBPs.

In summary, we have defined a site on bIGFBP-2 which is important for IGF binding. In addition, we have demonstrated that the N- and C-terminal domains are not connected by disulfide bonds, and we have solved the disulfide bonding pattern of the C-terminal domain of bIGFBP-2.

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