Contributions of the N- and C-terminal domains of IGF binding protein-6 to IGF binding

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Abstract

Insulin-like growth factors IGF-I and IGF-II are important mediators of growth. A family of six high affinity IGF binding proteins (IGFBPs) modulate IGF action. IGFBPs have three domains, of which the N- and C-domains are involved in high affinity IGF binding. IGFBP-6 is unique in its 20–100-fold IGF-II binding specificity over IGF-I. The aim of this study was to determine the contributions of the N- and C-domains of IGFBP-6 to its IGF binding properties. We confirmed that differential dissociation kinetics are responsible for the IGF-II binding preference of IGFBP-6. The N-domain has rapid association kinetics, similar to full-length IGFBP-6, but both IGF-I and -II dissociate rapidly from this domain, thereby reducing its binding affinity for IGF-II ∼ 50-fold. However, the N-domain binds IGF-I and -II with similar affinities and it has a similar IGF-I binding affinity to full-length IGFBP-6. This suggests that the C-domain confers the IGF-II binding preference of IGFBP-6; indeed, IGF-I bound inconsistently with very low affinity to the C-domain. Coincubation studies showed that isolated N- and C-domains of IGFBP-6 do not strongly cooperate to enhance IGF binding. The results of the binding studies are supported by the effects of the IGFBP-6 domains on IGF-induced colon cancer cell proliferation; the N-domain inhibited IGF-I- and -II-induced proliferation with ∼20-fold lower potency than IGFBP-6 and it was equipotent in inhibiting IGF-I- and IGF-II-induced proliferation. Coincubation of C-domain had no additional effect on N-domain-induced inhibition of proliferation. In conclusion, both the N- and C-domains of IGFBP-6 are involved in IGF binding, the C-domain is responsible for the IGF-II binding preference of IGFBP-6 and intact IGFBP-6 is necessary for high affinity IGF binding.

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Introduction

Insulin-like growth factors (IGFs) are important mediators of physiological growth (Jones & Clemmons 1995, Lupu et al. 2001). At the cellular level, IGFs stimulate proliferation, survival and differentiation, and they also have insulin-like metabolic effects. Most of these actions are mediated by the IGF-I receptor, although IGFs also bind to the IGF-II/mannose 6-phosphate and insulin receptors. Aberrant regulation of the IGF system is implicated in a range of diseases including cancer, atherosclerosis and diabetes (Bach 1999b).

The actions of IGFs are regulated by a family of six high affinity IGF binding proteins (IGFBPs 1–6) (Bach & Rechler 1995, Jones & Clemmons 1995, Firth & Baxter 2002). IGFBPs either inhibit or potentiate IGF actions, depending on the conditions of study. More recently, IGF-independent actions have also been described for some IGFBPs. All IGFBPs have three domains. The N- and C-domains are highly conserved among IGFBPs and contain internal disulfide linkages. A linker domain that is not conserved between IGFBPs joins these domains. Both the N- and C-domains contain determinants of high affinity IGF binding (Firth & Baxter 2002), although the specific roles of each of these domains has not been conclusively established. In contrast, the linker domain is probably not directly involved in IGF binding.

The distinctive feature of IGFBP-6 is its 20–100-fold higher binding affinity for IGF-II than IGF-I (Roghani et al. 1989, Bach 1999a). In contrast, IGFBPs 1–5 bind IGF-I and -II with...
approximately equal affinity, although IGFBP-2 has a slight IGF-II binding preference (Bach et al. 1993). Concomitant with its IGF-II binding specificity, IGFBP-6 is a relatively specific inhibitor of IGF-II actions (Bach 1999a) and overexpression of IGFBP-6 inhibits growth of IGF-II dependent neuroblastomas (Grellier et al. 1998) and rhabdomyosarcomas (Gallicchio et al. 2001) in vivo.

In this study, we used biosensor technology, which permits real-time kinetic analysis, to study the contributions of the N- and C-domains of IGFBP-6 (N-BP-6 and C-BP-6, respectively) to high affinity IGF binding and the IGF-II binding specificity of IGFBP-6. To correlate the IGF binding properties of these domains with their effects on IGF actions, we also studied modulation of IGFBP-6. The N-BP-6 binds IGF-II with ~50-fold lower affinity than full-length IGFBP-6 and has no IGF-II binding preference. Consistent with this, N-BP-6 equipotently inhibited IGF-I- and IGF-II-induced proliferation. IGF-II but not IGF-I bound to C-BP-6 with low affinity. These results indicate that the C-domain is responsible for the IGF-II binding preference of IGFBP-6.

Materials and methods

Expression of IGFBP-6 and its domains

A cDNA encoding full-length IGFBP-6 cDNA (amino acids 28–240 based on proIGFBP-6, SWISS-PROT accession number P24592) was excised from pGex-2T-BP-6 (Marinaro et al. 1999) and cloned into the pProEx HTb expression vector (Life Technologies, Melbourne, Australia) using SalI and BamHI restriction sites. This vector encodes a 28-amino acid N-terminal peptide containing a His6-tag (Fig. 1).

N- and C-terminal domain constructs

A cDNA encoding N-BP-6 from amino acids 28–106 was generated by PCR using the forward primer ATG GGA TCC GGG CCA TGC CCA GGC TGC GGG CAA GGG, which encodes a BamHI restriction site (underlined), and the reverse primer AGC TCT AGA CGG AAG GCA GCG GCC TCG GCC GAG which encodes a XbaI site and a stop codon (italics). PCR products were digested with BamHI and XbaI and the cDNA subcloned into pProEx HTb.

The C-terminal domain cDNA encoding amino acids 161–240 of C-BP-6 was also synthesized using PCR with the forward primer ATG GGA TCC GGC CCA TGC CGT AGA CAT CTG GAC and the reverse primer AGC TCT AGA CGG AAG GCA GCG GCC TCG GCC GAG and subcloned into pProEx HTb (Headey et al. 2003). DNA sequencing confirmed the correct sequences of the N- and C-domain cDNAs.

Expression and purification

JM109 Escherichia coli transformed with the IGFBP-6, N-BP-6 or C-BP-6 plasmids were grown in LB broth and protein expression was induced with IPTG (1 mM) for 4 h. Cells were harvested and lysed by sonication in a buffer containing 3 M guanidine HCl (GdnHCl), 10 mM imidazole, 50 mM NaH2PO4 (pH 7.8), His6-tagged proteins were purified by Ni-NTA chromatography (Qiagen, Clifton Hill, Australia). After three washes with 1 M GdnHCl, 300 mM NaCl, 20 mM imidazole and 50 mM NaH2PO4 (pH 7.8), proteins were eluted with 250 mM imidazole and 50 mM NaH2PO4 (pH 7.8). The eluate was dialyzed in 10 mM NaH2PO4 (pH 2.6) followed by dialysis in 50 mM Na acetate (pH 3.9 for N-BP-6 and pH 4.4 for C-BP-6). Proteins were further purified using SP-sepharose cation exchange chromatography (Amersham, Castle Hill, Australia) with a 0–1 M NaCl gradient in 50 mM Na acetate, (pH 3.9 for N-BP-6 and pH 4.4 for C-BP-6). Fractions containing the protein of interest were then concentrated and desalted. Identities of the constructs were confirmed by electrospray ioniz-
ation mass spectrometry and the correct disulfide linkages verified by peptide mapping as described previously (Neumann & Bach 1999).

**IGF binding studies**

Binding studies were performed using surface plasmon resonance on the BiaCore 2000 biosensor (BIAcore AB, Uppsala, Sweden) as described previously (Marinaro et al. 1999).

**Immobilization**

IGFBP-6, N-BP-6 and C-BP-6 were immobilised on separate channels of a CM5 sensor chip (BIAcore AB, Uppsala, Sweden) using the amine coupling method. One channel was left blank to act as a reference. Final resonance values were ~2000 RU for immobilized proteins.

**Binding**

The sensor chip allows buffer to flow sequentially over all four channels, enabling simultaneous measurement of binding on these channels. IGF-I or -II (1–400 nM; a generous gift from Eli Lilly, Indianapolis, IN, USA or purchased from GroPep, Adelaide, Australia) were injected over the surface of the sensor chip at a flow rate of 10 µl/min for 5 min in HBS buffer (0·01 M HEPES, 0·15 M NaCl, 0·005% P20, pH 7·4). Following IGF injection, dissociation was evaluated by passing buffer alone over the chip at 10 µl/min for 5 min. After each run, the chip was regenerated with two 30 s injections of 10 mM HCl. All experiments were performed at 20 °C. Six to 11 independent binding experiments were performed with two to three separate chips. Within most experiments, multiple concentrations of IGF-I or -II were evaluated.

To determine whether N- and C-BP-6 interacted to facilitate IGF binding, IGF-I or -II plus N-BP-6 were incubated in HBS buffer for 20 min and then injected over immobilized C-BP-6. Additionally, IGF-I or -II was incubated with C-BP-6 prior to injection over immobilized N-BP-6. Injection and regeneration protocols were the same as those described above.

**Kinetic modelling**

Kinetic modelling was performed using the BIAevaluation software version 3·0 (BIAcore AB). All modelling was performed after correcting for the effects of bulk refractive index by subtracting the reference channel signal from the active channels. The ‘two state reaction (conformational change)’ model was used to derive kinetic constants since this model provided a better fit of the experimental data than a 1:1 binding model, as has previously been noted for IGFBP-2:IGF binding (Carrick et al. 2001). This model assumes a bipphasic interaction, with rapid binding being followed by a second slower process that is envisaged as a binding-induced conformational change. Accordingly, the model estimates two association and dissociation rate constants. Where possible, modelling was performed simultaneously on a series of curves derived from a range of IGF concentrations.

**Proliferation of LIM 1215 human colon cancer cells**

Cell number was measured using the MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-dipheyltetrazolium bromide) assay as described previously (Leng et al. 2001). LIM 1215 human colon cancer cells (1×10⁶) were plated into wells of a 96-well plate in RPMI 1640, insulin (25 U/l), α-thioglycerol (10⁻⁵ M), hydrocortisone (1 mg/ml) and 5% fetal calf serum and allowed to attach overnight. After washing twice with phosphate buffered saline, cells were incubated for 24 h in serum-free media without insulin at 37 °C. Cells were then incubated in a serum-free medium containing 0·1% BSA ± IGF-I or IGF-II (100 ng/ml, ~13 and 14 nM respectively) and/or IGFBP-6 (0·4–40 nM), N-BP-6 (8·9–890 nM) or C-BP-6 (8·3–830 nM) for 96 h at 37 °C. MTT (10 µl of 5 mg/ml) was then added for 4 h. Media was removed and cells solubilized in 200 µl acidified isopropanol/0·04 M HCl followed by vigorous shaking on a rocker for 30 min at room temperature. Absorbance, reflecting cell number, was quantified in a plate reader at 540 nm. Within each of 4–6 independent experiments, 4 replicates were performed.

**Statistics**

Results are shown as mean ± S.E.M. Cell proliferation experiments were initially analyzed by analysis of variance. Post-hoc analyses were performed using the Fisher’s PLSD correction for multiple comparisons.
Results

IGF binding by IGFBP-6 and its domains

The association phase of IGF binding by IGFBP-6 was dominated by the fast first association rate, which was similar for both IGF-I and IGF-II (Fig. 2, Table 1). However, dissociation of both ligands was very rapid, resulting in a ~50-fold reduction in IGF-II binding affinity. The IGF-II binding specificity of wild-type IGFBP-6 was not seen for N-BP-6, which bound both IGF-I and -II with similar affinity (Table 1).

C-BP-6 bound IGF-II with a first association rate that was ~4-fold slower than that of IGFBP-6 (Fig. 2A, Table 1). The first dissociation rate of C-BP-6 was ~5-fold faster than that of IGFBP-6, while its second dissociation rate was ~7-fold faster. The affinity of C-BP-6 for IGF-II was therefore ~280-fold lower than that of IGFBP-6. In contrast to IGF-II, there was either no response or a small response (~10 RU) when IGF-I was passed over the C-BP-6 channel. Given the inconsistencies of these small responses, estimates of kinetic constants were not obtained for IGF-I.

In order to determine whether the N- and C-domains interact with IGFs to confer the high binding affinity and IGF-II binding preference of IGFBP-6, preincubation experiments were performed in which IGFs were coincubated with a domain at equimolar concentrations prior to injecting the mixture over a sensor-chip surface on which the other domain was immobilized. No binding interaction between N-BP-6 and C-BP-6 was observed in the absence of IGFs (Fig. 3).

When the IGF-II/N-BP-6 mixture was injected over the C-BP-6 channel, an increased response was evident with slow association and dissociation kinetics (Fig. 3A). However, the kinetics of N-BP-6/IGF-II binding to C-BP-6 were similar to those of IGF-II alone. In contrast to the results with IGF-II, there was little binding of IGF-I to C-BP-6, whether or not it was preincubated with N-BP-6 (Fig. 3B).

When IGF-II was coincubated with C-BP-6 and injected over the N-BP-6 channel, a large increase in response was observed (Fig. 4A). However, despite the increased response, the kinetics were similar in the presence and absence of C-BP-6. In contrast, only a small increase in maximum response was observed when IGF-I was coincubated with C-BP-6 prior to injection over the N-BP-6 channel (Fig 4B).

Proliferation of LIM 1215 human colon cancer cells

After 96 h, IGF-II (14 nM) increased LIM 1215 cell number to 170 ± 17% of control (Fig. 5A).
Coincubation of IGFBP-6 inhibited IGF-II-induced proliferation in a dose-dependent manner \((P=0.02)\). IGFBP-6 (40 nM, i.e. ~2.8-fold molar excess over IGF-II) decreased cell numbers to 112 ± 5% of control. Coincubation with N-BP-6 also inhibited IGF-II-induced proliferation \((P=0.01)\), although a concentration of 890 nM was necessary to decrease cell number to 115 ± 7% of control. The similar inhibitory effect of a ~20-fold higher concentration of N-BP-6 than IGFBP-6 is consistent with their relative IGF-II binding affinities. In contrast, C-BP-6 had no effect on IGF-II induced proliferation at concentrations of up to 830 nM \((P=0.62)\). Coincubation of C-BP-6 had no additional effect on inhibition of IGF-II actions by N-BP-6.

After 96 h, IGF-I (13 nM) increased LIM 1215 cell number to 190 ± 14% of control (Fig. 5B). As demonstrated previously \((Leng et al. 2001)\), IGFBP-6 had no effect on IGF-I-induced proliferation of LIM 1215 cells \((P=0.88)\), even when present in ~3-fold molar excess. The effect of a higher concentration of IGFBP-6 was not investigated. Interestingly, however, N-BP-6 inhibited IGF-I actions at high concentrations \((P=0.03, \text{Fig. 5B})\), with potency that was similar to its effects on IGF-II (Fig. 5A). As observed for IGF-II, C-BP-6 had no effect on IGF-I-induced proliferation at concentrations of up to 830 nM \((P=0.75)\) and coincubation of C-BP-6 had no further effect on inhibition of IGF-I actions by N-BP-6.

### Discussion

The results presented in this paper confirm that differential dissociation kinetics are responsible for the IGF-II binding preference of IGFBP-6. N-BP-6 has similar rapid association kinetics to full-length IGFBP-6, but both IGF-I and -II dissociate rapidly, so that the binding affinity for IGF-II is decreased ~50-fold. However, the N-domain binds IGF-I and -II with similar affinities and has a similar IGF-I binding affinity to full-length IGFBP-6. This suggests that the C-domain confers the IGF-II binding preference of IGFBP-6; indeed, IGF-I bound inconsistently with very low affinity to C-BP-6. Kinetic analysis of coincubation studies suggests that isolated N- and C-domains of IGFBP-6 do not cooperate strongly to enhance IGF binding. The results of the binding studies are supported by those showing the effects of IGFBP-6 domains on IGF-induced colon cancer cell proliferation, where N-BP-6 inhibited IGF-II-induced proliferation with ~20-fold lower potency than IGFBP-6 and was equipotent in inhibiting IGF-I and IGF-II-induced proliferation. Further, coincubation of C-BP-6 had no additional effect on N-BP-6 induced inhibition of proliferation.

The IGF binding affinity of IGFBP-6, as measured by biosensor in the present study, was lower than that previously determined by solution assay \((Bach et al. 1993, Marinaro et al. 1999)\). Such differences are commonly observed \((Forbes et al. 1998, Marinaro et al. 1999, Carrick et al. 2001)\).
Indeed, the affinity of IGF-II for IGFBP-6 determined by biosensor in this study (\(K_a\) \(\sim 1 \text{ nM}^{-1}\)) is similar to that determined previously for IGFBP-6 using this method (Marinaro et al. 1999). The difference between biosensor and solution assay affinities may be methodological; for example, the IGFBP is immobilized in biosensor experiments, whereas it is mobile in solution assays.

Many studies have shown that both the N- and C-domains of IGFBPs are involved in high affinity IGF binding. Structural studies identified an N-domain region of IGFBP-5 containing a number of hydrophobic amino acids that are involved in IGF binding (Kalus et al. 1998, Zeslawski et al. 2001). This region is highly conserved in all IGFBPs and substitution of five of these hydrophobic residues in IGFBP-3 and IGFBP-5 decreased their IGF-I binding affinities more than
1000-fold and greatly reduced the inhibitory effects of these IGFBPs on IGF actions (Imai et al. 2000). N-domain fragments of IGFBPs generally retain IGF binding but with 10–1000-fold lower affinity than full-length IGFBPs (Andress et al. 1993, Cheung et al. 1994, Hashimoto et al. 1997, Firth et al. 1998, Kalus et al. 1998, Qin et al. 1998, Standker et al. 2000, Twigg et al. 2000, Carrick et al. 2001, Galanis et al. 2001). Kinetic studies show that the N-domains of IGFBP-2 (Carrick et al. 2001), IGFBP-3 (Galanis et al. 2001) and IGFBP-5 (Kalus et al. 1998) associate with IGFs at least as rapidly as the full-length IGFBPs, but that dissociation is more rapid, resulting in lower binding affinities. The present study shows similar kinetics for IGF binding by the N-domain of IGFBP-6.

C-domains of IGFBPs also play a role in high affinity IGF binding. Full-length IGFBPs have higher IGF binding affinities than isolated N-domains (Andress et al. 1993, Firth et al. 1998, Kalus et al. 1998, Qin et al. 1998) and deletion (Forbes et al. 1998) or point mutation (Bramani et al. 1999) of C-domains of IGFBPs results in decreased IGF binding affinity. The precise nature of this role has been controversial, since some studies have shown that isolated C-terminal fragments of IGFBP-3 (Lalou et al. 1996), IGFBP-4 (Qin et al. 1998) and IGFBP-5 (Kalus et al. 1998) do not bind IGFs using solution assays, Western ligand blot and biosensor analysis, respectively. In contrast, a number of studies clearly demonstrate IGF binding by isolated C-domains of IGFBPs. Specifically, C-terminal fragments of IGFBP-2 bind IGFs with only ~10-fold lower affinity than full-length IGFBP-2 (Wang et al. 1988, Ho & Baxter 1997, Carrick et al. 2001). The C-domain of IGFBP-3 has also been shown to bind IGFs, with different studies reporting 3- to 1000-fold lower affinity than full-length IGFBP-3 (Galanis et al. 2001, Vorwerk et al. 2002, Payet et al. 2003).

The relative IGF binding affinities of isolated N- and C-domains appear to differ between different IGFBPs. The C-domain of IGFBP-2 bound IGFs with higher affinity than the N-domain, and had slower dissociation kinetics (Carrick et al. 2001). One study showed similar findings for IGFBP-3 (Galanis et al. 2001) although another showed equal binding affinities and dissociation kinetics for the N- and C-domains of IGFBP-3 (Vorwerk et al. 2002). In contrast, the C-domains of IGFBP-4 (Standker et al. 2000) and IGFBP-5 (Kalus et al. 1998) had lower binding affinities than their N-domains. The C-domain of IGFBP-6 in the present study therefore resembles those of IGFBP-4.
and IGFBP-5 in that it binds IGFs with lower affinity than its N-domain.

Do N- and C-domains of IGFBPs cooperate to mediate high affinity IGF binding? It has been shown recently that coincubation of the N- and C-domains of IGFBP-3 substantially enhanced IGF binding affinity, to the extent that it was only 5–10-fold lower than that of intact IGFBP-3 (Payet et al. 2003). This cooperativity was confirmed functionally as the two domains effectively inhibited IGF-I-stimulated DNA synthesis at 5-fold molar excess, whereas a 25-fold molar excess of either domain alone resulted in only weak inhibition. In contrast, although coincubation of the N- and C-domains of IGFBP-2 resulted in increased biosensor signal when passed over immobilized IGFs, high affinity binding was not observed (Carrick et al. 2001). Similarly to IGFBP-2, we found that preincubation of IGF-II with either the N- or C-domain of IGFBP-6 prior to passing over the other immobilized domain increased the biosensor signal. However, kinetic analysis revealed no change in IGF-II binding kinetics following preincubation. The biosensor signal depends on the mass of bound analyte, so that the enhanced signal may have been at least in part due to binding of a larger IGF-II/domain complex to the immobilized ligand. Alternatively, limitations in the biosensor modelling may have precluded detection of a small increase in affinity. Nevertheless, in contrast to IGFBP-3, the proliferation experiments clearly demonstrate that coincubation of N- and C-BP-6 was no more effective than N-BP-6 alone in inhibiting IGF-dependent cell proliferation. It is apparent that discrepancies between the binding properties of the N- and C-domains of different IGFBPs are beginning to emerge. Whether these represent true differences between IGFBPs or reflect differences in preparations or assay methodologies requires further study.

The distinctive functional property of IGFBP-6 is its IGF-II binding preference (Roghani et al. 1989, Bach 1999a). Although we previously postulated that differences in the disulfide bonds of the N-domain of IGFBP-6 may underlie this unique binding specificity (Neumann & Bach 1999), our results suggest that the C-domain mediates the IGF-II binding preference of IGFBP-6. The evidence for this is three-fold, since the N-domain has no IGF-II binding preference, its affinity for IGF-I is similar to that of full-length IGFBP-6 and the C-domain of IGFBP-6 had very low, if any, affinity for IGF-I. Preincubation of IGF-I with N-BP-6 would result in formation of a complex with increased mass compared with IGF-I alone and would therefore be expected to increase the biosensor signal if it bound to C-BP-6. The lack of enhancement in the biosensor signal reinforces the notion that C-BP-6 binds IGF-I with very low affinity.

The results of the present study do not define the molecular basis for the IGF-II binding preference of C-BP-6. We have recently determined the binding site for C-BP-6 on IGF-II using nuclear magnetic resonance (Headey et al. 2004); most of the residues in the binding site are conserved in IGF-I, but detailed mutational analysis of residues that differ may help to determine the basis of the IGF-II binding preference. We have also used nuclear magnetic resonance to solve the three-dimensional structure of C-BP-6 and identify its IGF-II binding site (Headey et al. 2004a). The binding site is consistent with deletion analysis of IGFBP-2, which indicated that amino acids 222–236 in its C-domain are important for IGF binding and may also contribute to its modest IGF-II binding preference (Forbes et al. 1998). Further mutational studies of IGFBP-6 based on these observations will also help to determine the molecular basis of the IGF-II binding preference of IGFBP-6.

In conclusion, this study shows that both the N- and C-domains of IGFBP-6 are involved in high affinity IGF binding, with the C-domain appearing to confer the unique IGF-II binding specificity of IGFBP-6. However, the presence of both the N- and C-domain of IGFBP-6 is insufficient to restore high affinity IGF binding, indicating that an intact IGFBP is necessary. It is unlikely that this is due to the linker region containing additional binding determinants, but rather that linkage of the N- and C-domains optimizes presentation of their IGF binding sites. This supports the notion that IGFBP proteases potentiate IGF action through cleavage of linker domains, thereby producing isolated N- and C-terminal fragments with reduced IGF affinity (Bunn & Fowlkes 2003).

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