Binding site for the C-domain of insulin-like growth factor (IGF) binding protein-6 on IGF-II; implications for inhibition of IGF actions

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Abstract Insulin-like growth factors (IGFs) are important mediators of growth and IGF-binding proteins (IGFBPs) 1–6 regulate IGF actions. As IGFBP C-terminal domains contribute to high-affinity IGF binding, we have defined the binding site for the C-domain of IGFBP-6 on IGF-II using NMR. This site lies adjacent to and between the binding sites for the IGFBP N-domain and IGF-I receptor (IGFIR), which have previously been found on opposite sides of the IGF molecule. The C-domain is therefore likely to interfere with IGF binding to the IGFIR, providing a structural basis for the potent inhibitory effects of intact IGFBPs on IGF actions.

Keywords: Insulin-like growth factor; Binding protein; Structure; Nuclear magnetic resonance; Interaction surface

1. Introduction

The insulin-like growth factor (IGF) system is the major control pathway of physiological growth in mammals [1]. IGF-I and -II, the ligands in this system, are widely expressed and play an important role in cell proliferation, differentiation and survival. Dysregulation of the IGF system is associated with many diseases such as cancer and diabetes [1–3], and there is considerable interest in the development of IGF-based therapeutics for these diseases.

A family of six high-affinity IGF-binding proteins (IGFBPs 1–6) regulates IGF activity [4–6]. IGFBPs inhibit IGF actions by competing with the IGF-I receptor (IGFIR) for IGF binding. IGFBPs can also enhance IGF actions in some situations by mechanisms that are incompletely understood. The distinctive functional feature of IGFBP-6, the subject of the present study, is its 20–100-fold higher binding affinity for IGF-II over IGF-I, resulting in IGFBP-6 being a relatively specific inhibitor of IGF-II actions [7]. IGFBP-6 inhibits growth of IGF-II-dependent tumors in vitro and in vivo [8,9], which supports the view that inhibiting IGF actions may decrease cancer growth.

IGFBPs consist of three domains of approximately equal size [4,6]. The N- and C-terminal domains of all IGFBPs each share a high degree of sequence homology [10]. Isolated N- and C-domains bind IGFs with lower affinity than full-length IGFBPs, implicating both of these in high-affinity IGF binding [6]. In contrast, there is little homology among the central L-domains of IGFBPs and these are not thought to be directly involved in IGF binding.

The IGF-I residues involved in binding to the N-terminal domain of IGFBP-5 have been identified recently [11,12]. Although the C-domains of IGFBPs are also important in high-affinity IGF binding [6,13,14], the IGF residues involved in this binding interaction are unknown, precluding a complete understanding of the molecular mechanisms by which IGFBPs modulate IGF actions. We therefore identified the C-domain interactive surface of IGF-II by monitoring changes in the NMR spectrum of ^{15}N-labelled IGF-II following the addition of unlabelled C-domain of IGFBP-6 (C-BP-6).

2. Materials and methods

2.1. Samples

C-BP-6, expressed as a His_6-tagged protein, was purified and characterized as described previously [15]. Recombinant ^{15}N-labelled IGF-II was expressed, purified and characterized as described previously [16,17].

2.2. NMR spectroscopy and C-domain interaction with IGF-II

To investigate the C-BP-6 binding site on IGF-II, ^{15}N–^{1}H HSQC spectra of 0.6 mM ^{15}N-labelled IGF-II in 10 mM sodium acetate and 0.05% (w/v) sodium azide at pH 3.5 were recorded in the presence and absence of unlabelled C-BP-6 (0.3 mM). Spectra were recorded at 25 °C on a Bruker DRX-600 using a triple-resonance probe equipped with triple-axis gradients and an Avance 500 spectrometer equipped with a cryoprobe. NMR data were processed in XWINNMR version 3.1 (Bruker Biospin) and analyzed using XEASY version 1.3 [18]. A pH of 3.5 was used for the direct observation of ^{15}N–^{1}H HSQC spectra of ^{15}N-labelled IGF-II as these spectra show significant line broadening at higher pH [19] and this is the lowest pH at which binding still occurs. In preliminary experiments, ^{15}N–^{1}H HSQC spectra of 0.3 mM ^{15}N-labelled C-BP-6 in the presence of 0.3 mM unlabelled IGF-II were recorded at pH 4.5, 4.0, 3.5 and 3.0. Maximal IGF-II-induced broadening of peaks from C-BP-6 occurred at pH 4.0 but significant

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Abbreviations: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; C-BP-6, C-domain of IGFBP-6; IGFIR, IGF-I receptor; IGF-II/M6PR, IGF-II/mannose-6-phosphate receptor

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broadening was still evident at pH 3.5. At pH 3.0 the spectrum began to resemble that of ^{15}N-labelled C-BP-6 in the absence of IGF-II. Therefore, pH 3.5 was selected for experiments investigating the C-BP-6 binding site on IGF-II. IGF-II ^{15}N–^{1}H assignments were based on those of Torres et al. [19]. The majority of cross-peaks from IGF-II backbone amides showed some line broadening upon addition of unlabelled C-BP-6.

3. Results and discussion

3.1. The C-BP-6 binding site on IGF-II

NMR spectra of the IGFs show broader resonances than would be predicted for proteins of their size [19], an effect attributed to oligomerization and/or conformational exchange. Unambiguous assignments based on published chemical shifts were made for all cross-peaks in the ^{15}N–^{1}H HSQC spectrum of free IGF-II, with the exception of Leu8, Cys9, Arg30, Arg40, Val43 and Glu67. IGF-II residues affected by C-BP-6 binding were identified from changes in ^{15}N–^{1}H HSQC spectra of ^{15}N-labelled IGF-II upon addition of unlabelled C-BP-6. The majority of backbone ^{15}N–^{1}H IGF-II resonances in the structured regions of the molecule showed some line broadening upon addition of unlabelled C-BP-6 (Fig. 1A). It is important to note, however, that numerous resonances in the spectrum of IGF-II that arise from structured regions of the protein (e.g., those around 8.8–9 ppm/124–126 ppm, including Asp23, and 8.5 ppm/111 ppm, from Gly41, as well as several less well-resolved peaks in Fig. 1A) are still clearly visible in spectra of the complex, confirming that the disappearance of resonances in the complex is not simply a consequence of non-specific line broadening of all resonances from structured regions of IGF-II, which would leave only peaks from flexible regions of the ligand still visible. The largest decreases in backbone cross-peak intensities were observed for IGF-II residues Thr7, Gly10, Gly11, Glu12, Leu13, Val14, Asp15, Thr16, Gln18, Cys21, Phe28, Ile42, Glu44, Cys46, Cys47, Phe48, Arg49 and Cys51 (Fig. 1A). This is consistent with these residues having a significant exchange contribution to their observed transverse relaxation rates as a result of intermediate chemical exchange between the bound and free forms. The exchange contribution is greatest for resonances that undergo the largest changes in chemical shift upon ligand binding, which, in the absence of allosteric effects, are usually those residues located at the binding interface. Intermediate exchange occurs when the lifetime of the bound and free states is approximately equal to the differences in chemical shift and/or transverse relaxation rates between the free and ligand-bound forms. As a consequence, the affected resonances broaden upon ligand addition and may even disappear [20]. Intermediate chemical exchange is commonly observed for proteins with a K_d in the μM range. Our NMR observations are thus consistent with the measured K_d for the C-BP-6/IGF-II interaction, determined by surface plasmon resonance (results not shown).

As a consequence of the disappearance or severe broadening of resonances from backbone amides of IGF-II upon complex formation with C-BP-6, it was not practical to utilize the method of saturation transfer [21,22] to map the binding interface. In this method, magnetization is transferred from the larger binding partner, in this case C-BP-6, to the ligand being observed by NMR. Generally speaking, this approach provides a more precise picture of the binding surface than that obtained by mapping chemical shift perturbations, which usually identify a larger interaction surface than X-ray crystallographic studies [23,24]. Thus, it should be borne in mind in interpreting our results that the actual contact surface between IGF-II and C-BP-6 is likely to be slightly smaller than inferred from the observed spectral perturbations.

When mapped onto the solution structure of IGF-II solved previously by us [19], the majority of residues affected by C-BP-6 binding are found on a continuous surface which forms a putative C-BP-6 binding site (Fig. 1B). The binding site of IGF-I to mini-IGFBP-5, which is part of the N-domain, is already known [12]. IGF-I and IGF-II share 67% amino acid identity and the residues within the N-domain binding site of IGF-I are completely conserved in IGF-II. Moreover, of the 18 IGF-II residues affected by incubation with the C-domain of IGFBP-6, 15 are identical in IGF-I. As shown in Fig. 1B,

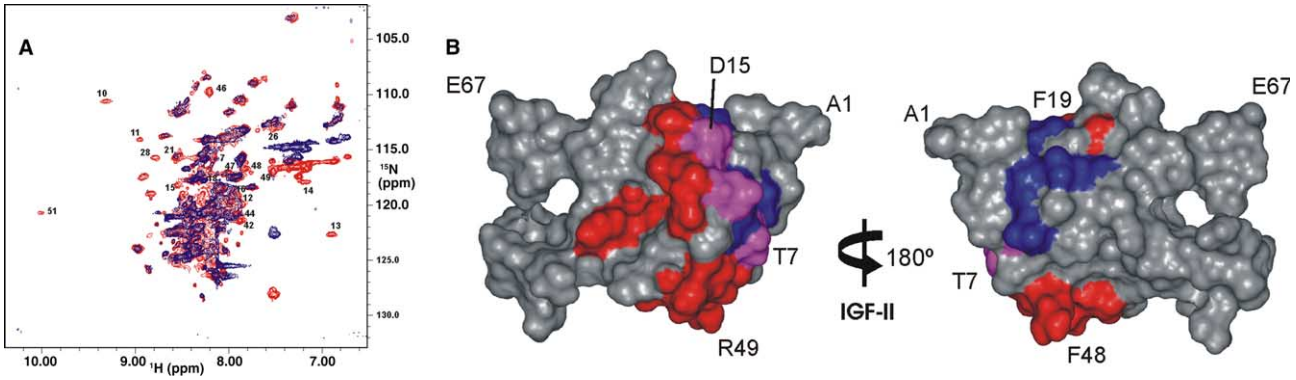

Fig. 1. (A) Comparison of ^{1}H–^{15}N HSQC of IGF-II in the presence (blue) and absence (red) of C-BP-6 (C-BP-6:IGF-II 0.5). The positions of some side chain resonances in the ^{15}N dimension are slightly different in the two spectra because different sweep widths were used; these peaks are folded from the top of the spectrum. Their chemical shifts in the ^{1}H dimension (around 6.8–7.6 ppm) are not affected. Residues labelled are those where the peak intensity for free IGF-II decreased by >80% upon C-BP-6 addition (% decreases in intensity were averaged from spectra acquired at 500 and 600 MHz). (B) Surfaces of IGF-II showing residues involved in N-domain binding (blue) and C-domain binding (red), and residues common to both domains (magenta). Note that the 11 residues that contribute to the N-domain binding surface [12] appear to form a discontinuous surface interrupted by the N-terminus; this arises because IGF-II contains an additional three residues at the N-terminus compared with IGF-I, and the first five residues are disordered in the solution structure [19]. When IGF-II interacts with the N-domain of IGFBP-6, these N-terminal residues most likely shift to expose the continuous N-domain binding surface identified in IGF-I [12]. This figure was prepared using InsightII.

the N- and C-domains bind to largely distinct but adjacent surfaces of the IGF molecule. The N- and C-domains of full-length IGFBPs are joined by a non-conserved, largely unstructured linker region, which is not believed to be directly involved in IGF binding. Based on our observations, the N- and C-domains are brought close together upon IGF binding, and it is likely that this flexible linker region loops out from the IGF molecule and does not directly interact with it.

The proposed C-BP-6 binding site on IGF-II explains site-directed mutagenesis studies that have identified a number of IGF residues important for IGFBP binding. These residues are largely distinct from residues important for binding to the IGFIR. In particular, mutation of IGF-II residues Glu6, Phe26 and Phe48–Ser50 substantially decreases IGF-II binding to IGFBPs [16,25,26] as does mutation in IGF-I of the equivalents of Glu6, Thr7, Gln18 and Phe19 in IGF-II [27]. Mutation in IGF-I of the equivalents of Leu8, Gly10, Leu13, Val20 and Phe28 in IGF-II reduces binding to IGFBP-1 substantially and to IGFBP-3 to a lesser extent [28]. From these results, two patches of surface-exposed hydrophobic residues that are involved in IGFBP binding were proposed. One patch, including the IGF-II equivalents Glu6, Phe19 and Leu53, was subsequently identified as the N-domain binding site, since the IGF-I/mini-IGFBP-5 crystal structure showed that these side chains were inserted deep into a cleft on mini-BP-5 [12]. The other patch includes the IGF-II equivalents Gly10, Val14, Phe28 and Val43. Extending this patch to include the adjacent Cys9 and Cys48, which were not mutated because of their disulfide link, results in a broader patch of residues that corresponds well to the C-domain binding site determined by NMR in this study (Fig. 1B).

3.2. IGFBP inhibition of IGF binding to IGF receptors

The mitogenic effects of IGFs are exerted mainly through the IGFIR. A number of IGF residues are important for binding to the IGFIR. In particular, mutation of Tyr27, Val43 or Tyr59 markedly reduces binding [29,30]. Mutation of Phe26 has a lesser effect [29,30], although mutation of the equivalent Phe in IGF-I had a more dramatic effect [31]. Aromatic residues including Phe26, Tyr27 and Phe28 form a surface patch on the opposite face of IGF-II to the mini-BP-5 binding site, and it was suggested that this may explain the relative inability of mini-BP-5 to completely inhibit IGFIR-dependent IGF actions [11,12].

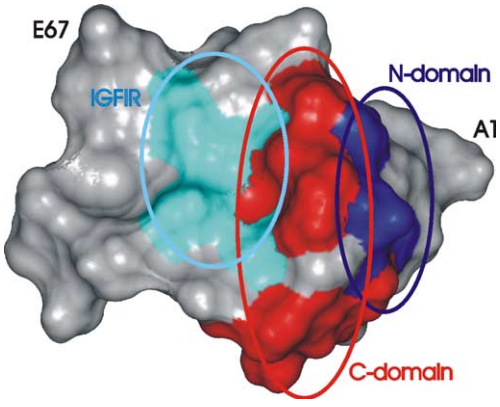

Fig. 2. Schematic of the surface of IGF-II showing the binding site for the IGFBP C-domain (red) lying between those for the IGFIR (IGFIR, cyan) and the IGFBP N-domain (blue). There is overlap between residues identified as interacting with the C-domain and either the N-domain or the IGFIR. Thr7, Glu12, Asp15, and Cys51 of IGF-II are implicated in interactions with both the C- and N-domains; of these, Glu12 and Asp15 are directly involved in binding to the N-domain [12] and are shown in blue, while Thr7 and Cys51 are not in direct contact and are shown in red. Both the C-domain and IGFIR are implicated in interactions with Phe28, which is shown in cyan. Residues 1 and 67 are labelled for orientation. This figure was prepared using InsightII.

In contrast, the surface of IGF-II that binds to C-BP-6 lies between the mini-BP-5 and IGFIR binding sites and overlaps with the latter at Phe28 (Fig. 2). Steric hindrance by IGFBP C-domains may therefore interfere with IGFIR binding. Alternatively, binding of IGFBP C-domains to this region may prevent dissociation of the IGF β-strand from the body of the molecule, a process that is important for binding of insulin to its receptor and may therefore also be important for IGF binding to the IGFIR [32]. The role of IGFBP C-domains in complete inhibition of IGFIR actions can be explained structurally by either of these alternatives.

IGF-II also binds with high affinity to the IGF-II/mannose 6-phosphate receptor (IGF-II/M6PR), which is thought to result predominantly in IGF-II clearance [5]. It appears that the binding sites on IGF-II for C-domains of IGFBPs and IGF-II/M6PR partially overlap, since mutagenesis of Phe48, Arg49 and Ser50 of IGF-II to their equivalent insulin residues (Thr, Ser, Ile, respectively) significantly decreases binding both to IGFBPs 1-6 [25] and to the IGF-II/M6PR [29]. In contrast, mutation of Ala54 and Leu55 of IGF-II completely abrogates IGF-II/M6PR binding [29], but has only modest effects on IGFBP binding [25]. Our NMR data are consistent with these findings, as Phe48 and Arg49 were strongly affected by C-BP-6 addition whereas Ala54 and Leu55 were not.

3.3. Conclusions

The C-domains of IGFBPs 1-6 are highly conserved. As a consequence, the binding site identified on IGF-II for C-BP-6 is likely to be largely the same for all IGFBPs. A number of general conclusions can therefore be drawn about IGF:IGFBP interactions as follows. The proximity of the IGF binding sites for the C- and N-domains of IGFBPs is consistent with the view that the L-domain acts as a flexible linker region between the two domains. Since C-BP-6 interacts with IGF-II residues that are adjacent to and overlap the IGFIR binding site, it also provides a structural basis for IGFBP inhibition of IGF binding to the IGFIR. Since excess IGF activity is implicated in diseases such as cancer, this knowledge also provides a framework for the development of high affinity therapeutic IGF antagonists.

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