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Letter to the Editor: ^{1}H, ^{13}C and ^{15}N resonance assignments of the C-terminal domain of insulin-like growth factor binding protein-6 (IGFBP-6)

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Biological context

Insulin-like growth factor II (IGF-II) is a widely expressed polypeptide involved in normal growth and development. However, many tumours over-express IGF-II, leading to increased cell proliferation and survival. IGF binding protein-6 (IGFBP-6) is one member of a family of six GFBPs that act as regulators of the IGFs. IGFBP-6 is unique among the IGFBPs in having a 20–100 fold higher affinity for IGF-II over IGF-I and appears to act primarily as an inhibitor of IGF-II actions (Bach, 1999). Rhabdomyosarcomas that over-express IGFBP-6 grow more slowly in nude mice than control tumours (Gallicchio et al., 2001).

The IGFBPs consist of three domains of approximately equal size (Figure 1A). Both the N- and C-terminal domains are cysteine-rich and highly homologous in sequence across IGFBPs, and contain the IGF binding sites. The central (L) domains are divergent in amino acid sequence. There are only two partial 3D structures available for IGFBPs: (i) An NMR structure of a sub-domain within the N-terminal domain of IGFBP-5 (Kalus et al., 1998), and (ii) a crystal structure of the same sub-domain bound to IGF-I (Zeslawski et al., 2001). These structures reveal a hydrophobic IGF binding site within a novel protein fold. However, the lower affinity of this sub-domain for the IGFs (Kalus et al., 1998) implies that other binding sites contribute to the high-affinity IGF binding of IGFBP-5. As yet no structure exists for the C-terminal domain of any IGFBP. Mutagenesis and sequential deletion studies of IGFBPs 1-5 have shown the importance of the C-terminal domain for IGF binding (Baxter, 2000).

Additional interest in the C-domains of the IGFBPs arises from the observation that the C-domains of IGFBP-3, -5 and -6 inhibit an IGFBP-4 specific protease (Fowlkes et al., 1997). Furthermore, the disulphide linkages of the C-terminal domain of IGFBP-6 match those of a cathepsin protease inhibitor with which it has a moderate degree of sequence homology, indicating that they may share a common fold (Gunčar et al., 1999).

Methods and experiments

DNA encoding residues 161-240 of IGFBP-6 (numbering based on proIGFBP-6) was synthesized by PCR and cloned into the pProEX HTb (Life Technologies) expression vector, which encodes an N-terminal 28-residue sequence containing a His_6-tag and protease cleavage site, producing a construct of 108 residues. The fusion protein was expressed in *E. coli* strain BL21 using M9 minimal media supplemented with $^{15}NH_4Cl$ (1 g l^{-1}) and $^{13}C_6$-glucose (2 g l^{-1}). A ^{15}N-labelled sample was also produced. Lysis of bacteria was performed using 3M guanidine HCl. The protein was purified using Ni-NTA and SP-FF Sepharose cation exchange chromatography. The sequence containing the His_6-tag was retained in order to maximize the solubility of the C-terminal domain. Mass spectrometry established the presence of a 12 053 Da protein, consistent with loss of the N-terminal Met. Correct disulphide linkages were confirmed using tryptic cleavage followed by mass spectrometry. IGF binding

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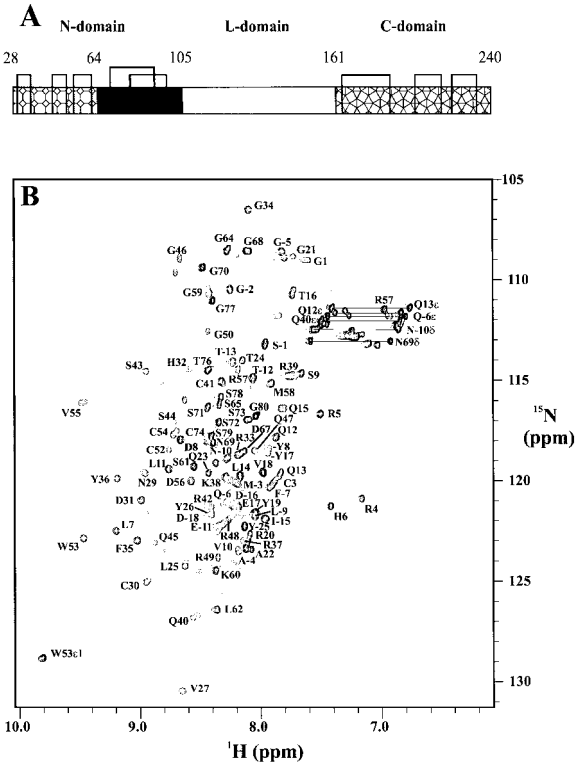

Figure 1. (A) The domain structure of IGFBP-6. Square brackets denote disulphide linkages. Residue numbering (28-240) is based on proIGFBP-6 (SWISS-PROT accession number P24592). (B) 2D [^{15}N, ^{1}H]-HSQC spectrum of the C-terminal domain of ^{15}N-IGFBP-6. The C-terminal domain comprises residues 161 to 240 of IGFBP-6, here numbered 1 to 80. The residues of the pProEx HTb vector leader sequence are numbered −1 to −25. Resonances were not identified for the first two residues of the leader sequence (−26 and −27).

studies showed that the C-domain bound IGF-II with a K_D in the low μM range.

Samples for NMR contained 1 mM protein in 10 mM sodium acetate (pH 4.5), 0.02% sodium azide in 95% H_2O/5% $^{2}H_2O$. The ^{13}C incorporation was > 95% but the ^{15}N incorporation rate was only 50% in both the ^{13}C- /^{15}N- and ^{15}N-labelled samples. Spectra were recorded at 25 °C on a Bruker DRX-600 spectrometer. The ^{1}H chemical shifts were referenced to TMS at 0 ppm and the ^{13}C and ^{15}N chemical shifts were referenced indirectly using the ^{13}C/^{1}H and ^{15}N/^{1}H ratios. NMR data were processed in XWIN-NMR (Bruker) and analysed using XEASY software (Bartels et al., 1995). An HSQC spectrum recorded on the ^{15}N-labelled sample is shown in Figure 1B.

Sequential connectivities were initially identified using HNCA and ^{15}N NOESY-HSQC experiments. Assignments were subsequently verified using HN-CACB, HNCO and HN(CA)CO spectra. Assignments of the H^{α} and H^{β} chemical shifts were obtained using HNHA and HAHB(CO)NH spectra. Non-aromatic side chain ^{1}H and ^{13}C frequencies were assigned using a ^{13}C NOESY-HSQC spectrum. A number of the aromatic ^{1}H chemical shifts were assigned from 2D TOCSY and NOESY spectra.

Extent of assignments and data deposition

Backbone ^{1}H, ^{13}C and ^{15}N resonances were assigned for all non-proline residues, as well as the ^{1}H and ^{13}C resonances of all seven proline residues. Ninety-six percent of ^{13}CO resonances were assigned, the exceptions being Gly residues 1, 50 and 80 and also Cys74. The H^{α}, H^{β}, C^{α} and C^{β} resonances could be assigned for all residues. All non-aromatic side-chain ^{13}C resonances were assigned. The Tyr residue at the beginning of the leader sequence and five of the six histidines of the His_6-tag were not assigned. The ^{1}H, ^{15}N, ^{13}C chemical shifts have been deposited in the BioMagResBank, Madison, WI, U.S.A. (accession number 5545).

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