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High-yield bacterial expression and structural characterization of recombinant human insulin-like growth factor binding protein-2

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Abstract

The diverse biological activities of the insulin-like growth factors (IGF-1 and IGF-2) are mediated by the IGF-1 receptor (IGF-IR). These actions are modulated by a family of six IGF-binding proteins (IGFBP-1–6; 22–31 kDa) that via high affinity binding to the IGFs ($K_D \sim 300\text{--}700\text{ pM}$) both protect the IGFs in the circulation and attenuate IGF action by blocking their receptor access. In recent years, IGFBPs have been implicated in a variety of cancers. However, the structural basis of their interaction with IGFs and/or other proteins is not completely understood. A critical challenge in the structural characterization of full-length IGFBPs has been the difficulty in expressing these proteins at levels suitable for NMR/X-ray crystallography analysis. Here we describe the high-yield expression of full-length recombinant human IGFBP-2 (rhIGFBP-2) in *E. coli*. Using a single step purification protocol, rhIGFBP-2 was obtained with >95% purity and structurally characterized using NMR spectroscopy. The protein was found to exist as a monomer at the high concentrations required for structural studies and to exist in a single conformation exhibiting a unique intra-molecular disulfide-bonding pattern. The protein retained full biologic activity. This study represents the first high-yield expression of wild-type recombinant human IGFBP-2 in *E. coli* and first structural characterization of a full-length IGFBP.

Keywords

Recombinant human IGF- binding protein-2 (rhIGFBP-2); E coli Expression; Protein Purification; Protein Structure; Secondary Structure; G-matrix Fourier transform (GFT) NMR

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Introduction

The insulin-like growth factor (IGF) system plays an essential role in organism growth, cell differentiation and function [1]. This system includes two peptide hormones, IGF-1 and -2; the IGF-1 and IGF-2 receptors; six soluble IGF-binding proteins (IGFBPs; numbered 1–6); IGFBP proteases. IGF-1 and -2 are small signaling proteins (~7.5 kDa) whose actions are mediated by the IGF-1R, and access to the receptor is regulated by the IGFBPs, which range in size from 22–31 kDa and share overall sequence and structural homology with each other [2]. The IGFBPs bind strongly to IGFs ($K_D \sim 300\text{--}700\text{ pM}$) such that IGFBP proteolysis is required to dissociate the IGFs from the complex, enabling them to bind and activate their cell surface receptors.

In recent years, the IGF system in general and IGFBPs in particular have become the focus as important targets of cancer therapeutics [1,2]. Epidemiologic studies indicate high circulating levels of IGF-1 are associated with increased risk of multiple cancers [3–7], and that high IGFBP-3 levels are inversely associated with cancer risk [8–10]. Some IGFBPs have direct stimulatory/inhibitory actions independent of their IGF-sequestering actions (reviewed in 11). IGFBP-2 expression has been linked to colon, adrenal, ovarian and prostate cancer cell proliferation [12–14]. Elevated IGFBP-2 correlates with more aggressive, metastatic glioblastoma, likely due to $\alpha 5\beta 1$ integrin engagement by its C-terminal RGD motif [15].

While the role of IGFBPs in biological systems has been extensively studied, a detailed understanding of their interaction with IGFs and other proteins is lacking. The three-dimensional (3D) structures of full-length IGFBPs have not yet been determined, though structural information is available on their individual domains [16–20]. All IGFBPs contain three structural domains of nearly equal size. The N- and C-terminal domains are highly conserved, contain, 16–18 spatially conserved cysteine residues, forming 8–9 disulfide bonds [2], and are required for IGF binding [21]. The central domain exhibits little homology among the IGFBPs and is susceptible to protease cleavage [22].

We have undertaken structural and functional studies of IGFBP-2 as a model system [23]. Key among the challenges in the structural characterization of full-length IGFBPs is obtaining suitable quantities of these proteins in soluble form for NMR/X-ray crystallography studies (~20–30 mg/ml) [18]. NMR spectroscopy, requires expensive isotope labeling schemes necessitating high yields in minimal media. IGFBPs and their fragments have been expressed in: *E. coli* [24], yeast [25], mammalian cells [26], and insect cells [27]. However, *E. coli* is the most preferred host organism due to lower costs and the ease with which different engineered forms of proteins can be produced. Our earlier efforts produced microgram quantities of homogeneous rhIGFBP-2 (R281C) from *E. coli* [23]. Here, we present a method for high yield production of monomeric, wild-type, hIGFBP-2 in soluble form (~30 mg/ml) for structural and functional studies. Preliminary NMR analysis indicates ~30% secondary structure content and ~70% unstructured regions. This development opens up new avenues for structural and functional studies in this protein family.

Materials and Methods

Materials and Reagents

cDNA encoding human IGFBP-2 (Cys²⁸¹) was obtained from Dr. Jörg Landwehr (Hoffman-La Roche, Switzerland). SCC-9 and DU145 cells were obtained from the ATCC (Manassas, VA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO). DMEM and His-Select™ nickel affinity gel were purchased from Sigma. NVP-AEW541

was kindly provided by Dr. Francesco Hofmann, Novartis (Basel, Switzerland). IGF-1 was generously provided by Genentech, Inc. (San Francisco, CA). IGFBP-2 polyclonal antibody was from Millipore, Inc. (Billerica, MA). [³H]thymidine was from GE Healthcare (Piscataway, NJ). For NMR studies ¹⁵NH₄Cl and ¹³C glucose were purchased from Cambridge Isotope Laboratories.

Generation of human IGFBP-2 construct

[Arg²⁸¹]6His•IGFBP-2, in which Cys²⁸¹ was changed to Arg²⁸¹ (Fig. S1 of Supporting Information) was created using the QuikChange[®] site directed mutagenesis kit from Stratagene. PCR was performed using [Cys²⁸¹]6His•IGFBP-2 plasmid (IPTG inducible T7 expression vector pET15b from Novagen) as a template with the following primers: 5'-GGAGGCTCGCGGGGTGC-3' (coding) and 5'-GCACCCCGCGAGCCTCC-3' (noncoding). Underlined residues represent the sequence that was mutated to encode arginine. Following PCR, the reaction was digested with DpnI to remove all but newly synthesized plasmid, and transformed into XL1-Blue *E. coli*. All resulting colonies were screened by DNA sequencing; re-sequencing confirmed the identity of [Arg²⁸¹]6His•IGFBP-2.

Expression and purification of IGFBP-2

IGFBP-2 constructs were transformed into BL21 Star[™] (DE3) *E. coli*. Selected colonies were incubated at 37°C in 1 ml starter culture of Luria-Bertoni (LB) medium containing 200 µg/ml ampicillin (amp). After a 1000-fold dilution into fresh LB/amp, the cells were regrown to midlog phase ($E_{600nm} \sim 0.5$) before protein expression was induced with 1 mM isopropyl β-D-thiogalactoside (IPTG) at 15°C for 24 h. Cells were harvested by centrifugation, freeze-thawed to lyse cells, solubilized in His-equilibration buffer (50 mM Na Phosphate, 0.3M NaCl, pH 7.5) with 6 M guanidine HCl, and sonicated on ice 3× for 10 min. Following centrifugation, the supernatant was incubated at 4°C for 1 h with 10 ml of a 50% slurry of equilibrated His-Select[™] Nickel Affinity agarose (Sigma-Aldrich) and packed into a column. The column was washed with two column volumes each of His-equilibration buffer containing 6M, 4M, 2M, 1M, and finally no guanidine HCl. The beads were then washed and eluted with 200 mM imidazole in His-equilibration buffer. The eluate was diluted 10-fold in AC buffer (50 mM HEPES, 150 mM NaCl, pH 7.4) and added to slurry of IGF-1-Sepharose overnight at 4°C. Following three washes with AC buffer and 10-fold diluted AC buffer, protein was eluted with 0.5 M acetic acid and lyophilized. The sample was further purified by reverse phase HPLC on a C4 column as previously described [23,28]

Mass Spectrometry

1 µl IGFBP-2 (1 µg/µl) was mixed with 1 µl sinapinic acid (20 mg/ml) and spotted on a gold-coated, stainless steel plate, air-dried and analyzed using an Applied Biosystems Voyager-DE MALDI-TOF mass spectrometer (Foster City, CA), equipped with a 337-nm nitrogen laser. External mass calibration was performed using insulin (bovine) (5,734.59 Da), thioredoxin (*E. coli*) (11,674.48 Da), apomyoglobin (horse) (16,952.56 Da). Mass accuracy was ±1 Da/1000 Da.

Circular Dichroism (CD) analysis

For CD analysis, IGFBP-2 was reconstituted in the experimental buffer (10 mM Tris buffer, pH 6.0). CD measurements were carried on a Jasco Model J- instrument at 298K using 0.1 cm quartz cuvette. Data were collected by using Jasco software and processed using K2D2 [29].

Preparation of isotope labeled samples for NMR

Protein samples for NMR studies were prepared using a slightly modified protocol (provided in Supporting Information). Briefly, cells were lysed using His-equilibrium buffer containing 8 M Urea, 5 mM DTT and 1 mM PMSF. Protein was eluted from Ni-beads under denaturing conditions with 300 mM imidazole and subjected to slow dialysis to remove urea first followed by removal of the reducing agent. The protein thus obtained was found to have purity > 95% as verified by SDS-PAGE analysis and not subjected to further purification. Isotope labeled samples ($^{13}\text{C}/^{15}\text{N}$ labeled) for NMR studies were prepared using cells grown in minimal medium (M9) containing $^{15}\text{NH}_4\text{Cl}$ and ^{13}C glucose as the sole sources of nitrogen and carbon, respectively, and supplemented with vitamin trace metal mixture [30].

NMR data collection, processing and analysis

All experiments were carried out at 285K on an AV-800MHz Bruker spectrometer equipped with Z-axis pulsed field gradient and triple resonance cryogenic probe. The following spectra were acquired: 2D [^{15}N , ^1H] -HSQC, 2D [^{15}N , ^1H] TROSY, 3D HNCO and GFT (3.2) D HA(CA)CO(N)H (for secondary structure identification) using a 0.8 mM (~30 mg/ml) hIGFBP-2 sample. For estimating the rotational correlation time of the molecule, ^{15}N -T₁, and ^{15}N -T₂ relaxation times were measured. All NMR data were processed and analysed using the software packages, NMRPipe [31] and XEASY [32], respectively.

Ligand and Immunoblot Analysis

Purified IGFBP-2 (100 ng) was resolved on 12.5% non-reducing polyacrylamide gels, transferred to nitrocellulose and subjected to ligand blot analysis (with 40 ng of biotinylated-IGF-1) as previously described [23]. A detailed protocol for the experiment is provided in the Supplementary material. To re-probe ligand blots for immunoblot analysis, antibodies were removed from the nitrocellulose via application of Re-Blot Plus-strong stripping solution according to the manufacturers' instructions (GE Healthcare, Piscataway, NJ). Immunoblots with IGFBP-2 polyclonal antibody were blocked, probed, and visualized as previously described [23].

IGF-1 binding assay

IGFBP-2 purified from *E. coli* was tested for its IGF-binding *in-vitro* using a His-tag pull down assay. rhIGFBP-2 (0.5 mg) was loaded onto a Ni-NTA affinity column pre-equilibrated with His-equilibrium buffer. IGF-1 (0.1 mg in 1.5 mL of His-equilibrium buffer) was applied to IGFBP-2 bound Ni-NTA resin for 2 hours and washed with 10 volumes of His-equilibrium buffer to remove unbound IGF-1. Absence of non-specific interactions between IGF-1 and the Ni-NTA resin was confirmed by loading IGF-1 to Ni-NTA resin in the absence of IGFBP-2. IGFBP-2/IGF-1 complexes bound to Ni-NTA beads were analyzed by SDS-PAGE, followed by staining with Coomassie Blue.

Tissue Culture and [^3H]Thymidine Incorporation

SCC-9 (human oral squamous cell carcinoma cell line) [33] and DU145 (human prostate carcinoma cell line) [34] cells were cultured as previously described [23], and maintained at 37° C in a humidified 5% CO₂-95% air incubator. For [^3H]thymidine incorporation assays, cells grown in 48-well dishes to 60–80% confluency were washed 3× with PBS and incubated with serum free medium (SFM) for 24 h. Cells were subsequently treated for 2 h with inhibitors in SFM followed by treatment for 21 h with IGF-1 and IGFBP-2 in fresh SFM. Medium was then replaced and [^3H]thymidine was added for 6 h followed by cell processing as previously described [35].

Results

Structural characterization by CD and NMR spectroscopy

Protein yields up to 15 mg/L of LB medium and 10 mg/L of M9 medium were obtained. MALDI-TOF MS confirmed the presence of a single protein with a molecular ion mass of 33,800.02 (predicted MW 33,783.48 Da) for rhIGFBP-2 (Fig. S2 of Supporting Information). The protein was soluble at 1 mM (~35 mg/ml). The purified sample was subjected to structural analysis by CD and NMR spectroscopy. Fig 1 shows the 2D [¹⁵N, ¹H] HQSC spectrum of IGFBP-2 (a 2D [¹⁵N-¹H] TROSY spectrum is shown in Fig. S3 Supporting Information). Notably, the protein lacked significant secondary structure as evident from the low ¹H chemical shift dispersion. That is also evident from the CD spectrum (Fig. S4 of Supporting Information). The lower secondary structure content in the protein is due to the fact that the central domain, which accounts for ~35% of the protein length, is known to be disordered [2]. To estimate the secondary structure content, the method of CSSI-PRO [36] was used. The GFT (3,2)D HA(CA)CO(N)HN spectra acquired for this purpose is shown in Fig 2. Based on the distribution of $\Omega(^{13}\text{C}') - 4 * \Omega(^1\text{H}^\alpha)$ chemical shifts [36], the secondary structure content of the protein was estimated to be ~25% helix, ~5% beta-strands and ~70% loops consistent with the secondary structure content in individual domains of different IGFBPs studied so far [16–20].

The spectral overlap in 2D [¹⁵N, ¹H] HQSC spectrum (Fig. 1) was resolved by acquiring 3D HNCO spectrum. A total number of 255 spin systems corresponding to about 90% of expected peaks (excluding residues belonging to the N-terminal His-tag and prolines) were observed. A single set of peaks observed in the NMR spectra points towards a unique conformation and absence of heterogeneity due to scrambling of the intra-molecular disulfide bonds. Further, a rotational correlation time of 22 ns was obtained at 285 K measured using ¹⁵N-T₁ and T₂ relaxation times [37], indicating a monomeric state and ruling out any oligomerization arising from inter-molecular disulfide bonds.

Ligand-blotting and IGF-1 Binding

Ligand blotting revealed labeling of IGFBP-2 with biotinylated IGF-1 (Fig. 3). The blot was stripped and reprobed with an IGFBP-2 antibody as a loading control. The high affinity interaction of rhIGFBP-2 with IGF-1 was further validated by His-tag pull down assays as described above. IGFBP-2 was found to bind IGF-1 by noting the presence of both IGFBP-2 and IGF-1 in the complex eluted from the Ni-NTA resin (Fig S5; Lane D). The biological activity of the protein was also tested in cell-based assays as described below.

[³H]Thymidine Incorporation

IGFBP-2 alone did not affect SCC-9 cell [³H]thymidine incorporation, but reduced IGF-1 (10 nM) stimulated [³H]thymidine incorporation (Fig 4A). In DU145 cells, IGFBP-2 significantly increased [³H]thymidine incorporation (Fig 4B), which was unaffected by addition of the IGF-1R tyrosine kinase inhibitor NVP-AEW541 (Fig 4C). This presumably reflects an IGF-independent action, given that treatment of cells with NVP-AEW541 did not reduce IGFBP-2 stimulated [³H]thymidine incorporation. An alternative explanation is that IGFBP-2 increased DNA synthesis via integrin engagement [38]. It is well documented that IGFBP-2 has IGF-independent actions in prostate cancer cells (such as DU145) but not in other cell lines or in normal prostate epithelial cells [39]. We have utilized SCC-9 cells as a control cell line that does not respond to IGFBP-2, other than by its IGF-dependent effects [23].

Discussion

In recent years, a renewed interest in the role of the IGF system in cancer has surfaced [2]. Despite growing evidence of IGFBP involvement in cancer, a detailed understanding of the structure-function relationship in this protein family is lacking. The goal of the present study was to develop a scheme for efficient expression of full length, functional protein in bacteria suitable for structural analyses. While full length IGFBP-3 expression in bacteria was reported in 1998 [40], the efficient expression of other IGFBPs has been difficult. The original cloning of IGFBP-2 with a 19th cysteine at position 281, instead of an arginine, was the result of a single base change occurring during DNA sequencing [41]. This nucleotide change may have been generated during cDNA synthesis or it may reflect different allelic forms of the IGFBP-2 gene [42]. This substitution does not affect binding to IGF-1 or IGF-2 [14], nor the biologic activities of IGFBP-2 [42]. However, we recently found that the presence of the extra cysteine resulted in the formation of soluble nanotubular structures by the C-terminal 41 residue fragment of this protein [43]. Thus, it is of importance to develop a construct expressing the wild-type protein containing 18 cysteines for structural and functional studies. In previous studies involving GST fusion constructs of IGFBPs, it has been reported that cleavage of the GST sequence has not always been successful [44–45], hence a His-tag construct was used in the present study. In addition, modification of our previously described protocol [23], enabled us to improve yields by: 1) employing BL21 (DE3) StarTM *E. coli*, 2) inducing protein expression at 15°C, 3) extracting IGFBP-2 from inclusion bodies using 8 M Urea or 6 M guanidine-HCl, 4) increasing nickel- bead volumes and 5) employing an efficient re-folding protocol.

In proteins containing multiple cysteines, a key challenge is to conserve the unique pattern of intra-molecular disulfide bonds required for protein function. This was achieved using a re-folding protocol, initially involving removal of denaturing agent, followed by reducing agent through dialysis. The fact that the majority of molecules fold with a unique pattern of intra-molecular disulfide bonds is evident from the single set of peaks observed in the NMR spectra (Fig 1). Disulfide bond scrambling would have led to protein molecules with different conformations resulting in severe line broadening of NMR signals. A unique conformation was further verified by comparing NMR spectra of protein samples prepared (see Methods) with samples prepared under non-denaturing conditions (Fig. S6 of Supporting Information). Identical spectra were observed for both samples, indicating correct refolding of samples prepared under denaturing conditions. The unfolding-refolding protocol also helps prevent oligomerization due to inter-molecular disulfide bonding. Since all IGFBPs contain 16–18 conserved cysteines [2], this protocol can be extended to other IGFBPs.

While the structure of any full-length IGFBP is not known, studies involving individual IGFBP domains and their complex with IGFs have provided some insight into the molecular mechanisms of IGFBP-IGF interactions [16–20]. It is now understood that there are intra-domain disulfide bonds within the N and C-domains but no inter-domain disulfide bonds [16–20]. Both the N- and C-domains of the IGFBPs are required for high-affinity IGF binding, whereas isolated N- and C-domain fragments have significantly lower affinities than full-length IGFBPs [16–20]. Furthermore, a recent study has revealed cooperativity between the N- and C-terminal domains in binding IGF-1 [47]. This brings out the importance of analyzing the structural and dynamic relationships among the IGFBP domains in the full-length forms and underscores the notion that the IGFBPs, as IGF antagonists, have an excellent potential as cancer therapeutics in IGF-sensitive-IGF-1R expressing tumors. Notably, the IGFBPs do not bind insulin and thus do not interfere with insulin-insulin receptor interactions. In this regard, expression in *E. coli* provides a platform to

rapidly generate engineered forms of these proteins, test their activities and carry out structural analysis using X-ray/NMR spectroscopy.

Conclusions

In summary, we have developed a method for high-yield expression of functional, monomeric human IGFBP-2 in *E. coli*. The high-solubility of the protein containing a single conformation provides new opportunities to carry out structure-based functional studies in this protein family. Expression in *E. coli* allows for generating different engineered forms of the protein for testing their efficacy in binding IGFs and other proteins. This will pave the way for developing novel IGFBP-based cancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. NMR spectroscopy
2D [^{15}N - ^1H] HSQC spectrum of purified rhIGFBP-2 recorded at a ^1H resonance frequency of 800 MHz at 285 K.

Figure 2. Secondary structure by NMR

GFT (3,2)D HA(CA)CO(N)HN spectra of purified rhIGFBP-2 spectra for estimating the secondary structure content in the protein.

Figure 3. Ligand and immunoblot analyses of 6His•IGFBP-2

Purified 6His•IGFBP-2 was resolved on a 12.5% acrylamide SDS gel under non-reducing conditions, transferred to nitrocellulose, and ligand blotted with biotinylated IGF-1. Blots were stripped and re-probed with a polyclonal anti-IGFBP-2 antibody.

Figure 4. Effect of IGFBP-2 on IGF-1 stimulated [³H]thymidine incorporation
 SCC-9 (A) and DU145 (B–C) cells were pretreated with inhibitor for 2h (C), treated as indicated for 21 h and processed for [³H]thymidine incorporation. Error bars represent standard deviation between three or more independent experiments. Significant differences were observed (**p<0.01, ***p<0.001, compared to unstimulated, unless indicated by brackets). Figures A–C are representative of three or more independent experiments.