

RESEARCH ARTICLE

IGF-dependent dynamic modulation of a protease cleavage site in the intrinsically disordered linker domain of human IGFBP2

Garima Jaipuria¹ | Divya Shet^{1,2} | Shahid Malik¹ | Monalisa Swain^{1,3} |
 Hanudatta S. Atreya[†] | Charles A. Galea⁴ | Mark G. Slomiany⁵ |
 Steven A. Rosenzweig⁵ | Briony E. Forbes⁶ | Raymond S. Norton^{4,7} |
 Somnath Mondal^{1,8} 

¹NMR Research Centre, Indian Institute of Science, Bangalore, Karnataka, India

²Nanobiophysics Lab, Raman Research Institute, Sadashivnagar, Bangalore, Karnataka, India

³Frederick National Laboratory for Cancer Research, Frederick, Maryland, USA

⁴Medicinal Chemistry, Monash Institute of Pharmaceutical Sciences, Parkville, Australia

⁵Department of Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina, USA

⁶Flinders Health and Medical Research Institute, Flinders University, South Australia, Australia

⁷ARC Centre for Fragment-Based Design, Monash University, Parkville, Australia

⁸Univ. Bordeaux, Institut Européen de Chimie et Biologie and INSERM U1212, ARNA Laboratory, Bordeaux, France

Correspondence

Garima Jaipuria and Somnath Mondal, NMR Research Centre, Indian Institute of Science, Bangalore 560012, Karnataka, India.
 Email: garimajaipuria@gmail.com and somomsom@gmail.com

Funding information

Council of Scientific and Industrial Research, India; DAE-BRNS; Department of Science and Technology, Ministry of Science and Technology, India; Indian Institute of Science; Ministry of Human Resource Development, India; National Health and Medical Research Council of Australia; National Institutes of Health, Grant/Award Number: CA138313

Abstract

Functional regulation via conformational dynamics is well known in structured proteins but less well characterized in intrinsically disordered proteins and their complexes. Using NMR spectroscopy, we have identified a dynamic regulatory mechanism in the human insulin-like growth factor (IGF) system involving the central, intrinsically disordered linker domain of human IGF-binding protein-2 (hIGFBP2). The bioavailability of IGFs is regulated by the proteolysis of IGF-binding proteins. In the case of hIGFBP2, the linker domain (L-hIGFBP2) retains its intrinsic disorder upon binding IGF-1, but its dynamics are significantly altered, both in the IGF binding region and distantly located protease cleavage sites. The increase in flexibility of the linker domain upon IGF-1 binding may explain the IGF-dependent modulation of proteolysis of IGFBP2 in this domain. As IGF homeostasis is important for cell growth and function, and its dysregulation is a key contributor to several cancers, our findings open up new avenues for the design of IGFBP analogs inhibiting IGF-dependent tumors.

KEYWORDS

conformational dynamics of intrinsically disordered protein, disordered protein, function-dynamics relationship of disordered protein, human IGF-binding protein-2, human insulin-like growth factor system, intrinsically disordered protein, NMR, protein-NMR, protein-protein interaction, proteolysis

Garima Jaipuria, Divya Shet, Shahid Malik, Monalisa Swain, and Somnath Mondal contributed equally to this study.

[†] Deceased.

1 | INTRODUCTION

The insulin-like growth factor (IGF) system plays an essential role in cell growth, differentiation, and function, and, in recent years, has

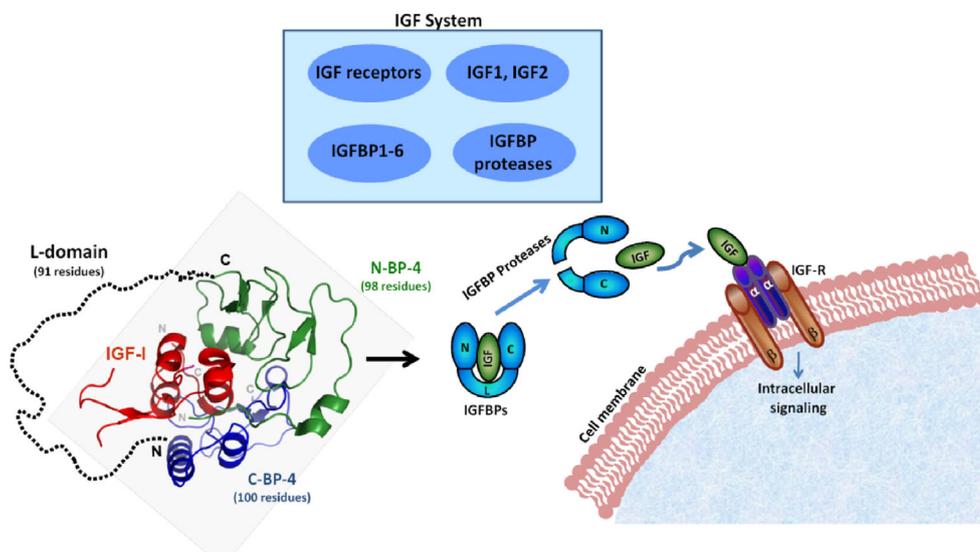


FIGURE 1 Schematic of the components in the insulin-like growth factor system (top box) and their functional regulation (lower right diagram). IGFBPs tightly bind and sequester IGFs in the serum and inhibit their interaction with the IGF receptor (IGF-1R). IGFBP protease cleavage within the disordered linker domain of the IGFBP releases IGF allowing it to bind to the IGF-R, stimulating intracellular IGF signaling. The crystal structure of the N- and C-domains of IGFBP4 (N-BP-4 [green] and C-BP-4 [blue], respectively) bound to IGF-1 (PDB ID: 2DSR, red) is shown, with the intrinsically disordered linker domain (L-domain) drawn as a dotted line for illustration. In the case of IGFBP2, the N-, C-, and the L-domains are each approximately 100 residues in length.

become an important target for cancer therapeutics, with more than 30 anti-cancer drugs focusing on this system.^{1–5} This system consists of two peptide hormones: IGF-1 and -2; the receptors: IGF-1R and IGF-2R; six soluble, high-affinity IGF-binding proteins (IGFBPs; numbered 1–6) and IGFBP proteases (Figure 1). IGF-1 and -2 are small proteins (~7.5 kDa) that circulate in the bloodstream and function in signaling by binding to IGF receptors.^{5–7} The biological activities of the IGFs are modulated by IGFBPs, which bind IGFs with higher affinities than the IGF-1R, thereby restricting their delivery to the IGF-1R.^{8,9} Proteolysis of the IGFBPs dissociates IGFs from the complex, enabling them to bind to and activate their receptors (Figure 1).

The biological actions of the IGF: IGFBP: IGF-R axis have been studied extensively.^{1–3,8,10} However, a comprehensive understanding of the structural basis for IGF–IGFBP interactions is still lacking. The IGFBPs consist of structured globular N- and C-domains joined by a central (linker) domain.^{2,3} The three-dimensional structures of full-length IGFBPs have not yet been determined, although structures are available for the N- and C-terminal domains of the different IGFBPs either free in solution or bound to IGFs.^{2,3,10–20} These studies have shown that all IGFBPs interact similarly with IGFs and that binding sites for the IGFs are located primarily in the N- and C-terminal domains of IGFBPs.^{2,3,21} The intrinsically disordered linker domain, which contains several posttranslational modification motifs²² and the IGFBP-protease cleavage sites,^{3,23,24} has been proposed to merely tether the N- and C-terminal domains.^{3,12} However, deletion of the linker domain from full-length proteins results in the loss of IGF-binding affinity,³ indicating that this domain may contribute to IGF binding in the full-length proteins. Further, in some of the IGFBPs, proteolysis in the linker domain requires the binding of IGFs.^{7,23,25–30}

Despite its apparent importance in IGFBP function, the structure of the linker domain and its interaction with IGFs have not been characterized. Here, we have investigated the interaction of the linker domain of human IGFBP2 (L-hIGFBP2; residues A97–C191) with IGFs using NMR spectroscopy and surface plasmon resonance (SPR). Our study confirms that L-hIGFBP2 is intrinsically disordered and shows that it retains moderate binding affinity to IGFs ($K_d \sim 4 \mu\text{M}$). IGF binding has a specific effect on the dynamics of L-hIGFBP2 residues that interact with IGF-1, but also on the protease cleavage sites. This altered dynamics may explain the IGF-dependent proteolysis of IGFBP2 in this domain. Our understanding of the role of dynamics in the functional regulation of the IGF-system offers new insights that will help guide the design and development of IGFBP-based analogs for inhibiting IGF–IGF-1R signaling and growth of IGF-dependent tumors.

2 | RESULTS

2.1 | NMR studies of L-hIGFBP2

L-hIGFBP2 was cloned, overexpressed, and purified as described in Section 4. The purified protein had a molecular mass of 12.2 kDa as verified by MALDI-TOF mass spectrometry (expected: 12.211 kDa) and migrated at ~20 kDa on SDS-PAGE (Figure S1). Such aberrant mobility on SDS-PAGE is typical of intrinsically disordered proteins (IDPs)³¹ and has been described for the linker domain of IGFBP5.³² The 2D [¹⁵N-¹H] heteronuclear single quantum coherence (HSQC) spectrum of recombinant L-hIGFBP2 (Figure 2A) shows a unique

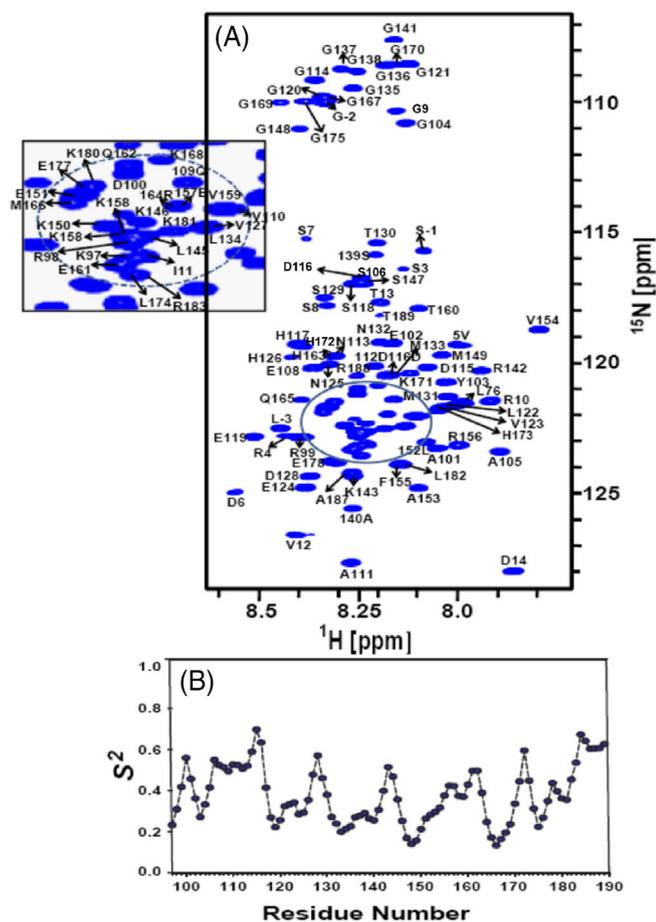


FIGURE 2 (A) Two dimensional [^{15}N - ^1H] HSQC NMR spectrum of L-hIGFBP2 acquired at ^1H resonance frequency of 800 MHz at 20°C in 50 mM Na-phosphate buffer (pH 6.0) and 100 mM NaCl. The peaks are numbered according to full-length protein (i.e., starting from 97 to 191), the peaks numbered -5 to -1 are a part of the N terminal tag while peak numbers 1–14 belong to the additional C-terminal residues. (B) The predicted order parameter of L-hIGFBP2 as calculated with TALOS+⁷³ using the observed ^1H , ^{15}N , $^{13}\text{C}^\alpha$, $^1\text{H}^\alpha$, C' chemical shifts³⁸

^{15}N - ^1H correlation for each residue, indicating that conformational averaging is fast on the NMR time scale. Comparison of the 2D [^{15}N - ^1H] HSQC spectra (Figure S2) for L-hIGFBP2 (in red) and full-length hIGFBP2 (in blue)³³ shows mainly overlap of all residues in the linker region regardless of whether they are part of the full-length protein or in isolation, implying that the linker domain is also disordered in the full-length protein.

The limited ^1H chemical shift dispersion of the 2D [^{15}N - ^1H] HSQC spectrum implies that L-hIGFBP2 is unstructured.^{34,35} In support of this, $^3J_{\text{HNH}\alpha}$ coupling constants (measured using G-matrix Fourier transform [GFT] [3,2] D HNHA³⁶), a lack of deviations of backbone chemical shifts from random coil values, and secondary structure propensity prediction³⁷ also indicate an overall lack of ordered secondary structure (Figure S3). The NH bond order parameter estimation based on the chemical shifts of the backbone ($^1\text{H}^\alpha$, $^{13}\text{C}^\alpha$, $^{13}\text{C}'$, ^{15}N) and sidechain ($^{13}\text{C}^\beta$) nuclei³⁸ confirms a high degree of

disorder in L-hIGFBP2 (Figure 2B), in agreement with the general consensus that the linker domains of all IGFBPs are disordered (Figure S4).^{3,39} The backbone ^1H amide exchange rates could not be obtained from 2D ^{15}N - ^1H HSQC spectra, as the decrease in intensity of cross-peaks in the 2D spectrum upon dissolving the protein in 100% $^2\text{H}_2\text{O}$ was too fast to be measurable. The exchange rates were therefore characterized from 2D ^{13}CO - ^{15}N EXSY at pH 6 and 20°C , which indicated an upper limit of $k_{\text{ex}} \sim 1 \text{ s}^{-1}$ for k_{ex} for all residues for a 1:1 mixture of H_2O and $^2\text{H}_2\text{O}$ (Figure S5).⁴⁰

2.2 | Binding of ^{15}N L-hIGFBP2 to IGF-1 and IGF-2

Dissociation constants (K_d) of $4.1 \pm 2.2 \mu\text{M}$ for IGF-1 and $3.7 \pm 1.5 \mu\text{M}$ for IGF-2 were estimated from surface plasmon resonance (SPR) based on the association and dissociation rates (Figures 3A,B). The binding of IGF-1 and IGF-2 to L-hIGFBP2 was also monitored using NMR. To identify L-hIGFBP2 residues involved in binding IGFBPs, a sample of ^{15}N -labeled L-hIGFBP2 was titrated with unlabeled IGF-1. Figure 3C shows an overlay of the 2D [^{15}N - ^1H] HSQC spectrum of the complex with that of free (unbound) L-hIGFBP2, with residues undergoing significant shifts highlighted in the inset. For 1 mM IGFBP2 protein, the peaks stop moving after the addition of 1 mM IGF-1, showing a clear 1:1 ratio for binding. Therefore, the binding is both very dynamic (fast on and off rates) and of moderate affinity (K_d of $4.1 \pm 2.2 \mu\text{M}$ as measured from SPR; Figure 3A). However, a substantial change in the overall dynamics of the protein was observed, as discussed below. Assignment of the shifted resonances was based on inspection of their position for the unbound L-hIGFBP2 spectrum. HSQC spectra of free IGFBP2 protein before the addition of IGF-1 and after the addition of increasing molar ratios of IGF-1 to IGFBP2 are shown in Figure S9. The magnitude of $^{15}\text{N}/^1\text{H}$ chemical shift perturbations for each residue of L-hIGFBP2 (Figure 3D) shows that residues K150-E161 and Q165 undergo the largest chemical shift changes upon binding IGF-1, with an average shift of $0.032 \pm 0.017 \text{ ppm}$. Similar shifts were observed when IGF-1 bound to full-length IGFBP2 (Figure S6). A region containing these residues (K150-E161) is predicted to be a molecular recognition feature (MoRF) (a short binding region located within a longer intrinsically disordered region)⁴¹ and is located adjacent to the protease cleavage sites of hIGFBP2 (Figure 3E). To confirm the involvement of these residues in binding IGF-1, we prepared a mutant form of L-IGFBP2 with deletion of K150-E161 (L-hIGFBP2[desK150-E161]), which showed weaker binding to IGF-1 (Figure S7).

We also identified residues in IGF-1 involved in binding L-hIGFBP2. For this purpose, ^{15}N -labeled IGF-1 in 50 mM Na-phosphate buffer (pH 6.0) was titrated at 20°C with a solution of unlabeled L-hIGFBP2. An overlay of the 2D [^{15}N - ^1H] HSQC spectrum of the L-hIGFBP2: IGF-1 complex with that of free IGF-1 is shown in Figure S8. Chemical shifts for most residues of IGF-1 remained unchanged, but cross-peaks for residues E3, T4, A13, V17, C18, G19, D20, R21, G22, M59, Y60, C61, and A62, which were very weak or absent in free IGF-1, exhibited a significant increase in intensity in the

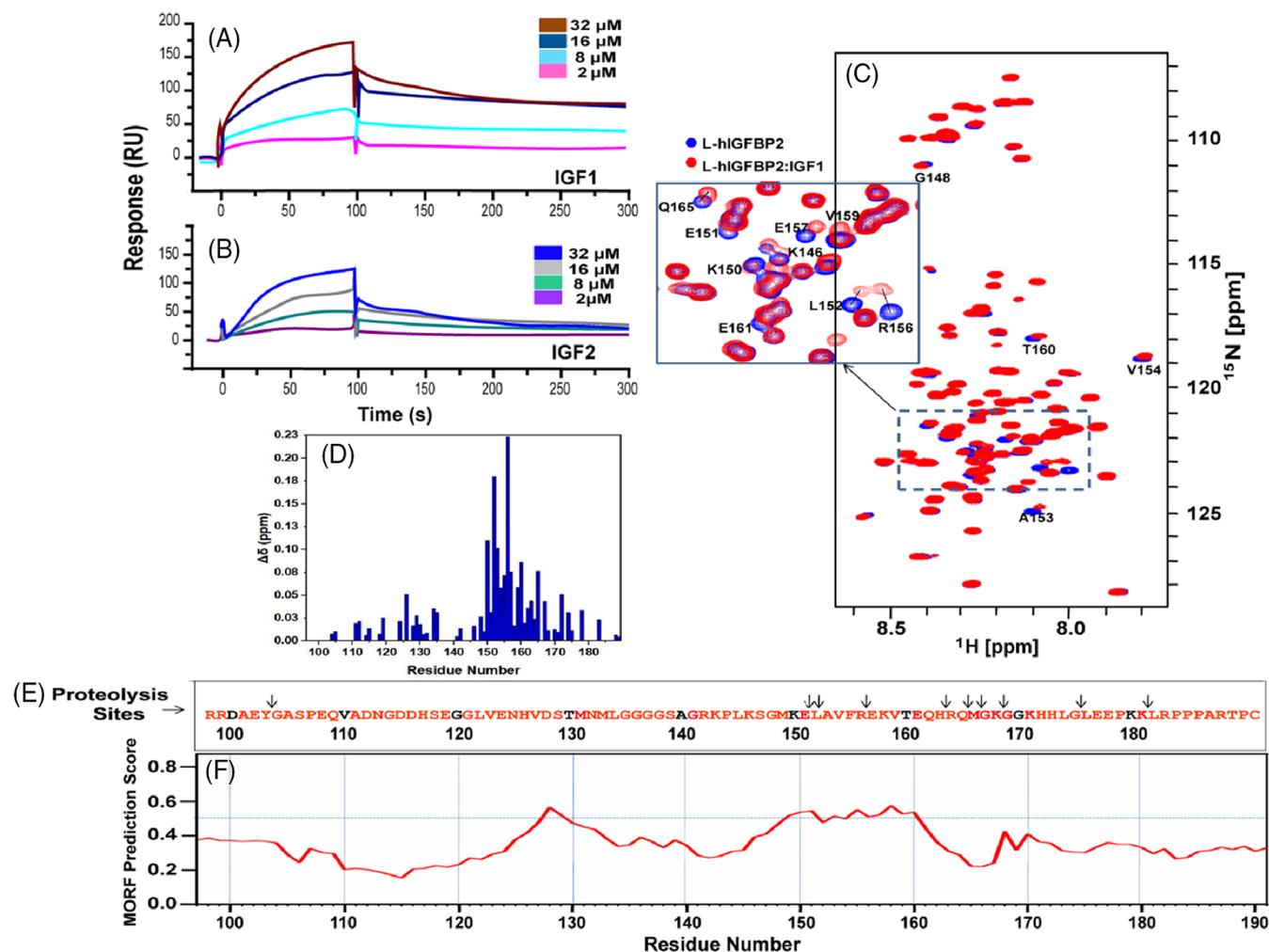


FIGURE 3 Characterization of the interaction of the IGFs with L-hIGFBP2. Sensorgrams of (A) IGF-1, (B) IGF-2 immobilized on the surface of a CM5 chip with L-hIGFBP2 as analyte. (C) Overlay of the 2D ^{15}N - ^1H HSQC spectra of L-hIGFBP2 (blue) and IGF-1: L-hIGFBP2 (red). Inset shows an expanded view of the region where residues show maximum chemical shift perturbations (residues 150–170). (D) Combined (^{15}N and ^1H) chemical shift difference plot for L-hIGFBP2 residues upon addition of IGF-1, calculated using $\Delta\delta(\text{ppm}) = \sqrt{(\delta(H)_{\text{bound}} - \delta(N)_{\text{free}})^2 + 0.17(\delta(N)_{\text{bound}} - \delta(N)_{\text{free}})^2}$. The dotted line is shown at 1 SD of the chemical shift differences. (E) Reported proteolysis sites on L-hIGFBP2.⁸ (F) Residues of the linker domain were analyzed for their predicted propensity to lie within MoRF motifs using the web-based program MoRFPred. A line between the peak centers of the red and blue signals for L152 and R156 residues shows the largest chemical shift deviations (in the insert, Figure 3C).

bound form (Figure S8). This implies that the dynamics of IGF-1 are also significantly affected upon binding L-hIGFBP2, even though its average conformation is unchanged (Figure S8).

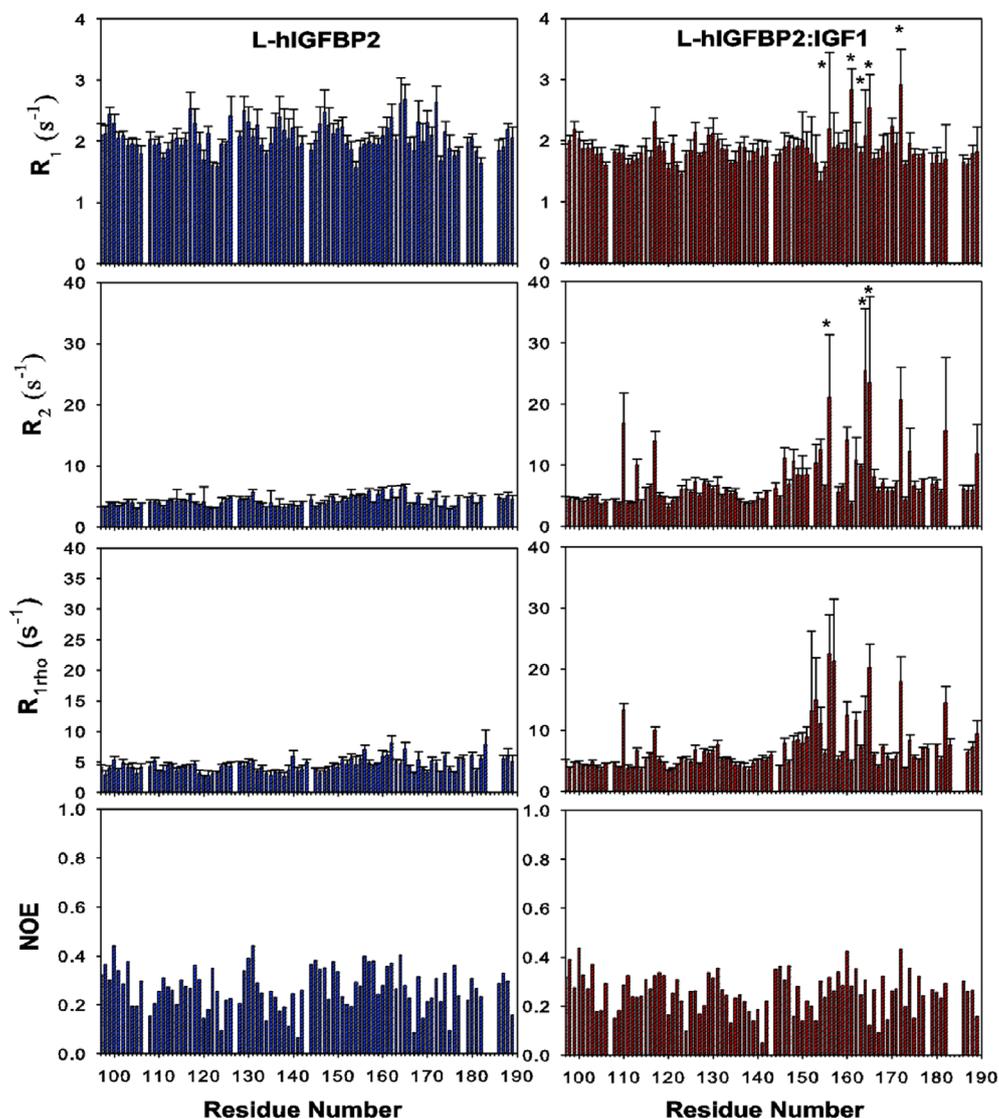
2.3 | Backbone dynamics of L-hIGFBP2 from ^{15}N relaxation

Insight into the functional regulation of IGF-1 by L-hIGFBP2 was achieved by studying the dynamics of L-hIGFBP2 in the free and IGF-1-bound forms using ^{15}N relaxation. Reduced spectral density mapping⁴² was used to examine dynamics in the microsecond–millisecond (μs – ms) and picosecond–nanosecond (ps–ns) regimes. The ^{15}N relaxation rates (R_1 , R_2 , $R_{1\rho}$) and ^{15}N - ^1H heteronuclear nuclear Overhauser

effects (HetNOE) were measured at a ^1H resonance frequency of 800 MHz (Figure 4). Based on ^{15}N R_1 and $R_{1\rho}$ relaxation values, an average overall rotational correlation time of ~ 3 ns for the disordered linker domain was obtained for unbound L-hIGFBP2. The average overall correlation time for the linker domain in full-length hIGFBP2 (32 kDa) determined by a similar method was ~ 4 ns at 293 K, implying that the disordered linker domain retains a high degree of flexibility in the full-length form, largely unaffected by the presence of the N- and C-domains.

The ^{15}N R_1 , R_2 , and ^{15}N - ^1H het-NOE values and a plot of the calculated spectral density functions— $J(0)$, $J(\omega_N)$, and $J(0.87^*\omega_H)$ for L-hIGFBP2 in free and IGF-1-bound forms are shown in Figures 4 and 5, respectively. Several important observations can be made. First, L-hIGFBP2 exhibits a high degree of flexibility in both the free and IGF-

FIGURE 4 R_2 , $R_{1\rho}$, and het-NOE plots for L-hIGFBP2 (blue) and IGF-1: L-hIGFBP2 (red) measured at ^1H resonance frequency of 800 MHz. Asterisks mark signals with overlap.



1-bound forms, as reflected by the $J(0.87\omega_H)$ and $J(0)$ values, with the latter being significantly less than $2/5\tau_c$ for most residues (where τ_c is the rotational correlation time of a rigid isotropically tumbling protein of equivalent size).⁴³ Second, the ^{15}N relaxation rates for residues K150-E161, Q165, and M166 of the linker domain are significantly perturbed by IGF-1 in complex with L-hIGFBP2, as the large complex causes a great increase in correlation time (t_c) which in turn causes the fast T_2 relaxation (Figure 4). In the full-length hIGFBP2 complex, the intensities of cross-peaks corresponding to these residues in the 2D [^{15}N , ^1H] HSQC spectrum are reduced owing to the formation of a large complex which causes an increase in correlation time (t_c), resulting in the fast T_2 relaxation and increased NMR line-width (Figure S6). Third, large $J(0)$ values indicative of dynamics in the $\mu\text{-ms}$ regime are significantly enhanced for L-domain residues involved in binding IGF-1 (K150-E161), as well as those distant from the binding site (V110, N113, H117, H172, Q165, M166, L174, and L182). This increase in $J(0)$ values can be attributed to the larger size of the complex and slow conformational exchange in the $\mu\text{-ms}$ regime and is quantified by the exchange rate, R_{ex} , which was

estimated by measuring the ^{15}N transverse relaxation rate in the rotating frame ($R_{1\rho}$) at 800 MHz for both the unbound and bound forms of L-hIGFBP2. Values of R_{ex} calculated using the difference in $J(0)$ values obtained with R_2 and $R_{1\rho}$ ⁴³ are plotted in Figure 5. Notably, an overall increase in R_{ex} is observed in the IGF-bound complex for residues of L-hIGFBP2 both close to and distant from the binding site. This implies that in the IGF-1-bound complex, L-hIGFBP2 populates an ensemble of alternate conformations that interconvert on the $\mu\text{-ms}$ timescale. The region bound to IGF-1 exhibits a “reduced” level of conformational dynamics, such that some of the ns-ps timescales have now entered the $\mu\text{-ms}$ timescale (and therefore now entered the exchange regime in these experiments). Interestingly, it is known that proteolytic cleavage of hIGFBP2 by the pregnancy-associated plasma protein-A (PAPP-A) is enhanced in the IGF-1-bound state.²⁹ The disordered linker domain of hIGFBP2 has a helical propensity, as indicated by the ^{13}C chemical shift predictions shown in Figure S3f. The region K150-E161 is also predicted by AlphaFold⁴⁴ to have a helical propensity, as shown in Figure 7; this region binds to IGF-1 and shows a change in exchange in $\mu\text{-ms}$ timescale due to the formation of the

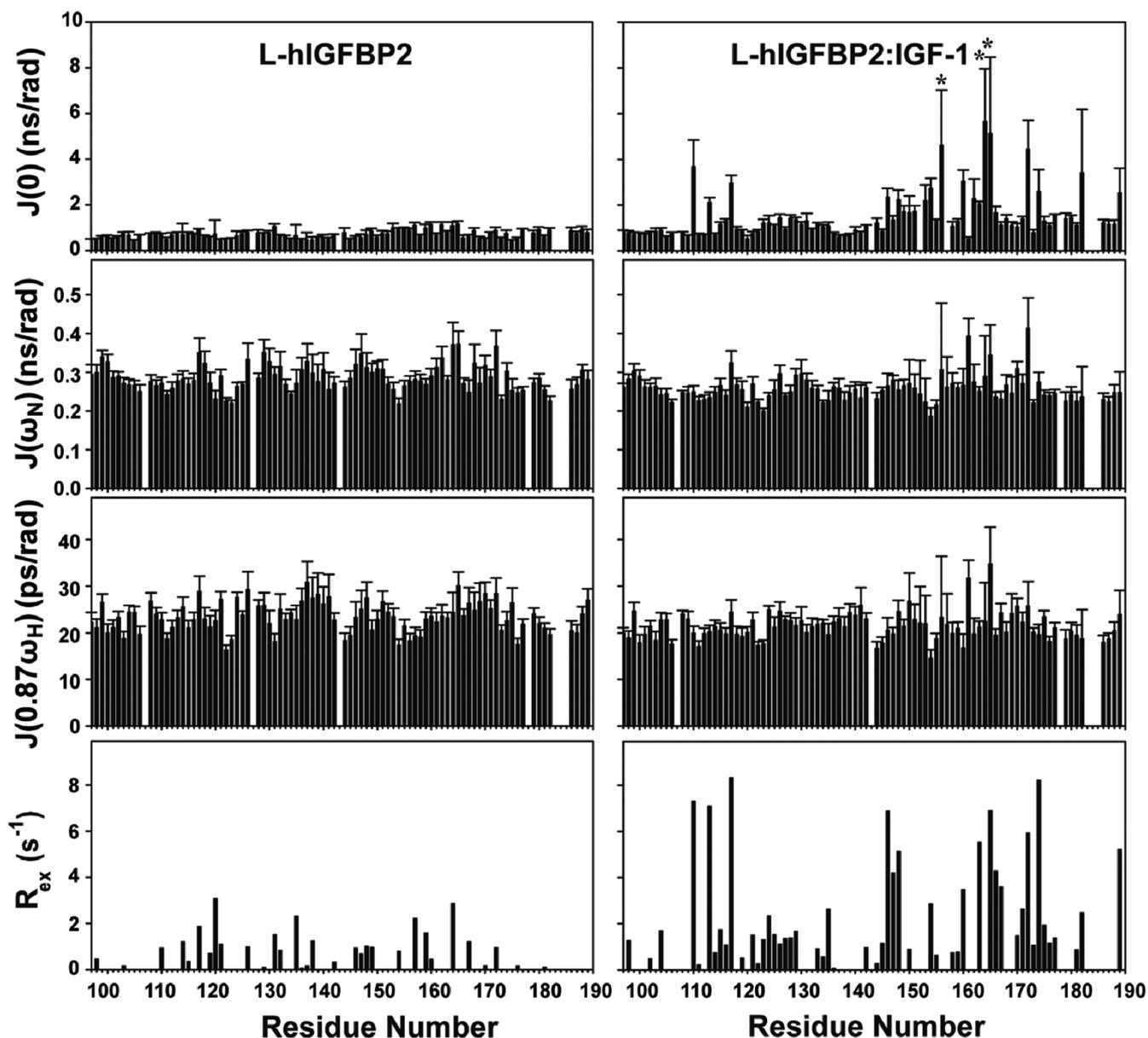


FIGURE 5 Spectral density function values at $J(0)$, $J(\omega_N)$, and $J(0.87\omega_H)$ frequencies for L-hIGFBP2 (left) and the L-hIGFBP2:IGF-1 (right) complex at a ^1H resonance frequency of 800 MHz. A-asterisks mark signals with overlap.

larger complex upon binding. The IGF-1 bound complex has a higher correlation time (t_c) which in turn causes the fast T_2 relaxation, resulting in a higher relaxation rate, as the relaxation times and relaxation rates are simple inverses of each other. The majority of the L-hIGFBP2 residues recognized by proteases (Figure 3) show increases in R_{ex} upon IGF binding (Figure 5). This explains the IGF-dependent dynamic modulation of a protease cleavage site region in the intrinsically disordered linker domain of hIGFBP2.

To estimate the conformational entropy associated with binding IGF-1, approximate backbone NH order parameters (S^2) using $J(0)$ and $J(\omega_N)$ ^{42,45} were calculated for L-hIGFBP2. $J(0)$ and $J(\omega_N)$ calculated from $R_{1\rho}$ were used to avoid the effect of conformational exchange when estimating S^2 values. Order parameters were calculated for both free and bound forms of L-hIGFBP2 and the change in conformational

entropy (ΔS) was estimated using the calculated S^2 values (for residues with $S^2 < 1$) (Equation 2; Figure 6).⁴³ The overall ΔS value (summed over all residues) of ~ 100 J/mole (0.024 kcal/mol) implies an increase in entropy for the system upon IGF-1 binding. The contribution of the conformational entropy to the free energy of binding is given by $-\Delta S$, which yields a contribution of -7 kcal/mol.

3 | DISCUSSION

In recent years, the concept of “fuzzy complexes” in IDPs has been described,^{46,47} which proposes that functionally important regions of IDPs in protein complexes can retain their structural disorder. In fuzzy complexes, dynamic regulation ensues when the ensemble average

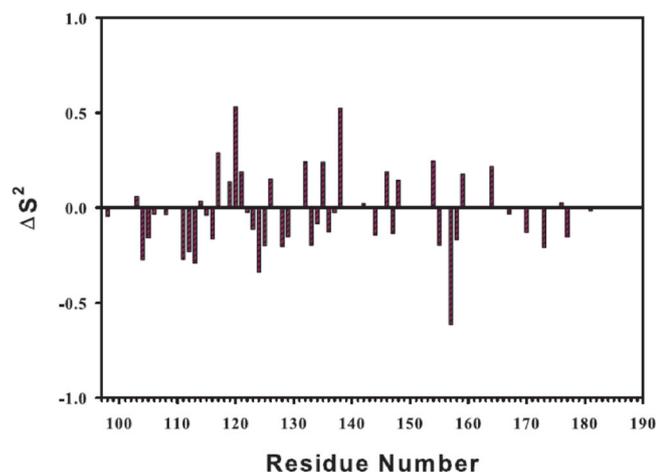


FIGURE 6 The difference in S^2 values (calculated using Equation 1) between the free and bound forms of L-hIGFBP2 as estimated from $J(0)$ and $J(\omega_N)$ calculated from $R_{1\rho}$

population of conformers of the IDP and/or their flexibility is affected upon ligand binding. The current study involving the intrinsically disordered linker domain of human IGFBP2 exemplifies such a case.

In the IGF system, proteolysis plays a crucial role in regulating the bioavailability of IGFs.²³ IGFBP levels are regulated by proteolysis following their secretion from the cell and the resulting proteolytic fragments have reduced affinity for IGF ligands.²³ The net effect is an increase in IGFs availability for interaction with the IGF-1R. Thus, efforts to reduce protease action could have a beneficial effect on reducing IGF-1R activity in cancer. Proteolysis of IGFBPs has been observed to be both IGF-dependent and IGF-independent; IGF-dependent proteolysis has been observed for IGFBP2²⁹ and in IGFBP4,^{23,25,26} whereas IGFBP3 and IGFBP5⁴⁸ undergo IGF-independent proteolysis.^{23,49} K150-E161 residues in IGFBP2 are more ordered (less disorder disposition, Figure S4), and our experimental results show an increase in dynamics for those residues in IGFBP2 after binding with IGF-1. As these K150-E161 residues in the linker region become more flexible after binding, the conformational ensemble populated by the linker domain of IGFBP2 shifts so that it is more readily recognized by the protease and/or is more amenable to proteolysis. This highlights an interesting link between binding of IGF-1 and proteolysis for hIGFBP2, which is not the case for IGFBP3 and IGFBP5. An example of an IGF-dependent protease action on hIGFBP2 is PAPP-A, which cleaves hIGFBP2 in an IGF-dependent manner at a single site between Gln165 and Met166, to yield two proteolytic fragments having weak IGF binding affinity.²⁹ Another recent finding shows that ParD antitoxin hotspot binding to its cognate ParE toxin alters a disorder-to-order transition, lessening its interaction affinity and increasing its protease degradation kinetics.⁵⁰

The different susceptibilities of the different IGFBPs to proteolysis have been attributed to ligand-induced conformational changes. Our studies demonstrate that K150-E161, Q165, and M166 residues of L-hIGFBP2, which are involved in binding IGF-1, exhibit enhanced conformational exchange upon IGF binding (Figure 4). Moreover, this

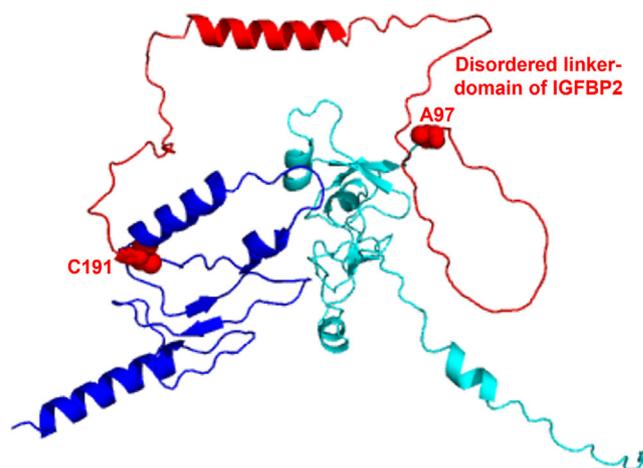


FIGURE 7 Predicted structure of full-length IGFBP2 from AlphaFold protein structure database where the disordered linker domain is represented in red, starting from A97 residue to C191 as marked, with N- and C-terminal domains of FL-IGFBP2 represented in cyan and blue, respectively. The helix in the linker domain was predicted by Alpha Fold only (<https://alphafold.ebi.ac.uk/>)⁴⁴ and consistent with the fact that K150-E161 is more ordered in IGFBP2 as we can see from Figure S4.

enhanced conformational exchange is not confined to the binding site but extends to some distant residues (V110, N113, H117, H172, Q165, M166, L174, and L182) (Figure 4) and is accompanied by an increase in conformational entropy. This implies distant dynamic regulation,^{51–54} where changes in protein dynamics induced by ligand binding extend to residues distant from the ligand-binding site, even in the absence of a well-defined conformational change. The increase in μ s-ms motions of residues in the vicinity of protease cleavage sites of L-hIGFBP2 provides an interesting link to proteolytic cleavage upon binding IGF-1 as it is well known that changes in conformational dynamics upon ligand binding are important for regulation of proteolysis.^{55–57} In the presence of IGF-1, the conformational ensemble populated by the linker domain of IGFBP2 shifts so that it is more readily recognized by the protease and/or is more amenable to proteolysis. Considering the current findings, we, therefore, propose that dynamic regulation in the linker domain of IGFBP2 plays an important role in its susceptibility to PAPP-A proteolytic cleavage.

These results have significant implications for the development of IGFBPs (mutants and/or chimeras) as antagonists of IGF-1R activation that can block IGF-1R-mediated tumor progression.^{2,3} Most current cancer therapeutics target the IGF-signaling pathway and focus on blocking the IGF-1R directly (kinase inhibitors) and/or its downstream effectors.⁵⁸ However, a drawback of this approach is the resulting high serum IGF-1 levels in response to targeted inhibition of IGF-1R and adverse side effects and/or toxicities arising from potential interference with the insulin pathway.⁵⁹ It has been suggested recently that therapeutics targeting the interaction of IGFs with IGFBPs may overcome these serious drawbacks.^{3,5,60} For example, IGFBPs engineered to be protease resistant by mutating or deleting the protease cleavage sites in the linker domain should act as IGF antagonists.

Recently, in separate studies, engineered protease-resistant hIGFBP2 and hIGFBP4 were found to inhibit tumor growth in breast cancer.^{61–63} Interestingly, the engineered protease-resistant form of hIGFBP2 lacking residues 114–170 (des[114–170]) retains high-affinity binding to IGF-1 and IGF-2, with only a 1.6–2-fold reduction in affinity compared to full-length hIGFBP2. The present study may now explain the loss in binding affinity of des(114–170)⁶² toward both IGFs compared to the full-length protein by the fact that residues K150–E161 of the linker domain, which facilitates IGF binding (Figure S7), were deleted from the construct. This suggests that, in addition to alteration of the protease cleavage sites, more potent IGFBP-based antagonists could be designed by considering the binding affinity of the linker domain for the IGFs and taking into account the resulting change in dynamics upon binding. These studies will facilitate the development of future IGFBP-based antagonists.

In summary, our studies of the intrinsically disordered linker domain of human IGFBP2 provide new insights into the regulatory mechanisms in the IGF system. Contrary to currently held models, the intrinsically disordered linker domain of IGFBP2 is involved in binding IGF-1. L-hIGFBP2 does not undergo a well-defined conformational change upon binding its ligand, but binding is accompanied by a significant change in dynamics on both the millisecond–microsecond and picosecond–nanosecond time scales. This is an example of functional regulation in an intrinsically disordered protein complex by dynamic regulation, which is being recognized increasingly in recent years.^{46,47}

4 | MATERIALS AND METHODS

4.1 | Cloning, expression, and purification of L-hIGFBP2

The primers were designed for L-hIGFBP2 (residues 97–191 of the full-length protein) and its L-hIGFBP mutants (L-hIGFBP2 [desK150–E161]) lacking the C-terminal tag and residues 150–161. Oligonucleotide strands 5' CAT GGT ACC GAT GAT GAT AAA AAG CGC CGG GAC GCC GAG TAT G 3' with enterokinase cleavage site and 5' GAC GAA TTC TTA GGG AGT CCT GGC AGG GGG TGG TCG CA 3' were used as forward and reverse primers for L-hIGFBP2 [desK150–E161]. The insert was cloned between Kpn1 and Xho1 restriction sites of the pET 32a vector having a thioredoxin tag fused to the protein.

The oligonucleotide strands 5' ATTG GGA TCC GAG AAG CGC CGG 3' and 3' ATTG GAA TTCTTA CAG GGA GTC CTG 5' with BamH1 and EcoR1 restriction sites on forward and reverse primers, respectively, were synthesized by MWG-Eurofins and used to amplify L-hIGFBP2 at 65°C in a thermo-cycler. Following amplification, PCR products were purified using a Sigma Gel elution kit after analyzing on 1% agarose gel in 1× TAE buffer. The vector used was an IPTG-inducible pGEX6P-1 with a GST tag. The vector and L-hIGFBP2 were digested with BamH1 and EcoR1, producing staggered ends, and treated with ligase at 16°C for 16 h. The ligated product was used to transform *E. coli* Top10 cells, and the desired clone was obtained using

a Sigma miniprep plasmid isolation kit. The clone was confirmed by sequencing. During the process of cloning the following C-terminal tag was introduced: KNSRVDSSGRIVTD. This tag did not affect the binding of IGF-1 as verified using the construct L-hIGFBP2 [desK150–E161], which did not contain these additional residues. At the N-terminal end, cleavage by HRV 3C protease to separate the GST tag from the protein resulted in the following residues added to the N-terminus: GPLGS.

The L-hIGFBP2 construct was transformed into *E. coli* BL21(DE3) competent cells. The transformed colony was inoculated in 50 ml of primary culture (LB broth with 100 µg/ml ampicillin) and incubated at 37°C for 16 h, 200 rpm. The cells were transferred to minimal media and grown to the mid-log phase (optical density at 600 nm [OD₆₀₀] ~ 0.6). At this OD, the expression of L-hIGFBP2 was induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 5 h at 37°C, 200 rpm.

Cells were harvested by centrifugation at 6000 rpm and resuspended in phosphate-buffered saline (PBS) (150 mM NaCl, 2.5 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and 1 mM PMSF. Lysis by sonication was carried out on the ice for 10 min (30 s pulse) and the lysate was centrifuged at 8000 rpm for 40 min. The supernatant was collected and allowed to bind to the preequilibrated GST bind resin for 2 h at 4°C on a rotator. The resin was washed three times with each of 10 bed volume of PBS buffer, high salt (25 mM HEPES, 0.05% NaN₃, 0.5 M NaCl, 0.1% Triton X-100, pH 7.5) and low salt (25 mM HEPES, 0.5% NaN₃, 0.1 M NaCl, 0.1% TritonX-100, pH 7.5) buffer. The cleavage buffer (PBS) was used to wash the beads before adding HRV 3C protease to perform on-column cleavage at 4°C for 16 h.

L-hIGFBP2[desK150–E161] was expressed in *E. coli* BL21 cells at 30°C and induced for 4 h with 0.5 mM IPTG. For the purification of thioredoxin fused L-hIGFBP2[desK150–E161], the cell pellet was resuspended in buffer A (20 mM Tris pH 8.0, 100 mM NaCl, 10 mM imidazole, and 10% glycerol) containing 0.3 mg/ml lysozyme and incubated for 20 min in the presence of EDTA-free protease inhibitor cocktail from Roche. The cell resuspension was sonicated, and the cell lysate was clarified by centrifuging at 14 000 rpm for 30 min at 4°C. The supernatant was loaded onto a preequilibrated Ni-NTA HisTrap column (GE Healthcare, 5 ml) with a flow rate of 0.25 ml/min. Unbound proteins were removed by extensive washing with buffer A followed by a wash with buffer A containing 0.5 M NaCl. The fusion protein was eluted with buffer A containing 250 mM imidazole and exchanged with enterokinase cleavage buffer (50 mM Tris-Cl, pH 8.0, 1 mM CaCl₂, and 50 mM NaCl). The thioredoxin tag was removed by incubating the fusion protein in enterokinase at 23°C for 16 h and passing the mixture back onto the Ni-NTA HisTrap column (GE Healthcare, 5 ml). Unbound protein was eluted in buffer A. The protein was concentrated using a Millipore centricon with a 3 kDa molecular weight cut-off.

4.2 | Expression and purification of full-length hIGFBP2

A ¹⁵N-labeled sample of full-length hIGFBP2 was prepared as described previously.³³ Briefly, the hIGFBP2 construct was

transformed into *E. coli* BL21(DE3) Star™ competent cells. The transformed colony was inoculated in 10 ml primary culture of LB medium containing 200 µg/ml ampicillin and incubated at 37°C for 16 h, 200 rpm. The cells were then diluted 100-fold into fresh LB (amp) and grown up to a cell density corresponding to OD₆₀₀ ~ 0.6. The cells were centrifuged and the cell pellet transferred to 1 L minimal media followed by growth at 37°C up to a cell density corresponding to OD₆₀₀ ~ 0.8 before inducing the protein expression with 0.5 mM IPTG at 25°C for 6 h. Cells were harvested by centrifugation, solubilized in Buffer-I (Phosphate-buffer [50 mM sodium phosphate, 0.3 M NaCl, pH 8.0] containing 8 M urea, 5 mM DTT, and 1 mM PMSF) and then sonicated on ice. The supernatant was incubated at 4°C for 2 h with 1 ml of preequilibrated His-Select™ Nickel affinity agarose (Sigma-Aldrich) followed by washing and elution of the protein with 200 mM imidazole in Buffer-I. The eluate was dialyzed in Buffer-I to remove urea, followed by removal of DTT. The dialyzed protein was exchanged with phosphate buffer (50 mM sodium phosphate, 50 mM NaCl, pH 6.0) and concentrated to 500 µl for NMR experiments.

4.3 | SPR studies of L-hIGFBP2 and its mutants

SPR studies to estimate the binding affinity of IGF-1 and IGF-2 to L-hIGFBP2 and its mutant (lacking the residues 150–161) were carried out on a Biacore 3000 instrument. IGF-1 (10 µg/µl) in 10 mM sodium acetate buffer, pH 4.0, was immobilized at a flow rate of 2 µl/min onto the activated CM-5 sensor chip using the amine coupling kit. A total of 520 response units (RU) of IGF-1 were coupled on separate flow cells. The immobilization of pure IGF-2 (5.5 µg/µl) was carried out on a separate chip at 25°C at a flow rate of 2 µl/min and 350 response units of IGF-2 were coupled on the flow cell. Binding experiments were carried out for different concentrations of L-hIGFBP2 and its mutants in HBS buffer (10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.5) at a flow rate of 10 µl/min. Regeneration of the biosensor surface before the next reading was done by passing 5 µl of 2 M MgCl₂ across the chip. The data were fit to the Langmuir 1:1 binding model⁶⁴ using the fitting procedures in BIA-evaluation software 3.0.1 to calculate dissociation constants.

4.4 | Sequence-specific resonance assignments

NMR spectra were acquired at 20°C on an 800 MHz NMR spectrometer equipped with a triple-resonance cryogenic probe. The following spectra (with measurement times) were acquired for sequence-specific resonance assignment of backbone and side-chain nuclei: 2D HSQC (0.6 h), 3D HNCO (4.2 h), 3D HNCACB (13 h), 3D CBCACONH (13 h), 3D HBHACONH (6.8 h), 3D (H)C(CO)NH TOCSY (10.4 h), 3D ¹³C-TOCSY-Methyl COSY (11.2 h), reduced dimensionality (RD) 3D HN(CA)NH (12.4 h), GFT (3,2)D HNHA (6 h). Data were processed with NMRPipe⁶⁵ and analyzed using XEASY.⁶⁶ Resonance assignments were obtained using a semi-automated approach in the program AUTOASSIGN.⁶⁷ Sequence-specific resonance assignments

were aided by amino acid selective unlabeled of Arg, Asn, Thr, Ser, Gly, and Ala and reduced dimensionality.^{68–71} The complete chemical shifts assignments have been deposited in the BioMagResBank (accession code: 19475).⁷²

4.5 | Backbone dynamics of L-hIGFBP2 from ¹⁵N relaxation

The experiments for dynamics studies of ¹⁵N-labeled hL-IGFBP2 in the presence and absence of IGF-1 were performed at 20°C on a Bruker 800 MHz NMR spectrometer equipped with a cryogenic probe. For R₁ measurements, eight different time points were collected with relaxation delay periods of 0.01, 0.05, 0.1, 0.2, 0.4, 0.8, 1, and 1.5 s. R₂ and R_{1ρ} measurements were carried out at eight different time points with relaxation delay periods of 0.01, 0.03, 0.05, 0.1, 0.13, 0.17, 0.2, and 0.23 s. For the R₁, R₂, R_{1ρ} experiments: two scans with an interscan relaxation delay of 2.5 s, 256 points, and a spectral window of 24 ppm (65 ms acquisition time) were used in the ¹⁵N dimension. The ¹H dimension was acquired using 2048 points (t_{max} = 106 ms) over a 12-ppm spectral width. For R_{1ρ}, a 4 ms spin-lock block with a field strength of 2 kHz was used. The same data sets with identical time points, relaxation delays, and experimental time were recorded for the IGF-1-bound form of L-hIGFBP2. All the relaxation experiments were processed with a shifted sine-bell function and zero-filled with twice the time domain points in both dimensions. [¹⁵N-¹H] Het-NOE spectra for the free and bound form of L-hIGFBP2 were recorded with 32 transients for 23 h each with 2048 and 256 points in the direct and indirect dimensions, respectively. An interscan delay of 5 s was used and acquisition times were 80 ms in the direct and 65 ms in the indirect dimension.

An approximate S² to estimate the conformational entropy of the NH bond vector was calculated using Equation (1) and the entropy was calculated using Equation (2).

$$S^2 = \frac{5}{2} \frac{[J(0) - J(\omega_N)] [1 + \omega_N^2 t_m^2]}{\omega_N^2 t_m^3} \quad (1)$$

$$\Delta S^{\text{conf}} = -k_B \ln \pi \left(3 - (1 + 8S)^{1/2} \right) \quad (2)$$

The entropy estimated in this manner is considered as an upper limit due to the inherent assumption that the NH bond fluctuations for each residue are independent of others.⁴⁵

ACKNOWLEDGMENTS

The NMR Research Centre supported by the Department of Science and Technology (DST), India, and the SPR facility at IISc are gratefully acknowledged. Hanudatta S. Atreya acknowledges research grant support from DAE-BRNS. Raymond S. Norton acknowledges fellowship support from the National Health and Medical Research Council of Australia. Steven A. Rosenzweig was supported by the NIH grant CA138313. Garima Jaipuria acknowledges support from IISc and

CSIR. The authors acknowledge the assistance provided by Ms. Kerrie McNeil (University of Adelaide, Australia) in the preparation of labeled IGF-1, and thank Dr. Cameron Mackereth (IECB, Bordeaux, France) for his helpful suggestions to improve the quality of the manuscript.

PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/prot.26350>.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

ORCID

Somnath Mondal  <https://orcid.org/0000-0002-5657-8077>

REFERENCES

1. LeRoith D, Roberts CTJ. The insulin-like growth factor system and cancer. *Cancer Lett.* 2003;195(2):127-137. doi:10.1016/s0304-3835(03)00159-9
2. Rosenzweig SA, Atreya HS. Defining the pathway to insulin-like growth factor system targeting in cancer. *Biochem Pharmacol.* 2010;80(8):1115-1124. doi:10.1016/j.bcp.2010.06.013
3. Forbes B, McCarthy P, Norton R. Insulin-like growth factor binding proteins: a structural perspective. *Front Endocrinol.* 2012;3:38.
4. Li T, Forbes ME, Fuller GN, Li J, Yang X, Zhang W. IGFBP2: integrative hub of developmental and oncogenic signaling network. *Oncogene.* 2020;39(11):2243-2257. doi:10.1038/s41388-020-1154-2
5. Wei L, Weng X, Huang X, Peng Y, Guo H, Xu Y. IGFBP2 in cancer: pathological role and clinical significance (review). *Oncol Rep.* 2021;45(2):427-438. doi:10.3892/or.2020.7892
6. LeRoith D, Holly JMP, Forbes BE. Insulin-like growth factors: ligands, binding proteins, and receptors. *Mol Metab.* 2021;52:101245. doi:10.1016/j.molmet.2021.101245
7. Allard JB, Duan C. IGF-binding proteins: why do they exist and why are there so many? *Front Endocrinol.* 2018;9:117.
8. Denley A, Cosgrove LJ, Booker GW, Wallace JC, Forbes BE. Molecular interactions of the IGF system. *Cytokine Growth Factor Rev.* 2005;16(4-5):421-439. doi:10.1016/j.cytogfr.2005.04.004
9. Mancarella C, Morrione A, Scotlandi K. Novel regulators of the IGF system in cancer. *Biomolecules.* 2021;11(2):273. doi:10.3390/biom11020273
10. Baxter RC. IGF binding proteins in cancer: mechanistic and clinical insights. *Nat Rev Cancer.* 2014;14(5):329-341. doi:10.1038/nrc3720
11. Chandrashekar IR, Yao S, Wang CC, et al. The N-terminal sub-domain of insulin-like growth factor (IGF) binding protein 6 structure and interaction with IGFs. *Biochemistry.* 2007;46(11):3065-3074. doi:10.1021/bi0619876
12. Galea CA, Mobli M, McNeil KA, et al. Insulin-like growth factor binding Protein-2: NMR analysis and structural characterization of the N-terminal domain. *Biochimie.* 2012;94(3):608-616. doi:10.1016/j.biochi.2011.09.012
13. Kuang Z, Yao S, Keizer DW, et al. Structure, dynamics and heparin binding of the C-terminal domain of insulin-like growth factor-binding Protein-2 (IGFBP-2). *J Mol Biol.* 2006;364(4):690-704. doi:10.1016/j.jmb.2006.09.006
14. Kuang Z, Yao S, McNeil KA, et al. Cooperativity of the N- and C-terminal domains of insulin-like growth factor (IGF) binding protein 2 in IGF binding. *Biochemistry.* 2007;46(48):13720-13732. doi:10.1021/bi701251d
15. Kalus W, Zwickstetter M, Renner C, et al. Structure of the IGF-binding domain of the insulin-like growth factor-binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. *EMBO J.* 1998;17(22):6558-6572. doi:10.1093/emboj/17.22.6558
16. Sitar T, Popowicz GM, Siwanowicz I, Huber R, Holak TA. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. *Proc Natl Acad Sci U S A.* 2006;103(35):13028-13033. doi:10.1073/pnas.0605652103
17. Siwanowicz I, Popowicz GM, Wisniewska M, et al. Structural basis for the regulation of insulin-like growth factors by IGF binding proteins. *Structure (London, England: 1993).* 2005;13(1):155-167. doi:10.1016/j.str.2004.11.009
18. Zesławski W, Beisel HG, Kamionka M, et al. The interaction of insulin-like growth factor-I with the N-terminal domain of IGFBP-5. *EMBO J.* 2001;20(14):3638-3644. doi:10.1093/emboj/20.14.3638
19. Kibbey MM, Jameson MJ, Eaton EM, Rosenzweig SA. Insulin-like growth factor binding Protein-2: contributions of the C-terminal domain to insulin-like growth Factor-1 binding. *Mol Pharmacol.* 2006;69(3):833-845. doi:10.1124/mol.105.016998
20. Qin X, Strong DD, Baylink DJ, Mohan S. Structure-function analysis of the human insulin-like growth factor binding Protein-4 *. *J Biol Chem.* 1998;273(36):23509-23516. doi:10.1074/jbc.273.36.23509
21. Kineman RD, Del Rio-Moreno M, Sarmiento-Cabral A. 40 Years of IGF1: understanding the tissue-specific roles of IGF1/IGF1R in regulating metabolism using the Cre/LoxP system. *J Mol Endocrinol.* 2018;61(1):T187-T198. doi:10.1530/JME-18-0076
22. Firth SM, Baxter RC. Cellular actions of the insulin-like growth factor binding proteins. *Endocr Rev.* 2002;23(6):824-854. doi:10.1210/er.2001-0033
23. Bunn RC, Fowlkes JL. Insulin-like growth factor binding protein proteolysis. *Trends Endocrinol Metab.* 2003;14(4):176-181. doi:10.1016/s1043-2760(03)00049-3
24. Monti SM, De Simone G, Langella E. The amazing world of IDPs in human diseases. *Biomolecules.* 2021;11(2):333. doi:10.3390/biom11020333
25. Conover CA, Kiefer MC, Zapf J. Posttranslational regulation of insulin-like growth factor binding Protein-4 in Normal and transformed human fibroblasts. Insulin-like growth factor dependence and biological studies. *J Clin Invest.* 1993;91(3):1129-1137. doi:10.1172/JCI116272
26. Conover CA, Durham SK, Zapf J, Masiarz FR, Kiefer MC. Cleavage analysis of insulin-like growth factor (IGF)-dependent IGF-binding Protein-4 proteolysis and expression of protease-resistant IGF-binding protein-4 mutants. *J Biol Chem.* 1995;270(9):4395-4400. doi:10.1074/jbc.270.9.4395
27. Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. *Prog Growth Factor Res.* 1995;6(2-4):273-284. doi:10.1016/0955-2235(95)00012-7
28. Gaidamauskas E, Gyrop C, Boldt HB, et al. IGF dependent modulation of IGF binding protein (IGFBP) proteolysis by pregnancy-associated plasma protein-a (PAPP-A): multiple PAPP-A-IGFBP interaction sites. *Biochim Biophys Acta.* 2013;1830(3):2701-2709. doi:10.1016/j.bbagen.2012.11.002
29. Monget P, Mazerbourg S, Delpuech T, et al. Pregnancy-associated plasma protein-a is involved in insulin-like growth factor binding Protein-2 (IGFBP-2) proteolytic degradation in bovine and porcine preovulatory follicles: identification of cleavage site and characterization of IGFBP-2 degradation. *Biol Reprod.* 2003;68(1):77-86. doi:10.1095/biolreprod.102.007609
30. Bach LA. What happened to the IGF binding proteins? *Endocrinology.* 2018;159(2):570-578. doi:10.1210/en.2017-00908
31. Uversky VN, Oldfield CJ, Midic U, et al. Unfoldomics of human diseases: linking protein intrinsic disorder with diseases. *BMC Genomics.* 2009;10(1):S7. doi:10.1186/1471-2164-10-S1-S7

32. Sung M, Jeong MS, Jang SB. Biological characterization and structure based prediction of insulin-like growth factor binding Protein-5. *Biochem Biophys Res Commun*. 2010;403(2):230-236. doi:10.1016/j.bbrc.2010.11.016
33. Swain M, Slomiany MG, Rosenzweig SA, Atreya HS. High-yield bacterial expression and structural characterization of recombinant human insulin-like growth factor binding Protein-2. *Arch Biochem Biophys*. 2010;501(2):195-200. doi:10.1016/j.abb.2010.06.006
34. Malik SA, Mondal S, Atreya HS. Enhanced stability of an intrinsically disordered protein against proteolytic cleavage through interactions with silver nanoparticles. *RSC Adv*. 2019;9(49):28746-28753. doi:10.1039/c9ra05514b
35. Mondal S, Thirupathi R, Rao LP, Atreya HS. Unraveling the dynamic nature of protein-graphene oxide interactions. *RSC Adv*. 2016;6(58):52539-52548. doi:10.1039/c6ra03759c
36. Barnwal RP, Rout AK, Chary KVR, Atreya HS. Rapid measurement of 3J(H N-H alpha) and 3J(N-H Beta) coupling constants in polypeptides. *J Biomol NMR*. 2007;39(4):259-263. doi:10.1007/s10858-007-9200-8
37. Marsh JA, Singh VK, Jia Z, Forman-Kay JD. Sensitivity of secondary structure propensities to sequence differences between alpha- and gamma-synuclein: implications for fibrillation. *Protein Sci*. 2006;15(12):2795-2804. doi:10.1110/ps.062465306
38. Berjanskii M, Wishart DS. A simple method to predict protein flexibility using secondary chemical shifts. *J Am Chem Soc*. 2005;127(43):14970-14971. doi:10.1021/ja054842f
39. Rosenzweig SA. What's new in the IGF-binding proteins? *Growth Horm IGF*. 2004;14(5):329-336. doi:10.1016/j.ghir.2004.02.003
40. Thakur A, Chandra K, Dubey A, D'Silva P, Atreya HS. Rapid characterization of hydrogen exchange in proteins. *Angew Chem Int Ed Engl*. 2013;52(9):2440-2443. doi:10.1002/anie.201206828
41. Disfani FM, Hsu W-L, Mizianty MJ, et al. MoRFpred, a computational tool for sequence-based prediction and characterization of short disorder-to-order transitioning binding regions in proteins. *Bioinformatics (Oxford, England)*. 2012;28(12):i75-i83. doi:10.1093/bioinformatics/bts209
42. Lefevre JF, Dayie KT, Peng JW, Wagner G. Internal mobility in the partially folded DNA binding and dimerization domains of GAL4: NMR analysis of the N-H spectral density functions. *Biochemistry*. 1996;35(8):2674-2686. doi:10.1021/bi9526802
43. Yang D, Kay LE. Contributions to conformational entropy arising from bond vector fluctuations measured from NMR-derived order parameters: application to protein folding. *J Mol Biol*. 1996;263(2):369-382. doi:10.1006/jmbi.1996.0581
44. Jumper J, Evans R, Pritzel A, et al. Highly accurate protein structure prediction with AlphaFold. *Nature*. 2021;596(7873):583-589. doi:10.1038/s41586-021-03819-2
45. Bracken C, Carr PA, Cavanagh J, Palmer III AG. Temperature dependence of intramolecular dynamics of the basic leucine zipper of GCN4: implications for the entropy of association with DNA. *J Mol Biol*. 1999;285(5):2133-2146. doi:10.1006/jmbi.1998.2429
46. Tompa P, Fuxreiter M. Fuzzy complexes: polymorphism and structural disorder in protein-protein interactions. *Trends Biochem Sci*. 2008;33(1):2-8. doi:10.1016/j.tibs.2007.10.003
47. Fuxreiter M. Fuzziness: linking regulation to protein dynamics. *Mol Biosyst*. 2012;8(1):168-177. doi:10.1039/C1MB05234A
48. Dong C, Zhang J, Fang S, Liu F. IGFBP5 increases cell invasion and inhibits cell proliferation by EMT and Akt signaling pathway in glioblastoma multiforme cells. *Cell Div*. 2020;15:4. doi:10.1186/s13008-020-00061-6
49. Laursen LS, Overgaard MT, S e R, et al. Pregnancy-associated plasma protein-a (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A. *FEBS Lett*. 2001;504(1-2):36-40. doi:10.1016/s0014-5793(01)02760-0
50. Snead KJ, Moore LL, Bourne CR. ParD antitoxin hotspot alters a disorder-to-order transition upon binding to its cognate ParE toxin, lessening its interaction affinity and increasing its protease degradation kinetics. *Biochemistry*. 2022;61(1):34-45. doi:10.1021/acs.biochem.1c00584
51. Wand AJ. Dynamic activation of protein function: a view emerging from NMR spectroscopy. *Nat Struct Biol*. 2001;8(11):926-931. doi:10.1038/nsb1101-926
52. Cooper A, Dryden DT. Allostery without conformational change. A plausible model. *Eur Biophys J*. 1984;11(2):103-109. doi:10.1007/BF00276625
53. Popovych N, Sun S, Ebricht RH, Kalodimos CG. Dynamically driven protein allostery. *Nat Struct Mol Biol*. 2006;13(9):831-838. doi:10.1038/nsmb1132
54. Werbeck ND, Shukla VK, Kunze MBA, et al. A distal regulatory region of a class I human histone deacetylase. *Nat Commun*. 2020;11(1):3841. doi:10.1038/s41467-020-17610-w
55. Fontana A, Fassina G, Vita C, Dalzoppo D, Zamai M, Zamboni M. Correlation between sites of limited proteolysis and segmental mobility in thermolysin. *Biochemistry*. 1986;25(8):1847-1851. doi:10.1021/bi00356a001
56. Hubbard SJ, Eisenmenger F, Thornton JM. Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Sci*. 1994;3(5):757-768. doi:10.1002/pro.5560030505
57. Hubbard SJ. The structural aspects of limited proteolysis of native proteins. *Biochim Biophys Acta*. 1998;1382(2):191-206. doi:10.1016/s0167-4838(97)00175-1
58. Gualberto A, Pollak M. Emerging role of insulin-like growth factor receptor inhibitors in oncology: early clinical trial results and future directions. *Oncogene*. 2009;28(34):3009-3021. doi:10.1038/onc.2009.172
59. Yu H, Rohan T. Role of the insulin-like growth factor family in cancer development and progression. *J Natl Cancer Inst*. 2000;92(18):1472-1489. doi:10.1093/jnci/92.18.1472
60. Weroha SJ, Haluska P. The insulin-like growth factor system in cancer. *Endocrinol Metab Clin North Am*. 2012;41(2):335-350. doi:10.1016/j.ecl.2012.04.014
61. Ryan AJ, Napoletano S, Fitzpatrick PA, Currid CA, O'Sullivan NC, Harmey JH. Expression of a protease-resistant insulin-like growth factor-binding Protein-4 inhibits tumour growth in a murine model of breast cancer. *Br J Cancer*. 2009;101(2):278-286. doi:10.1038/sj.bjc.6605141
62. Soh C-L, McNeil K, Owczarek CM, et al. Exogenous administration of protease-resistant, non-matrix-binding IGFBP-2 inhibits tumour growth in a murine model of breast cancer. *Br J Cancer*. 2014;110(12):2855-2864. doi:10.1038/bjc.2014.232
63. Xiao Y, Zhu S, Yin W, Liu X, Hu Y. IGFBP-4 expression is adversely associated with lung cancer prognosis. *Oncol Lett*. 2017;14(6):6876-6880. doi:10.3892/ol.2017.7014
64. Drescher DG, Ramakrishnan NA, Drescher MJ. Surface Plasmon resonance (SPR) analysis of binding interactions of proteins in inner-ear sensory epithelia. *Methods Mol Biol (Clifton, NJ)*. 2009;493:323-343. doi:10.1007/978-1-59745-523-7_20
65. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR*. 1995;6(3):277-293. doi:10.1007/BF00197809
66. Bartels C, Xia T, Billeter M, G ntert P, W ttrich K. The program XEASY for computer-supported NMR spectral analysis of biological macromolecules. *J Biomol NMR*. 1995;6(1):1-10. doi:10.1007/BF00417486
67. Moseley HN, Monleon D, Montelione GT. Automatic determination of protein backbone resonance assignments from triple resonance nuclear magnetic resonance data. *Methods Enzymol*. 2001;339:91-108. doi:10.1016/s0076-6879(01)39311-4

68. Krishnarjuna B, Jaipuria G, Thakur A, D'Silva P, Atreya HS. Amino acid selective unlabeled for sequence specific resonance assignments in proteins. *J Biomol NMR*. 2011;49(1):39-51. doi:[10.1007/s10858-010-9459-z](https://doi.org/10.1007/s10858-010-9459-z)
69. Dubey A, Mondal S, Chandra K, Atreya HS. Rapid identification of amino acid types in proteins using phase modulated 2D HN (CACB) and 2D HN (COCACB) rapid identification of amino acid types in proteins using phase modulated 2D HN (CACB) and 2D HN (COCACB). *J Magn Reson*. 2016;267(April):22-29. doi:[10.1016/j.jmr.2016.04.004](https://doi.org/10.1016/j.jmr.2016.04.004)
70. Chandra K, Jaipuria G, Shet D, Atreya HS. Efficient sequential assignments in proteins with reduced dimensionality 3D HN(CA)NH. *J Biomol NMR*. 2012;52(2):115-126. doi:[10.1007/s10858-011-9598-x](https://doi.org/10.1007/s10858-011-9598-x)
71. Jaipuria G, Krishnarjuna B, Mondal S, Dubey A, Atreya HS. Amino acid selective labeling and unlabeled for protein resonance assignments. *Adv Exp Med Biol*. 2012;992:95-118. doi:[10.1007/978-94-007-4954-2_6](https://doi.org/10.1007/978-94-007-4954-2_6)
72. Ulrich EL, Akutsu H, Doreleijers JF, et al. BioMagResBank. *Nucleic Acids Res*. 2008;36(Database issue):D402-D408. doi:[10.1093/nar/gkm957](https://doi.org/10.1093/nar/gkm957)
73. Shen Y, Delaglio F, Cornilescu G, Bax A. TALOS+: a hybrid method for predicting protein backbone torsion angles from NMR chemical shifts. *J Biomol NMR*. 2009;44(4):213-223. doi:[10.1007/s10858-009-9333-z](https://doi.org/10.1007/s10858-009-9333-z)

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Jaipuria G, Shet D, Malik S, et al. IGF-dependent dynamic modulation of a protease cleavage site in the intrinsically disordered linker domain of human IGFBP2. *Proteins*. 2022;1-12. doi:[10.1002/prot.26350](https://doi.org/10.1002/prot.26350)