Heparin-binding mechanism of the IGF2/IGF-binding protein 2 complex

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Abstract

IGF1 and IGF2 are potent stimulators of diverse cellular activities such as differentiation and mitosis. Six IGF-binding proteins (IGFBP1–IGFBP6) are primary regulators of IGF half-life and receptor availability. Generally, the binding of IGFBPs inhibits IGF receptor activation. However, it has been shown that IGFBP2 in complex with IGF2 (IGF2/IGFBP2) stimulates osteoblast function *in vitro* and increases skeletal mass *in vivo*. IGF2 binding to IGFBP2 greatly increases the affinity for 2- or 3-carbon *O*-sulfated glycosaminoglycans (GAGs), e.g. heparin and heparan sulfate, which is hypothesized to preferentially and specifically target the IGF2/IGFBP2 complex to the bone matrix. In order to obtain a more detailed understanding of the interactions between the IGF2/IGFBP2 complex and GAGs, we investigated heparin-binding properties of IGFBP2 and the IGF2/IGFBP2 complex in a quantitative manner. For this study, we mutated key positively charged residues within the two heparin-binding domains (HBDs) in IGFBP2 and in one potential HBD in IGF2. Using heparin affinity chromatography, we demonstrate that the two IGFBP2 HBDs contribute differentially to GAG binding in free IGFBP2 and the IGF2/IGFBP2 protein complex. Moreover, we identify a significant contribution from the HBD in IGF2 to the increased IGF2/IGFBP2 heparin affinity. Using molecular modeling, we present a novel model for the IGF2/IGFBP2 interaction with heparin where all three proposed HBDs constitute a positively charged and surface-exposed area that would serve to promote the increased heparin affinity of the complex compared with free intact IGFBP2.

Key Words

- ▶ IGF2/IGFBP2 complex
- ▶ glycosaminoglycans
- ▶ heparin affinity
- ▶ ternary binding model

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Introduction

Insulin-like growth factor 1 (IGF1) and IGF2 are small polypeptides that have diverse autocrine, paracrine, and endocrine effects in various mammalian tissues (Clemmons 1997). A family of six high-affinity IGF-binding proteins (IGFBP1–IGFBP6) ensures the tight regulation of IGF half-life and the interaction with specific receptors (Bayes-Genis *et al.* 2000, Firth & Baxter 2002, Forbes *et al.* 2012). The IGFBPs are homologs, structurally highly similar, and yet functionally very distinct. They all

have similar N- and C-terminal domains that are connected by a variable linker region (Clemmons 2001, Bach *et al.* 2005, Forbes *et al.* 2012). The N- and C-terminal domains adopt distinct tertiary structures and both are required for high-affinity IGF binding, presumably in a cooperative fashion (Kuang *et al.* 2007). *In vitro* solution assays generally indicate that purified native or recombinant IGFBPs have a higher affinity for IGF peptides than do IGF receptors, making all six IGFBPs capable of binding

IGFs and preventing receptor interaction and activation (Firth & Baxter 2002, Denley *et al.* 2005). IGF2 exerts its action through the insulin receptor and the IGF receptor 1 (IGF1R) and is cleared from circulation by the IGF2R (Butler *et al.* 1998, Frasca *et al.* 1999, Bayes-Genis *et al.* 2000, Williams *et al.* 2007, Brown *et al.* 2008). In addition, certain IGFBPs have been shown to increase local IGF activity by targeting IGF and IGFBP complexes (IGF/IGFBP) to the cell membrane and the extracellular matrix (ECM) surrounding the cells (Conover 1992, Jones *et al.* 1993).

We had previously shown that IGFBP2 when complexed with IGF2 had increased binding to human osteoblast ECM while IGFBP2 alone and IGFBP2 in complex with IGF1 (IGF1/IGFBP2) showed little or no binding (Khosla *et al.* 1998, Conover & Khosla 2003). IGFBP2 was unique among the six IGFBPs in exhibiting IGF2-enhanced matrix binding (Conover & Khosla 2003). Similarly, Arai *et al.* (1996) demonstrated that IGF/IGFBP2 complexes were able to bind heparin-sepharose and ECM produced by human fibroblasts. These investigators further suggested that binding of IGF/IGFBP2 was principally to heparin-like glycosaminoglycans (GAGs). Our results indicated that heparin, heparan sulfate, and high salt inhibited IGF2-enhanced IGFBP2 binding to human osteoblast ECM while chondroitin sulfate, integrins, and mannitol did not. These observations further supported binding to highly sulfated GAGs particularly abundant in bone (Conover & Khosla 2003). Moreover, in a matrix-rich environment, the IGF2/IGFBP2 complex was as effective as IGF2 alone in stimulating osteoblast proliferation, whereas IGFBP2 alone had no effect (Conover 2008). The mechanism for this IGF2/IGFBP2 stimulation of osteoblastic cell receptors is unclear. No evidence of proteolytic cleavage and no apparent change in IGFBP2 affinity for IGF with matrix association were observed (Conover & Khosla 2003). This is in contrast to IGFBP3 and IGFBP5 binding to cell surface and matrix, respectively, which results in reduced affinity for IGFs, thus potentially releasing the growth factors (Clemmons 2001).

IGFBP2 contains two heparin-binding domains (HBDs), one in the C-terminal domain and the other located in the linker region (Hodgkinson *et al.* 1994, Russo *et al.* 2005, Kuang *et al.* 2006). In addition, IGF2 possesses a putative HBD that is not present in IGF1 (Cardin & Weintraub 1989, Conover & Khosla 2003). The aim of this study was to investigate heparin-binding properties of the IGF2/IGFBP2 complex and evaluate these at the structural level. To this end, we employed an *Escherichia coli*-based co-expression system to produce a panel of murine *Igfbp2*

variants in complex with human IGF2, with mutations of key positively charged HBD residues. The heparin-binding properties of these IGF2/IGFBP2 complexes were then determined using heparin affinity chromatography as a surrogate model for bone ECM GAGs. In conjunction with structural modeling, the results lead us to propose a new model for the ternary IGF2/IGFBP2/heparin interaction.

Subjects and methods

Generation of expression plasmids

DNA encoding two fusion proteins were cloned into a pET-Duet 1 plasmid (Merck, Darmstadt, Germany): i) human IGF2 with an N-terminal thioredoxin, spaced by six His residues and a tobacco etch virus (TEV) protease recognition site and ii) murine IGFBP2 with an N-terminal maltose-binding protein, spaced by a TEV protease recognition site and a six-Gly linker. Site-directed mutagenesis of the co-expression vector was used to generate IGF2 and IGFBP2 mutations. Selected Lys, Arg, or His residues were changed to Ala using the Quikchange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. Reverse complementary sets of mutagenesis primers were purchased from TAG Copenhagen (Copenhagen, Denmark). Forward primer sequences: linker-HBD (IGFBP2 K162A K163A, 5'-CTC-AGTCTGGAGGAGCCCGCAGCGCTGCGCCCGCCTCCC-GCCAGGACC-3'), C-term-HBD (IGFBP2 K209A H210A K216A, 5'-CCCCAACTGTGACGCCGCGGGCCGGTACAA-CCTTGCGCAGTGCAAGATGTCTCTGAACGG-3'), IGF2 HBD (IGF2 R37A R38A R40A, 5'-CGCAAGCCGTGTGAG-CGCTGCTAGCGCTGGCATCGTTGAGGAGTG-3'), and IGFBP2 control mutation (R188A R193A, 5'-GGATCTCCAC-CATGGCTCTCCGGATGATGCTGGCCCCCTGG-3'). The resulting plasmids were verified by sequencing (Eurofins, Luxembourg, Luxembourg).

Protein expression

Fusion proteins were co-expressed in *E. coli* Rosetta-gami B (DE3) (Novagen) using buffered LB medium (10 g/l NaCl, 10 g/l tryptone, 5 g/l yeast extract, 10 g/l D-glucose, 1.5 g/l KH₂PO₄, and 10 g/l K₂HPO₄, added with 100 mg/l ampicillin and 34 mg/l chloramphenicol) at pH 7. Cultures were grown at 37 °C to OD_{600 nm} ~1 in an aerated 2 l bioreactor (Sartorius BBI Systems, Melsungen, Germany) with pH regulation before induction with 0.5 mM isopropyl B-D-1-thiogalactopyranoside (IPTG). Protein expression was continued at 25 °C for 19 h before harvest by centrifugation at 4000 g and 4 °C for

30 min. Cells were resuspended in lysis buffer (20 mM Tris, 50 mM NaCl, 5 mM imidazole, 2 mM MgSO₄, 1 mM β-mercaptoethanol, 1 g/l lysozyme, 25 U/ml benzonase, and 0.5% (volume/volume) protease inhibitor cocktail III (Merck), pH 7.5) and lysed by repeated freeze–thaw cycles. Cell debris was removed by centrifugation at 90 000 *g* for 45 min at 4 °C.

Protein purification

The complexes of IGF2 and IGFBP2 fusion proteins were purified by immobilized metal ion affinity chromatography using a Ni-NTA column (Qiagen). IGF2/IGFBP2 fusion proteins containing fractions were dialyzed against a TEV compatible buffer (20 mM Tris, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 1% glycerol, pH 8) and 5 µg/ml added recombinant, modified TEV protease (Kapust *et al.* 2001). Liberated IGF2/IGFBP2 complexes were further purified by cation-exchange chromatography using a Source 15S resin (GE Healthcare, Little Chalfont, Bucks, UK) at pH 6.5. Finally, the purified IGF2/IGFBP2 preparations were buffer exchanged using a Fast Desalting HR 10/10 column (GE Healthcare) into buffers containing 20 mM acetate, 20 mM MES or 20 mM phosphate, and 50 mM NaCl at pH 5.5, 6.5, or 7.5 respectively. To obtain free IGFBP2, the individual protein complexes were separated by size-exclusion chromatography (Superdex 75 HR 10/30 (GE Healthcare)) at pH 3 (50 mM citrate and 20% (v/v) acetonitrile) followed by buffer exchange as described previously. Human IGFBP3 and IGF1/IGFBP3 complex were kind gifts from Andreas Sommer (Insmed, Bridgewater, NJ, USA), and human IGFBP4 and IGF2/IGFBP4 complex were essentially cloned, expressed, and purified as described above (M T Overgaard, in preparation).

Protein analysis

Purified proteins were analyzed by SDS–PAGE in presence or absence of 50 mM dithiothreitol (DTT) and stained with Coomassie brilliant blue G250. Secondary structure of protein was assayed using circular dichroism (CD) on a Chirascan Plus spectropolarimeter (Applied Photophysics, Leatherhead, Surrey, UK). CD spectra of 200 µg/ml protein complexes in acetate buffer at pH 5.5 were acquired in the range of 195–260 nm at 25 °C using a 0.5 mm light-path cuvette. CD measurements were subtracted from buffer scan, baseline corrected, adjusted for CD signal intensity using the absorbance signal measurements, and converted to corrected mean residue weighted molar ellipticity ($[\theta]_{MRW}^*$ (dg × m²/dmol)). Protein identity

was confirmed by peptide mass fingerprinting using MALDI–TOF mass spectrometry of tryptic peptides on a Bruker Reflex III. To investigate the protein disulfide bridge pattern, alkylated samples (100 mM iodoacetic acid for 0.5 h), with or without prior DTT reduction, were similarly digested with trypsin and analyzed by MALDI–TOF MS.

Heparin affinity chromatography

Relative heparin affinities of protein complexes and IGFBPs alone were analyzed using heparin affinity chromatography at various pH values. A fast protein liquid chromatography system (ÄKTApurifier 10, GE Healthcare) equipped with a 1 ml HiTrap Heparin HP column (GE Healthcare), u.v.-280 nm absorption (UV-900, GE Healthcare), and a conductivity detector (pH/C-900, GE Healthcare) was used at a flow rate of 1 ml/min. Heparin affinity was assayed in 20 mM acetate, pH 5.5, and 50 mM NaCl for all experiments, except for the experiments with varying pH, where 20 mM MES or 20 mM phosphate, and 50 mM NaCl at pH 6.5 or 7.5, respectively, were employed. The ionic strength – measured as the retention conductance (κ_r (mS/cm)) – necessary to elute the bound proteins was a measure of relative heparin affinity. Triplicate measurements of κ_r for IGFBP2 variants with and without IGF2 were subjected to a two-way ANOVA with the Bonferroni's pairwise comparison test of all possible

Complex	IGF2	IGFBP2	Cartoon
IGF2/IGFBP2	WT	WT
IGF2/IGFBP2_linker-HBD	WT	K162A K163A
IGF2/IGFBP2_C-term-HBD	WT	K209A H210A K216A
IGF2 HBD/IGFBP2	R37A R38A R40A	WT
IGF2/IGFBP2_Ctrl	WT	R188A R193A

Figure 1

Overview of protein complexes investigated. The first column shows the nomenclature used throughout the text. The second and third columns illustrate the specific mutations introduced in IGF2 or IGFBP2 respectively. The numbering refers to the sequences of mature human IGF2 and murine IGFBP2 respectively (Fig. 2). The fourth column shows cartoons of the individual complexes, which will be used to aid nomenclature. IGFBP2 (N-terminal domain, blue; linker region, white; and C-terminal domain, green), IGF2 (red), and mutations (black circles).

combinations with a mutational variant and IGF2-binding state as independent variables (GraphPad Software, Prism 5, La Jolla, CA, USA). The triplicate measurements of κ_r for the binary complexes at various pH levels were analyzed in an equivalent analysis with pH and protein variants as independent variables.

Homology structural modeling

MODELLER v.9.10 was used for the generation of a three-dimensional structural model of the IGF2/IGFBP2 complex using the structures of human IGF1 in complex with human IGFBP4 N- and C-terminal domains (PDB ID: 2DSR), C-terminal domain of human IGFBP2 (PDB ID: 2H7T), and IGF2 (PDB ID: 1IGL) as templates (Torres *et al.* 1995, Eswar *et al.* 2006, Kuang *et al.* 2006, Sitar *et al.* 2006). Clustal W sequence alignments of template sequences, and murine IGFBP2 (ID P47877) and human IGF2 (ID P01344), respectively, were used to generate 50 models. The best-ranked model was further refined using the MODELLER loop refinement algorithm. Stereochemical quality of the generated models was inspected using the SAVES server (<http://nihserver.mbi.ucla.edu/SAVES/>), where the final model passed three-dimensional benchmarking with Verify_3D as well as being in agreement with allowed Ramachandran conformations (Eisenberg *et al.* 1997).

Results

Protein expression, purification, and analysis

Four different IGF2/IGFBP2 variants were made by exchanging positively charged residues in putative HBDs

to Ala, three involving mutations IGFBP2 (linker-HBD, C-terminal domain HBD, and a control variant) and one with mutations in the proposed IGF2 HBD (Figs 1 and 2). Rosetta-gami (DE3) cells, which have a favorable cytosolic environment for disulfide formation, were used for co-expression of murine IGFBP2 and human IGF2, both as fusion proteins. The first step of the purification employed Ni^{2+} affinity selectively binding IGF2 fusion proteins (which include a hexa-histidine sequence), and thereby simultaneous co-purification of IGFBP2 fusion proteins capable of forming a complex with IGF2. The following specific proteolytic cleavage with TEV protease, subsequent cation exchange, and size-exclusion chromatography steps ensured removal of non-complexed IGF2, released fusion protein affinity tags, non-cleaved fusion proteins, and TEV protease. Denaturing SDS-PAGE analysis of the purified complexes demonstrated identical electrophoretic mobility for all variants of IGFBP2 both under reducing and non-reducing conditions respectively (Fig. 3A). The IGFBP2 variants all displayed an identical small decrease in electrophoretic mobility under reducing conditions, supporting the presence of an intact disulfide bond pattern – further analyzed below. IGF2 HBD variant displayed a slightly increased mobility relative to IGF2, consistent with the substitution of three positively charged Arg residues for Ala. CD was used to probe for potential structural differences between the IGF2/IGFBP2 variants (Fig. 3B). The CD spectra were highly similar for all complexes, indicating no significant differences in the secondary structure content of protein.

MALDI-TOF analysis of tryptic peptides generated from reduced samples confirmed the introduction of

Figure 2

Amino acid sequence alignments for homology modeling with annotations added. (Upper and middle panels) Sequences for the N-terminal domain (Glu1–Arg84) and C-terminal domain (Arg170–Gln271 plus adjacent linker region residues Pro161–Ala169) from mature murine IGFBP2 aligned with the corresponding parts of mature human IGFBP4. (Lower panel) Mature human IGF2 is aligned with human IGF1. IGFBP2 linker (Lys162–Arg165) and C-terminal domain (Lys209–His210, Lys216, and His253) HBD residues are colored in gray along with the proposed IGF2 HBD residues (Arg37–Arg40).

The charged residues mutated to Ala in this study are boxed. IGF2-binding residues of IGFBP2 are highlighted in orange. IGF2 residues involved in IGF1R (turquoise) and IGF2R (olive green) binding are also highlighted (Butler *et al.* 1998, Clemmons 2001, Kuang *et al.* 2006, Williams *et al.* 2007, Brown *et al.* 2008). Disulfide bridges identified in this study are shown with black lines. Identified disulfide clusters are shown with gray lines. Numbering corresponds to mature murine IGFBP2 and human IGFBP4, IGF1, and IGF2 as indicated.

mutations (data not shown). Analysis of iodoacetic acid-treated, non-reduced proteins showed no alkylation of any Cys-containing peptides, strongly indicating that no free Cys-SH was present (data not shown). Furthermore, tryptic peptide mass fingerprinting of non-reduced protein complexes identified Cys–Cys crosslinked peptides consistent with the previously reported IGFBP disulfide structures for the N-terminal domain of IGFBP4 and IGFBP5 and the C-terminal domain of IGFBP2 (and other IGFBPs; Smith *et al.* 1989, Kalus *et al.* 1998, Zesławski *et al.* 2001, Mark *et al.* 2005, Siwanowicz *et al.* 2005, Sitar *et al.* 2006, Brown *et al.* 2008, Nili *et al.* 2012). This pattern can be described by numbering the Cys residues consecutively. The N-terminal domain Cys–Cys linkages are C1–C5, C2–C6, C3–C7, C4–C8 (forming a cluster of three crosslinked tryptic peptides), C9–C11, and C10–C12. The C-terminal

domain linkages are C13–C14, C15–C16, and C17–C18 disulfides, the latter two being expected to result in a three-peptide cluster upon tryptic digestion. In addition, the IGF2 disulfide connections (C1–C4, C2–6, and C3–C5) are expected to connect three tryptic peptides in one cluster.

We specifically verified the IGFBP2 C9–C11 and C10–C12 linkages (Cys48–Cys61 and Cys55–Cys81, respectively, using mature murine IGFBP2 sequence numbering) in the N-terminal domain, and the C13–C14 (Cys173–Cys207) disulfide in the C-terminal domain (Fig. 2). Further delineation of the disulfide patterns was impaired by the close proximity of the Cys residues. However, the expected peptide clusters from tryptic digest of IGFBP2 and IGF2 were identified (Fig. 2). Importantly, this observation was consistent for all IGF2/IGFBP2 variants.

Heparin affinity chromatography

The relative heparin-binding strength of the IGF2/IGFBP2 complex, free IGFBP2, and variants was analyzed by heparin affinity chromatography with the retention conductances (κ_r (mS/cm)) taken as a measure of their respective heparin affinities. Free IGFBP2 and complexes were eluted in single peaks as detected by u.v. absorption (examples of IGF2/IGFBP2 elution from the heparin column are shown in Supplementary Figure 1, see section on supplementary data given at the end of this article).

Initially, the protein complexes were investigated (Fig. 4). The retention conductance, κ_r , was determined as the conductivity at the chromatographic elution peak. Mutations of positively charged HBD residues to Ala generally decreased IGF2/IGFBP2 heparin affinities, as seen by elution at a lower κ_r . However, the specific disruption of HBDs affected the heparin affinity of the complexes to different extents, with the general order of affinities being IGF2/IGFBP2 = IGF2/IGFBP2_Ctrl > IGF2/IGFBP2_C-term-HBD > IGF2/IGFBP2_linker-HBD > IGF2 HBD/IGFBP2. The observed differences were all statistically significant ($P < 0.01$). These observations demonstrated differential contributions of the individual HBDs. The IGFBP2 C-terminal domain HBD contributed less than the linker-HBD to the IGF2/IGFBP2 heparin affinity. Further, the proposed IGF2 HBD contributed significantly more than the two IGFBP2 HBDs to the IGF2/IGFBP2 complex heparin affinity. As a method of validation and a point of reference, the human IGF1/IGFBP3 and human IGF2/IGFBP4 complexes were also analyzed as these were previously shown to exhibit strong and no significant GAG binding respectively. The IGF1/IGFBP3 complex bound to the heparin column with a higher affinity than

Figure 3
Analysis of protein-folding integrity. (A) SDS–PAGE analysis of the purified protein complexes. (B) Far–u.v. CD spectra of protein complexes in acetate buffer at pH 5.5 and 25 °C. θ^*_{MRW} is the corrected mean residue weight ellipticity.

Figure 4

Heparin affinities of IGF/IGFBP complexes. Columns indicate mean and bars the s.d. of triplicates at pH 5.5. Lettering indicates statistically unique values as determined from two-way ANOVA with pairwise comparisons ($P < 0.01$). IGF2/IGFBP4 did not bind to the column at the applied loading conditions ($\kappa_r \sim 8$ mS/cm). Cartoons are added to aid the nomenclature with IGFBP3 N- and C-terminal domains in brown and orange respectively. IGFBP4 N- and C-terminal domains are in sea green and turquoise color, respectively, also IGF1 is in gray color. See Fig. 1 for additional color legend. Lines indicate κ_r for loading conditions (dotted) and PBS (solid).

the IGF2/IGFBP2 complex, while the IGF2/IGFBP4 complex showed no measurable heparin binding at the column loading conditions. Mutation of surface exposed, charged residues not part of any proposed HBD (IGF2/IGFBP2_Ctrl) resulted in a heparin-binding affinity indistinguishable from that of the WT IGF2/IGFBP2 complex and thus confirmed that the protein complex interaction with the heparin column was governed by specific interactions and not overall charges, i.e. true affinity chromatography and not ion-exchange chromatography. In support of this was also the ion-exchange capacity of the applied HiTrap Heparin column which was ~ 25 -fold less than that of the Source 15S column used during purification, and the protein complexes displayed an ~ 2.3 -fold greater affinity for the HiTrap Heparin column compared with the Source 15S column as measured by κ_r (data not shown).

Then, we investigated the significance of the individual IGFBP2 HBDs in the free IGFBP2 variants by the same method (Fig. 5). Both HBD-targeted variants displayed a decreased heparin affinity, demonstrating that both HBDs

are needed for maintaining the heparin affinity of free IGFBP2. The control variant, IGFBP2_Ctrl, displayed the same affinity as the WT IGFBP2. Interestingly, the order of affinities was IGFBP2 > IGFBP2_linker-HBD > IGFBP2_C-term-HBD implying a reversed significance of the two HBDs in free IGFBP2 relative to the IGF2/IGFBP2 complex. Again, all observed differences were statistically significant ($P < 0.01$). In case of free IGFBP2, the C-terminal domain HBD appeared to contribute significantly more to the overall heparin affinity than the linker-HBD. This result also implied that different IGFBP2 HBDs might be relevant for targeted GAG binding of the free IGFBP2 and IGF2/IGFBP2 complex respectively. Finally, free IGFBP2 showed significantly (all $P < 0.01$) decreased heparin affinity relative to their respective complexes with IGF2, and this was consistent across WT, control, and mutated proteins (compare Figs 4 and 5).

The relative impact of the IGF2 HBD mutation was surprising, why free IGFBP2, IGF2/IGFBP2, and IGF2 HBD/IGFBP2 mutated complex were compared separately (Fig. 6). In general, binding of IGF2 increases the heparin affinity of IGFBP2. However, binding of IGF2 with a dysfunctional HBD resulted in a significantly decreased affinity compared with the free IGFBP2. As the C-terminal

Figure 5

Heparin affinities of free IGFBPs. Information in graph is the same as described for Fig. 4. Lettering indicates statistically unique values as determined from two-way ANOVA with pairwise comparisons ($P < 0.01$).

Figure 6
Heparin affinities of IGFBP2, IGF2/IGFBP2, and IGF2 HBD mutated complex. Information in the graph is the same as described for Fig. 4. Lettering indicates statistically unique values as determined from two-way ANOVA with pairwise comparisons ($P < 0.001$).

HBD of IGFBP2 contributes less to the overall heparin affinity of the complex than it does for the free binding protein, IGF2 binding appears to partly mask the IGFBP2 C-terminal domain HBD. This would further enforce the importance of the IGF2 HBD in the increased heparin affinity demonstrated by the complex compared with free IGFBP2.

The pH dependency of IGF2/IGFBP2 heparin affinity

Bone ECM shows local pH gradients during remodeling and these could be part of IGF2/IGFBP2 targeting to this tissue (Arnett 2003). To further investigate this hypothesis, heparin affinities of the IGF2/IGFBP2 variants were measured at different pH levels. The results demonstrated decreased heparin affinity with increasing pH (Fig. 7). Most complexes displayed a similar pH dependency in the range of pH 5.5–7.5, as indicated by the parallel courses of their heparin affinities as a function of pH. The IGF2/IGFBP2_C-term-HBD was an exception

demonstrating a slightly lower pH dependency. As the His210 residue was mutated to Ala in the IGF2/IGFBP2_C-term-HBD, this observation supported that His210 was involved in the heparin interactions of the other complexes and that this residue changes its protonation state in the pH interval of 5.5–7.5. Furthermore, the residual pH dependence of the IGF2/IGFBP2_C-term-HBD complex implied that at least one other His residue contributes to the IGF2/IGFBP2 heparin affinity. This was most likely His253, which has been demonstrated to be part of the HBD of the isolated IGFBP2 C-terminal domain (Kuang *et al.* 2006). All observed differences, i.e. between protein variants and pH levels, were statistically significant (all $P < 0.001$).

Structural modeling of IGF2/IGFBP2

Homology modeling based on the structure of human IGF1 complexed to the N- and C-terminal domains of IGFBP4 was used to create a structural model of the IGF2/IGFBP2 complex (Fig. 8). The sequence alignment input is shown in Fig. 2. The C-terminal eight residues of the linker domain (Pro161–Ala169) of IGFBP2 (covering the linker-HBD) were included in the model to illustrate the proximity to the C-terminal domain start. Noteworthy, the C-chain of IGF1, including the residues aligning to the IGF2 HBD, is not resolved in the template structure, indicating a high

Figure 7
Heparin affinities of IGF2/IGFBP2 variants as a function of pH. Note that the IGF2/IGFBP2_C-term-HBD lacked His209 and demonstrated reduced pH sensitivity. Notations are as given in Fig. 1. Connecting lines drawn to guide the eye. Error bars indicate s.d. (in most cases smaller than the symbol sizes). All values were statistically significant and different from each other as determined from two-way ANOVA with pairwise comparisons ($P < 0.001$).

flexibility of this loop. However, adjacent residues are clearly defined and support the homology model as a good estimate for the position of the IGF2 HBD (see Subjects and methods). The IGF2/IGFBP2 structural model served to tentatively evaluate the HBDs and IGF2-binding site properties. All residues predicted to be involved in heparin binding (gray) were surface exposed in the homology model, including the proposed IGF2 HBD (Fig. 8B). Interestingly, the three HBDs together form an elongated positively charged surface consisting of the IGF2 HBD flanked by the IGFBP2 linker and C-terminal domain HBDs on either side (Fig. 8B and C). A heparin molecule or equivalent GAG polymer may be thought to wrap around the complex across the IGFBP2 C-terminal domain, IGF2, and the IGFBP2 linker-HBDs and bind via electrostatic interactions with the exposed positive charges (Fig. 8C). The IGFBP2 C-terminal domain HBD Lys209 and His210 were in close proximity to the IGF2-binding surface with

the possibility of especially Lys209 being partly buried by IGF2, albeit still somewhat surface exposed. This may potentially explain the decreased heparin binding of the IGF2 HBD/IGFBP2 complex compared with free IGFBP2, as hypothesized above (Fig. 6).

IGFBP2 residues previously identified to be involved in IGF2 binding (orange) were consistently found buried in the interface between IGFBP2 and IGF2 or the interface of the two IGFBP2 domains (Fig. 8A). IGF2 residues involved in IGF1R and IGF2R binding (Arg24/Phe26/Tyr27/Val43 (turquoise) and Leu8/Thr16/Phe19/Asp56/Leu53 (olive green) respectively) were partly buried by the IGFBP2 domains (Figs 2 and 8). Only two of four IGF1R-binding residues in IGF2 (Tyr27/Arg24 (turquoise)) were partially surface exposed in the IGF2/IGFBP2 model. It was noteworthy that the IGF2 IGF1R-binding residues were positioned away from the IGF2 HBD in the modeled IGF2 structure (Fig. 8B). Similarly, only two of the five

Figure 8

Three-dimensional, structural model of the IGF2/IGFBP2 complex based on homology modeling to the human IGF1/IGFBP4 complex. (A and B) Surface rendered model ((A) IGF2 shown as cartoons), highlighting the different features of the complex. IGFBP2 N- and C-terminal (including linker domain residues Pro161–Ala169) domains (blue and green respectively), IGF2-binding residues (orange), IGF2 (red), HBD residues (gray),

IGF1R-binding residues (turquoise) and IGF2R-binding residues (olive green), attachment points for the linker region (purple), and control mutations (black). (C) Surface rendering with charge plot. The modeled IGFBP2 N-terminal domain is in good agreement with the one presented by Galea *et al.* (2012).

IGF2R-binding residues in IGF2 (Asp52/Leu53 (olive green)) were surface exposed in the model, and furthermore, these residues were adjacent to the suggested IGF2/IGFBP2 combined heparin-binding surface. The latter could be indicative of IGF2/IGFBP2 heparin binding further burying IGF2 residues interacting with IGF2R. The model was validated through spatial and energy calculations as detailed above, and furthermore, the IGFBP2 N-terminal domain was in good agreement with a recently published homology model (Galea *et al.* 2012). In addition, the disulfide bridges identified by peptide mass fingerprinting were consistent with those of the model (not highlighted).

Discussion

In this study, we employed site-directed mutagenesis, affinity chromatography, and structural modeling to determine heparin-binding interactions of full-length IGFBP2 in presence and absence of IGF2. We found that the two HBDs in IGFBP2 contribute differentially depending on IGF2 binding, while the previously uncharacterized HBD of IGF2 contributes the greatest to the heparin-binding affinity of the IGF2/IGFBP2 complex.

The results from heparin affinity chromatography not only confirm the importance of the IGFBP2 HBDs, but also emphasize a pivotal role for the IGF2 HBD. Of the three, the HBD in IGF2 contributes to the greatest extent to the affinity of IGF2/IGFBP2 for heparin. Combined with the insights offered by homology modeling of the IGF2/IGFBP2 complex, we propose a model of binding in which the IGF2 HBD aligns with the IGFBP2 linker and C-terminal domain HBDs to form a larger GAG-binding surface. This is consistent with previous observations that GAG affinity of the complex is greater than that for either protein alone (Arai *et al.* 1996, Conover & Khosla 2003, Beattie *et al.* 2008). One limitation is, however, that the homology model does not include the flexible IGFBP2 linker domain, thus the model can neither account for the location of the IGFBP linker nor imply the significance or influence on heparin affinity. The pivotal role of the IGF2 HBD in the IGF2/IGFBP2 complex could explain as to why IGFBP2 bound to IGF2 and not to IGF1 shows targeted GAG binding, as IGF1 lacks an equivalent HBD motif (Fig. 2; Conover & Khosla 2003). In binding with IGFBP2, IGF2 creates a more favorable interaction between IGFBP2 linker-HBD and heparin. The structural model predicted the IGF2-binding sites in IGFBP2 to potentially conflict with the C-terminal domain HBD residues, and this was consistent with the heparin affinity measurements indicating that the IGFBP2 HBDs in the

binary complex were partly masked by the IGF2. The differential contribution of IGFBP2 HBDs could indicate that free IGFBP2 binds to GAGs primarily via the C-terminal domain HBD, while the linker region and IGF2 HBDs govern the binding of the binary complex. Furthermore, the affinity of these interactions may very well be GAG specific, as the IGFBP2 linker and C-terminal domain HBDs differ both at the primary and tertiary structural levels, thus opening for the possibility of IGF2-dependent and GAG-specific targeting to ECM.

The IGF1R-binding residues of IGF2 (Arg24, Phe26, Tyr27, and Val43) appear not to interfere with heparin binding, but are as mentioned somewhat buried in the model, similar to the structure of the IGF1/IGFBP4 and IGF1/IGFBP5 complexes (Zesławski *et al.* 2001, Sitar *et al.* 2006). For IGFBP3 and IGFBP5, where the free IGFBP has a higher affinity for GAGs than the IGF/IGFBP complex the decrease in IGF affinity upon GAG binding would be a plausible mechanism for IGF activation in the ECM. In the case of IGF2/IGFBP2, however, where the complex has been shown to be equally good in stimulating osteoblasts grown on ECM as the free IGF2 (Conover & Khosla 2003), release of IGF2 upon GAG binding seems implausible, as the complex demonstrates a higher GAG affinity than the free IGFBP2. The presented IGF2/IGFBP2 model may give us an alternative mechanism for explaining the high IGF activity of the complex. In this model, GAG binding induces a conformational change in the IGF2/IGFBP2 complex, exposing the partially buried IGF1R-binding residues of IGF2. Thus, the IGF2/IGFBP2 complex is targeted to cell surface by cell surface GAG binding, and a ternary complex of IGF2, IGFBP2, and GAGs can activate nearby IGF1R.

The pivotal role of the IGF2 HBD and its central position in the suggested IGF2/IGFBP2 combined heparin binding surface would support the latter interpretation, as would the decreased heparin-binding affinity of the free IGFBP2 compared to the IGF2/IGFBP2 complex. Finally, this mode of interaction would also leave IGF2 protected from IGF2R inactivation during the signaling event.

A recent study by Kuang *et al.* (2007) has suggested that heparin interacts with IGFBP2 and IGF1 to form larger complexes rather than leading to dissociation of the IGF1/IGFBP2 complex.

The proposed structural model suggests that IGF2/IGFBP2 GAG binding would further bury the adjacent IGF2 residues involved in IGF2R binding, indicating that IGF2/IGFBP2 GAG binding potentially plays a role in protecting IGF2 from clearance by IGF2R-mediated endocytosis (Bayes-Genis *et al.* 2000, Williams *et al.* 2007).

While the heparin affinity column method inherently focuses on heparin, it should be noted that this polysaccharide is only one representative of the extremely variable GAGs. Heparin also has a very high negative charge density, and less sulfonated GAGs would elute at a lower κ_r . The binding surface will accommodate similar sulfonated polysaccharides in accordance with the potential regulatory role of ECM GAG composition. In support of this view, our investigation of the IGF2/IGFBP2 heparin affinity dependence on the pH demonstrated that adding or removing a few charges by changing the protonation state of His residues can significantly alter the GAG affinity. These results were in excellent agreement with a recent study by Kuang *et al.* (2006) that also, using nuclear magnetic resonance (NMR)-followed sucrose octasulfate titration, identifies pH-dependent protonation states of the IGFBP2 C-terminal domain HBD His residues. Hence, the pH of tissue ECM is a possible regulatory parameter of GAG affinity. Furthermore, cell type-specific GAGs show great structural variability and may differ in response to IGFBP2. Interestingly, Shen *et al.* has recently demonstrated an IGF1-independent GAG-binding activity for the linker-HBD in vascular smooth muscle cells. In this study, IGFBP2 was shown to bind to the proteoglycan receptor protein tyrosine phosphatase β via the linker-HBD independent of IGF1 binding. This binding in turn enhanced IGF1-stimulated vascular smooth muscle cell migration and proliferation (Shen *et al.* 2012).

Our results challenge some of the conclusions drawn from previous studies utilizing isolated IGFBP2 domains, and in doing so emphasize the importance of analyzing protein–protein and protein–GAG interactions in an integrated manner. Further studies will be needed to delineate the underlying molecular mechanism of the suggested IGF1R activation by the IGF2/IGFBP2/GAG ternary complex. The heparin-binding mechanism delineated in this study may lay the foundation for therapeutic intervention of IGFBP2-associated diseases, such as certain types of cancer, using heparin or heparin analogs for prevention of IGFBP2-mediated IGFR1 activity. Knowledge of the molecular details of GAG binding may also spur development of potential new anabolic therapies targeting bone ECM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0184>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

C A C and M T O conceived and designed the study. J L and M T S performed experiments. J L, M T S, and M T O analyzed data. All authors wrote the manuscript.

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