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IGFBP-3 and IGFBP-5 associate with the cell binding domain (CBD) of fibronectin

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ABSTRACT

We have used Surface Plasmon Resonance (SPR) – based biosensor technology to investigate the interaction of the six high affinity insulin-like growth factor binding proteins (IGFBP 1–6) with the cell binding domain (CBD) of fibronectin. Using a biotinylated derivative of the ninth and tenth TypeIII domains of FN ($^{9-10}$FNIII), we show that IGFBP-3 and -5 bind to FN-CBD. We show that this binding is inhibited by IGF-I and that, for IGFBP-5, binding occurs through the C-terminal heparin binding domain of the protein. Using site-directed mutagenesis of $^{9-10}$FNIII, we show both the "synergy" and RGD sites within these FN domains are required for maximum binding of both IGFBPs. We discuss the possible biological consequences of our results.

Introduction

IGFBP-5 is an important regulator of the activity of both insulin-like growth factors (IGF-I and IGF-II) [1]. The affinity of IGFBP-5 for both IGFs exceeds that of cell surface IGF-I and IGF-II receptors (IGF-IR and IGF-IIR) and as such most IGFs are sequestered in binding complexes with IGFBP-5 and other high affinity IGFBPs [2]. This disparity in affinity of IGFBPs and IGFRs for IGFs requires the proteolysis of IGFBPs to release IGFs and allow binding to cell surface receptors [3]. IGFBP-5 and IGFBP-3 bind to other biomolecules such as components of the extracellular matrix – including heparin, osteopontin, PAI-1 and vitronectin [4–7]. Previous yeast two-hybrid studies have suggested that the interaction between IGFBP-5 and FN is mediated via the C-terminal 10th–11th Type I domains of FN [8].

In this study we have used Surface Plasmon Resonance (SPR) biosensor technology to further characterise the interaction of IGFBP-5 and IGFBP-3 with FN. In contrast to previous studies, we demonstrate conclusively that both IGFBP-3 and -5 interact with the FN cell binding domain (CBD), comprising the 9th–10th FN Type III domains ($^{9-10}$FNIII), which harbour the integrin α5β1-binding synergy (PHSRN) and RGD sites, respectively [9]. Using C-terminal mutants we show that residues within the heparin binding domain of IGFBP-5 are important for binding, although there exist subtle differences in the residues which are required for interaction with $^{9-10}$FNIII compared with those required for heparin binding. In contrast to previous studies we demonstrate that binding of IGFBP-3 and -5 to $^{9-10}$FNIII is inhibited following co-incubation with IGF-I. RGD peptide had little effect on the binding of either IGFBP-3 or -5 to $^{9-10}$FNIII, although mutagenesis within the PHSRN or RGD motifs of $^{9-10}$FNIII inhibited IGFBP binding. We discuss the possible biological consequences of our data in relation to regulation of IGF activity in the pericellular environment.

Materials and methods

Materials. Mouse (m) IGFBP-5 expression, mutagenesis and purification has been described previously [10,11]. mIGFBP-1, mIGFBP-2, mIGFBP-3, hIGFBP-4, and mIGFBP-6 were supplied by R&D Systems (Abingdon, UK) The $^{9-10}$FNIII wild type cDNA cloned into pRSET was from Prof. H. Mardon, University of Oxford. Mutation of the $^{9-10}$FNIII cDNA template as directed by the amino acid substitutions described in Table 1 was made following the Quikchange™ protocol (Stratagene, Amsterdam, Netherlands). The $^{9-10}$FNIII construct used in this study was a stable mutant (substituting Pro^{1408} for Leu as described in [12]) extended at the C-terminus with a GGC tripeptide [13]. Using this $^{9-10}$FNIII construct as a template, and showing only the sense strand, $^{9-10}$FNIII-PHAAA was achieved using 5′-GAA GAT CGG GTG CCC CAC GCT GCG GCT TCC ATC ACC CTC ACC AAC C; $^{9-10}$FNIII-KGD was achieved using 5′-GTG TAT GCT GTC ACT GGC AAA GGA GAC AGC CCC GCA AGC; and $^{9-10}$FNIII-GG was achieved using 5′-CCC ATT GAT TGG CCA ACA ATC AAC AGG TGG CGT TTC TGA TGT TCC GAG GGA CC (mutations underlined). The $^{9-10}$FNIII proteins were biotinylated via the sulfhydryl group of the C-terminal cysteine with PEO_2-maleimide activated biotin (Pierce, UK) according to the manufacturer's

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Table 1
Average affinity and R_{max} values were obtained by non-linear regression of analyte concentration v Req as described in Methods section. IGFBP-3 and -5 were analysed over the concentration range 0–100 nM, in duplicate with randomised injection. The substitution levels for the $^{9-10}$FNIII ligands are reported in the Methods section. This experiment was repeated three times and the values shown are mean ± SD. Differences in average affinity and R_{max} values are indicated by different letters, $p < 0.05$.

	BP-3	BP-5
Average equilibrium affinities (nM)		
Fn^{9-10}	50 ± 13	17 ± 3.9
Fn KGD	27 ± 8	9 ± 1.9
FnPHAAA	37 ± 4.2	10 ± 1.8
FN GG	34 ± 6	15 ± 1.6
R_{max}		
Fn^{9-10}	489 ± 32 a	825 ± 64 a
Fn KGD	358 ± 31 b	190 ± 12 b
FnPHAAA	248 ± 13 c	131 ± 9 c
FN GG	301 ± 30 b	172 ± 16 b

recommendations Biotin incorporation was estimated using the HABA (4′-hydroxyazobenzene-2-carboxylic acid) method (Pierce, UK).

Methods. Binding of IGFBP to streptavidin (SA)-immobilised biotinylated ligand using Biosensor 3000 instrumentation has been described previously [14]. For binding biotinylated $^{9-10}$FNIII ligand, 10 μg/ml ligand in HBS-EP buffer was applied to the surface of SA coated biosensor chips to provide substitution densities between 50 and 500 resonance units (RUs) (50–500 pg mm^{-2}) of protein. Wt and mutant IGFBPs as analyte were present at 0–100 nM and were passed at a flow rate of 30 μl min^{-1} across $^{9-10}$FNIII derivatised biosensor chips. Association, dissociation and regeneration conditions were described previously [14]. The stoichiometry of IGFBP–$^{9-10}$FNIII interaction is unknown and an average affinity for this interaction was therefore derived by non-linear regression analysis of equilibrium binding of at different analyte concentrations as described previously [14,15]. For IGF competition experiments IGFBP-3 and -5 were co-incubated with 10 μM IGF-I overnight at 4 °C prior to analysis. The effect of mutagenesis of $^{9-10}$FNIII was investigated by immobilisation of biotinylated $^{9-10}$FNIII proteins at the following levels –$^{9-10}$FNIII = 603 RUs; $^{9-10}$FNIII-KGD = 560RUs; $^{9-10}$FNIII-PHAAA = 610 RUs; $^{9-10}$FNIII-GG insert = 548 RUs. Wt IGFBP-3 and -5 were present at 100 nM and were injected (5×) at 30 μl min^{-1} for 5 min. Binding data are presented as the ratio IGFBP bound/ligand substitution level.

Statistics. Analysis was performed with GraphPad Prism™ using repeated measures ANOVA followed by post hoc Tukeys' *t*-test. Differences were considered significant at $p < 0.05$.

Results

Fig. 1 indicates that of the six IGFBPs only IGFBP-3 and IGFBP-5 bind to SA-immobilised biotinylated $^{9-10}$FNIII. In these and replicate experiments, IGFBP-5 appears to bind to a greater extent than IGFBP-3 and this may be a function of the faster association rate for the former binding protein. The right hand column shows sensorgrams generated following co-incubation of IGFBPs with IGF-I and indicates that IGF-I inhibits binding of both IGFBP-3 and -5 to $^{9-10}$FNIII is reduced.

In Fig. 2 we show the results of the binding of a panel of IGFBP-5 mutants to immobilised $^{9-10}$FNIII and compare this with data obtained previously for binding of IGFBP-5 mutants to SA-immobilised biotinylated heparin, wherein positively charged residues within the 201–218 region of IGFBP-5 were shown to be important [11]. Here, initial studies indicated that a mutant IGFBP-5 lacking the C-terminal domain (residues 1–168) did not bind to immobilised $^{9-10}$FNIII (data not shown). Although cumulative mutagenesis of positively charged residues within the C-terminal 201–218 region of IGFBP-5 result in decreased binding to $^{9-10}$FNIII (see Fig. 2), it is clear that for $^{9-10}$FNIII, only more highly mutated

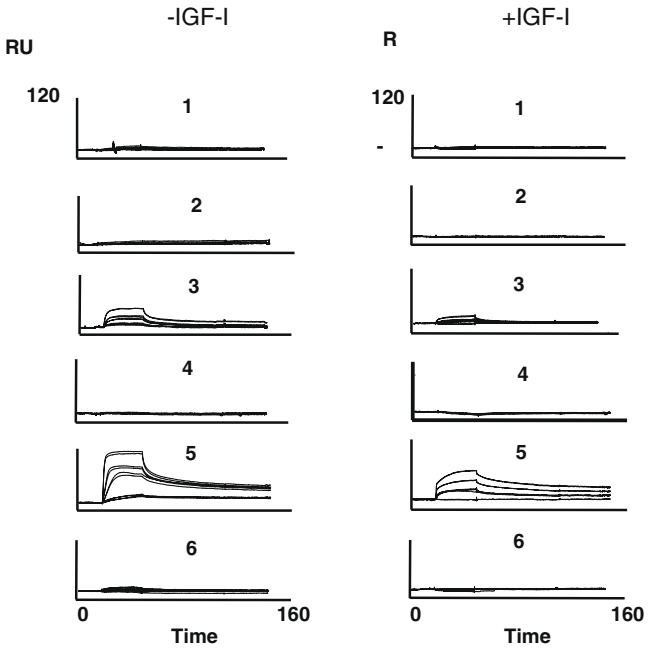

Fig. 1. IGFBP-3 and -5 bind $^{9-10}$FNIII in an IGF-I displaceable manner. IGFBPs 1–6 were analysed at 0, 6.25, 12.5, 25, 50 and 100 nM against 88 RUs of biotinylated $^{9-10}$FNIII. Injections were performed in a randomised order and in duplicate for each IGFBP concentration. The control flow cell contained 114 RUs BSA and the response in this cell was subtracted automatically. Responses for zero analyte have also been subtracted. Association and dissociation were for 3 and 15 min, respectively. In competition experiments IGF-I was present at 10 μM.

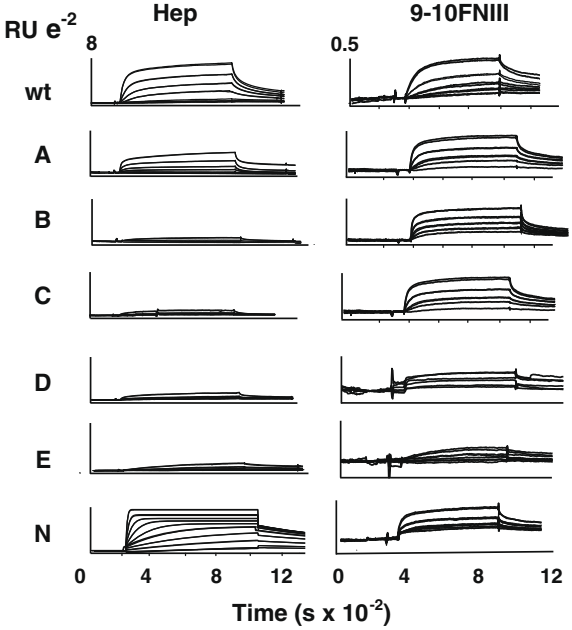

Fig. 2. Binding of IGFBP-5 mutants to heparin and $^{9-10}$FNIII. Wt and mutant IGFBP-5 proteins (concentrations as for Fig. 1) were analysed against 524 RUs of $^{9-10}$FNIII. Control flow cells contained 488 RUs BSA. Injection of wt and mutant proteins were randomised and duplicated. Response in control flow cells were subtracted automatically and responses for zero analyte have also been subtracted. Association and dissociation were for 8.3 and 15 min, respectively. For clarity only the first 2 min of the dissociation phase are shown.

IGFBP-5 proteins show significantly compromised binding. Therefore, although IGFBP-5 mutants B and C have greatly compromised binding to heparin, they retain almost wild type affinity for $^{9-10}$FNIII. Mutants D and E show compromised binding to $^{9-10}$FNIII, similar to results previously observed for heparin binding [11].

We next examined the binding of IGFBP-3 and -5 to mutant $^{9-10}$FNIII proteins. Preliminary experiments indicated that isolated ^{9}FNIII and ^{10}FNIII domains did not inhibit IGFBP-3/5 binding to immobilised $^{9-10}$FNIII (data not shown). Similarly RGD peptide did not affect binding of either IGFBP-3 or IGFBP-5 to immobilised $^{9-10}$FNIII. However, mutagenesis of the RGD motif to KGD ($^{9-10}$FNIII-KGD) impaired the ability of both IGFBP-3 and IGFBP-5 to bind to $^{9-10}$FNIII (21% and 75%, respectively – see Fig. 3). Mutation of the synergy site ($^{9-10}$FNIII-PHAAA) impaired the ability of both IGFBP-3 and -5 to bind to $^{9-10}$FNIII (48% and 85%, respectively). In addition, increasing the distance between the ^{9}FNIII and ^{10}FNIII domains via insertion of two glycine residues in the linker region ($^{9-10}$FNIII-GG, having intact RGD and synergy sites) decreases the binding to IGFBP-3 and -5 (by 33% and 77%, respectively). Equilibrium binding affinity analysis conducted over a range of IGFBP concentrations (0–100 nM), revealed no significant differences in the average affinities of the fibronectin constructs for BP-5 or BP-3 – see Table 1. This suggests that the ratio of the rate constants ($K_a/K_d = K_D$) is similar for the interaction of all the $^{9-10}$FNIII proteins with IGFBP-5. However the stoichiometry of interaction between $^{9-10}$FNIII proteins and IGFBP-5 is unknown and further investigations are required to confirm these findings.

Discussion

Our initial experiments clearly demonstrated that of the six well characterised IGFBPs, only IGFBP-3 and IGFBP-5 bound to $^{9-10}$FNIII (Fig. 1). In addition we show that pre-incubation with a molar excess of IGF-I inhibits the interaction of both IGFBP-3 and -5 with immobilised $^{9-10}$FNIII. In this respect this data is similar to our previous findings which reported binding of IGFBP-3 and -5 to immobilised heparin [14] Two earlier studies [8,16] have used a yeast two hybrid methodology to demonstrate the binding of IGFBP-3 and IGFBP-5 to various FN fragments. Gui and Murphy [16] initially isolated a clone encoding the cell binding domain (CBD), heparin binding domain (HBD) and fibrin binding domain (FBD) from the C-terminal region of FN which bound both glycosylated (g) and non-glycosylated (ng) IGFBP-3. Further analysis using commercially available FN fragments indicated that IGFBP-3 bound to a 40 kDa C-terminal fragment of FN encompassing the HBD and FBD. Although these authors showed that this binding could be competed by soluble heparin they noted that this may simply be due to heparin association with the HBD of IGFBP-3 [4] and they were therefore unable to assign the IGFBP-3 binding region of this FN fragment to either (or both) the HBD or FBD. In contrast to our studies, they reported that IGFBP-3 did not bind to a commercially available 120 kDa FN fragment which contained the CBD present in $^{9-10}$FNIII. However, the blotting and solid phase binding methods used in this study to monitor binding (where ligand is removed in repeated washing) may not detect those complexes which display high dissociation rates. Examination of Fig. 1 shows that both IGFBP-3 and -5 show, at least qualitatively, rapid dissociation from immobilised $^{9-10}$FNIII. This is confirmed in so far that the K_D reported for interaction between IGFBP-3 and the 40 kDa FN fragment (0.3 nM) is 150-fold lower than that reported for IGFBP-3 binding to immobilised $^{9-10}$FNIII (see – Table 1). Alternatively it is well known that the conformation of the 9th and 10th TypeIII domains within the CBD of FN is critical for interaction with cognate ligands [12] and using a mutagenesis strategy we have confirmed that this is the case for IGFBP-3 and IGFBP-5 binding to $^{9-10}$FNIII (see Fig. 3). It may be that these criteria are not met in assays involving the 120 kDa FN fragment analysed previously [16] and this, together with the other arguments outlined above may explain the discrepancy between our results and those reported previously. This is an area which requires further investigation.

A yeast two hybrid strategy has also indicated that IGFBP-5 bound to FN fragments [8]. In this instance a mutagenic strategy was used to identify clones containing the 10th and 11th Type I FN domains bound to IGFBP-5. These domains form the greater part of the extreme C-terminal FBD of FN and as the 40 kDa IGFBP-3 binding fragment also contains this domain it is interesting to hypothesise that this site binds both IGFBP-3 and IGFBP-5. Indeed IGFBP-5 was shown to be an effective competitor of IGFBP-3 binding to full length FN [16]. Whether this competition occurs at the C-terminal FBD requires further investigation.

In these earlier studies it was shown that IGF-I was unable to displace IGFBP-3 or -5 from the full length FN protein. Although it is not possible from studies with full length FN protein to identify which region of FN bound to IGFBP-3/5, we clearly demonstrated that pre-incubation with IGF-I displaced IGFBP-3 and -5 from $^{9-10}$FNIII (see Fig. 1). We have indicated above that this may be due to affinity differences in the interaction of different FN domains with IGFBPs although it may be that these IGFBP-3 and -5 bind via different determinants to the separate heparin/fibrin and CBD domains in FN. Indeed it is possible that the full length fibronectin molecule can bind IGFBP-3 and -5 at both domains simultaneously and that these domains may have differing affinities for the IGFBPs. Our data suggest that IGFBP-3 and -5 bind to $^{9-10}$FNIII fibronectin domains with an affinity (10–50 nM see Table 1) which is 100-fold less than the affinity of both IGFBPs for IGF-I This may account for the different results with respect to IGF-I displaceable binding of IGFBP reported in previous studies [8,16].

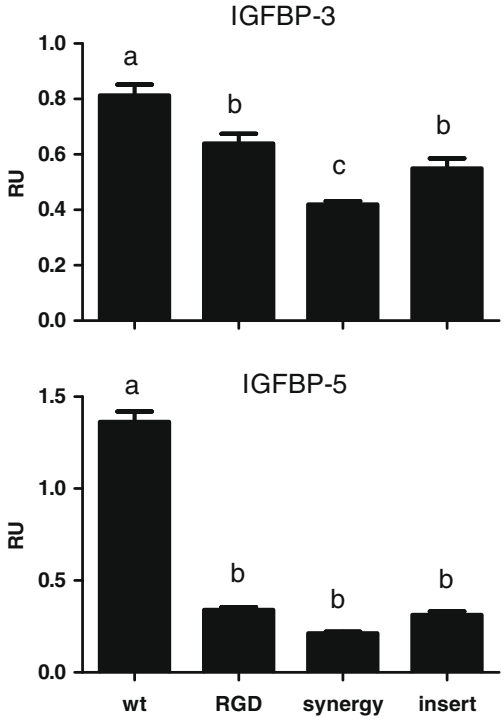

Fig. 3. Binding of IGFBP-3 and -5 to immobilised $^{9-10}$FNIII domain (FN 9–10), KGD, PHAAA and GG insert mutants. For details of $^{9-10}$FNIII mutants see methods section. IGFBP-3 or -5 was injected at 100 nM (5×) across SA biosensor chips derivatised with biotinylated $^{9-10}$FNIII or mutants as described in Methods section. Binding data are evaluated as the ratio bound analyte/immobilised ligand. Mean ± SD ($n = 5$) for each protein. Data are analysed with repeated measures ANOVA and post hoc Tukeys' t-test. Columns with different superscripts indicate significance at $p < 0.05$.

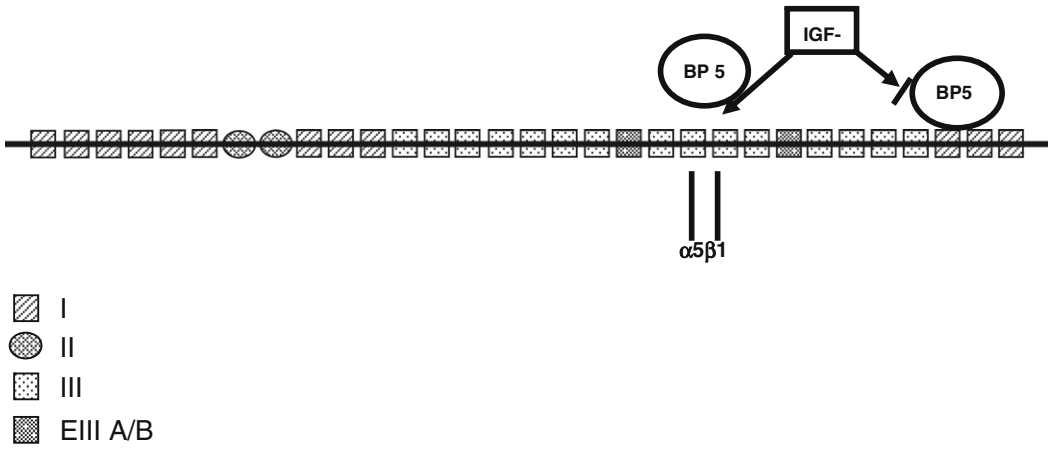

Fig. 4. Putative association of IGFBP-5 with FN. IGFBP-5 is shown binding, in an IGF-I displaceable manner to the 9th and 10th TypeIII Fn domain. This domain also contains the cell binding domain and is shown occupied with α5β1 integrin. IGFBP-5 is also shown associated with the 10th and 11th Type I domains which form part of the fibrin binding domain of fibronectin. This interactions is not displaceable by IGF-I as reported in Ref. [8].

The results of our IGFBP-5 mutagenesis studies (Fig. 2) show different binding characteristics for IGFBP-5 interaction with $^{9-10}$FNIII and heparin. For example mutant B has greatly compromised binding to heparin but retains almost wild type activity toward $^{9-10}$FNIII. This argues that residues K207 and K211 are critical residues in binding $^{9-10}$FNIII see [11] for a description of IGFBP-5 mutants. Earlier studies have also shown a differential effect of mutagenesis within the IGFBP-5 (201–218) region on binding to various extracellular proteins [7,17]. In the context of the ECM this phenomenon may influence the availability of both IGF-I and IGFBP-5 in the pericellular environment and influence both the IGF-dependant and IGF- independent actions IGFBP-5 – reviewed in [1]. Interestingly, limited mutagenesis studies within the C-terminal region of IGFBP-3 have also demonstrated differential effects on ECM and cell binding [18,19] and a more comprehensive analysis of IGFBP-3 mutagenesis and subsequent ECM interactions seems warranted.

Our data also suggest that the structural integrity of the $^{9-10}$FNIII module is important in binding to IGFBP-3 and IGFBP-5. Mutations within the RGD and "synergy" sites compromised binding of both IGFBP-3 and IGFBP-5. In addition the insertion mutant "GG" also displayed greatly reduced binding to both IGFBPs. In general terms, mutagenesis of $^{9-10}$FNIII had greater effects on IGFBP-5 than IGFBP-3 binding (see Fig. 3). The structural integrity and orientation of RGD and synergy sites within the CBD of FN are also required for high affinity integrin binding [12,20,21]. We have recently used biosensor methodology to show that full length α5β1 integrin interacts with immobilised $^{9-10}$FNIII [13] and a previous biosensor based study reported a K_D of 1.7 nM for the interaction between the "headpiece" structure of α5β1 integrin and immobilised $^{7-10}$FNIII a value which was similar to that found for full length recombinant α5β1.

We believe there are many other important physiological consequences of our data which are primarily associated with the observation that IGFBP-5/3 and some cell surface integrins are both able to bind the CBD of FN. Perhaps the most obvious is the potential for both IGFBPs to displace cells from fibronectin in the ECM. Indeed, McCaig et al. [22] found that exogenous IGFBP-5 decreased the binding of the breast epithelial cell line Hs578T to FN coated surfaces. Such a process may be involved in apoptosis in breast epithelial cells wherein IGFBP-5 removes cells from adjacent extracellular matrix. In support of this, IGFBP-5 has been shown to be upregulated both in vivo and in vitro during cellular apoptosis in the mammary gland [23,24] In this fashion, IGFBP-5 may exhibit a dual apoptotic function by neutralising the cell survival factor – IGF-I – and by disrupting cell interaction with extracellular matrix.

A complicating factor in this model are the numerous reports of the IGF-enhancing activity IGFBP-5 and -3. Such experiments have usually been performed in tissue culture and have been shown to be dependant on cell type and specific culture conditions [5]. In these circumstances, association of IGFBPs with ECM may provide a low affinity sink for IGFs. It could be postulated that displacement of IGFBPs by IGFs from ECM binding sites, for example the CBD of FN, may render the IGFBPs more susceptible to proteolysis, in turn releasing IGFs to act at cell surface receptors. Experiments to examine this hypothesis are currently underway in our laboratories.

We have used the data from this and previous studies to construct a possible model for the interaction of IGFBP-5 with FN (see Fig. 4).

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