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Differential dissociation kinetics explain the binding preference of insulin-like growth factor binding protein-6 for insulin-like growth factor-II over insulin-like growth factor-I

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Abstract Insulin-like growth factor binding protein-6 binds insulin-like growth factor-II with a marked preferential affinity over insulin-like growth factor-I. The kinetic basis of this binding preference was studied using surface plasmon resonance. Binding of insulin-like growth factor-I and insulin-like growth factor-II to immobilized insulin-like growth factor binding protein-6 fitted a two-site binding kinetic model. Insulin-like growth factor-I and insulin-like growth factor-II association rates were similar whereas the dissociation rate was ~60-fold lower for insulin-like growth factor-II, resulting in a higher equilibrium binding affinity for insulin-like growth factor-II. The equilibrium binding affinities of a series of insulin-like growth factor-II mutants were also explained by differential dissociation kinetics. *O*-glycosylation had a small effect on the association kinetics of insulin-like growth factor binding protein-6. The insulin-like growth factor binding properties of insulin-like growth factor binding protein-6 are explained by differential dissociation kinetics.

Key words: Insulin-like growth factor binding protein-6; Biosensing technique; Kinetic; Glycosylation

1. Introduction

Insulin-like growth factors (IGF-I and IGF-II) are important mediators of physiological growth [1]. Their actions are modulated by a family of six structurally related, high affinity binding proteins (IGFBPs 1–6), which may enhance or inhibit IGF actions under different conditions [1,2]. IGFBP-6 has the highest binding affinity of the IGFBPs for IGF-II [1,2]. It differs from the other IGFBPs in that it binds IGF-II with a 20–100-fold higher affinity than IGF-I [3–7]. IGFBP-6 is therefore a relatively specific inhibitor of IGF-II actions [6,8,9]. However, the kinetic basis of the IGF-II binding preference of IGFBP-6 has not been previously studied.

Surface plasmon resonance (SPR) technology allows real-time measurement of binding between a ligand in solution which is passed over a sensor chip to which an interacting molecule is immobilized [10]. Using this technology, both association and dissociation kinetics can be determined. The kinetics of IGF binding to IGFBPs other than IGFBP-6 have been previously determined by SPR [11–14] and some of these studies suggested two-site binding kinetics [11,12]. IGFBP-3 had high and low affinity binding sites for IGFs, where the high affinity site accounted for 90% of IGF binding [11]. Binding of IGFBP-2 to immobilized IGF-II fitted a two-site model, with two association and two dissociation rates [12]. In contrast, IGFBP-2 bound immobilized IGF-I with single-site association and dual-site dissociation kinetics. These findings suggest a more complex interaction between IGFs and IGFBPs than previously believed.

In this study, SPR was used to investigate the kinetic basis for the binding preference of IGFBP-6 for IGF-II over IGF-I. Kinetics of binding of IGF-II mutants with decreased binding affinities for IGFBP-6 [7] were also studied. Further, IGFBP-6 is *O*-glycosylated and we have previously shown that enzymatic deglycosylation of IGFBP-6 had no effect on the IGF binding, measured by solution competition studies with [^{125}I]IGF-II [15]. The role of *O*-glycosylation of IGFBP-6 was further studied by comparing the kinetics of IGF binding to glycosylated and non-glycosylated IGFBP-6.

2. Materials and methods

2.1. IGFBPs

Recombinant human glycosylated IGFBP-6 (gIGFBP-6) was expressed in A293 kidney fibroblasts stably transfected with the mammalian expression vector, phBP6-E3 [8], using lipofectamine (Life Technologies, Glen Waverley, Australia) [16]. gIGFBP-6 was purified to homogeneity by IGF-II affinity chromatography and reverse-phase FPLC as previously described [16,17]. The identity and purity of gIGFBP-6 were confirmed by N-terminal Edman sequencing.

Non-glycosylated IGFBP-6 (n-gIGFBP-6) was expressed as a soluble GST fusion protein in *Escherichia coli* transformed with pGex-2T (Pharmacia, Piscataway, NJ, USA) [17]. The fusion protein was bound to a glutathione-agarose column and n-gIGFBP-6 was cleaved from the column with thrombin. n-gIGFBP-6 was further purified by IGF-II affinity chromatography and reverse-phase FPLC. Identity and purity were confirmed by electrospray ionization mass spectrometry as previously described [17].

2.2. SPR analysis of interactions between IGFs and IGFBP-6

SPR analysis was performed using the BIAcore 2000 system (BIAcore AB, Uppsala, Sweden). gIGFBP-6 and n-gIGFBP-6 were immobilized by amine coupling onto separate channels on a CM5 sensor chip (BIAcore AB) as described below. The geometry of the CM5 sensor chip allows the same IGF sample in solution to sequentially flow over each IGFBP preparation and a control channel, to which no IGFBP was immobilized. The control channel was included to measure differences in the bulk refractive index due to changes in buffers.

Prior to immobilization of IGFBP-6, the carboxy-methylated dextran surface of the CM5 chip was activated by exposure to equal volumes of 0.39 M *N*-ethyl-*N*′-(3-diethyl-aminopropyl) carbodiimide and 0.1 M *N*-hydroxysuccinimide. gIGFBP-6 or n-gIGFBP-6 (10 μg/ml) in HBS buffer (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 0.005% P20 detergent) or HBS buffer alone (control) were injected onto separate channels of the activated surface (10 μl/min, 20°C). Residual active coupling groups were inactivated by exposure to 1 M ethanolamine. Following immobilization, the chip was washed for 30 min with HBS buffer. The net increase in the signal (682 and 1642 resonance unit

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(RU) of gIGFBP-6 and n-gIGFBP-6, respectively, where 1000 RU is approximately equivalent to 1 ng of protein/mm^2) represents the amount of immobilized protein.

IGF-I and IGF-II (1.31–134 nM, kind gifts from Lilly, Indianapolis, IN, USA) in HBS were passed over the chip at 10 μl/min for 10 min ($n=4$ for each IGF). A series of IGF-II mutants ([R^{54}, R^{55}]IGF-II, [L^{27}]IGF-II and [T^{48}, S^{49}, I^{50}]IGF-II, generously provided by Dr K. Sakano (Daiichi Pharmaceuticals, Tokyo, Japan)) with differential binding affinities for IGFBPs as measured by a solution competition assay [7] was also studied as above ($n=2$ for each mutant). Following injection of each of the IGFs, the kinetics of dissociation were evaluated by passing HBS buffer alone over the chip at 10 μl/min for 10 min. After each run, the chip was regenerated with 0.01 M HCl to elute the remaining bound IGFs. All experiments were performed at 20°C.

Similar binding kinetics were observed at flow rates of 20 μl/min and 50 μl/min (results not shown), suggesting minimal effects of mass transport limitation on binding of IGFs to IGFBPs [10].

2.3. Data analysis

Kinetic constants were derived using BIAevaluation software version 2.1 (BIAcore AB). All binding curves were corrected for background by the subtraction of the signal obtained from the control channel. Apparent kinetic constants were generated by fitting both the experimental association and dissociation curves separately to either a one-site and two-site model. Experimental data for each ligand best-fitted a two-site model. The relative contribution of each binding site to the total binding was calculated and is described as a weighting factor. Data are shown as mean ± S.E.M. of four runs for IGF-I and IGF-II and two runs for the IGF-II analogues.

Equilibrium association constants (K_a) were calculated using the weighted average of the two association rates ($k_{on(app)}$) as described by Hobba et al. [12]. The contribution of the second dissociation rate (k_{off2}) to the total dissociation was always less than 15% and therefore did not substantially affect the total dissociation. The equilibrium association constant was therefore defined as $k_{on(app)}/k_{off1}$ [12].

2.4. Solution assays

Competitive binding studies were carried out as previously described [7]. IGFBPs were incubated with [^{125}I]IGF-II (2×10^4 cpm, specific activity 168 μCi/μg) ± unlabelled IGF-I (0.0013–10.5 nM), IGF-II (0.0067–10.3 nM) or IGF-II mutants (0.001–11 nM) for 18 h at 4°C in 0.1 M $NaPO_4$/0.1% bovine serum albumin (BSA)/0.02% Na azide (pH 7.4). Free tracer was removed by incubation with ice-cold 5% charcoal, 2% BSA in Dulbecco's phosphate buffered saline, followed by centrifugation and counting of bound radioactivity in the supernatant. Points were measured in duplicate. IGF binding affinities were determined using Prism software (GraphPad, San Diego, CA, USA).

3. Results

3.1. Dissociation kinetics explain the IGF-II binding preference of IGFBP-6

Previous studies using solution binding assays have shown that IGFBP-6 has a 20–100-fold preferential binding affinity for IGF-II over IGF-I [3–7]. Similarly, gIGFBP-6 bound IGF-II with a 67-fold IGF-II binding preference as assessed

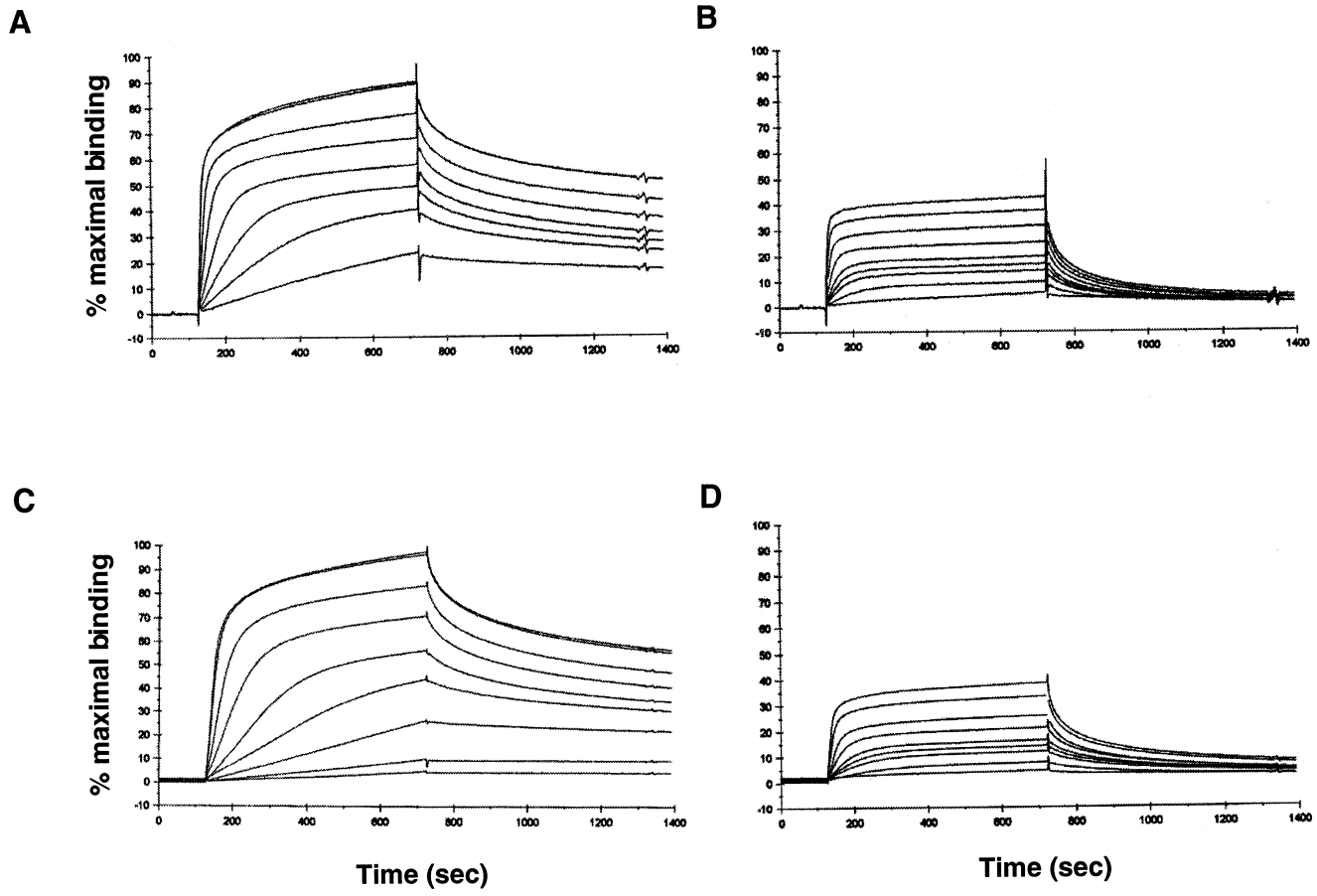

Fig. 1. Sensorgrams of IGF-II (A, C) and IGF-I binding (B, D) to glycosylated (A, B) and non-glycosylated IGFBP-6 (C, D). IGF-II (1.34, 2.68, 6.69, 13.4, 26.8, 53.5, 107 and 134 nM from bottom to top curve) or IGF-I (1.31, 2.61, 6.54, 13.1, 26.1, 52.3, 105 and 131 nM from bottom to top curve) were passed over immobilized IGFBP-6 for 10 min (association phase). The buffer flow was then switched to HBS for 10 min (dissociation phase). Sensorgrams were normalized so that maximal binding of IGF-II to g or n-gIGFBP-6 was set to 100%. Maximal binding was 140 RU and 410 RU for gIGFBP-6 and n-gIGFBP-6, respectively. Results have been corrected for refractive index changes due to buffer change-over.

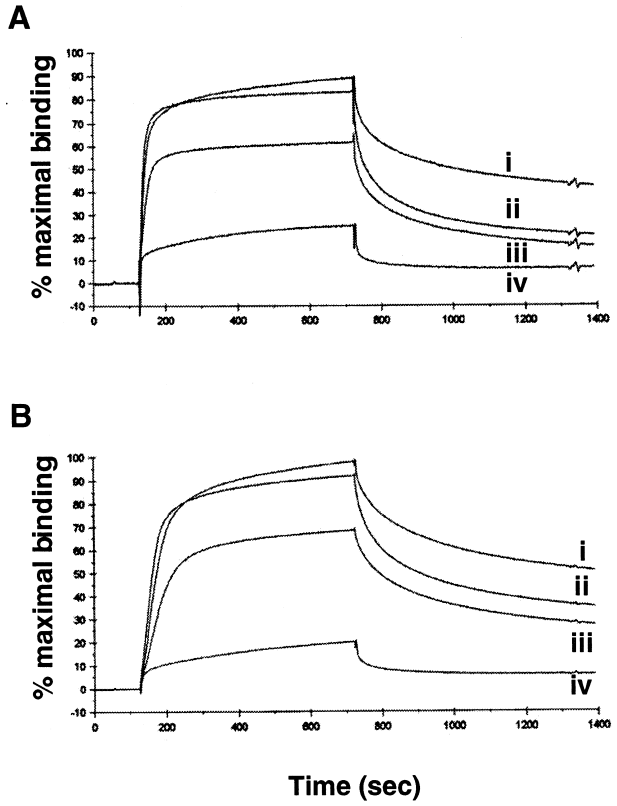

Fig. 2. Sensorgrams of binding of IGF-II and IGF-II mutants to IGFBP-6. Shown are normalized sensorgrams for the interaction of gIGFBP-6 (A) and n-gIGFBP-6 (B) with 50 nM of (i) IGF-II, (ii) [R^{54}, R^{55}]IGF-II, (iii) [L^{27}]IGF-II and (iv) [T^{48}, S^{49}, I^{50}]IGF-II. Sensorgrams were normalized so that maximal binding of IGF-II to g or n-gIGFBP-6 was set to 100%. Maximal binding was 120 RU and 350 RU for gIGFBP-6 and n-gIGFBP-6, respectively. Results have been corrected for refractive index changes due to buffer changeover.

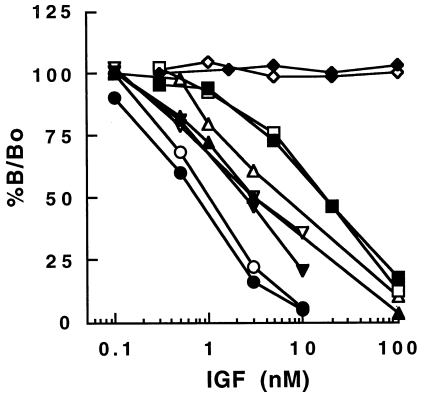

Fig. 3. A charcoal adsorption competition assay of IGF binding by glycosylated and non-glycosylated IGFBP-6. gIGFBP-6 (closed) and n-gIGFBP-6 (open) were incubated with [^{125}I]IGF-II (3×10^4 cpm/well) ± unlabelled IGF-II (circles), IGF-I (square), [R^{54}, R^{55}]IGF-II (triangles), [L^{27}]IGF-II (inverted triangles) or [T^{48}, S^{49}, I^{50}]IGF-II (diamonds). Results are shown as a percentage of specific binding in the absence of unlabelled IGF-II (% B/Bo). Total specific binding (Bo) was 12.6% and 11.7% of the total counts for gIGFBP-6 and n-gIGFBP-6, respectively. Duplicate measurements for each point differed by less than 5%. Non-specific binding was 5.8%.

by SPR analysis (Table 1). Typical sensorgrams for IGF binding to gIGFBP-6 are shown in Fig. 1.

The IGF binding kinetics of gIGFBP-6 best-fitted a two-site model. For both IGF-I and IGF-II, ~70% of the total association was due to the rapid association component (Table 1). The weighted association rates ($k_{on(app)}$) for binding of IGF-I and IGF-II to gIGFBP-6 were similar (Table 1). The difference in the equilibrium binding affinities of IGF-I and IGF-II for gIGFBP-6 was due to differing dissociation rates. The major dissociation rate (k_{off1}) was ~60-fold higher for IGF-I than IGF-II (Table 1). Although the minor dissociation rate (k_{off2}) was 10-fold lower for IGF-I than IGF-II, this accounted for less than 15% of the total dissociation.

Fig. 2 shows a typical sensorgram for binding of IGF-II and IGF-II mutants, [L^{27}]IGF-II, [R^{54}, R^{55}]IGF-II and [T^{48}, S^{49}, I^{50}]IGF-II, to IGFBP-6. The A-domain mutant, [R^{54}, R^{55}]IGF-II, has previously been shown to have a ~5-fold lower binding affinity than IGF-II for IGFBP-6 using solution assays [7]. The B-domain mutant, [L^{27}]IGF-II, has a ~3-fold reduced binding affinity for IGFBP-6 [7], whereas [T^{48}, S^{49}, I^{50}]IGF-II does not substantially bind to IGFBP-6 [7]. Using SPR, the order of maximal binding was IGF-II > [R^{54}, R^{55}]IGF-II > [L^{27}]IGF-II > [T^{48}, S^{49}, I^{50}]IGF-II. Although the rapid association constant for [T^{48}, S^{49}, I^{50}]IGF-II was 3-fold higher than that of IGF-II, it only accounted for 33% of the total association compared with 62–85% for IGF-I, IGF-II and other mutants (Table 1). The weighted apparent association rates of the three IGF-II mutants were similar to or slightly greater than that of IGF-II (Table 1). However, the equilibrium binding constants for all three mutants were lower than that of IGF-II because of a more rapid dissociation (Table 1). This was especially apparent for [T^{48}, S^{49}, I^{50}]IGF-II which had dissociation kinetics similar to those of IGF-I. Similarly to IGF-I, the differential binding affinities of IGF-II mutants for gIGFBP-6 are therefore determined by differential dissociation rates.

Table 1
Binding kinetics of glycosylated IGFBP-6

	$k_{on1}\times10^5$ (M^{-1}/s)	$k_{on2}\times10^5$ (M^{-1}/s)	$k_{on(app)}\times10^5$ (M^{-1}/s)	Weighting factor[a]	$K_{off1}\times10^{-3}$ (s^{-1})	$k_{off2}\times10^{-3}$ (s^{-1})	K_A (nM^{-1})[b]	Relative affinity (% of IGF-II)
IGF-I	8.9 ± 1.3	0.30 ± 0.04	5.6 ± 0.8	0.62	20.3 ± 0.8	1.1 ± 0.1	0.028 ± 0.003	1.5
IGF-II	9.3 ± 0.4	0.31 ± 0.00	6.3 ± 0.3	0.67	0.34 ± 0.01	11.6 ± 0.1	1.88 ± 0.16	100
[R^{54}, R^{55}]IGF-II	13.6 ± 2.5	1.3 ± 0.2	11.3 ± 1.7	0.82	0.90 ± 0.20	15.6 ± 0.4	1.29 ± 0.08	69
[L^{27}]IGF-II	9.2 ± 0.8	1.0 ± 0.2	8.0 ± 0.6	0.85	0.94 ± 0.01	13.8 ± 0.6	0.85 ± 0.08	45
[T^{48}, S^{49}, I^{50}]IGF-II	29.7 ± 1.0	0.72 ± 0.01	10.1 ± 2.6	0.33	11.9 ± 3.9	0.2 ± 0.1	0.085 ± 0.006	4.5

[a]Weighting factor indicated the proportion of total binding due to k_{on1} (where total binding = 1).
[b]$K_A = k_{on(app)}/k_{off1}$ since k_{off2} contributed less than 15% to the total dissociation in all instances.

Table 2
Binding kinetics of non-glycosylated IGFBP-6

	$k_{on1} \times 10^5$ (M^{-1}/s)	$k_{on2} \times 10^5$ (M^{-1}/s)	$k_{on(app)} \times 10^5$ (M^{-1}/s)	Weighting factor[a]	$K_{off1} \times 10^{-3}$ (s^{-1})	$k_{off2} \times 10^{-3}$ (s^{-1})	K_A (nM^{-1})[b]	Relative affinity (% of IGF-II)
IGF-I	6.5 ± 0.1	0.31 ± 0.03	4.6 ± 0.1	0.67	19.7 ± 0.0	1.3 ± 0.1	0.023 ± 0.001	4.2
IGF-II	2.8 ± 0.0	0.18 ± 0.00	2.0 ± 0.0	0.70	0.36 ± 0.01	10.6 ± 0.1	0.55 ± 0.01	100
[R^{54}, R^{55}]IGF-II	3.5 ± 2.4	0.3 ± 0.03	2.9 ± 1.5	0.80	0.68 ± 0.04	12.6 ± 1.4	0.43 ± 0.2	78
[L^{27}]IGF-II	1.2 ± 0.1	0.6 ± 0.05	1.1 ± 0.5	0.82	0.92 ± 0.01	14.4 ± 1.7	0.12 ± 0.05	22
[T^{48}, S^{49}, I^{50}]IGF-II	12.4 ± 4.3	0.42 ± 0.04	3.6 ± 0.3	0.28	15.8 ± 2.0	0.4 ± 0.1	0.022 ± 0.001	4.0

[a]Weighting factor indicated the proportion of total binding due to k_{on1} (where total binding = 1).
[b]$K_A = k_{on(app)}/k_{off1}$ since k_{off2} contributed less than 15% to the total dissociation in all instances.

3.2. *O*-glycosylation modestly increases the association kinetics of IGF-II binding to IGFBP-6

To confirm that n-gIGFBP-6 expressed in *E. coli* was correctly folded, the stoichiometry of high affinity IGF-II binding to gIGFBP-6 and n-gIGFBP-6 was compared. The stoichiometry of IGF:IGFBP-6 binding in solution is ~1:1 [4]. Assuming the same stoichiometry, ~80% of the expected binding of IGF-II to both gIGFBP-6 and n-gIGFBP-6 was experimentally observed. This result confirms the correct folding of n-gIGFBP-6 since it is likely to be required for high affinity binding. Further, this result indicates that immobilization of IGFBP-6 to the sensor chip surface did not substantially impair binding.

The association kinetics of IGF-I binding to n-gIGFBP-6 and gIGFBP-6 were similar (Fig. 1, Table 1 and 2). In contrast, the $k_{on(app)}$ of IGF-II binding to n-gIGFBP-6 was ~3-fold lower than that of gIGFBP-6 (Table 1 and 2). The dissociation kinetics of IGF binding to n-gIGFBP-6 were similar to those of gIGFBP-6 (Table 1 and 2). Because of the lower association rate of IGF-II binding to n-gIGFBP-6, the equilibrium association constant was also ~3-fold lower than that of gIGFBP-6 and the IGF-II binding preference of n-gIGFBP-6 over IGF-I was reduced to 24-fold.

Similarly to IGF-II, the association rates of IGF-II mutant binding to n-gIGFBP-6 were 3–7-fold lower than those of gIGFBP-6 whereas the dissociation rates were similar. Although the equilibrium affinity constants were therefore modestly lower for n-g than gIGFBP-6, the relative binding affinities of the IGF-II mutants and IGF-II were similar for both IGFBP-6 preparations.

3.3. Solution competition assay of IGF binding to gIGFBP-6 and n-gIGFBP-6

The results obtained using SPR were compared with competition solution assays using [^{125}I]IGF-II as tracer and the same IGF preparations used for the SPR studies (Fig. 3). The binding affinity of gIGFBP-6 for IGF-II was ~8-fold higher when measured by a charcoal binding assay compared with SPR (Table 3). Although charcoal assays were performed at 4°C and SPR at 20°C, the binding affinity of IGFBP-6 for IGF-II was unchanged when measured by the charcoal assay at 20°C (not shown), indicating that the temperature difference did not underlie the binding affinity difference.

Similarly to previous studies [3–7], IGF-I bound to gIGFBP-6 with ~25-fold lower affinity than IGF-II. [L^{27}]IGF-II and [R^{54}, R^{55}]IGF-II had 3–5-fold lower binding affinities for gIGFBP-6 than IGF-II, whereas [T^{48}, S^{49}, I^{50}]IGF-II did not bind to gIGFBP-6, which is consistent with our previous studies [7].

We have previously shown that enzymatic deglycosylation of IGFBP-6 did not affect IGF binding affinities when using [^{125}I]IGF-II as tracer in solution assays [15]. The lack of effect of *O*-glycosylation on IGF binding by IGFBP-6 using solution assays was confirmed in the present study (Fig. 3). The relative binding affinities of IGF-II mutants for n-gIGFBP-6 were similar to those for gIGFBP-6 (Table 3).

4. Discussion

Using SPR, the results of the present study show that the preferential binding affinity of IGFBP-6 for IGF-II over IGF-I is a result of the lower dissociation rate of IGF-II. Similarly, differential dissociation rates determined the binding affinities of IGF-II mutants. This suggests that, whereas association of IGFs with IGFBP-6 is relatively independent of small changes in the IGF sequence, dissociation is determined by specific amino acids in the IGF sequences. This is exemplified by the IGF-II analogue [T^{48}, S^{49}, I^{50}]IGF-II, the association rate of which was slightly higher than that of IGF-II, but which dissociated far more rapidly. In contrast to the present findings regarding IGFBP-6, differences in both association and dissociation rates accounted for the decreased binding affinities of IGF-I mutants for IGFBP-1 and the IGF-I receptor [13,18] and IGFBP-3 [11].

Significant alteration of high affinity ligand binding is a relatively uncommon consequence of protein glycosylation [19]. *O*-glycosylation slightly affected the binding of IGF-II and IGF-II mutants but not IGF-I to IGFBP-6 as assessed

Table 3
Comparison of IGF binding affinities for IGFBP-6 using a solution binding assay and SPR

	Glycosylated IGFBP-6		Non-glycosylated IGFBP-6	
	Solution (nM^{-1})	SPR (nM^{-1})	Solution (nM^{-1})	SPR (nM^{-1})
IGF-II	14.1 ± 0.1	1.88 ± 0.16	12.5 ± 0.1	0.55 ± 0.01
IGF-I	3%	1.5%	5%	4%
[R^{54}, R^{55}]IGF-II	22%	69%	17%	78%
[L^{27}]IGF-II	30%	45%	30%	22%
[T^{48}, S^{49}, I^{50}]IGF-II	0%	5%	0%	4%

Binding affinities of IGF-I and IGF-II mutants are shown as a percentage of IGF-II.

by SPR. Association rates and therefore equilibrium binding affinities of IGF-II and mutants were 3–6-fold lower for n-gIGFBP-6 than gIGFBP-6. In contrast, dissociation rates were similar for n-gIGFBP-6 and gIGFBP-6. However, IGF-II binding affinities measured with charcoal assays were similar for both gIGFBP-6 and n-gIGFBP-6 performed at either 4°C or 20°C. It is possible that more subtle changes in binding are detectable by SPR because of its inherent sensitivity. These subtle changes may also be missed in solution assays due to the need for iodinated ligand, which may have slightly altered binding properties. Alternatively, immobilization of gIGFBP-6 and n-gIGFBP-6 to the sensor chip surface may have resulted in slightly different availabilities of IGF-II binding sites on each.

The binding affinity of gIGFBP-6 measured by SPR was 8-fold lower than that measured by a solution binding assay. The difference in binding affinity cannot be explained by the SPR and solution experiments being performed at different temperatures, since the binding affinity of IGFBP-6 was unaffected by temperature using solution assays. However, it has previously been shown that the association rates of some ligand binding protein interactions are lower when measured by SPR than in solution [10].

The sensitivity of SPR allowed low affinity binding of IGFBP-6 by the IGF-II mutant [T^{48}, S^{49}, I^{50}]IGF-II to be detected. As previously reported [7], no binding of this mutant was detected by a charcoal assay. Insulin was shown to bind with low affinity to IGFBP-3 using SPR [11], despite its inability to compete with iodinated IGFs for binding to IGFBPs using solution assays. These findings suggest that SPR is a superior method for the detection of low affinity binding. However, IGF-I binding to IGFBP-6 is readily detectable in solution assays despite having a similar low binding affinity to [T^{48}, S^{49}, I^{50}]IGF-II for IGFBP-6 using SPR. Other as yet unexplained factors must also underlie these observations.

IGF bound to IGFBP-6 with two-site kinetics in the present study. It is not clear whether this indicates two distinct physical binding sites for IGFs on IGFBP-6 or complex binding kinetics on a single physical site. It is noteworthy that competition curves for binding to IGFBP-6 of unlabelled IGF-I and IGF-II are not parallel when [^{125}I]IGF-I is used as a tracer and the presence of high and low affinity binding sites on IGFBP-6 has previously been suggested on this basis [3,6]. However, the $\sim$1:1 stoichiometry of binding would suggest that IGFs do not simultaneously bind at two sites on IGFBP-6. An alternative possibility is that the presence of a second low affinity site may be a SPR artefact due to IGFBP-6 being randomly linked to the sensor chip by amine coupling, with subsequent impairment of IGF binding of a proportion of the linked molecules. This is unlikely, however, since (i) the IGFBP-6 stoichiometry was $\sim$0.8 for IGF-II (approximating 1:1 binding), (ii) the contribution of the second association site in IGFBP-6 (30–40%) was greater than that observed for IGFBP-3 ($\sim$10%) [11] and is greater than would be expected for an amine coupling effect, (iii) a previous study in which IGFs were immobilized to the sensor chip and bound by soluble IGFBP-2 also suggested two-site kinetics [12]. Artefactual multiple-site binding using SPR may be due to mass transport effects limiting the ligand delivery to the sensor surface. However, this is unlikely because the binding kinetics of IGFBP-6 were unaffected by the flow rate.

In conclusion, the binding preference of IGFBP-6 for IGF-II over IGF-I is due to a slower dissociation of IGF-II rather than a more rapid association. *O*-glycosylation of IGFBP-6 modestly increases association kinetics without affecting the dissociation. SPR enables the detection of low affinity binding of some IGF-II mutants to IGFBP-6 which is not detectable by competitive binding studies in solution. SPR analysis of IGF binding to IGFBP-6 indicates dual-site kinetics. Further studies are necessary to determine whether this reflects two distinct binding sites or complex interactions such as multiple-step binding at a single-site.

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