Critical evaluation of a theory of molecular recognition using human insulin-like-growth-factor-I fragment 21–40 and its complementary peptide

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Using solid-phase methods we have synthesized human insulin-like-growth-factor-I (IGF-I) fragment 21–40 (IGF-I 21–40) and the peptide derived from the 5' → 3' translation of the complementary nucleic acid of this peptide, 'I-FGI 20–40' (the complementary peptide). According to a recently proposed theory of molecular recognition, these two peptides should bind specifically to each other. We have tested this theory by using both solid- and solution-phase direct-binding assays for this complementary-peptide pair. We have also investigated the ability of I-FGI 20–40 to interfere with native IGF-I binding during radioimmunoassay (r.i.a.), radio-receptor (r.r.a.) assay and ligand-blot analysis of IGF-binding proteins. We have obtained no evidence of any interaction between IGF-I 21–40 and I-FGI 20–40 in either solid- or solution-phase assays. In addition, I-FGI 20–40 does not interfere in the assays used to detect IGF-I binding antibodies (r.i.a.), receptors (r.r.a.) or binding proteins (ligand blots). Our data therefore question the universality of this particular theory of molecular recognition.

INTRODUCTION

In recent years, evidence has accumulated from several laboratories in support of a 'molecular recognition code' whereby peptides encoded by complementary sequences of nucleic acids possess the ability to bind specifically and with high degrees of affinity to each other [1–5] (for a recent review see [6]). This phenomenon has been reported for the *c-raf* protein fragment 356–376 [7], the *N*-terminal 20 amino acid residues of the pro-[Arg⁸]vasopressin/bovine neurophysin II precursor [8], residues B22–B27 of insulin [9], ribonuclease-S-peptide [10] and the cell-surface-binding domain of fibronectin [11]. In each instance direct binding between peptide pairs was reported, and the term 'complementary peptides' has been employed to describe peptides which are related in this manner [12]. In addition to this evidence, the structural relationship between complementary-peptide pairs has been confirmed by the observation that antibodies raised to complementary peptides have, at their binding sites, structural similarity to the original target peptide [13–15]. For example, antibodies raised to the complementary peptide of γ -endorphin were shown to bind to the putative δ -opiate receptor of the NG 108-15 neuroblastoma × glioma hybrid cell line, thus mimicking a property of the original target peptide [16]. In total, 12 different complementary peptide pairs have been shown to interact, and using complementary-peptide methods, seven different receptor proteins have been purified [17].

Nevertheless, despite this body of supportive evidence, the area remains controversial in that some reports have questioned the universality of the theory. For example, two recent studies have found no evidence for interaction between the octapeptide angiotensin II and the corresponding complementary peptide [18,19], thus contradicting earlier observations with these two peptides [20,21]. Similarly no evidence of binding between human parathyroid hormone fragment 1–40 (hPTH 1–40) and its corresponding complementary peptide was obtained [22]. Finally, the reported inability of antibodies to the complementary peptide

of human-adrenocorticotrophic-hormone fragment 1–24 (hACTH 1–24) to recognize the ACTH receptor and the finding by the same author of no interaction between this complementary peptide pair [23] contradicts several previous reports [24–27].

Because of these conflicting data, and owing to the potential significance of the molecular-recognition theory in the areas of drug design, evolution of polypeptide ligand-receptor genes and idiotypic/anti-idiotypic antibody networks [12,28], we have re-examined this theory using human-insulin-like-growth-factor-I fragment 21–40 (IGF-I 21–40) and its complementary peptide (I-FGI 20–40). We report data on the binding of this peptide pair in solid- and solution-phase assays and of the behaviour of I-FGI 20–40 in radioimmuno- (r.i.a.) and radio-receptor (r.r.a.) assays for IGF-I. In addition, we examine the ability of I-FGI 20–40 to interfere with the interaction of ¹²⁵I-IGF-I and its soluble binding protein(s) during ligand-blot analysis.

MATERIALS AND METHODS

Peptide design, synthesis and purification

The peptides used in the present study were IGF-I 21–40 and its corresponding complementary peptide I-FGI 20–40. Fig. 1 illustrates the nucleic acid and peptide sequences of IGF-I 21–40 and the derived nucleic acid and peptide sequence of its complementary peptide. In addition, a 15-mer of sequence QRYKGISRGERLATH was used as a control peptide throughout these studies. All three peptides were synthesized by solid-phase methods [29] using *N*-(9-fluorenylmethoxycarbonyl) ('fMoc') chemistry on an automated 431A peptide synthesizer (Applied Biosystems, Warrington, Cheshire, U.K.).

Peptides were purified and analysed by reverse-phase h.p.l.c. [C₈ columns; 4.6 mm × 30 mm (analytical); 1 cm × 25 cm (preparative); Brownlee], using a model 151A peptide purification and analysis h.p.l.c. system and standard trifluoroacetic acid/acetonitrile/water eluting buffer systems (Applied Biosystems). Identity and purity of peptides was confirmed by

Abbreviations used: IGF-I, insulin-like growth factor I; I-FGI, the peptide complementary to IGF-I; r.r.a., radio-receptor assay; r.i.a., radioimmunoassay; hPTH 1–40, human-parathyroid-hormone fragment 1–40; hACTH 1–24, human-adrenocorticotrophic-hormone fragment 1–24; PBS, phosphate-buffered saline; IGFBP, IGF-binding proteins; f.a.b., fast atom bombardment.

Fig. 1. Nucleic acid and peptide sequence of IGF-I 21–40 and the corresponding complementary peptide I-FGI 20–40

The IGF-I 21–40 sequence is from [41] and the complementary peptide sequence is derived by the 5' → 3' translation of the complementary nucleic acid as indicated in the Figure and as described in [24]. N and C indicate the N- and C-termini respectively.

amino acid analysis, fast-atom-bombardment (f.a.b.) m.s. (M-Scan, Ascot, Berks., U.K.) and analytical reverse-phase h.p.l.c.

In some studies, two additional peptides were used as controls. These were bovine-growth-hormone fragment 63–74 (sequence: GKNEAQQKSDLE) and 165–175 (sequence: FRKDLHKTETY) and were provided by Dr. D. Morrell (Institute of Child Health, London W.C.1, U.K.).

Direct studies of peptide binding

Solid-phase binding studies of peptides were performed by absorption of test and control peptides at 10 or 100 µg/well in 100 µl of phosphate-buffered saline (0.06 M-NaH₂PO₄/0.154 M-NaCl, pH 7.4; PBS) on to 96-well flexible poly(vinylidene chloride) microtitre plates (Flow Laboratories).

Plates were washed three times and blocked in PBS containing 0.05% Tween-20; 100 µl of ¹²⁵I-IGF-I or ¹²⁵I-IGF-I 21–40 in PBS/0.05% Tween-20 (90000 c.p.m./well) was added and plates were incubated for 16 h at 4 °C. Plates were then washed five times in PBS 0.05% Tween and wells were cut directly into tubes for determination of radioactivity by γ-radiation counting. Binding is expressed as the percentage of total counts added.

C.d.

Peptides were scanned from 260 to 190 nm on a Jasco J600 spectropolarimeter using cells with a path length of 0.02 cm. Peptides were present at a concentration of 0.33 mg/ml in sodium phosphate, pH 7.4. Spectra were run at both 20 and 4 °C. The ellipticity data were calculated using a mean residue weight of 103 for I-FGI 20–40 and 114 for IGF-I 21–40.

IGF-I r.i.a.

The details of the IGF-I r.i.a. have been published previously [30]. Peptides were tested for their ability to compete with IGF-I in r.i.a. over the concentration range indicated in the relevant Figure legend. All peptide, tracer and first-antibody dilutions were made in r.i.a. buffer [0.06 M-NaH₂PO₄ (pH 7.4)/0.154 M-NaCl/0.5% (w/v) BSA/0.1% NaN₃].

IGF-I r.r.a.

Human placental membranes were used as a source of IGF-I receptors and were prepared as described previously [31]. Membranes were suspended at a concentration of 1 mg/ml in 20 mM-Tris/HCl/20 mM-CaCl₂, pH 7.4, and 100 µg aliquots were added to assay tubes. ¹²⁵I-IGF-I (40000 c.p.m./tube) and test peptides were diluted in 20 mM-Tris/HCl/1% (w/v) BSA (pH 7.4), 100 µl aliquots were added to membrane suspensions and incubations were continued for 16 h at ambient temperature. After the addition of 500 µl of 0.9% (w/v) NaCl, membranes were pelleted by centrifugation at 1700 g for 30 min. The

supernatants were decanted, and bound radioactivity was determined by γ-radiation counting.

Western ligand blotting of IGF-binding proteins (IGFBP)

Medium which had been conditioned by a rat-liver-derived cell line (clone 9; A.T.C.C. no. CRL 1439) was used as the source of IGFBP. Details of the method by which cells were propagated and medium collected are available from J.B. on request. The production of rat IGFBP-2 by this cell line has been previously described [32]. Ligand-blot analysis of secreted proteins was performed by the method originally described by Hossenlopp *et al.* [33]. Competing peptides were present in the binding buffer at the concentrations indicated in the relevant Figure legend.

Iodination of peptides

Both IGF-I and IGF-I 21–40 were iodinated by the Iodogen method [34]. Labelled peptides were separated from free iodine by gel filtration over a 3 ml bed volume of Sephadex G-10. IGF-I was iodinated to a sp. radioactivity of approx. 250 Ci/g and IGF-I 21–40 to one of approx. 50 Ci/g.

RESULTS

The authenticity and purity of the complementary peptides IGF-I 21–40 and I-FGI 20–40 were confirmed by m.s. (Fig. 2a), amino acid analysis (Table 1) and analytical reverse-phase h.p.l.c. (Fig. 2b). IGF-I 21–40 ($M_r + H^+ = 2275.6$) and I-FGI 20–40 ($M_r + H^+ = 2158.2$) both gave major signals corresponding to true molecular ions after f.a.b.m.s. analysis (Fig. 2a). In addition, analytical reverse-phase h.p.l.c. indicated that peptides were eluted as a single peak at 18% acetonitrile (IGF-I 21–40) or 25% acetonitrile (I-FGI 20–40) (Fig. 2b). Final confirmation of peptide identity was by amino acid analysis (Table 2). Reverse-phase h.p.l.c. analysis of two of the control peptides used in this study, (1) QRYKGISRGERLATH and (2) GKNEAQQKSDLE, indicated that purity was at least 90%. Purity of the third control peptide (3) FRKDLHKTETY was estimated at approx. 80% by reverse-phase h.p.l.c. analysis (results not shown).

The results of direct binding studies between ¹²⁵I-IGF-I 21–40 and I-FGI 20–40 are shown in Table 2. In these experiments I-FGI 20–40, control peptides 1 and 2 and BSA (10 µg of each) were absorbed into wells of microtitre plates, and the binding of ¹²⁵I-IGF-I 21–40 was examined. As indicated, no significant difference was observed in binding of ¹²⁵I-IGF-I 21–40 to its complementary peptide compared with binding to BSA or control peptides 1 and 2. The amount of radioactivity which bound in these experiments was only 0.2%–0.3% of added c.p.m. (200–300 c.p.m.). It should be noted that similar results were obtained when the binding of ¹²⁵I-IGF-I was examined (results not shown). In addition, no differences in binding were seen

Fig. 2. Characterization of synthetic peptides

(a) F.a.b.m.s.; (b) reverse-phase h.p.l.c. Peptide sequences are given in Fig. 1.

when wells were coated with 100 μ g of control and test peptides (results not shown).

C.d. spectroscopic analysis of the peptides IGF-I 21-40 and I-FGI 20-40 is shown in Fig. 3. The spectrum obtained for each of these peptides [(1) IGF-I 21-40; (2) I-FGI 20-40] indicate that, in solution (10 mM-sodium phosphate, pH 7.4), each exists in a random-coil conformation [35]. Furthermore, comparison of the summed spectra for IGF-I 21-40 and I-FGI 20-40 [3] with the spectrum obtained with an equimolar mixture of the two peptides [4] shows little difference in overall profile and only slight quantitative changes in absorption maxima. These results suggest no high-affinity interaction between these peptides in solution.

Fig. 3. Near-u.v. scanning c.d. spectra of peptides

1, IGF-I 21-40; 2, I-FGI 20-40; 3, sum of spectra 1 + 2; 4, spectra of equimolar mixture of two peptides.

The spectra shown in Fig. 3 were recorded at 20 °C. Identical results were obtained in experiments conducted at 4 °C.

The lack of interaction between IGF-I 21-40 and I-FGI 20-40 in both solid-phase (Table 2) and solution-phase (Fig. 3) assays was further manifested by the inability of I-FGI 20-40 to inhibit 125 I-IGF-I binding in three separate assay systems (Figs. 4-6). Fig. 4 indicates that at concentrations up to 100 μ g/ml, I-FGI 20-40 had no effect on 125 I-IGF-I binding in a specific r.i.a. Under the conditions of r.i.a., 50% inhibition of 125 I-IGF-I binding was seen at approx. 3 ng of unlabelled IGF-I/ml. In a similar vein, an r.r.a. for IGF-I, using human placental

Table 1. Amino acid analysis of peptides IGF-I 21-40 and IGF-I 20-40

Compositions are given as residues/molecule; n.d., not determined.

IGF-I 21-40			IGF-I 20-40		
Amino acid	Found	Theoretical	Amino acid	Found	Theoretical
Asp	1.21	1	Thr	0.97	1
Thr	0.88	1	Glu	0.95	1
Ser	2.51	3	Pro	4.05	4
Glu	0.89	1	Gly	1.93	2
Pro	2.60	2	Ala	2.89	3
Gly	2.68	3	Cys	n.d.	1
Ala	1.04	1	Val	1.89	2
Tyr	2.09	2	Ile	1.83	2
Phe	2.29	2	Leu	1.87	2
Lys	1.12	1	Lys	1.19	1
Arg	3.93	3	Arg	1.55	2

Table 2. Binding of ^{125}I -IGF-1 21–40 to its complementary peptide

Peptides/proteins were coated on to the surface of 96-well microtitre plates and binding of ^{125}I -IGF-1 21–40 was determined as described in the Materials and methods section. Control peptides: 1, QRYKGISRGERLATH; 2, GKNEAQQKSDLE. Data are expressed as percentages of added radioactivity (90000 c.p.m./well) bound and represent the means $\pm$ S.E.M. of binding observed in ten different wells.

Peptide/protein coated on well	Radioactivity bound (%)
I-FGI 20–40	0.286 ± 0.033
BSA	0.261 ± 0.019
Control peptide 1	0.240 ± 0.034
Control peptide 2	0.285 ± 0.039

membranes as a source of Type I IGF receptors, indicated that, over the concentration range 1–3000 ng/ml, there was no dose-dependent inhibition of ^{125}I -IGF-I binding to its receptor by I-FGI 20–40 (Fig. 5). When using this assay, 50% inhibition of ^{125}I -IGF-I binding was seen at approx. 30 ng of unlabelled IGF-I/ml. Insulin, at this concentration caused no displacement of ^{125}I -IGF-I binding and, indeed, at concentrations up to 10 $\mu\text{g}/\text{ml}$ resulted in only a small (approx. 10%) decrease in ^{125}I -IGF-I binding, indicating that binding of IGF-I under these conditions is to the type 1 receptor. Finally, the ability of I-FGI 20–40 to inhibit the interaction between ^{125}I -IGF-I and an IGFBP secreted by a rat liver cell line (clone 9) was examined by ligand-blot analysis. Fig. 6(a) indicates that ^{125}I -IGF-I binds to a 30 kDa protein that is secreted by this cell line [32] and that this binding is inhibited by the inclusion of 2.5 μg of unlabelled IGF-I/ml during the binding incubation (Fig. 6b). I-FGI 20–40 (Fig. 6c) (at 5 $\mu\text{g}/\text{ml}$) had no effect on this binding. Both control peptides 2 and 3 (Figs. 6d and 6e) were also without effect.

Fig. 4. Cross-reactivity of I-FGI 20–40 (■) and control peptide 1 (□) in an IGF-I r.i.a.

IGF-I (○) was assayed over the concentration range 0.5–16 ng/ml and the other two peptides over the range 1 ng–100 $\mu\text{g}/\text{ml}$ as indicated in the Figure. Triplicate determinations were made at each peptide concentration, and seven separate experiments were performed. Mean values are plotted. For clarity, S.D. values have been omitted.

Fig. 5. Cross-reactivity of I-FGI 20–40 (■) and control peptide 1 (□) in an IGF-I radio-receptor assay

Human placental membranes were used as the source of IGF-I receptors and assays were performed as described in the Materials and methods section. Peptides were tested over the concentration range 1–3000 ng/ml; triplicate determinations were made at each peptide concentration, and five separate experiments were performed. The results are presented as mean values and, as in Fig. 4, standard deviations have been omitted for clarity.

DISCUSSION

We have used several techniques, direct solid-phase and solution-phase assays of peptide binding (Table 2 and Fig. 3 respectively) and interference of complementary peptide in established binding assays for IGF-1 to determine whether any binding of the complementary peptide pair IGF-I 21–40 and I-FGI 20–40 was evident. We have found no evidence indicating that these peptides bind to each other, despite the fact that several of these methods have been used previously to demonstrate binding of complementary peptide pairs. For example, binding of complementary peptides by immobilization of one member of the pair in a microtitre well followed by incubation with the ^{125}I -labelled partner is a method which has previously been used to demonstrate the binding of ^{125}I -ACTH 1–24 to its complementary peptide [25] and ^{125}I -insulin to a complementary peptide hexamer to residues B22–B27 of the insulin molecule [9].

In the former study, 200 μg of peptide was coated on to the wells of microtitre places, although in the latter study with the insulin-binding hexamer plates were coated with only 0.5 μg of peptide. At 10 or 100 $\mu\text{g}/\text{well}$, therefore, the amount used in the

Fig. 6. Cross-reactivity of I-FGI 20–40 (c) and the control peptides GKNEAQQKSDLE (d) and FRKDLHKTETY (e) during Western-ligand-blot analysis of ^{125}I -IGF-I binding to IGF-binding protein 2

Ligand blots were performed as described in the Materials and methods section in the presence of (a) no competing peptide; (b) unlabelled IGF-I (2.5 $\mu\text{g}/\text{ml}$); and (c–e) test and control peptides (5 $\mu\text{g}/\text{ml}$). The autoradiograph shown was exposed for 14 days.

present study lies between these values. Binding of labelled peptide was found to approach maximum at around 1 nM-¹²⁵I-ACTH or 10 nM-¹²⁵I-insulin, whereas in the present study labelled peptides (¹²⁵I-IGF-I or ¹²⁵I-IGF-I 21–40) were used at concentrations of 1–5 nM, with no binding apparent for either peptide at these concentrations. In both previous studies [9,25] binding of complementary peptide pairs was performed in the presence of a PBS solution containing 0.1% BSA. For the data reported in Fig. 3, binding of peptides was carried out in a buffer containing PBS/0.05% Tween-20. However identical results were obtained in PBS/0.1% BSA.

C.d. spectrophotometry is a technique which has also been used previously to demonstrate an interaction between complementary peptide pairs in solution. Fassina *et al.* [7] used the 20-mer *c-ras* protein fragment 356–375 and a complementary peptide designed by using a computer program to generate a peptide which was a hydropathic mirror image of *c-ras* 356–375. The method of design of the complementary peptide was thus different from that reported in our study, although these authors also synthesized a complementary peptide which was derived by translation of the appropriate anti-sense nucleic acid. Although they did not report any c.d. spectrophotometric data on the binding of this anti-sense peptide to the *c-ras* fragment 356–375, they did show, by affinity chromatography, that their computer-generated peptide had approx. 50-fold greater affinity for *c-ras* 356–375 than the anti-sense complementary peptide. Other differences [concentration of peptide used and the pH of the buffer (20 mM-ammonium acetate, pH 5.7, versus 10 mM-sodium phosphate, pH 7.4, in the present study)] may account for the different results obtained in the present study.

Given that the solid-phase and solution-phase binding data suggest that I-FGI 20–40 does not bind either to IGF-I 21–40 or to native IGF-I, it is perhaps not surprising that I-FGI 20–40 does not show any reactivity in a specific IGF-I r.i.a. (Fig. 4).

We also investigated the activity of I-FGI 20–40 in r.r.a.s using human placental membranes as a source of type I IGF receptors. In contrast with the r.i.a. described above, the interaction between IGF-I and the binding molecule in RRA (i.e. the type I receptor) has been studied in some detail. Replacement of residues 27–37 of hIGF-I with a tetraglycine bridge resulted in a 30-fold increase in affinity for the type I receptor [36]. Further studies indicated that tyrosine residues at positions 24 and 31 are involved in the high-affinity binding of IGF-I to type I receptors in human placental membranes [37,38]. It follows, therefore, that binding of the complementary peptide I-FGI 20–40 to this region of the IGF-I molecule might be expected to inhibit hormone binding to receptor. Inspection of the data in Fig. 5 indicates that there is no significant concentration-dependent inhibition of ¹²⁵I-IGF-I binding to human placental membranes by I-FGI 20–40. Other studies have shown that complementary peptides to partial sequences of the insulin molecule [9], fibronectin [11] and the angiotensin II octapeptide [21] inhibit the binding of these polypeptides to their receptors. The lack of any evidence of direct binding between I-FGI 20–40 and IGF-I or IGF-I 21–40 provides the most likely explanation for the inability of I-FGI 20–40 to inhibit ¹²⁵I-IGF-I binding to placental membranes and also for the lack of any effect on the interaction between ¹²⁵I-IGF-I and IGF binding protein (see Fig. 6).

At the level of peptide chemistry it is difficult to rationalize the binding of complementary peptides. An early report [39] indicated that, in a majority of cases, hydrophobic amino acid residues were complemented with hydrophilic residues. This is well illustrated for the two peptides under consideration here. Inspection of Fig. 1 indicates that hydrophilic residues in IGF-I 21–40 are complemented by hydrophobic amino acids in I-FGI 20–40. By contrast, weakly hydrophilic or hydrophobic amino

acids are partnered by similar residues (e.g. S at 33, 34 and 35 in IGF-I 21–40 are complemented by G, A and T respectively). If peptides interact in an elongated conformation as indicated in Fig. 1 and as has been suggested previously [10], then it is difficult to envisage how the direct apposition of hydrophilic and hydrophobic residues could enhance high-affinity binding between peptides. Nevertheless, this hydropathic anti-complementarity displayed by complementary peptide pairs has been used by one group to design complementary peptides solely on the basis of the hydropathic profile displayed by the target peptide [7]. This may be an alternative approach for the synthesis of an effective complementary peptide to IGF-I 21–40.

A further consideration in the design of complementary peptides is the amount of sequence degeneracy which can be tolerated in a complementary peptide without binding to target peptide being significantly affected [10,40]. For example, in one study [10] inversion of the N to C sequence of a complementary peptide had little effect on the interaction between target and complementary peptide. In a further remarkable development [26] it was reported that 3' → 5' translation of a nucleic acid complementary to the sequence of ACTH 1–24 resulted in a peptide (3' → 5' complementary peptide) which, like the 5' → 3'-translated peptide possessed the ability to bind to ACTH 1–24. The ability of both 5' → 3' and 3' → 5' complementary peptides to bind to target peptide has been rationalized on the basis that it is the second base of a triplet codon which determines the hydropathic nature of an amino acid residue and thus the hydropathic profile of 5' → 3' and 3' → 5' complementary peptides is similar. Therefore, by arguments used previously [10,40], both of these peptides might be expected to bind to target peptides.

In summary, we have found no evidence of binding of the complementary peptide pair – IGF-I 21–40 and I-FGI 20–40. No binding was observed in either solid-phase (Table 2) or solution-phase (Fig. 3) assays. Furthermore no evidence of interaction between I-FGI 20–40 and native IGF-I was observed using three different assay systems (Figs. 4–6). Although further studies of the activity of the 3' → 5' complementary peptide and both the 5' → 3' and 3' → 5' inverted sequences in this system are needed, the data obtained to date question the universality of application of this particular molecular-recognition theory.

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