Insulin-like growth factor (IGF)-II binding to IGF-binding proteins and IGF receptors is modified by deletion of the N-terminal hexapeptide or substitution of arginine for glutamate-6 in IGF-II

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Recombinant insulin-like growth factor-II (IGF-II) and two structural analogues, des(1–6)IGF-II and [Arg⁶]-IGF-II, were produced to investigate the role of N-terminal residues in binding to IGF-binding proteins (IGFBPs) and hence the biological properties of the modified peptides. The growth factors were modelled on two previously characterized variants of IGF-I, des(1–3)IGF-I and [Arg³]-IGF-I, which both show substantially decreased binding to IGFBPs and were expressed as fusion proteins in *Escherichia coli*. The biological activities of the corresponding analogues of IGF-I and IGF-II were compared in rat L6 myoblasts and H35B hepatoma cells. In the L6-myoblast protein-synthesis assay, the IGF-II analogues, des(1–6)IGF-II and [Arg⁶]-IGF-II, were slightly more potent than IGF-II but about 10-fold less potent than IGF-I and 100-fold less potent

than the respective IGF-I analogues, des(1–3)IGF-I and [Arg³]-IGF-I. In H35 hepatoma cells the anabolic response measured was the inhibition of protein breakdown, and the potency order was insulin $\gg$ [Arg³]-IGF-I $>$ des(1–3)IGF-I $>$ [Arg⁶]-IGF-II $>$ des(1–6)IGF-II $>$ IGF-I $>$ IGF-II. Binding of the IGFs and their analogues to the type 1 IGF receptor in L6 myoblasts and to the insulin receptor in H35 hepatoma cells did not fully explain the observed anabolic potency differences. Moreover, binding of all four analogues to the IGFBPs secreted by L6 myoblasts and H35B hepatoma cells was greatly decreased compared with the parent IGF. We conclude that the observed anabolic response to each IGF was determined by their relative binding to the competing cell receptor and IGFBP binding sites present.

INTRODUCTION

Insulin-like growth factor (IGF)-II is a 67-amino-acid polypeptide that shows considerable structural and functional homology with IGF-I and also insulin (Humbel, 1990). IGF-I and IGF-II are potent mitogens for many different cell types, and in this role both growth factors appear to act through the type 1 IGF receptor (Humbel, 1990). IGF-II also binds to its own specific receptor, the type 2 IGF receptor, which has been shown to be identical with the cation-independent mannose 6-phosphate receptor (McDonald et al., 1988). In addition to their receptor binding, the actions of IGF-I and IGF-II are controlled by a group of binding proteins (IGFBPs), which are secreted by most cells in culture and found in many biological fluids. We have described a truncated variant of IGF-I, des(1–3)IGF-I, which was more potent than IGF-I in its anabolic actions in cultured cells (Francis et al., 1986, 1988). The explanation for this increased potency was established as a decreased binding to inhibitory IGFBPs secreted by the cells (Szabo et al., 1988; Ross et al., 1989). The importance of Glu³ in the binding of IGF-I to the IGFBPs has been confirmed by using both chemically synthesized and recombinantly expressed analogues of IGF-I (Bagley et al., 1989; King et al., 1992; Francis et al., 1992). Furthermore, we also established that these IGF-I analogues are more potent growth factors *in vivo* than native IGF-I (Tomas et al., 1992).

Here we report the preparation of recombinant IGF-II and two analogues modified at the N-terminus, [Arg⁶]-IGF-II and des(1–6)IGF-II, and compare their biological properties in cul-

tured cells with IGF-I and with the corresponding structural analogues of IGF-I, [Arg³]-IGF-I and des(1–3)IGF-I.

MATERIALS AND METHODS

Materials

Recombinant IGF-I, des(1–3)IGF-I and [Arg³]-IGF-I were provided by GroPep Pty. Ltd., Adelaide, South Australia, Australia. A synthetic cDNA encoding human IGF-II was kindly provided by Dr. A. G. Mackinlay, School of Biochemistry, University of New South Wales, Kensington, N.S.W., Australia. Restriction enzymes were purchased from Kabi Pharmacia Pty. Ltd., North Ryde, Sydney, N.S.W., Australia, and the 'Mutagene' *in vitro* mutagenesis kit was purchased from Bio-Rad, Richmond, CA, U.S.A. All other molecular-biology materials, including synthetic oligonucleotides, were from Bresatec Ltd., Thebarton, South Australia, Australia. Carrier-free Na¹²⁵I and [4,5-³H]leucine were obtained from Amersham Australia Pty. Ltd., Sydney, N.S.W., Australia. Highly purified pig IGFBP-3 was kindly provided by Dr. Paul Walton, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, South Australia, Australia, and recombinant H64A subtilisin BPN' by Genentech Inc., South San Francisco, CA, U.S.A.

Plasmid construction and bacterial strain

A 216 bp *Pvu*II fragment of synthetic human IGF-II (hIGF-II) was cloned into the *Sma*I site of the PTZ18 cloning vector. For this fragment to be expressed in *Escherichia coli*, the arginine

Abbreviations used: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; des(1–3)IGF-I, des(Gly-Pro-Glu)-IGF-I; des(1–6)IGF-I, des(Ala-Tyr-Arg-Pro-Ser-Glu)-IGF-I; h, human; pGH, pig growth hormone.

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codons between positions 70 and 120 bp had to be changed from the low-frequency AGG to the high-frequency CGT codon by site-directed mutagenesis so as to complement the specific tRNA species present (Figure 1). To clone the insert into the restriction vector, we introduced codons for Val-Asn (GTAAAC), creating a *HpaI* restriction site as well as the H64A mutant subtilisin BPN' cleavage motif, Phe-Ala-His-Tyr, and two stop codons. All changes introduced by site-directed mutagenesis were made by the procedure described in the 'Mutagene' kit and confirmed by dideoxy-DNA sequencing.

By using the optimized hIGF-II clone, two mutant IGF-II constructs, [Arg⁶]-IGF-II and des(1-6)IGF-II, were obtained by annealing independent oligonucleotides (Figure 1). The complete hIGF-II or mutant sequence was cloned into the *HpaI*-*HindIII* cut p[Met¹]-pGH(1-11)-Val-Asn-IGF-I expression vector (pGH, pig growth hormone) as described by Francis et al. (1992) and Upton et al. (1992). The resultant constructs, p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II, p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-[Arg⁶]-IGF-II and p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-des(1-6)IGF-II, were then transformed into *E. coli* JM101 (lac I^a) and selected on minimal agar containing 29 µM ampicillin (Upton et al., 1992).

Fermentation and isolation of inclusion bodies

The bacterial strain *E. coli* JM101, transformed with the required plasmid, p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II, p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-[Arg⁶]-IGF-II or p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-des(1-6)IGF-II, was grown on minimal media and a single colony was used to inoculate in turn a 20 ml starter culture and a 15 litre fermentor (Chemap, Volketswil, Switzerland; King et al., 1992). Cells were

induced with 0.32 mM isopropyl β-D-galactoside, and inclusion-body harvesting was carried out as previously described (King et al., 1992).

Refolding of the reduced IGF-II fusion proteins

Washed inclusion bodies in 25 g batches were solubilized by homogenization at 10% (w/v) in 8 M urea containing 40 mM glycine, 0.1 M Tris, 5 mM ZnCl₂ and 40 mM dithiothreitol at pH 9.0 and desalted on a column of Sephadex G-25 (Kabi Pharmacia), by using this solvent containing 1.6 mM dithiothreitol (Francis et al., 1992). The desalted and reduced inclusion-body extract was chromatographed on an anion-exchange column essentially as described by Upton et al. (1992). This and all other stages of peptide production were monitored by analysis on a microbore C₄ reverse-phase h.p.l.c. column (2.1 mm diameter × 10 cm; Brownlee Laboratories, Santa Clara, CA, U.S.A.) with a gradient of 15–55% acetonitrile over 40 min in the presence of 0.1% (v/v) trifluoroacetic acid and at a flow rate of 0.5 ml/min. Eluted protein was detected by its A₂₁₅. The reduced IGF-II fusion proteins were then folded as described previously (Francis et al., 1992; Upton et al., 1992), except that the final urea concentration used was 4 M. The refolded IGF-II fusion proteins were concentrated and partially purified by Fast Flow S (Kabi Pharmacia) cation-exchange and C₁₈ reverse-phase chromatography as described previously (Francis et al., 1992).

Cleavage of IGF-II fusion proteins and purification of the IGF-II peptides

The freeze-dried refolded IGF-II fusion proteins were dissolved in a solution containing 8 M urea and 20 mM Tris adjusted to pH 8.5 with HCl. The solubilized fusion protein was diluted to a concentration of 200 µg/ml in a solution containing 2.5 M urea, 5 mM CaCl₂, 200 mM NaCl and 20 mM Tris and adjusted to pH 8.5. Cleavage of the fusion protein was initiated by addition of 0.03% (w/w) H64A subtilisin BPN' and terminated after 18 h by acidification to pH 2.1 with trifluoroacetic acid.

The acidified IGF-II peptides were purified by reverse-phase h.p.l.c. on a Waters Delta Prep 3000 chromatography system (Millipore-Waters, Milford, MA, U.S.A.) using a C₄ PrepPak 500 cartridge (47 mm diameter × 300 mm) essentially as described previously (Francis et al., 1992). The amount and purity of each peptide was determined by analysis of its A₂₁₅ and A₂₈₀ on a microbore C₄ and Novapak C₁₈ h.p.l.c. column respectively (Francis et al., 1992).

N-terminal protein sequence analysis was performed by Edman degradation with a gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.; model 470A) by the methods of Hunkapiller et al. (1983).

Electrospray mass spectrometry was carried out on peptide samples by using a VG Biotech Quattro mass spectrometer (VG Biotech Ltd., Altrincham, Cheshire, U.K.).

Computer modelling of the molecular structure of IGF-II was based on the atomic co-ordinates of Blundell et al. (1978) and performed with the Desktop Molecular Modeller (Crabbe and Appleyard, 1989).

IGF binding to cell receptors

Binding to the type-1 IGF receptors on rat L6 myoblasts or to type-2 receptors on rat L6 myoblast and H35 hepatoma cells was measured with ¹²⁵I-labelled recombinant human IGF-I and human IGF-II respectively (Ross et al., 1989). Briefly, radio-labelled ligand was added to myoblast or hepatoma monolayers

Figure 1 Nucleotide and amino acid sequence of the *HpaI*-*HindIII* fragment in the construct for expression of hIGF-II

The oligonucleotides used to optimize hIGF-II (oligo 1 and oligo 2) as well as those used to direct the mutants (oligo 3 and oligo 4) are shown and the regions altered are indicated by the shaded or hatched boxes. The amino acids are numbered and stop codons are indicated by an asterisk.

in the presence of increasing concentrations of unlabelled peptides in a total volume of 0.5 ml and incubated at 2 °C for 18 h. Binding was expressed as the percentage of bound radioactivity occurring in the absence of added unlabelled IGF peptide.

Effect of IGF peptides on protein synthesis

Stimulation of protein synthesis in rat L6 myoblasts by growth factors was measured as described previously (Francis et al., 1986). Stimulation of protein synthesis was measured as the increased incorporation of [³H]leucine into total cell protein over an 18 h incubation period compared with a buffer control.

Effect of IGF peptides on intracellular protein breakdown

Confluent monolayers of rat H35 hepatoma cells in 24-place multiwells were prelabelled with [³H]leucine for 18 h, incubated with Dulbecco's Minimal Essential Medium containing unlabelled leucine for a 2 h period, and then the rates of intracellular protein breakdown were measured over a 4 h period (Ballard et al., 1986). Breakdown rates were expressed as the percentage of initial labelled protein degraded to trichloroacetic acid-soluble radioactivity during the measurement period.

Measurement of binding to IGF-BPs

L6 myoblast and H35 hepatoma IGF-BPs were prepared from serum-free Dulbecco's Minimal Essential Medium conditioned by confluent monolayers of cells for 24 h. The collected medium was centrifuged at 10000 *g* for 5 min, filtered through a 0.22 µm-pore filter and stored at -20 °C until used. The relative binding of the various IGF peptides to IGF-BPs present in conditioned medium was measured as described previously (Szabo et al., 1988). Binding was expressed as the percentage of ¹²⁵I-labelled hIGF-I or hIGF-II bound in the absence of added unlabelled IGF peptide.

Analysis of data from biological assays

Data from the various assays were analysed by a computer-fitted logistic dose-response function using the TableCurve software version 3.0 (Jandel Scientific, Corte Madera, CA., U.S.A.), and the ED₅₀ value was determined from the fitted curve by computation.

RESULTS

Fusion protein expression, folding and cleavage

Recombinant IGF-II and two analogues, [Arg⁶]-IGF-II and des(1-6)IGF-II, were expressed as fusion proteins in transformed *E. coli* JM101 cells under control of the *trc* promoter and in conjunction with the *lac* operator. These fusion-protein constructs contain the sequence of human IGF-II or one of its analogues linked to an N-terminal extension peptide derived from [Met¹]-pGH by a short peptide sequence that includes the unique protease cleavage motif for a genetically engineered protease, H64A subtilisin BPN'. The fusion proteins [Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II, [Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-[Arg⁶]-IGF-II and [Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-des(1-6)IGF-II, were produced as inclusion bodies, with yields of approx. 6 g wet wt. containing 0.5 g of peptide/litre of fermentation medium.

Production of the IGF-II peptides involved a similar strategy to that used previously for human IGF-I and its analogues (King et al., 1992; Francis et al., 1992) or chicken IGF-I (Upton et al., 1992). Downstream processing of each of the IGF-II peptides was similar, and only the production of IGF-II is described below. As previously reported for chicken IGF-I (Upton et al., 1992), the IGF-II peptides were rapidly degraded by bacterial proteases present in the inclusion-body paste unless the latter were removed by anion-exchange chromatography. The fusion

Figure 2 Downstream processing of recombinant IGF-II

Analysis on a microbore C₄ reverse-phase h.p.l.c. system after (a) p[Met¹]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II inclusion bodies from *E. coli* were solubilized, desalted by gel-filtration and partial purification by anion-exchange chromatography, (b) refolding in a redox buffer, (c) cleavage of the fusion protein with H64A subtilisin BPN', and (d) final purification to homogeneity as described in the Materials and methods section. Elution of protein was monitored as A₂₁₅ (—) and performed with a gradient of acetonitrile (----) in 0.1% (v/v) trifluoroacetic acid. The elution position of recombinant IGF-II is indicated by the arrows.

Figure 3 Stimulation of protein synthesis in rat L6 myoblasts by IGF-I (○), [Arg³]-IGF-I (□), des(1-3)IGF-I (△), IGF-II (●), [Arg³]-IGF-II (■) and des(1-6)IGF-II (▲)

Values are means of triplicate determinations on three cultures at each peptide concentration. S.E.M. values are indicated by the descending bars where they are larger than the symbols.

proteins were stable provided that this modification to the purification protocol was introduced (Figure 2). The inclusion bodies after solubilization and desalting under denaturing and reducing conditions contained a major and a minor absorbance peak, as observed for all of the IGF constructs so far expressed in *E. coli* (Figure 2a). The major peak eluted at 25.5 min on *C*₄ h.p.l.c. for the unfolded IGF-II fusion protein gave rise to two peaks eluted at 21 min and 23 min during the refolding reaction (Figures 2a and 2b). These two species probably represent different disulphide-bond isomers, a result confirmed by their co-elution at 25.5 min on *C*₄ h.p.l.c. after reduction (results not shown). A yield of 890 mg of refolded IGF-II fusion protein was obtained from 25 g wet wt. of inclusion bodies.

The desired IGF-II peptides were released by cleavage of the fusion protein at a sensitive Tyr-Ala bond that linked the two fusion partners. This was achieved with H64A subtilisin, which cleaves peptide bonds on the C-terminal side of the tetrapeptide Phe-Ala-His-Tyr (Carter et al., 1989). Both major fusion-protein substrate peaks gave rise to a single major product that was eluted at 15.2 min on *C*₄ h.p.l.c. (Figure 2c). Purification of the subtilisin digest through several reverse-phase steps yielded 150 mg of homogeneous IGF-II (Figure 2d). N-terminal sequence analysis of the final products gave only the expected sequence for each IGF-II peptide. Electrospray mass spectrometry of all three IGF-II peptides gave the expected mass values (results not shown).

Biological activity and receptor-binding properties in L6 myoblasts

The biological activities of IGF-II, [Arg³]-IGF-II and des(1-6)IGF-II were compared with those of IGF-I and the corresponding IGF-I analogues, [Arg³]-IGF-I and des(1-3)IGF-I. In rat L6 myoblasts all peptides stimulated protein synthesis; the maximal responses were less than the 400% stimulation observed with 10% (v/v) foetal bovine serum.

The order of potency and concentration required to give 25% of the response observed with 10% (v/v) foetal-bovine serum was: des(1-3)IGF-I (0.05 nM); [Arg³]-IGF-I (0.05 nM); IGF-I

Figure 4 Binding of IGFs to type 1 and type 2 IGF receptors on rat L6 myoblast cells

Competition for binding of (a) ¹²⁵I-labelled IGF-I and (b) ¹²⁵I-labelled IGF-II by IGF-I (○), [Arg³]-IGF-I (□), des(1-3)IGF-I (△), IGF-II (●), [Arg³]-IGF-II (■) and des(1-6)IGF-II (▲). Values are means of triplicate determinations on three cultures at each peptide concentration. S.E.M. values are indicated by the descending bars where they are larger than the symbols.

(0.66 nM); [Arg³]-IGF-II (5.0 nM); des(1-6)IGF-II (6.6 nM); and IGF-II (7.6 nM) (Figure 3). This anabolic response in L6 myoblasts has been shown to follow binding to the type 1 IGF receptor (Ewton et al., 1987). IGF binding to the type 1 IGF receptor is shown in Figure 4(a). The effective dose for half-maximal competition to binding of the tracer (ED₅₀) was: des(1-3)IGF-I (0.39 nM); IGF-I (0.44 nM); [Arg³]-IGF-I (1.09 nM); IGF-II (2.09 nM); [Arg³]-IGF-II (3.7 nM); and des(1-6)IGF-II (4.1 nM).

Rat L6 myoblasts also possess specific type 2 IGF receptors. Accordingly the functional properties of the IGF-II analogues were further tested by their abilities to compete with ¹²⁵I-labelled IGF-II for binding to this receptor (Figure 4b). Surprisingly, [Arg³]-IGF-II was almost 3 times as potent and des(1-6)IGF-II less than half as potent as IGF-II itself, with ED₅₀ values of 2.3 nM, 17 nM and 6.6 nM respectively. As expected, none of the IGF-I-related peptides showed any competition with ¹²⁵I-labelled IGF-II binding at the concentrations tested.

The recombinant IGF-II prepared here was equipotent to bovine and pig IGF-II purified from natural sources (Francis et al., 1988, 1989) in the above assays (results not shown).

Figure 5 Inhibition of intracellular protein breakdown in rat H35 hepatoma cells by insulin (▽), IGF-I (○), [Arg³]-IGF-I (□), des(1–3)IGF-I (△), IGF-II (●), [Arg⁶]-IGF-II (■) and des(1–6)IGF-II (▲)

Values are means of triplicate determinations on three cultures at each peptide concentration. S.E.M. values are indicated by the descending bars where they are larger than the symbols.

Figure 6 Binding of IGFs to the insulin and type 2 IGF receptors on rat H35 hepatoma cells

Competition for binding of (a) ¹²⁵I-labelled insulin and (b) ¹²⁵I-labelled IGF-II by insulin (▽), IGF-I (○), [Arg³]-IGF-I (□), des(1–3)IGF-I (△), IGF-II (●), [Arg⁶]-IGF-II (■) and des(1–6)IGF-II (▲). Values are means of triplicate determinations on three cultures at each peptide concentration. S.E.M. values are indicated by the descending bars where they are larger than the symbols.

Figure 7 Binding of IGFs to IGF-BPs

Competition for binding of ¹²⁵I-labelled IGF-II to IGF-BPs in media conditioned by (a) rat L6 myoblasts and (b) rat H35 hepatoma cells by IGF-I (○), [Arg³]-IGF-I (□), des(1–3)IGF-I (△), IGF-II (●), [Arg⁶]-IGF-II (■) and des(1–6)IGF-II (▲). Values are means of triplicate determinations at each peptide concentration. S.E.M. values are indicated by the descending bars where they are larger than the symbols.

Biological activity and receptor binding in H35B hepatoma cells

As a result of the differences in receptor binding and biological potencies in myoblasts amongst the different analogues, we investigated their actions in another cell line, the rat H35 hepatoma. The anabolic response measured was the inhibition of protein breakdown (Figure 5). The basal rate of protein breakdown was 7.22%/4 h. Insulin was the most potent growth factor, with a concentration of 0.0021 nM achieving half the maximal inhibition of protein breakdown observed with 10% (v/v) foetal-calf serum. Equivalent responses were produced by [Arg³]-IGF-I (0.10 nM), des(1–3)IGF-I (0.16 nM), [Arg⁶]-IGF-II (0.22 nM), des(1–6)IGF-II (0.39 nM), IGF-I (0.42 nM) and IGF-II (0.94 nM).

The receptor binding of the IGFs in H35 hepatoma cells was examined to see if it explained the greater relative potency of IGF-II and its analogues in this cell line (Figures 6a and 6b). Binding to the insulin receptor, as indicated by the concentrations required to give half-maximal competition to ¹²⁵I-labelled insulin binding, decreased in the following order: insulin (2.5 nM); IGF-II (43 nM); [Arg⁶]-IGF-II (74 nM); des(1–3)IGF-I (165 nM); IGF-I (250 nM); des(1–6)IGF-I (325); and [Arg³]-IGF-I (370 nM).

We also evaluated binding to the specific type 2 IGF receptor

Table 1 Biological potencies of the IGFs in rat L6 myoblasts and H35 hepatoma cells expressed relative to IGF-II

The values have been calculated from the ED₅₀ in the various assays, and are expressed relative to IGF-II.

Cells	Assay	IGF-I	[Arg ⁶]-IGF-I	Des(1-3)IGF-I	IGF-II	[Arg ⁶]-IGF-II	Des(1-6)IGF-II
L6 myoblasts	Protein synthesis	11	150	160	1.0	1.5	1.3
	Type 1 IGF receptor	4.7	1.9	5.4	1.0	0.56	0.56
	Type 2 IGF receptor	< 0.001	< 0.001	< 0.001	1.0	2.9	0.42
	IGFBP	0.065	< 0.001	< 0.001	1.0	0.008	0.003
H35 hepatoma	Protein breakdown	2.2	8.6	6.0	1.0	4.3	2.7
	Insulin receptor	0.17	0.12	0.26	1.0	0.58	0.14
	Type 2 IGF receptor	< 0.001	< 0.001	< 0.001	1.0	1.9	0.26
	IGFBP	1.7	0.005	0.007	1.0	0.015	0.006

in these cells (Figure 6b). [Arg⁶]-IGF-II was approximately twice as potent and des(1-6)IGF-II was approximately one-quarter as potent as IGF-II in competing for ¹²⁵I-labelled IGF-II binding to these cells, with ED₅₀ values of 4.4 nM, 31 nM and 8.2 nM respectively.

Binding of IGF analogues to IGFBPs

We have reported previously that the biological potency of N-terminal variants of IGF-I in cultured cells was modified by their binding to IGFBPs secreted by those cells. In competition binding assays employing ¹²⁵I-labelled IGF-II as the radiolabelled ligand and conditioned medium from L6 rat myoblasts as the source of IGFBP, the order of potency, with the ED₅₀ values in parentheses, was: IGF-II (0.68 nM); IGF-I (10 nM); [Arg⁶]-IGF-II (86 nM); des(1-6)IGF-II (220 nM); [Arg³]-IGF-I (> 500 nM); and des(1-3)IGF-I (> 500 nM) (Figure 7a). Competition binding assays using radiolabelled IGF-I rather than IGF-II gave a qualitatively similar result (results not shown). The IGFBPs in conditioned medium from H35 hepatoma cultures gave a different order of potency: IGF-I (1.3 nM); IGF-II (2.2 nM); [Arg⁶]-IGF-II (150 nM); des(1-3)IGF-I (330 nM); des(1-6)IGF-II (370 nM); and [Arg³]-IGF-I (390 nM) (Figure 7b).

A summary of the biological assay data showing the potencies of the IGFs relative to IGF-II is given in Table 1.

DISCUSSION

Production of recombinant IGF-II analogues

Our main aim in this study was to examine whether the N-terminal region of IGF-II, in particular the glutamate residue at position 6, was involved in the association of IGF-II with IGFBPs and what effect this had on their biological activity in cultured cells. This relationship has previously been established for the equivalent structures in IGF-I (Bagley et al., 1989; King et al., 1992; Francis et al., 1992). To achieve this goal, we developed methods to prepare significant quantities of recombinant IGF-II and two analogues, des(1-6)IGF-II and [Arg⁶]-IGF-II, following their expression in *E. coli* as fusion proteins. These fusion proteins comprised an N-terminal partner derived from the first 11 amino acids of [Met¹]pGH linked to the IGF-II sequence by a short peptide that included a recognition motif, Phe-Ala-His-Tyr, for a genetically engineered mutant subtilisin, H64A subtilisin BPN' (Carter et al., 1989). After refolding the reduced fusion protein in a redox buffer, the desired IGF-II sequences were obtained in high yield after digestion with the H64A subtilisin BPN'.

Role of the IGF N-terminal region on IGF actions in L6 myoblasts

We have established the importance of the N-terminal region in IGF-I, in particular the glutamate at position 3, as a requirement for binding to IGFBPs (Szabo et al., 1988; Bagley et al., 1989). We have reported that N-terminal analogues modified at this site were more potent growth factors in L6 rat myoblasts as a result of their decreased binding to IGFBPs secreted into the extracellular medium (Ross et al., 1989; King et al., 1992; Francis et al., 1992). The substantially increased potency of two of these analogues, [Arg³]-IGF-I and des(1-3)IGF-I, in rat L6 myoblasts relative to IGF-I is shown in Figure 3 and Table 1. By contrast, the N-terminal analogues of IGF-II described in this paper, [Arg⁶]-IGF-II and des(1-6)IGF-II, only showed a small increase in their biological potency in L6 myoblasts compared with IGF-II (Table 1). This is despite the fact [Arg⁶]-IGF-II and des(1-6)IGF-II exhibited 1% or less of the affinity for the L6 myoblast IGFBPs compared with IGF-II. Since the stimulation of protein synthesis by both IGF-I and IGF-II is mediated through the type 1 IGF receptor in L6 myoblasts (Ewton et al., 1987; Ballard et al., 1988) the approx. 5-10-fold decreased binding to this receptor by the IGF-II peptides compared with IGF-I must contribute to their weaker biological potency. Moreover, the 2-fold decreased binding shown by [Arg⁶]-IGF-II and des(1-6)IGF-II relative to IGF-II partly explains the less than expected potency of the IGF-II analogues compared with IGF-II.

Another factor that may contribute to the potency differences between the IGF-I and IGF-II peptides is the large pool of type 2 IGF receptors present in L6 myoblasts (Beguino et al., 1985), since these may act as an inert 'sink' for IGF-II peptides, whereas IGF-I and its analogues show extremely low affinity for the type 2 IGF receptor (Figure 4b and Table 1). The effect of this would be to decrease selectively the concentration of free IGF-II peptide available to bind to the type 1 IGF receptor.

Role of the IGF N-terminal region on IGF actions in H35 hepatoma cells

H35 hepatoma cells offer a different comparison for IGF-I and IGF-II analogues, because these cells do not possess a type 1 IGF receptor. Rather, the actions of IGFs are mediated through the related type 1 insulin receptor (Mottola and Czech, 1984). Moreover, as with the L6 myoblasts, these cells possess a large number of type 2 IGF receptors, which are not involved in mediating the anabolic responses to added IGF-II (Massague et al., 1982). Protein breakdown in these cells was inhibited by [Arg⁶]-IGF-II and des(1-6)IGF-II with 4.4-fold and 2.4-fold

greater potency than IGF-II respectively. Interestingly, the corresponding analogues for IGF-I, [Arg³]-IGF-I and des(1-3)IGF-I, showed very similar changes to this in their potency relative to IGF-I, unlike the situation in L6 myoblast protein synthesis (Table 1). Both the IGF-I and IGF-II analogues show less than 1–2% binding to the IGFBP-1 present in H35B-hepatoma-conditioned-medium IGFBPs compared with IGF-I and IGF-II (Gargosky et al., 1990). Thus the potential inhibitory action of IGFBPs is similarly decreased for all the analogues in this cell type. However, the pattern of insulin-receptor binding by the IGF peptides in the hepatoma cells was different from that observed for the type 1 IGF receptor in L6 myoblasts (Table 1). For example, in H35B hepatoma cells IGF-II binding to the insulin receptor was about 6-fold higher than that of IGF-I, whereas the opposite was true for binding to the type 1 receptor in L6 myoblasts. Surprisingly, [Arg³]-IGF-I, which binds least well to the insulin receptor, is the most potent inhibitor of protein breakdown.

We conclude that in L6 myoblasts and H35 hepatoma cells the anabolic potency of the IGFs is determined mainly by their binding to the type 1 IGF or insulin receptors and the inhibitory IGFBPs. However, the type 2 receptor has the potential to act as an inert 'sink' for the IGF-II peptides and to decrease their availability to bind to the type 1 IGF receptor. Moreover, this property contributes to the lower than expected potency of the IGF-II peptides in both cell types.

Effect of IGF-II structure on type 2 IGF receptor and IGFBP binding

In this paper we describe the preparation of two analogues of IGF-II engineered at the N-terminus, [Arg⁶]-IGF-II and des(1-6)IGF-II, in an attempt to modify the IGFBP binding of IGF-II. We have found that binding to the type 2 IGF-II receptor by des(1-6)IGF-II was decreased to between 30 and 40% of that observed with IGF-II (Table 1). Luthi et al. (1992), who have also reported the production of recombinant des(1-6)IGF-II in *E. coli*, found a similar result using type 2 IGF-receptor-transfected baby-hamster kidney cells. They concluded that the decrease in receptor binding resulted from a local disturbance of the so-called A-domain region of the IGF-II molecule, which is involved in type 2 IGF-receptor binding. To examine this possibility, we carried out computer modelling of the three-dimensional structure of IGF-II using the atomic co-ordinates of Blundell et al. (1978) derived from pig insulin. Sakano et al. (1991) suggested that a linear stretch of the A-domain covering Phe⁴⁸-Arg⁴⁹-Ser⁵⁰ and Ala⁵⁴-Leu⁵⁵ was the most important region for binding of IGF-II to its receptor. Our modelling indicates that Glu⁶ in IGF-II lies within 0.4–0.5 nm (4–5 Å) of Ser⁵⁰, and the N-terminal residues Ala¹ and Tyr² are separated from Ala⁵⁴-Leu⁵⁵ by a similar distance. Hence the opportunity exists for individual residues in the N-terminal domain to influence type 2 IGF-receptor binding in a specific manner. This conclusion is supported by the properties of the two analogues that we have described here, des(1-6)IGF-II and [Arg⁶]-IGF-II, which show a 7-fold difference in their relative affinity for the type 2 IGF receptor (Table 1). Deleted residues other than Glu⁶ could be responsible for the decreased binding of des(1-6)IGF-II to this receptor, for example Arg³, since the increased positive charge in [Arg³]-IGF-II enhances the binding to the type 2 IGF receptor.

The decrease in IGFBP binding resulting from the changes made to the N-terminal sequence of IGF-II was predicted from our previous studies of the related IGF-I analogues (Ross et al., 1989; King et al., 1992; Francis et al., 1992). Luthi et al. (1992) found a similar decrease in the affinity of des(1-6)IGF-II for a non-glycosylated recombinant human IGFBP-3. We also found that des(1-6)IGF-II binding to IGFBP-3 purified from sheep or pig plasma was decreased approx. 10-fold compared with IGF-II; in fact the pattern of IGF binding to these IGFBPs was almost identical with that seen with the L6 myoblast IGFBPs reported here (results not shown). The critical role of Glu⁶ in IGF-II association with IGFBPs has been confirmed by the similar decrease in binding observed with [Arg⁶]-IGF-II (Table 1).

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