Kangaroo IGF-II is structurally and functionally similar to the human [Ser²⁹]-IGF-II variant

C A Yandell^{1,3}, G L Francis^{1,2}, J F Wheldrake^{1,3} and Z Upton^{1,2}

¹Cooperative Research Centre for Tissue Growth and Repair, PO Box 10065, Adelaide BC, 5000, South Australia, Australia

²CSIRO, Human Nutrition, Kintore Avenue, Adelaide, 5000, South Australia, Australia

³Department of Biological Sciences, Flinders University of South Australia, GPO 2100, Adelaide, 5001, South Australia, Australia

(Requests for offprints should be addressed to C Yandell at Cooperative Research Centre for Tissue Growth and Repair, PO Box 10065, Adelaide BC, 5000, South Australia, Australia)

Abstract

Kangaroo IGF-II has been purified from western grey kangaroo (*Macropus fuliginosus*) serum and characterised in a number of *in vitro* assays. In addition, the complete cDNA sequence of mature IGF-II has been obtained by reverse-transcription polymerase chain reaction. Comparison of the kangaroo IGF-II cDNA sequence with known IGF-II sequences from other species revealed that it is very similar to the human variant, [Ser²⁹]-hIGF-II. Both the variant and kangaroo IGF-II contain an insert of nine nucleotides that encode the amino acids Leu-Pro-Gly at the junction of the B and C domains of the mature protein. The deduced kangaroo IGF-II protein sequence also contains three other amino acid changes that are not observed in human IGF-II. These amino acid differences share similarities with the changes described in many of the IGF-IIs reported for non-mammalian species. Characterisation of human IGF-II, kangaroo IGF-II, chicken IGF-II and [Ser²⁹]-hIGF-II in a number of *in vitro*

assays revealed that all four proteins are functionally very similar. No significant differences were observed in the ability of the IGF-IIs to bind to the bovine IGF-II/cation-independent mannose 6-phosphate receptor or to stimulate protein synthesis in rat L6 myoblasts. However, differences were observed in their abilities to bind to IGF-binding proteins (IGFBPs) present in human serum. Kangaroo, chicken and [Ser²⁹]-hIGF-II had lower apparent affinities for human IGFBPs than did human IGF-II. Thus, it appears that the major circulating form of IGF-II in the kangaroo and a minor form of IGF-II found in human serum are structurally and functionally very similar. This suggests that the splice site that generates both the variant and major form of human IGF-II must have evolved after the divergence of marsupials from placental mammals.

Journal of Endocrinology (1999) **161**, 445–453

Introduction

The insulin-like growth factors (IGF-I and IGF-II) are mitogenic proteins that play an important role in the regulation of growth and development in vertebrates. The mature proteins, consisting of four domains (B, C, A and D) are structurally related to proinsulin and have been highly conserved throughout vertebrate evolution (for review see Duguay *et al.* 1995, 1996).

While IGF-I has an important role in mediating many of the effects of growth hormone postnatally, IGF-II appears to be important during embryonic development. Transgenic mice in which the IGF-II gene has been disrupted show impaired embryonic growth, attaining only 60% of the body weight of their normal littermates at birth (DeChiara *et al.* 1990). There is also considerable evidence that suggests that IGF-II plays a crucial role in the development of the placenta (Han *et al.* 1996, Reynolds *et al.* 1997). Indeed, developmental analysis of

the growth kinetics in mouse embryos carrying null mutations of IGF and IGF receptor genes, alone or in combination, suggested that the effect of IGF-II on placental growth is mediated by another receptor, originally termed receptor X (Wang *et al.* 1994). A more recent study has confirmed that placental growth is compromised only in mutants lacking IGF-II and that IGF-II acts via an unknown placenta-specific receptor which is distinct from the type-1 IGF and insulin receptors (Louvi *et al.* 1997).

The IGF-II gene is a single copy gene that is expressed as a family of RNA transcripts (Brissenden *et al.* 1984, Tricoli *et al.* 1984, Jansen *et al.* 1985). The heterogeneity of these transcripts originates from the use of multiple promoters, alternative splicing and differential use of polyadenylation sites. While the majority of IGF-II exists as the mature form of 7.5 kDa, high molecular weight (MW) isoforms of IGF-II have been detected in human serum (Zumstein *et al.* 1985, Gowan *et al.* 1987),

cerebrospinal fluid (Haselbacher & Humbel 1982) and malignant tissue extracts (Daughaday *et al.* 1988, Daughaday & Kapadia 1989). Variant forms of IGF-II have also been identified in human serum (Hampton *et al.* 1989, Straczek *et al.* 1990, Van den Brande *et al.* 1990) and placental tissue (De Ceuninck *et al.* 1995b).

IGF-II binds with high affinity to two very different membrane-bound receptors: (i) the type 1 IGF receptor, which contains intrinsic tyrosine kinase activity and is activated upon ligand binding, and (ii) the type 2 IGF receptor, a multifunctional receptor that has also been identified as the cation-independent mannose 6-phosphate receptor (CI-MPR). The full physiological role of IGF-II binding to the CI-MPR in particular is presently unclear and somewhat controversial. Interestingly, the avian and amphibian CI-MPRs lack the binding site for IGF-II but are able to bind mannose 6-phosphate-bearing ligands (Canfield & Kornfeld 1989, Clairmont & Czech 1989). However, the CI-MPR from the opossum, a species that recently diverged from placental mammals, can bind IGF-II, albeit with a 75-fold lower affinity than the bovine receptor (Dahms *et al.* 1993). Thus, the IGF-II binding property of the CI-MPR appears to have been acquired later in vertebrate evolution and may be limited to mammals.

While our knowledge of IGF structure and function in mammalian species has been well defined, the evolution of the individual components of the IGF systems in other species is less well understood. For example our knowledge of the IGF system in birds (except for commercially relevant species) or indeed in any species ancestral to eutherian (placental) mammals is remarkably limited. While both are mammals, one of the key differences between metatherians (marsupials) and eutherians is the evolution of different modes of foeto-maternal interactions during embryonic development. The metatherians are distinct in that, while the embryo is reliant on a yolk sac for nutrients, implantation and development of a placenta occur late in pregnancy (Renfree 1993, 1995). The embryo is born at an early stage of development and its survival is dependent on finding its way to the pouch and a mammary teat for nourishment. In eutherians on the other hand, implantation and placental invasion occur early in gestation, the embryo is born at a comparatively advanced stage of development and the young are generally quickly weaned. In the light of the convincing research that has been undertaken in eutherians pointing to a major role for IGF-II in fetal development (DeChiara *et al.* 1990, Wang *et al.* 1994) examination of the similarities, as well as differences, in these two classes of mammals may be beneficial in understanding the function of IGFs during embryogenesis. However, an important first step to this end is to gain an understanding of the basics of the IGF system in marsupials. We have previously reported the purification of IGFs from kangaroo serum and the characterisation of kangaroo IGF-I (kIGF-I) (Yandell *et al.* 1998). Here we report, for the first time, the

complete cDNA sequence and characterisation of a marsupial IGF-II.

Materials and Methods

Materials

Kangaroo blood was collected from wild western grey kangaroos (*Macropus fuliginosus*) at Peterborough, South Australia, Australia. Recombinant human IGF-II (rhIGF-II) and chicken IGF-II (cIGF-II) were supplied by GroPep Pty Ltd, Adelaide, South Australia, Australia. Bovine livers from freshly slaughtered cattle were obtained from Agpro Abattoir, Gepps Cross, South Australia, Australia and were snap frozen in liquid N₂ before being stored at -80 °C. Blood collected from female non-pregnant tammar wallabies (*M. eugenii*) was kindly donated by Professor Russell Baudinette, University of Adelaide, Department of Zoology, South Australia, Australia. Radioiodinated rhIGF-II was prepared to specific activities between 30 and 60 µCi/µg with chloramine-T and separated from reaction components by chromatography on a Sephadex G50 column as previously described (Francis *et al.* 1989). Recombinant [Ser²⁹]-hIGF-II was a generous gift from Dr C Lüthi, University of Zurich, Zurich, Switzerland. The mouse monoclonal antibody raised against rat IGF-II was generously provided by Dr K Nishikawa, Kanazawa Medical University, Uchinada, Ishikawa, Japan. Sheep anti-mouse Ig was obtained from Silenus Laboratories, Hawthorn, Victoria, Australia and carrier-free Na¹²⁵I was purchased from Amersham International, Amersham, Bucks, UK.

The Wizard PCR DNA Purification System was purchased from Promega, Madison, WI, USA while the RNeasy Mini Kit was purchased from Qiagen GmbH, Hilden, Germany. ELONGASE enzyme mix, 10 mM dNTP Mix, oligo(dT) and Superscript II RT were purchased from Gibco-BRL, Life Technologies, Eggenstein, Germany. Specific oligonucleotide primers as described below were synthesised by Gibco-BRL, Life Technologies. The Expand High Fidelity PCR System was obtained from Boehringer Mannheim, Castle Hill, New South Wales, Australia and the ThermoSequenase sequencing kit was obtained from Amersham Life Sciences, Cleveland, OH, USA.

Methods

IGF-II purification and characterisation kIGF-II was purified from western grey kangaroo serum as described by Yandell *et al.* (1998). Purity was assessed by N-terminal protein sequencing and quantitation was performed as described by Upton *et al.* (1995) using the calculated absorption coefficient of 31.69 g/l per cm at 214 nm.

kIGF-II was characterised in a number of *in vitro* assays where it was compared with hIGF-II, cIGF-II and the

human variant, [Ser²⁹]-hIGF-II. Before analysis the samples were dried under vacuum, dissolved in HCl (10 mM) and resuspended in the appropriate assay buffer.

IGF-II RIAs were carried out as described in Kita *et al.* (1996) using ¹²⁵I-hIGF-II as the radioligand and a monoclonal antibody raised against rat IGF-II. The amount of specific antiserum used was adjusted to produce 30–40% tracer binding. Non-specific binding was not subtracted and binding was expressed as the percentage of the control with no IGF-II added.

Protein synthesis was determined using confluent cultures of rat L6 myoblasts and was measured as the incorporation of [³H]leucine into total cell protein over a 16 h incubation period as previously described (Ballard *et al.* 1986). Responses are expressed as the percentage stimulation above that of the control with no added IGF-II.

Binding of IGF-II to purified bovine IGF-II (bIGF-II)/CI-MPR was measured as previously described (Scott & Baxter 1987). The IGF-II/Ci-MPR was purified from bovine liver by phosphomannan–Sephrose affinity chromatography as described in Dahms *et al.* (1993). Competition for binding with ¹²⁵I-hIGF-II to bIGF-II/Ci-MPR was again expressed as a percentage of the control with no added IGF-II.

Binding of IGF-II to IGF-binding proteins (IGFBPs) in human or tammar wallaby serum was carried out as described in Szabo *et al.* (1988). Briefly, radioligand and unlabelled IGF-II were added to 30 µl serum and incubated for 16 h at 4 °C. After incubation, bound radioligand was separated from free radioligand by the addition of a 0.5% (w/v) charcoal suspension for 30 min. The amount of free radioligand was determined by counting the radioactivity in the pellet after centrifugation. The results are expressed as a percentage of the control with no added IGF-II.

All concentrations of IGF-II were assayed in triplicate, with most experiments repeated at least three times as indicated in the figure legends. The IC₅₀ values for all assays were determined using TableCurve (Jandel Scientific, San Rafael, CA, USA). Statistical analysis was performed using repeated-measures ANOVA. A *P* value <0.05 was considered an indication of a significant difference between means.

Reverse transcription-polymerase chain reaction (RT-PCR) and sequence analysis The cDNA sequence for kIGF-II was obtained using mRNA extracted from kangaroo liver, followed by RT-PCR. RNA was extracted using the RNeasy Mini Kit. The quality of the RNA was assessed by ethidium bromide staining after agarose gel electrophoresis. First-strand cDNA was synthesised from total RNA using 0.5 µg oligo(dT) primer and 200 U Superscript II RT enzyme in a total volume of 20 µl according to the manufacturer's protocols.

Primer design and amplification Amplification of kIGF-II was performed using non-degenerate oligonucleotide primers. Primer IGF2–1 was based on the partial cDNA sequence of tammar wallaby IGF-II (S Wilcox, Royal Children's Hospital, Melbourne, Victoria, Australia, personal communication), while primer IGF2–2 was designed from the cDNA sequence of the E domain of human IGF-II. The primer pair 5' CTGTTATCACTCAC 3' (IGF2–1) and 5' AATCAGGGGACGGTG 3' (IGF2–2) amplify a fragment of approximately 489 bp that includes portions of the prepro-IGF-II molecule as well as the entire mature protein sequence. The PCR was carried out in a total volume of 50 µl containing 60 mM Tris–SO₄ (pH 9.1), 18 mM (NH₄)₂SO₄, 1.5 mM MgSO₄, 0.2 mM dNTP, 0.5 U ELONGASE, 200 ng oligonucleotide primers and 2.5 µl cDNA template. After denaturation at 94 °C for 3 min, the PCR reaction proceeded for 40 cycles of 1 min at 94 °C (denaturation), 1 min at 50 °C (annealing) and 1 min at 68 °C (extension).

The PCR product was purified by the Wizard PCR DNA Purification System prior to sequencing. Sequencing was performed using a radiolabelled terminator cycle sequencing kit, ThermoSequenase, with the PCR product sequenced in both the forward and reverse directions. Sequencing was performed on products from two independent RT-PCRs.

Results

Purification and cDNA sequencing of kIGF-II

IGF-II was purified from western grey kangaroo serum as previously described (Yandell *et al.* 1998). A yield of 106 µg IGF-II from 7 l of serum was obtained and was determined by N-terminal protein sequencing to be 95% pure. Electrospray mass spectroscopy revealed that kIGF-II isolated from serum had a molecular mass of 2921 Da, approximately 450 Da larger than that reported for hIGF-II (Rinderknecht & Humbel 1978). Previous attempts at determining the sequence of kIGF-II by Edman degradation N-terminal protein sequencing indicated that the A, B and D domains were highly conserved (Yandell *et al.* 1998). Hence, it was reasoned that the changes accounting for the mass differences between kIGF-II and hIGF-II must lie within the C domain. As endo-proteinase digestion and N-terminal protein sequencing require the use of relatively large amounts of protein, RT-PCR was used to obtain the cDNA sequence of kIGF-II.

RT-PCR primers were designed using the known sequence of the tammar wallaby (S Wilcox, personal communication) prepro-IGF-II signal peptide and a conserved region of the E domain from mammalian species. This primer set amplified a band of approximately 500 bp and subsequent sequencing of this product revealed it to be IGF-II (Fig. 1a). The theoretical molecular mass

(a)

```

Ala Tyr Arg Pro Ser Glu Thr Leu Cys Gly Gly Glu
5' GCT TAT CGG CCC AGT GAG ACC CTT TGT GGT GGG GAA
Leu Val Asp Thr Leu Gln Phe Val Cys Gly Asp Arg
CTG GTA GAC ACT CTC CAG TTT GTG TGT GGC GAC CGC
Gly Phe Tyr Phe Ser Leu Pro Gly Arg Pro Leu Ser
GGC TTC TAT TTC AGT CTT CCT GGC AGA CCC CTG AGC
Arg Val Ser Arg Arg Leu Asn Arg Gly Ile Val Glu
CGC GTG AGC CGT CGC CTG AAT CGC GGC ATC GTG GAA
Glu Cys Cys Phe Arg Ser Cys Asp Leu Ala Leu Leu
GAG TGT TGC TTC CGC AGC TGT GAT CTG GCC CTG CTG
Glu Thr Tyr Cys Ala Thr Pro Ala Lys Ser Glu
GAG ACC TAC TGT GCT ACC CCT GCC AAG TCC GAG 3'

```

(b)

```

          1          10          20          30
human      .AYRPSETLCGGELVDTLQFVCGDRGFYFS...RPASRVS
Ser29 human .-----RLPG-----
kangaroo   .-----LPG--L-----
chicken    A---GTA-----...--VG-NN

          40          50          60
human      RRS.RGIVEECCFRSCDLALLETYCATPAKSE
Ser29 human ----.-----
kangaroo   --LN-----
chicken    --IN-----KSV---

```

Figure 1 (a) The cDNA and deduced amino acid sequence of mature kIGF-II and (b) comparison of amino acid sequences of human, chicken, kangaroo and [Ser²⁹]-hIGF-II. Sequences are those reported by Rinderknecht & Humbel (1978) (human), Kallincos *et al.* (1990) (chicken) and Hampton *et al.* (1989) ([Ser²⁹]-hIGF-II) while the sequence for the kIGF-II is deduced from the present study and Yandell *et al.* (1998). Dashed lines represent identical sequence compared with hIGF-II. Periods were used to frame shift proteins for maximal alignment between sequences. Numbering is according to the human sequence.

calculated from the deduced amino acid sequence was the same as that obtained from the mass spectroscopy analysis of kIGF-II purified from serum. Comparison of the deduced amino acid sequence of kIGF-II and the hIGF-II amino acid sequence revealed that the A, B and D domains are indeed highly conserved (Fig. 1b). However, the C domain of kIGF-II contains an insert of Leu-Pro-Gly at position 30 (numbering using the human sequence), a substitution of Leu for Ala at position 32, Leu for Ser at position 39, as well as an insert of an Asn at position 40. An insertion of Leu-Pro-Gly in the C domain of IGF-II has also been described in an IGF-II variant, [Ser²⁹]-hIGF-II, found in human serum and placental tissue (Hampton *et al.* 1989, DeCeuninck *et al.* 1995b). In addition to the tripeptide insert, the human variant has a substitution of Arg for Ser²⁹. At the nucleotide level, the cDNA sequence of kIGF-II shares 100% identity with the partial tammar wallaby IGF-II cDNA sequence and 85% identity with [Ser²⁹]-hIGF-II.

Characterisation

In order to examine if the structural differences between kIGF-II and IGF-II from other species conferred any functional differences, kIGF-II was characterised in a number of *in vitro* assays and compared with hIGF-II, cIGF-II and the human variant, [Ser²⁹]-hIGF-II. The affinity of binding to the IGF-II/CI-MPR was examined by measuring competition with ¹²⁵I-hIGF-II for binding to bIGF-II/CI-MPR purified from liver (Fig. 2). Half-maximal competition for binding was observed at 61.5 ± 7.9 (mean $\pm$ s.e.m.), 57.9 ± 7.9 , 52.3 ± 3.8 and 51.4 ± 1.2 pM for kIGF-II, hIGF-II, cIGF-II and [Ser²⁹]-hIGF-II respectively. There were no significant differences between the mean IC₅₀ values of all four proteins ($P > 0.05$).

The ability of the proteins to stimulate protein synthesis in rat L6 myoblasts was also examined by measuring the incorporation of [³H]leucine into total protein (Fig. 3). All IGF-IIs were found to have a similar potency with

Figure 2 Competition for binding of ^{125}I -hIGF-II to purified hIGF-II/CI-MPR. The competing ligands were human (●), kangaroo (▲), chicken (■) and [Ser 29]-hIGF-II (▼). Binding is expressed as a percentage of the binding observed in the absence of competing IGF-II. Values are means $\pm$ S.E.M. for three separate experiments.

half-maximal effects observed at concentrations of 13.0 ± 1.2 , 10.3 ± 1.7 , 8.9 (no S.E.M. as only one experiment was performed) and 7.8 ± 1.0 nM for cIGF-II, kIGF-II, [Ser 29]-hIGF-II and hIGF-II respectively. Again there were no significant differences between these values ($P > 0.05$).

On the other hand, differences in the affinities of the IGF-II for human IGFBPs were observed in competitive binding studies using human serum (Fig. 4a). kIGF-II was the least effective ($P < 0.001$) at competing with ^{125}I -hIGF-II for binding to the IGFBPs in human serum. cIGF-II and [Ser 29]-hIGF-II were also significantly less effective than hIGF-II in their ability to bind to the human IGFBPs ($P < 0.05$) in this assay. The half-maximal inhibitory concentrations observed for kIGF-II, cIGF-II, [Ser 29]-hIGF-II and hIGF-II were 5.32 ± 0.25 , 3.81 ± 0.15 , 3.67 ± 0.13 and 2.89 ± 0.17 nM respectively.

To determine if the differences detected in IGFBP affinity were due to the heterologous nature of the assay, we also tested the ability of kIGF-II and hIGF-II to compete for binding to the IGFBPs found in the serum of a marsupial, the tammar wallaby (Fig. 4b). hIGF-II and kIGF-II were similar in their ability to bind to tammar wallaby IGFBPs with half-maximal competition observed at concentrations of 7.1 and 7.4 nM respectively.

The cross-reactivity of kIGF-II in an RIA using a monoclonal antibody raised against rat IGF-II was also examined (Fig. 5). This antibody has been routinely used

Figure 3 Dose-response curves of hIGF-II (●), kIGF-II (▲), cIGF-II (■) and [Ser 29]-hIGF-II (▼) in a rat L6 myoblast protein synthesis assay. Bioactivity is expressed as the percentage stimulation above that of cells incubated with medium alone. Values are means $\pm$ S.E.M. for two separate experiments for human, chicken and kangaroo IGF-II. Values for [Ser 29]-hIGF-II are the mean $\pm$ S.D. of one experiment.

in our laboratory for measuring the levels of IGF-II in rat and human sera. The concentration curves obtained from serial dilutions of kIGF-II and hIGF-II were parallel. However, the concentrations required for half-maximal binding with kIGF-II and hIGF-II, 24.4 ± 1.6 and 12.9 ± 0.4 pM respectively, were significantly different ($P < 0.05$).

Discussion

We have previously purified both IGF-I and IGF-II from western grey kangaroo serum and reported the characterisation and full sequence of IGF-I (Yandell *et al.* 1998). We have now obtained the complete cDNA sequence for kIGF-II and characterised the purified protein in a number of *in vitro* assays.

The molecular mass of the purified material as determined by electrospray mass spectrometry was 7921 Da, approximately 450 Da larger than that reported for hIGF-II (Rinderknecht & Humbel 1978). To confirm this result and to obtain the complete amino acid sequence we used RT-PCR of mRNA isolated from kangaroo liver to obtain the cDNA sequence of kIGF-II. The cDNA sequence of kIGF-II revealed 100% identity with the partial IGF-II cDNA sequence obtained previously from the tammar wallaby. This finding is not unexpected as the

Figure 4 Competition for binding of ^{125}I -hIGF-II to IGFBPs in (a) human serum and (b) tammar wallaby serum. The competing ligands were human (●), kangaroo (▲), chicken (■) and [Ser²⁹]-hIGF-II (▼). Binding is expressed as a percentage of the binding observed in the absence of competing IGF-II. Values are means $\pm$ S.E.M. for three separate experiments for the human serum. Values for the tammar wallaby serum are the mean $\pm$ S.D. of one experiment.

western grey kangaroo and tammar wallaby are closely related species. Comparison of the deduced amino acid sequence with hIGF-II revealed that kIGF-II contains six amino acids that are different from the hIGF-II sequence. One major difference is that the kangaroo protein has a three amino acid insert of Leu-Pro-Gly at position 30 (numbering based on human sequence). Comparison with

Figure 5 IGF-II RIA using antiserum raised against rat IGF-II. The radioligand was ^{125}I -hIGF-II and the competing ligands were hIGF-II (●) and kIGF-II (▼). Values are means $\pm$ S.E.M. for three separate experiments.

other known IGF-II sequences revealed that kIGF-II is similar to a minor IGF-II variant, [Ser²⁹]-hIGF-II, previously described in human serum (Hampton *et al.* 1989, Straczek *et al.* 1990) and placental tissue (De Ceuninck *et al.* 1995b). This variant also contains an insertion of Leu-Pro-Gly at position 30 along with a substitution of Arg for Ser at position 29. The variant mRNA arises from alternative splicing of a common RNA precursor and the sequence contains a typical acceptor site (5'-CTTC CAG-3') in the extra nine nucleotides that encode Leu-Pro-Gly (Jansen *et al.* 1985). The A and the G nucleotides of the AGC codon for Ser²⁹ most likely act as donor and acceptor splice sites respectively. Furthermore, nucleotide sequence analysis of the IGF-II gene indicates that there is a 1700 nucleotide intron at exactly this point (Dull *et al.* 1984). The kIGF-II cDNA sequence (Fig. 1a) differs slightly from the human variant cDNA sequence in this region, which suggests that alternate splicing may not occur, giving rise to only the larger mRNA transcript. Thus, we propose that the splice site that generates the major form of hIGF-II has evolved recently, occurring after the divergence of metatherians from eutherian mammals. The hypothesis that the IGF-II variant in humans arises from an ancestor gene in marsupials suggests that this variant may also be present in other mammalian species.

Surprisingly, we found no evidence for any higher MW IGF-II isoforms or variant IGF-IIs during the purification procedure. Nor did we identify any other RNA transcripts of IGF-II when cloning IGF-II from kangaroo liver. However, several variants of IGF-I were detected during

the purification of the IGF-I from kangaroo serum (Yandell *et al.* 1998). The difference(s) between the variant forms and the major circulating kIGF-I were not established. In contrast, human serum contains several IGF-II variants (Hampton *et al.* 1989, Straczek *et al.* 1990, Van den Brande *et al.* 1990) and higher MW isoforms of IGF-II (Zumstein *et al.* 1985, Gowan *et al.* 1987) that together comprise a minor component of total circulating IGF-II. The physiological role of both the high MW forms of IGF-II and IGF-II variants is, at present, unclear.

The deduced kIGF-II protein sequence also contains three other amino acid changes that are not observed in hIGF-II. These changes share similarities with the differences identified in many of the IGF-IIs reported for non-mammalian species. Indeed, all differences detected in the kIGF-II sequence, except the tripeptide insertion, are conservative changes arising from a one-nucleotide mutation of the cIGF-II sequence. Whether there are avian IGF-IIs which also contain an insert at the B–C hinge awaits sequencing of IGF-II cDNA or protein from other avian species.

Analysis of receptor/ligand binding using purified bIGF-II/CI-MPR indicated that kIGF-II binds to this receptor with affinity equal to all other IGF-IIs tested. This result is in agreement with the results reported by De Ceuninck *et al.* (1995a,b) who found that [Ser²⁹]-hIGF-II had equal affinity with hIGF-II for IGF-II/CI-MPRs on ovine placental membranes and chondrocyte membranes. However, it has also been demonstrated that [Ser²⁹]-hIGF-II has a 10% higher affinity than IGF-II for BHK cells overexpressing the IGF-II/CI-MPR (Lüthi *et al.* 1992). The small differences observed between these studies may be due to the use of the different assay systems as the residues proposed to be important for IGF-II binding to this receptor, residues 48–50 and 54–55 (Burgisser *et al.* 1991, Sakano *et al.* 1991), have been conserved in [Ser²⁹]-hIGF-II, kIGF-II and hIGF-II.

The ability of kIGF-II, hIGF-II, cIGF-II and [Ser²⁹]-hIGF-II to stimulate protein synthesis in L6 myoblasts was also examined. While there was a trend suggesting that kIGF-II was less potent (1.3-fold) than hIGF-II, this difference was not statistically significant. Nor were there any significant differences between the potencies of [Ser²⁹]-hIGF-II, hIGF-II and cIGF-II. Previous studies report that in HIGRI cells overexpressing the type 1 IGF receptor, [Ser²⁹]-hIGF-II was 1.4-fold less potent than hIGF-II at stimulating [³H]thymidine incorporation into DNA (Lüthi *et al.* 1992). Similarly, [Ser²⁹]-hIGF-II was 2- to 3-fold less potent than hIGF-II at stimulating growth in MCF-7 cells (Hampton *et al.* 1989) and at stimulating sulphation in cultured chondrocytes (De Ceuninck *et al.* 1995a).

The B–C hinge region of IGFs is believed to be important for binding to the type 1 IGF receptor (Humbel 1990). Arguably, the three amino acid insert at this

interface in both kIGF-II and [Ser²⁹]-hIGF-II may alter their affinities for this receptor. Indeed, [Ser²⁹]-hIGF-II has been shown to have a lower affinity than hIGF-II for type 1 IGF receptors on human placental membranes (Hampton *et al.* 1989, De Ceuninck *et al.* 1995b), HIGRI cells overexpressing the type 1 IGF receptor (Lüthi *et al.* 1992) and in cultured chondrocyte membranes (De Ceuninck *et al.* 1995a). As the mitogenic activity of IGF-II is mediated through its interaction with this receptor this may explain the lower potency of [Ser²⁹]-hIGF-II and kIGF-II.

There were significant differences in the ability of the four IGF-IIs to compete with ¹²⁵I-hIGF-II for binding to the IGFBPs present in human serum. In order of increasing affinity they were kIGF-II < cIGF-II = [Ser²⁹]-hIGF-II < hIGF-II. This finding contrasts with that reported by Lüthi *et al.* (1992), who found that the ability of [Ser²⁹]-hIGF-II to compete for binding with ¹²⁵I-hIGF-II to recombinant IGFBP-3 was 171% of that observed for hIGF-II. As IGF-II and IGFBP-3 in serum are likely to be found in a ternary complex with the acid-labile subunit this may explain the differences in apparent affinities between recombinant IGFBP-3 and IGFBPs in serum. Likewise, the differences between these two studies may also reflect the differences in the affinities of the IGF-IIs for the other IGFBPs that are present in human serum.

In contrast to the results obtained with human serum, the apparent affinity of kIGF-II for IGFBPs in tammar wallaby serum was no different from that of hIGF-II. The apparent affinity of both kIGF-II and hIGF-II for the IGFBPs in wallaby serum, however, appears to be lower than their affinity for IGFBPs present in human serum. This suggests that there may be differences in the number and ratios of the circulating IGFBPs present in the tammar wallaby, or alternatively there may be structural differences between the wallaby and human IGFBPs. It has previously been shown that the IGFBPs in tammar wallaby serum are similar in apparent size and circulating forms to those found in eutherian mammals (Carr *et al.* 1995). Furthermore, Northern analysis of total liver RNA from wallabies indicated that transcripts for IGFBP-1, -2 and -4 are present in this species. However, the extent of the homology between wallaby IGFBPs and mammalian IGFBPs is not known.

In this study it was revealed that kIGF-II shares structural homology with both non-mammalian and mammalian IGF-IIs. However, kIGF-II contains a tripeptide insertion in the C domain that is also found in a hIGF-II variant, [Ser²⁹]-hIGF-II. Characterisation of chicken, human, kangaroo and [Ser²⁹]-hIGF-II in a number of *in vitro* assays revealed that these IGF-IIs are also functionally similar. The finding that the major form of circulating IGF-II in the kangaroo is structurally and functionally similar to a minor IGF-II variant in humans is intriguing. Indeed, it is plausible that the differences in

IGF-II structure in eutherians, metatherians and non-mammalians arose due to a changing role for IGF-II during mammalian development. Hence, it will be interesting to determine if this is in some way linked to the changing role of the CI-MPR, as an IGF-II receptor, in eutherians.

Acknowledgements

The authors gratefully acknowledge Dr Morey Slodki for generously providing *Hansenula holstii* phosphomannan, Dr Christine Lüthi for the recombinant [Ser²⁹]-hIGF-II, Dr Stephen Wilcox for the partial cDNA sequence of wallaby IGF-II and Dr K Nishikawa for the anti-rat IGF-II antibody. We also wish to thank Mr Doug Evans for giving up his time to help us collect kangaroo blood, Professor Russell Baudinette and Ms Jayne Skinner for the tammar wallaby blood, Mr Larry Hick (Wollongong University) for the mass spectrometry analysis and Mr Darryl Lanthois (Agpro Abattoir) for providing the bovine liver. We are also grateful to Dr Andrew Dunbar for his assistance with the RT-PCR and Dr F John Ballard for helpful discussions.

References

- Ballard FJ, Read LC, Francis GL, Bagley CJ & Wallace JC 1986 Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. *Biochemical Journal* **233** 223–230.
- Brissenden J, Ullrich A & Francke U 1984 Human chromosomal mapping of genes for insulin-like growth factors I and II and epidermal growth factor. *Nature* **310** 781–784.
- Burgisser DM, Roth BV, Giger R, Lüthi C, Weigl S, Zarn J & Humbel RE 1991 Mutants of human insulin-like growth factor II with altered affinities for the type 1 and type 2 insulin-like growth factor receptor. *Journal of Biological Chemistry* **266** 1029–1033.
- Canfield WM & Kornfeld S 1989 The chicken liver cation-independent mannose 6-phosphate receptor lacks the high affinity binding site for insulin-like growth factor-II. *Journal of Biological Chemistry* **264** 7100–7103.
- Carr JM, Owens JA, Baudinette RV & Wallace JC 1995 Characterization of insulin-like growth factor binding proteins (IGFBPs) in the tammar wallaby, *Macropus eugenii*. *General and Comparative Endocrinology* **99** 50–59.
- Clairmont KB & Czech MP 1989 Chicken and *Xenopus* mannose 6-phosphate receptors fail to bind insulin-like growth factor II. *Journal of Biological Chemistry* **264** 16390–16392.
- Dahms NM, Brzycki-Wessell MA, Ramanujam KS & Seetharam B 1993 Characterization of mannose 6-phosphate receptors (MPRs) from opossum liver: opossum cation-independent MPR binds insulin-like growth factor II. *Endocrinology* **133** 440–446.
- Daughaday WH & Kapadia M 1989 Significance of abnormal serum binding of insulin-like growth factor II in the development of hypoglycaemia in patients with non-islet-cell tumours. *Proceedings of the National Academy of Sciences of the USA* **86** 6778–6782.
- Daughaday WH, Emanuele MA, Brooks MH, Barbato AL, Kapadia M & Rotwein P 1988 Synthesis and secretion of insulin-like growth factor II by a leiomyosarcoma with associated hypoglycaemia. *New England Journal of Medicine* **319** 1434–1440.
- De Ceuninck F, Poiraudou S, Pagano M, Tsagris L, Blanchard O, Willeput J & Corvol M 1995a Inhibition of chondrocyte cathepsin B and L activities by insulin-like growth factor-II (IGF-II) and its Ser²⁹ variant *in vitro*: possible role of the mannose 6-phosphate/IGF-II receptor. *Molecular and Cellular Endocrinology* **113** 205–213.
- De Ceuninck F, Willeput J & Corvol M 1995b Purification and characterization of insulin-like growth factor II (IGF-II) and an IGF-II variant from human placenta. *Journal of Chromatography B* **666** 203–214.
- DeChiara TM, Efstratiadis A & Robertson EJ 1990 A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345** 78–80.
- Duguay SJ, Chan SJ, Mommsen TP & Steiner DF 1995 Divergence of insulin-like growth factors I and II in the elasmobranch *Squalus acanthias*. *FEBS Letters* **371** 69–72.
- Duguay SJ, Lai-Zhang J, Steiner DF, Funkenstein B & Chan SJ 1996 Developmental and tissue-regulated expression of IGF-I and IGF-II mRNAs in *Sparus aurata*. *Journal of Molecular Endocrinology* **16** 123–132.
- Dull TJ, Gray A, Hayflick JS & Ulrich A 1984 Insulin-like growth factor-II precursor gene organisation in relation to insulin gene family. *Nature* **310** 777–781.
- Francis GL, Owens PC, McNeil KA, Wallace JC & Ballard FJ 1989 Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like growth factor- I and -II. *Journal of Endocrinology* **122** 681–687.
- Gowan LK, Hampton B, Hill DJ, Schlueter RJ & Perdue JF 1987 Purification and characterization of a unique high molecular weight form of insulin-like growth factor II. *Endocrinology* **121** 449–458.
- Hampton B, Burgess WH, Marshak DR, Cullen KJ & Perdue JF 1989 Purification and characterization of an insulin-like growth factor II variant from human plasma. *Journal of Biological Chemistry* **264** 19155–19160.
- Han VKM, Bassett N, Walton J & Challis JRG 1996 The expression of insulin-like growth factor (IGF) and IGF-binding protein (IGFBP) genes in the human placenta and membranes: evidence for IGF-IGFBP interactions at the foeto-maternal interface. *Journal of Clinical Endocrinology and Metabolism* **81** 2680–2693.
- Haselbacher G & Humbel RE 1982 Evidence for two species of insulin-like growth factor II (IGF II and 'big' IGF II) in human spinal fluid. *Endocrinology* **110** 1822–1824.
- Humbel RE 1990 Insulin-like growth factors I and II. *European Journal of Biochemistry* **190** 445–462.
- Jansen M, van Schaik FMA, van Tol H, Van den Brande JL & Sussenbach JS 1985 Nucleotide sequences of cDNAs encoding precursors of human insulin-like growth factor II (IGF-II) and an IGF-II variant. *FEBS Letters* **179** 243–246.
- Kallincos NC, Wallace JC, Francis GL & Ballard FJ 1990 Chemical and biological characterization of chicken insulin-like growth factor-II. *Journal of Endocrinology* **124** 89–97.
- Kita K, Tomas FM, Owens PC, Knowles SE, Forbes BE, Upton Z, Hughes R & Ballard FJ 1996 Influence of nutrition on hepatic IGF-I mRNA levels and plasma concentrations of IGF-I and IGF-II in meat-type chickens. *Journal of Endocrinology* **149** 181–190.
- Louvi A, Accili D & Efstratiadis A 1997 Growth-promoting interaction of IGF-II with the insulin receptor during mouse embryonic development. *Developmental Biology* **189** 33–48.
- Lüthi C, Roth BV & Humbel RE 1992 Mutants of human insulin-like growth factor II (IGF-II). Expression and characterization of truncated IGF-II and two naturally occurring variants. *European Journal of Biochemistry* **205** 483–490.
- Renfree MB 1993 Diapause, pregnancy and parturition in Australian marsupials. *Journal of Experimental Zoology* **266** 450–462.
- Renfree MB 1995 Monotreme and marsupial reproduction. *Reproduction, Fertilisation and Development* **7** 1003–1020.
- Reynolds TS, Stevenson KR & Wathes DC 1997 Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology* **138** 886–897.

- Rinderknecht E & Humbel RE 1978 Primary structure of human insulin-like growth factor II. *FEBS Letters* **89** 283–286.
- Sakano K, Enjoh T, Numata F, Fujiwara H, Marumoto Y, Higashihashi N, Sato Y, Perdue JF & Fujita-Yamaguchi Y 1991 The design, expression and characterization of human insulin-like growth factor II (IGF II) mutants specific for either the IGF-II/cation-independent mannose 6-phosphate receptor or IGF-I receptor. *Journal of Biological Chemistry* **266** 20626–20635.
- Scott CD & Baxter RC 1987 Purification and immunological characterization of the rat liver insulin-like growth factor-II receptor. *Endocrinology* **120** 1–9.
- Straczek J, Heulin MH, Chenut AM, Lasbennes A, Geschier C, Belleville F & Nabet P 1990 Purification and characterization of three molecular forms of insulin-like growth factor II from human Cohn paste IV. *Journal of Chromatography* **532** 237–248.
- Szabo L, Mottershead DG, Ballard FJ & Wallace JC 1988 The bovine insulin-like growth factor (IGF) binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-I for binding. *Biochemical and Biophysical Research Communications* **151** 207–214.
- Tricoli JV, Rall LB, Scott J, Bell GI & Shows TB 1984 Localization of insulin-like growth factor genes to human chromosomes 11 and 12. *Nature* **310** 784–786.
- Upton Z, Francis GL, Kita K, Wallace JC & Ballard FJ 1995 Production and characterization of recombinant chicken insulin-like growth factor-II from *Escherichia coli*. *Journal of Molecular Endocrinology* **14** 79–90.
- Van den Brande JL, Hoogerbrugge CM, Beyreuther K, Roepstorff P, Jansen J & van Buul-Offers SC 1990 Isolation and partial characterization of IGF-like peptides from Cohn fraction IV of human plasma. *Acta Endocrinologica* **122** 683–695.
- Wang Z-Q, Fung MR, Barlow DP & Wagner EF 1994 Regulation of embryonic growth and lysosomal targeting by the imprinted Igf2/Mpr gene. *Nature* **372** 464–467.
- Yandell CA, Francis GL, Wheldrake JF & Upton Z 1998 Purification, amino acid sequence and characterization of kangaroo IGF-I. *Journal of Endocrinology* **156** 195–204.
- Zumstein PP, Lüthi C & Humbel RE 1985 Amino acid sequence of a variant pro-form of insulin-like growth factor-II. *Proceedings of the National Academy of Sciences of the USA* **82** 3169–3172.

Received 9 November 1998

Accepted 29 January 1999