PRODUCTION AND CHARACTERIZATION OF NONMAMMALIAN INSULIN-LIKE GROWTH FACTORS

by

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SUMMARY

Little is known about the functions of insulin-like growth factors (IGFs) in growth and development in nonmammalian species, primarily due to the low amounts of pure peptide available for testing. In order to overcome this I have developed gene-fusion strategies and downstream processing procedures to produce recombinant nonmammalian IGFs in *Escherichia coli*.

Researchers from this laboratory have previously reported the purification and amino acid sequences of chicken IGF-I (cIGF-I) and chicken IGF-II (cIGF-II). Using that protein sequence information, I have produced milligram quantities of recombinant cIGF-I and cIGF-II, thus permitting thorough assessment of non-mammalian IGFs for the first time. I present evidence that recombinant cIGF-I has the same biological, IGF binding-protein (IGFBP)- and receptor-binding interactions as human IGF-I. *In vitro* analysis of recombinant cIGF-II, on the other hand, has revealed differences between cIGF-II and its human counterpart. Recombinant cIGF-II is less potent than hIGF-II in stimulating protein synthesis in rat myoblasts. This appears to be due to a decreased affinity for the type-1 IGF receptor. The human and chicken peptides are equipotent however, in studies assessing binding to the type-2 IGF receptor and to IGFBPs. Moreover, recombinant cIGF-II and hIGF-II are equipotent in both biological and receptor binding studies in chick embryo fibroblasts, suggesting that there may be a difference between mammalian and avian type-1 IGF receptors.

A recombinant analogue of cIGF-I with an eighteen amino acid N-terminal extension as well as a substitution of Arg for Glu at position 3 in cIGF-I has also been produced. This particular analogue was engineered because *in vitro* and *in vivo* studies with the human equivalent of this protein have shown that it is a more potent form of IGF-I. The increased potency is the result of decreased affinity for IGFBPs. The recombinant chicken homologue produced also exhibits decreased affinity for chicken or rat IGFBPs and enhanced *in vitro* biological activity.

The recent cloning of a cDNA for hagfish IGF suggests that the IGFs have a long evolutionary history and this in turn suggests that the IGFs are functionally important in all vertebrates. I have produced recombinant hagfish IGF and *in vitro* assessment indicates that while hagfish IGF is significantly less potent, it does indeed share functional properties with human IGF-I. Biological and receptor binding studies in mammalian and fish cell lines suggest that motifs important for IGF functions appear to have evolved prior to the Agnanthans diverging from the main line of vertebrate evolution.

While mammalian IGF binding proteins have been extensively studied, again little is known about these proteins in nonmammalian species. In order to understand more about the origins and evolutionary relationships of IGFs and their binding proteins, I have partially characterized the IGFBPs in non-mammalian sera. I show by both *in vitro* labelling with subsequent analytical gel filtration and by ligand blotting, that IGFBPs are present in hagfish and lamprey serum, indicating that the IGFBPs are very ancient proteins. Furthermore, I present evidence that the major serum IGFBP in lamprey is significantly smaller than the high molecular weight IGFBP3 complex in rat serum. In addition, I show that IGFBPs occur widely throughout vertebrates, as IGFBPs are detected in ligand blots of serum from marsupials, monotremes, reptiles, birds and turtles.

STATEMENT OF ORIGINALITY

This thesis contains no material which has been accepted for the award of any other degree or diploma in any University. To the best of my knowledge and belief it contains no material that has previously been published by any other person except where due reference is made. The author consents to the thesis being made available for photocopying and loan.

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Zee Upton

Erratum addendum

i) Agnatha and Agnathan have been incorrectly spelt as Agnantha and Agnanthan throughout the thesis.

ii) In method 2.2.4.7, page 46, it should be noted that the samples and the molecular mass standards were analyzed under non-reducing conditions.

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Publications arising from thesis research

Published manuscripts:

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Upton, F.Z., Chan, S.J., Steiner, D.F., Wallace, J.C. & Ballard, F.J. (1993). Evolution of insulin-like growth factor binding proteins. *Growth Regulation* **3**, 29-32.

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McMurtry, J.P., Francis, G.L., Upton, F.Z., Rosselot, G. & Brocht, D.M. (1993, in press). Development of a homologous radioimmunoassay for chicken insulin-like growth factor-I (cIGF-I). *Journal of Endocrinology.*

Upton, Z., Francis, G.L., Wallace, J.C. & Ballard, F.J. (1993, in press). Production and characterization of recombinant chicken insulin-like growth factors. *Proceedings, Australian Poultry Science* **4**, in press. (Sydney, Australia).

Upton, Z., Francis, G.L., Kita, K., Wallace, J.C. & Ballard, F.J. (1993, submitted). Chicken insulin-like growth factor (IGF)-II differs from human IGF-II in its receptor binding characteristics. *Journal of Endocrinology.*

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Upton, Z., Wallace, J.C. & Ballard, F.J. (1992). Production and characterization of recombinant chicken insulin-like growth factor-I from *Escherichia coli.*

Abstract *Proceedings: Biorecognition -International Industrial Biotechnology Conference*, 83. (Montreal, Canada).

Upton, Z., Francis, G.L., Wallace, J.C. & Ballard, F.J. (1993). Characterization and production of recombinant chicken insulin-like growth factor-II from *Escherichia coli*.. Abstract *Proceedings, Australian Poultry Science* **3**, 72. (Sydney, Australia).

Upton, Z., Chan, S.J., Steiner, D.F., Francis, G.L. & Ballard, F.J. (1994, in press). Recombinant hagfish IGF exhibits both IGF-I and IGF-II-II characteristics. Abstract. *Proceedings, Third International Symposium on Insulin-like Growth Factors*, in press. (Sydney, Australia).

ABBREVIATIONS

In addition to those abreviations accepted for use in *The Journal of Biological Chemistry* the following will be assumed;

BSA:	bovine serum albumin
CEF:	chick embryo fibroblasts
cpm:	counts per minute
DMEM:	Dulbecco's Modified Eagle's Medium
DTT:	dithiothreitol
E. coli:	*Escherichia coli*
FPLC:	fast pressure liquid chromatography
GH:	growth hormone
HFBA:	heptafluorobutyric acid
HPLC:	high performance liquid chromatography
IGF-I:	insulin-like growth factor-I
IGF-II:	insulin-like growth factor-II
cIGF-I:	chicken IGF-I
cIGF-II	chicken IGF-II
hIGF-I:	human IGF-I
hIGF-II:	human IGF-II
IGFBP:	insulin-like growth factor binding protein
SDS:	sodium dodecyl sulphate
SDS-PAGE:	SDS polyacrylamide gel electrophoresis
TFA:	trifluoroacetic acid

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1: INTRODUCTION

The insulin-like growth factors (IGFs) are a family of polypeptides with both structural and functional homology to insulin. In this chapter I will firstly review the structural characterization of the mammalian IGFs, the organization and expression of their genes, the interactions of IGFs with receptors and with specific carrier proteins and the functional characterization of these proteins *in vitro* and *in vivo*. I will then describe what is known about nonmammalian IGFs. The review of both mammalian and non-mammalian IGFs will encompass the literature available until the end of 1991. The most recent information relating to my PhD research will be included in the appropriate chapters.

1.2: MAMMALIAN IGFs

1.2.1: Original isolation of the IGFs

Three separate lines of investigation led to the discovery of what are now known as IGFs. The IGFs were originally defined as sulphation factors by Salmon and Daughaday (1957) who observed that growth hormone (GH) injected into rats stimulated incorporation of $^{35}SO_4$ into cartilage. This effect could not be mimicked *in vitro* thus leading to the formation of the "Somatomedin hypothesis" in which the sulphation factor was proposed to be produced in the body under the control of GH and to circulate in the blood to target tissues, hence mediating the effects of GH. These sulphation factors were later termed somatomedins. The IGFs were also identified as non-supressible insulin-like activity (NSILA) by Froesch *et al.* (1963) who identified a factor in serum which acted like insulin in adipose tissues, but whose activity could not be neutralized by an anti-insulin antibody. Finally, Dulak & Temin (1973) identified a factor produced by rat liver cells which could promote the growth of cells normally dependent on serum. This factor was termed multiplication stimulating activity (MSA). Following purification and characterization, these three factors, somatomedin, NSILA and MSA, were given the common name of IGF

(Daughaday *et al.* 1987a) due to their high degree of structural similarity with insulin (Rinderknecht & Humbel, 1976).

1.2.3: Structure of the IGFs

1.2.3.1: Sequences of IGFs

Two types of IGFs have been identified, IGF-I and IGF-II. Both are single chain molecules with 70 and 67 amino acid residues respectively (Rinderknecht & Humbel 1978a,b). Both forms of IGFs are closely related with 70% sequence similarity. Somatomedin C was found to be structurally identical to IGF-I (Klapper *et al.* 1983) whereas rat MSA was found to be more similar to IGF-II (Marquardt *et al.* 1981).

The amino acid sequences of mammalian IGFs are highly conserved with human (Rinderknecht & Humbel 1978a), bovine (Francis *et al.* 1986; Honegger & Humbel, 1986; Francis *et al.* 1988), porcine (Francis *et al.* 1989b) and guinea pig (Bell *et al.* 1990) IGF-I all being identical, while ovine (Francis *et al.* 1989a), rat (Tamura *et al.* 1989) and mouse (Bell *et al.* 1986) IGF-I differ by only 1, 3 and 4 residues respectively. Likewise, all the mammalian IGF-IIs characterized thus far have highly conserved amino acid sequences (Fig.1.1).

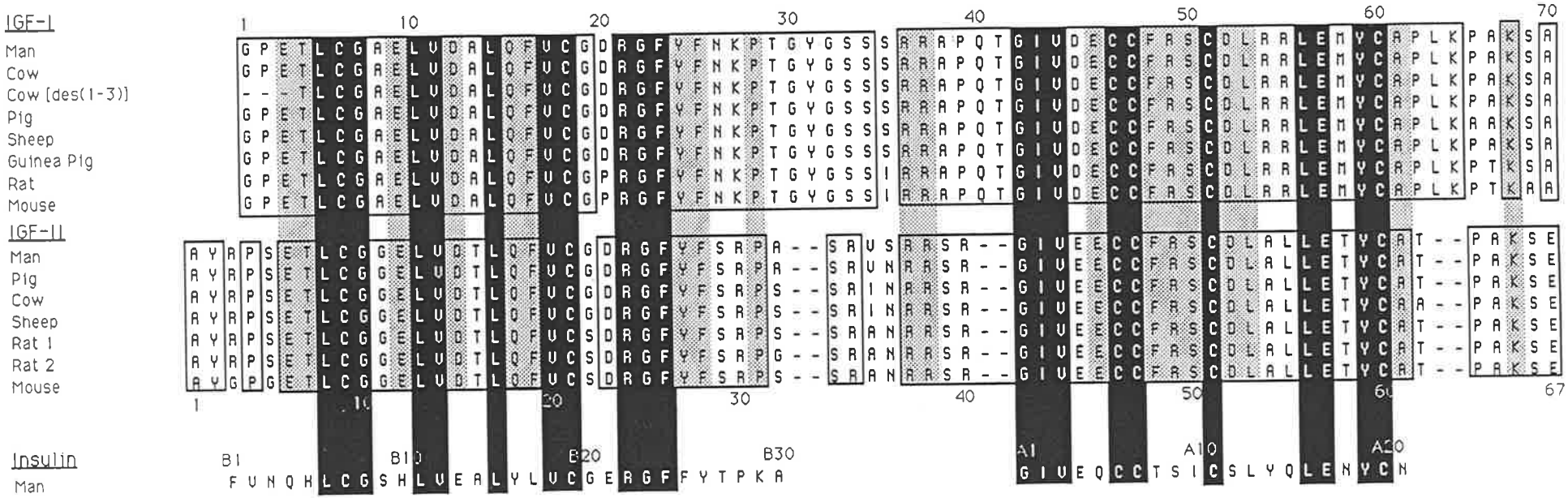
1.2.3.2: Structural similarities between IGFs and insulin

Not only do the IGFs exhibit a high degree of identity with each other, but also, they share similarity with insulin. Both the IGFs and insulin have a similar domain structure and all six cysteine residues, and hence the intramolecular disulphide bonds, are conserved. Indeed, IGF-I shares 25/51 amino acid residues with insulin. The IGFs differ from insulin, however, in that the C-peptide is not cleaved from the mature protein. Thus IGFs consist of a single polypeptide chain similar to that of proinsulin. In addition, there is a carboxy-terminal extension of 8 (IGF-I) or 6 (IGF-II) amino acids for IGF-I and IGF-II respectively, known as the D-peptide. The extensive similarity of IGFs to proinsulin suggests they evolved from a common ancestral gene.

FIGURE 1.1: Sequence comparison of insulin and insulin-like growth factors (IGF). Those residues conserved in IGF-I or in IGF-II are boxed, those residues conserved between IGF-I and IGF-II are shaded, while those residues also conserved in insulin are highlighted in black. The references from which these sequences were obtained are as follows:

IGF-I:	
Man	Rinderknecht & Humbel, 1978a
Cow	Honeggar & Humbel, 1986
Cow [des(1-30]	Francis *et al.* 1986
Pig	Francis *et al.* 1989b
Sheep	Francis *et al.* 1989a
Guinea pig	Bell *et al.* 1990
Rat	Tamura *et al.* 1989
Mouse	Bell *et al.* 1986

IGF-II:	
Man	Rinderknecht & Humbel, 1978b
Pig	Francis *et al.* 1989b
Cow	Honegger & Humbel, 1986
Sheep	Francis *et al.* 1989
Rat 1	Dull *et al.* 1984
Rat 2	Marquardt *et al.* 1981
Mouse	Stempien *et al.* 1986



Insulin C-peptide: R R E A E D L Q V G Q V E L G G G P G A G S L Q P L A L E G S L Q K R

1.2.4: IGF gene structure

1.2.4.1: IGF-I cDNA and gene structure

The sequence of a cDNA encoding IGF-I was first reported by Jansen *et al.* (1983). The sequence revealed that human IGF-I messenger RNA (mRNA) gives rise to a precursor protein of at least 130 amino acids which is subsequently proteolytically cleaved at both ends to form the mature peptide. More recently it has been established that two different IGF-I mRNAs encoding alternative E-domains are formed from the human IGF-I gene. The two prepro-mRNAs are referred to as IGF-Ia and IGF-Ib (Rotwein *et al.* 1986). Likewise, multiple forms of IGF-I mRNA are formed by the rat IGF-I gene (Roberts *et al.* 1987). Furthermore, both species reveal many mRNA species when tissues are probed by Northern analysis. These arise by alternative splicing of the 3' untranslated region and by mutiple polyadenylation.

The gene encoding human IGF-I has been localized to the long arm of chromosome 12 (12q22-q24.1)(Brissenden *et al.* 1984; Tricoli *et al.* 1984) and consists of 5 exons spanning over at least 40 kb. Exon 1 contains 5' untranslated sequences, while exons 2 and 3 encode the signal peptide, intact IGF-I, as well as the first 16 amino acids of the E-domain. Exons 4 and 5 contain sequences for the alternative amino acids in the E-domain, in addition to 3' untranslated sequences. Alternative splicing of the primary IGF-I gene transcript results in either a 153 amino acid IGF-Ia precursor protein containing a 35 amino acid E-domain (exons 1,2,3 and 5), or in a 195 amino acid IGF-Ib precursor protein with a 77 residue E-domain (exons 1,2,3 and 4). Likewise, the rat and mouse IGF-I genes also contain 5 exons that encode two prepro-IGFs which differ in their C-termini (Bell *et al.* 1986; Shimatsu & Rotwein, 1987a,b). It has been proposed that differential splicing may provide a mechanism for regulating IGF-I biosynthesis and function.

1.2.4.2: IGF-II cDNA and gene structure

The cloning and sequencing of a cDNA encoding human IGF-II revealed that the protein was derived from a 180 amino acid prepro-form. As was the situation with IGF-I, the precursor is processed at both ends to release the mature IGF-II peptide (Bell *et al.* 1985). The gene encoding human IGF-II has been localized to the short arm of chromosome 11 (11p15), in close proximity to the insulin gene (Brissenden *et al.* 1984; Tricoli *et al.* 1984; Bell *et al.* 1985). The human gene, extending over 30 kb, consists of 9 exons. Exons 1-6 contain the 5' untranslated sequences, while exons 7, 8 and 9 encode the sequences for the IGF-II precursor protein as well as the 3' untranslated sequences. Multiple promoters have been identified for both the human and the rat IGF-II gene, thus mutiple mRNA species arise from the genes. In the rat however, IGF-II mRNAs arise, not only due to the presence of the four promoters, but also due to variable RNA processing and polyadenylation (Daughaday & Rotwein 1989).

1.2.5: Variant IGFs

Several variant types of IGF molecules have been identified. A variant lacking the first three N-terminal amino acids of IGF-I has been isolated from bovine colostrum (Francis *et al.* 1986; Francis *et al.* 1988), human brain (Carlson-Skwirut *et al.* 1986; Sara *et al.* 1986), porcine uterus (Ogasawara *et al.* 1989) and from human platelets (Karey *et al.* 1989). Several IGF-II variants have been identified including a precursor form of IGF-II from the conditioned medium of a rat cell line (Yang *et al.* 1985), a large molecular weight form of IGF-II from human serum and cerebral spinal fluid (Hasselbacher & Humbel, 1982; Gowan *et al.* 1987), as well as two variants containing amino acid insertions (Zumstein *et al.* 1985; Jansen *et al.* 1985). Other IGF-I and IGF-II variants in human plasma have been identified by isolelectric focusing (Blum *et al.* 1986). As only one gene exists for IGF-I and for IGF-II, it is believed that these subforms of IGFs are caused by allelic variations, alternative splicing and different proteolytic processing of the proforms.

1.2.6: IGF gene expression

The IGFs genes have been demonstrated to be expressed in a wide variety of cells and tissues, thereby supporting previous observations that the IGFs appeared to be ubiquitous hormones, exerting paracrine, in addition to, endocrine functions. The expression of the genes is both tissue and developmentally specific (review, Sussenbach 1989).

1.2.6.1: IGF-I gene expression

In humans, IGF-I mRNA transcripts in a range of sizes from 1.0 to 7.5 kb, are expressed at low levels in both fetal and adult tissues. The liver, however, is the major site of expression and synthesis during postnatal growth. The developmental pattern of IGF-I gene expression in the liver correlates well with serum IGF-I levels, thus supporting the concept that the liver is the primary source of circulating IGF-I (Rotwein 1986; Rotwein *et al.* 1987). Moreover, GH stimulates the induction of IGF-I mRNA and production of IGF-I in liver cells (Mathews *et al.* 1986. On the other hand, fasting, protein malnutrition and diabetes all cause a decrease in IGF-I mRNA expression and IGF-I production in the liver (Bornfeldt *et al.* 1989). IGF-I gene expression is also regulated by a number of other factors including puberty (Zapf *et al.* 1981) and pregnancy (Bala *et al.* 1981; Hall *et al.* 1984). This is not surprising given that studies suggest that a number of hormones including estrogen (Murphy *et al.* 1987a), placental lactogen (Adams *et al.* 1983), fibroblast growth factor, angiotensin II and acute corticotropin, all modify IGF-I levels (Penhoat *et al.* 1989).

The findings from a number of studies suggest that the major IGF-I mRNA in human fetal tissues is the 7.5 kb transcript, whereas the predominant mRNA species in adults is the 1.1 kb transcript identified in the liver (Rotwein 1986; Han *et al.* 1987; Rotwein *et al.* 1987; Han *et al.* 1988; Sandberg *et al.* 1988). Hoppener *et al.* (1988) suggest that the 7.5 kb transcript may represent an IGF-Ia mRNA. Interestingly, the 7.5 kb transcript is also the predominant mRNA

species present in a number of tumours (Hoppener *et al.* 1988; Sandberg *et al.* 1988). In rats, IGF-Ia mRNA is the major form in all tissues with the exception of the liver, where IGF-Ib mRNA predominates (Lowe *et al.* 1988). Furthermore, both prepro-forms of mRNA are GH regulated, though IGF-Ib to a greater extent than IGF-Ia (Murphy *et al.* 1987a; Lowe *et al.* 1988). Expression of IGF-I is low in fetal rat liver, kidney and heart, but progressively rises postnatally. Conversely, IGF-I transcripts in lung, muscle and stomach are higher in fetal than in adult rats (Adamo *et al.* 1989). Taken together, these results suggest that there is both developmental and tissue-specific regulation of IGF-I gene expression. In particular, there appears to be a clear distinction between IGF-I gene expression in hepatic compared to other tissues.

1.2.6.2: IGF-II gene expression

Multiple forms of IGF-II mRNA are also found in humans. The four promoters present in the IGF-II gene, along with alternative splicing, give rise to an array of variously sized mRNA transcripts. Transcription from promoter 1 (P1) gives rise to a 5.3 kb mRNA containing exons 1, 2, 3, 7, 8 and 9, whereas initiation from P2 gives rise to a 5.0 kb transcript containing exons 4, 7, 8 and 9. Transcription from P3 gives rise to 6.0 and 4.8 kb transcripts containing exons 5, 7, 8 and 9, but differing in their 5' untranslated sequences. A 4.8 kb mRNA is formed by initiation of transcription from P4 (exons 6, 7, 8 and 9)(Sussenbach *et al.* 1991). The 5.3 kb transcript has only been found in adult liver. The 2.2 and 6.0 kb mRNA species are expressed in fetal and in non-hepatic adult tissues. Finally, the 4.8 kb transcript is expressed in both fetal and adult non-hepatic tissues. Overall, IGF-II gene expression is higher in the fetus than in the adult, suggesting the importance of this protein in early development (De Pagter-Holthuizen *et al.* 1987; De Pagter-Holthuizen *et al.* 1988; Sussenbach,1989). However, it has not yet been ascertained whether high expression correlates with increased IGF-II production (Graham *et al.* 1986; Haselbacher *et al.* 1987).

IGF-II gene expression in the rat also gives rise to an array of mRNA transcripts. Not only are these generated by the use of alternative promoters and differential splicing, but also by the use of alternative polyadenylation sites (Chiariotti *et al.* 1988). As with humans, IGF-II expression in the rat is also developmentally regulated with abundant IGF-II mRNA present in fetal tissues (Romanus *et al.* 1988). However, unlike humans, IGF-II expression is undetectable in adults, with the exception of the brain (Murphy *et al.* 1987b). This contrasts with the situation in humans where IGF-II is expressed in adult liver, hence explaining why IGF-II is present in the adult human circulation, but not in adult rat.

1.2.7: IGF receptors

1.2.7.1: Type-1 IGF receptor

The type-1 IGF receptor is thought to mediate most of the biological actions of IGFs. This receptor is a glycoprotein with an $\alpha 2\beta 2$ subunit composition maintained by disulphide bonds. The two extracellular α subunits (molecular mass = 130 kDa) bind the ligand, while the two β subunits (molecular mass = 95 kDa) span the cell membrane (Fig.1.2). The primary structure of the human type-1 IGF receptor as deduced from a placental cDNA, indicates that the receptor is synthesized as a precursor of approximately 1400 amino acids which is subsequently glycosylated and proteolytically processed to produce the mature α and β subunits (Ullrich *et al.* 1986).

The subunit structure is similar to that of the insulin receptor (Fig.1.2). Indeed, insulin can bind to the type-1 IGF receptor, albeit with considerably lower affinity (Massague & Czech, 1982). Likewise, IGFs can also bind to insulin receptors. The affinity of the type-1 IGF receptor is highest for IGF-I, 2- to 10-fold lower for IGF-II and 100- to 500-fold lower for insulin. A further similarity between the insulin and the type-1 IGF receptor is that both receptors exhibit intrinsic, ligand-stimulated tyrosine-kinase activity (Jacobs *et al.* 1983). However, recent studies with chimeric receptors consisting of the ligand-binding extracellular

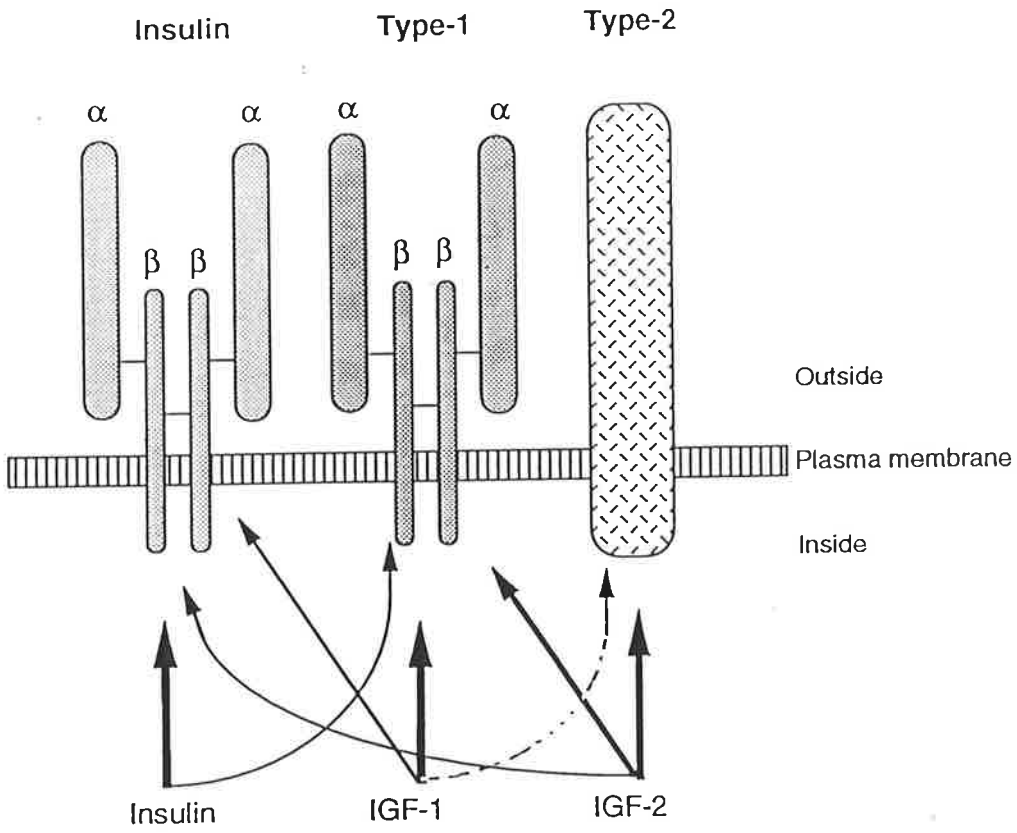


FIGURE 1.2: Schematic representation of the insulin, type-1 IGF and type-2 IGF receptor structures. The α and β subunits of the insulin and type-1 IGF receptor are connected by disulphide bonds. The thickness of the arrows indicates the relative affinities of insulin, IGF-I and IGF-II for the receptors.

domain of the insulin receptor and the intracellular domain of the type-1 IGF receptor have indicated that the receptors have distinct signalling potentials. While both the type-1 IGF and insulin receptors can mediate short term responses such as glucose transport, the chimeric receptor containing the type-1 IGF intracellular domain is more effective in stimulating protein synthesis than the insulin receptor (Lammers *et al.* 1989).

The tyrosine protein kinase domains are located in the cytoplasmic region of the β subunit, thus may mediate the biological actions of IGFs by phosphorylating endogenous cellular substrates. Several cellular substrates for the type-1 IGF receptor kinases have now been identified, including the phosphoprotein pp185. Moreover, pp185 is also an endogenous substrate for the insulin receptor kinases (Rothenberg *et al.* 1991; Sun *et al.* 1991). This protein, now designated as Insulin Receptor Substrate (IRS-1) has been proposed as a multi-site "docking" protein, which, when phosphorylated associates with cellular proteins such as phospatidylinositol 3' kinase through *src*-homology domains (Cantley *et al.* 1991).

1.2.7.2: Type-1 IGF receptor expression and regulation

Type-1 IGF receptor mRNA is widely distrubuted in fetal rat tissues. Indeed, mRNA for the receptor is detected early in organogenesis and is prominent in muscle and in the developing nervous system. The levels of mRNA decrease in most tissues during postnatal development with the exception of the liver, heart and kidney where mRNA levels increase postnatally (Rechler & Nissley, 1985; Werner *et al.* 1989).

The type-1 IGF receptor appears to be regulated according to receptor occupancy. IGF-I, IGF-II as well as insulin have all been demonstrated to downregulate the receptor (Rosenfeld 1982; Rosenfeld & Hintz, 1980). In addition, growth and nutritional state appear to modulate type-1 IGF receptor numbers (Bohannon *et al.* 1988; Caro *et al.* 1988). The mechanisms and sites controlling receptor recycling remain to be determined.

1.2.7.3: Type-2 IGF receptor

The type-2 IGF receptor is identical to the cation-independent mannose-6-phosphate receptor (Lobel *et al.* 1987), a receptor which acts as a lysosomal targeting protein. At this stage the functional significance of the IGF binding sites on this receptor remains an enigma. Moreover, the receptor is structurally unrelated to either the type-1 IGF or to the insulin receptor (Morgan *et al.* 1987). The type-2 IGF receptor consists of a single polypeptide chain with a large extracellular domain containing distinct binding sites for IGF-II and for mannose-6-phosphate, a single transmembrane domain and a short cytoplasmic region (Fig. 1.2). The receptor has a high affinity for IGF-II, binds IGF-I at very much lower affinity and does not recognize insulin (Roth 1988). As with the type-1 IGF receptor, the type-2 IGF receptor is developmentally regulated. Indeed, these receptors are expressed very early in development and have been detected on pre- and periimplantation mouse embryos (Mattson *et al.* 1988).

The signal transduction mechanisms of the type-2 IGF receptor are still to be elucidated. While the cytoplasmic domain lacks intrinsic protein kinase activity, a 14 amino acid segment of the cytoplasmic domain of the receptor has been shown to be coupled to a GTP binding protein (Nishimoto *et al.* 1989). The relationship of the GTP binding protein signaling mechanism to IGF-II function has not yet been defined.

A truncated, circulating form of the IGF-II receptor has been identified in human (Causin *et al.* 1988) , rat (Kiess *et al.* 1987) and monkey serum (Gelato *et al.* 1988), as well as in human urine (Causin *et al.* 1988). Furthermore, rat tissues also secrete the truncated receptor (Bobek *et al.* 1991). This soluble form of the receptor is approximately 10 kDa smaller than the membrane-bound form (Kiess *et al.* 1987), is truncated at the C-terminus (MacDonald *et al.* 1989) and is developmentally regulated with higher concentrations found in fetal and neonatal rat serum than in adult serum (Kiess *et al.* 1987). It has been proposed that proteolytic cleavage of the type-2 IGF receptor at the cell surface may

provide a mechanism for receptor turnover. Alternatively, the soluble receptor may be involved in regulating exogenous lysozymal enzyme activity in tissue repair or remodelling (Causin *et al.* 1988).

1.2.7.4: Regulation of IGF-II and the type-2 receptor by parental imprinting

Yet another puzzling aspect concerned with the type-2 IGF receptor, is that in mammals at least, the type-2 IGF receptor appears to be maternally imprinted (Barlow *et al.* 1991). That is, the maternally-inherited gene for the type-2 IGF receptor is selectively transcribed. Conversely, it is the paternal IGF-II gene which is expressed (De Chiara *et al.* 1991). The significance of the opposite genomic imprinting of IGF-II and its type-2 receptor has not yet been elucidated. However, Haig & Graham (1991) propose that the major function of the type-2 IGF receptor is to act as a "sink" for IGF-II. They suggest that the receptor produced by the maternal genome binds and degrades the IGF-II produced by the paternal genome before IGF-II can bind to the type-1 IGF receptor and exert its biological actions. They argue that it is in the father's interest to increase the expected fitness of the embryo, as larger offspring are more likely to survive and reproduce. On the other hand, this needs to be weighed up against the mother's interest, in that the more resources taken by one offspring, the fewer resources available to other offspring. Thus, Haig & Graham (1991) believe the inverse imprinting arrangement arose due to the evolutionary trade-off between the number of offspring produced and the size of the individual offspring.

1.2.8: IGF binding proteins

The biological actions of the IGFs are determined not only by their interactions with IGF receptors, but also by their interactions with a family of carrier proteins, now known as IGF binding proteins (IGFBPs). While the hormonal, nutritional and developmental regulation of IGFBPs has been extensively studied (Cohick & Clemmons, 1991), these aspects will not be covered in this literature review.

1.2.8.1: IGFBP structure

Thus far, six IGFBPs have been identified. The primary structures of all six have been determined and reveal striking similarities (Fig. 1.3). The proteins consist of 200 to 300 amino acids, 20 to 40 of which form a signal peptide. The IGFBPs have 18 conserved cysteine residues in the amino- and carboxy-terminal ends, suggesting the disulphide arrangement is important for the IGF-binding specificity. However, rat and human IGFBP6 respectively, lack 2 and 4 of the homologous cysteines. On the other hand, IGFBP4, contains 2 additional cysteines. IGFBP-3, -4, -5 and -6 are glycosylated to varying degrees, though the significance of this is not yet established. Both IGFBP-1 and -2 contain an RGD sequence in their carboxy-terminal ends. This motif is found in many extracellular proteins and often serves as a recognition sequence for cell surface integrin receptors (for review see McCusker & Clemmons, 1992).

1.2.8.2: Characterization of the IGFBPs

IGFBP1 has been purified from human amniotic fluid (Drop *et al.* 1984; Povoa *et al.* 1984) and decidua (Koistenen *et al.* 1986) and was the first IGFBP to be completely characterized. Sequence analysis of cDNAs from human and rat indicate 58% identity, and a deduced molecular mass of 25 kDa (Brewer *et al.* 1988; Murphy et al. 1990). IGF-I and IGF-II bind to IGFBP1 with similar affinity. Moreover, at pH 7.0, the affinity for IGF-I is 5-fold greater than what is observed for the type-1 IGF receptor.

IGFBP2 was originally purified from serum-free medium conditioned by rat liver cells (Mottola *et al.* 1986) and by bovine kidney cells (Szabo *et al.*1988). Sequence analysis of human (Binkert *et al.* 1989), bovine (Upton *et al.* 1991) and rat (Brown *et al.* 1989) cDNAs indicate approximately 85% identity and the deduced molecular mass is approximately 30 kDa. While IGFBP2 exhibits greater affinity for both IGF-I and IGF-II than what is observed for IGFBP1, its affinity is 3-fold higher for IGF-II than for IGF-I. Expression of IGFBP2 mRNA is

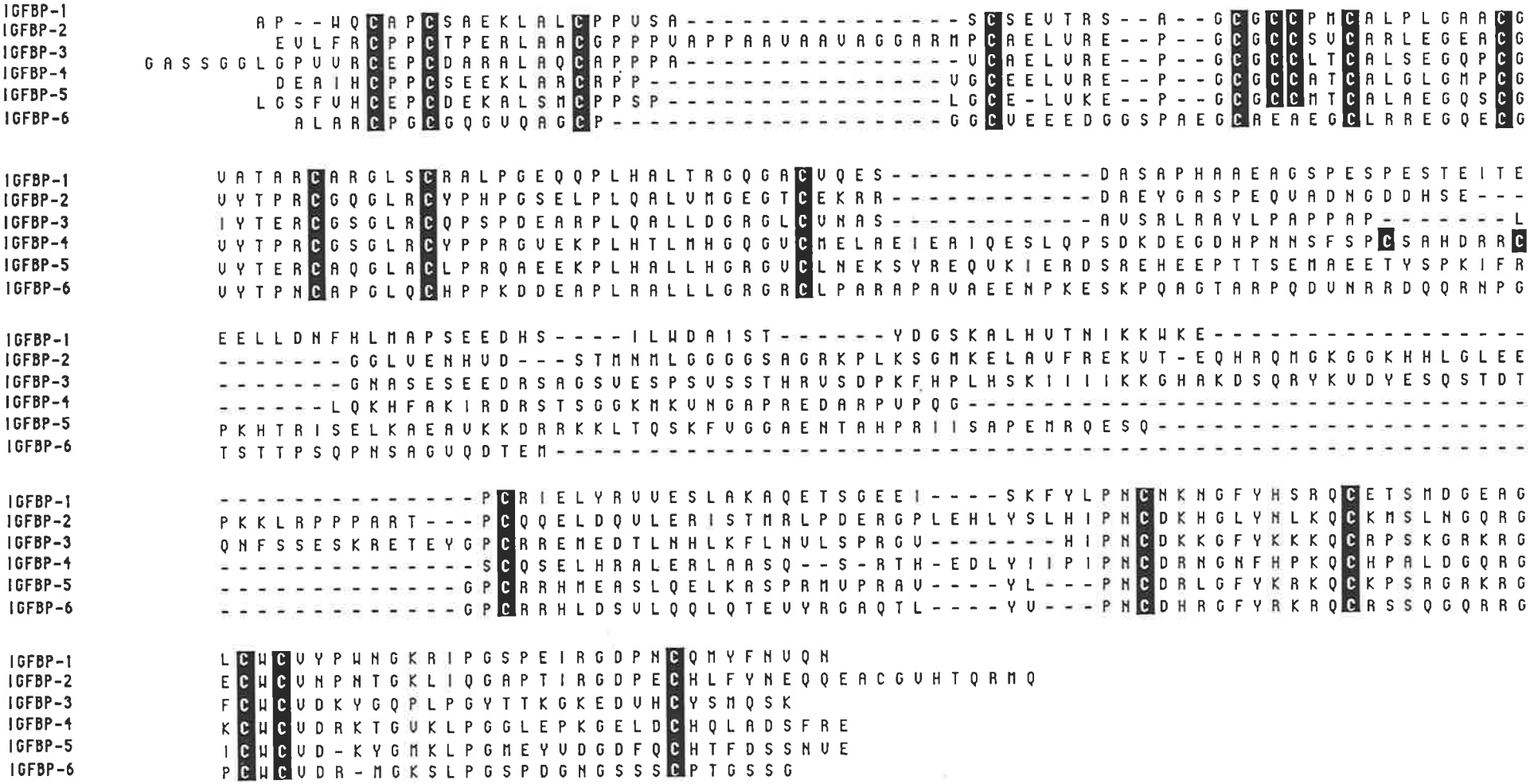


FIGURE 1.3: Sequence comparison of the six human IGFBPs. The references from which the sequences were obtained are described in the text.

higher in fetal than in adult tissues. In particular, high levels are expressed in fetal liver and brain tissues (Ooi *et al.* 1990; Orlowski *et al.* 1990).

IGFBP3 is the IGF-binding subunit of the 150 kDa complex which carries IGF in serum (Baxter & Martin, 1989). The 150 kDa complex is comprised of glycosylated IGFBP3, an 85 kDa acid labile subunit and IGF-I or -II. IGFBP3 was originally purified from human plasma (Martin & Baxter 1986) and cDNAs for human (Wood *et al.* 1988), rat (Albiston & Herrington, 1990), porcine (Shimasaki *et al.* 1990a) and bovine IGFBP3 (Conover, 1990) have subsequently been cloned. Again, considerable conservation of sequence is observed between species. IGFBP3 is the most abundant IGFBP present in serum and carries up to 95% of the IGFs in the circulation. Furthermore, IGFBP3 has a high affinity for both IGF-I and -II. Circulating levels of IGFBP3 are regulated by GH, however, this effect is presumed to be mediated by IGF-I since IGF-I infused into rats also increases IGFBP3 levels (Clemmons *et al.*1989; Zapf *et al.* 1989).

IGFBP4 has been isolated from human (Kiefer *et al.* 1991b) and rat (Shimonaka *et al.* 1989) serum, and from media conditioned by human osteosarcoma cells (Mohan *et al.* 1989). Human (La Tour *et al.* 1990) and rat (Shimasaki *et al.* 1990b) cDNAs reveal 92% identity, a deduced molecular mass of 24.5 kDa and the presence of a N-linked glycosylation site. However, IGFBP4 is generally present in a non-glycosylated state. Messenger RNA transcripts for IGFBP4 have been detected in a variety of adult rat and human tissues and in fetal fibroblasts. As is found with IGFBP3, IGFBP4 also has a high affinity for both IGF-I and IGF-II.

IGFBP5 has been purified from human bone (Bautista *et al.* 1991), human osteoblast-like cells (Andress & Birnbaum,1991) and from rat serum (Shimasaki *et al.* 1991), while cDNAs have been isolated from human (Kiefer *et al.* 1991a) and rat (Shimasaki *et al.* 1991b) libraries. The deduced amino acid sequences indicate a molecular mass of approximately 28.5 kDa and a N-linked glycosylation site. Of all the IGFBPs, IGFBP5 exhibits the highest affinity for IGFs.

Indeed, at pH 7.4, the affinity is 50-fold greater than what is observed for the type-1 IGF receptor.

IGFBP6 has been purified from a number of human sources (Forbes *et al.* 1990; Martin *et al.* 1990; Roghani *et al.* 1991) and cDNAs have been isolated from rat ovary, human placenta and human osteosarcoma cells (Kiefer *et al.* 1991b; Shimasaki *et al.* 1991a). The deduced molecular mass for IGFBP6 is approximately 22 kDa. Similar to IGFBP2, IGFBP6 preferentially binds IGF-II (Forbes *et al.* 1990; Martin *et al.* 1990).

1.2.8.3: Regulation of IGF bioavailabilty by IGFBPs

As mentioned earlier, the majority of the IGFs in serum circulate in a 150 kDa complex consisting of IGFBP3, an acid-labile subunit, and the IGF itself. Moreover, the association of IGFBP3 and the acid-labile subunit is completely dependent on the presence of IGFs (Baxter & Martin 1989). Furthermore, the half-life of IGF in serum appears to be dependent on the association with IGFBPs. This has been demonstrated by Ballard *et al.* (1991) who found that radiolabelled des(1-3)IGF-I, which has a reduced affinity for IGFBPs, is rapidly cleared from the circulation.

Only very low levels of the other IGFBPs have been detected in serum and it seems that most of the unsaturated IGF binding sites in serum are associated with these proteins (Zapf *et al.* 1990; Kiefer *et al.* 1991b). It is believed that these IGFBPs may be important for mediating the transport of IGFs from the vascular space to the extracellular fluids. Indeed, IGFBP-1,-2, -3 and -4, whether alone or bound to IGFs, have been shown to cross the vascular endothelium (Bar *et al.* 1990).

IGFBPs have been shown to both inhibit and enhance the biological actions of IGFs at the cellular level. While purified IGFBP-1, -2, -3 and -4 have been shown *in vitro* to inhibit IGF action by preventing receptor interactions (Blat *et al.* 1989; Burch *et al.* 1989; Mohan *et al.* 1989; Ross *et al.* 1989; Conover *et al.* 1990; Cheung *et al.* 1991), IGFBP-1, -2, -3, -5 and -6 have been shown to

potentiate the mitogenic activity of IGFs (Clemmons & Gardiner 1990; Conover *et al.* 1990; Bautista *et al.* 1991; Andress *et al.*1991). The potentiating ability of IGFBP-1, -2 and -3 is thought to involve association of the IGFBPs with the cell surface, however, the mechanism by which this occurs is not known (Clemmons *et al.* 1990; Conover *et al.* 1990; Conover *et al.* 1991). Likewise, it has not been established whether the potentiating effects of IGFBP-5 and -6 involve interactions with cell membranes.

The importance of IGFBPs in regulating the availability of IGFs has also been demonstrated by *in vitro* and *in vivo* studies where the truncated IGF-I analogue, des(1-3)IGF-I, has been shown to be more potent than IGF-I. Cultured cell studies have determined that the higher potency of des(1-3)IGF-I is the result of increased concentrations of free peptide being available for interactions with cell receptors (Ballard *et al.* 1987; Ballard *et al.* 1988; Szabo *et al.* 1988; Bagley *et al.* 1989). The enhanced potency of des(1-3)IGF-I compared to full-length IGF-I is mirrored in animal studies where exogenous administration of the analogue has been shown to increase organ weight, protect body protein reserves during nitrogen restriction and to produce enhanced beneficial effects following surgical trauma (Gillespie *et al.* 1990; Lemmey *et al.* 1991; Tomas *et al.* 1991). The enhanced potency of des(1-3)IGF-I *in vivo* is thought to be a result of poor binding of the analogue to IGFBPs, thus allowing rapid clearance of des(1-3)IGF-I from blood to tissues. Indeed, studies on plasma clearance and tissue clearance of des(1-3)IGF-I support this explanation (Ballard *et al.* 1991). The slow plasma clearance of IGF-II, its higher affinity for IGFBPs, and its low biological potency, can be explained by the same hypothesis.

Yet another mechanism by which IGFBPs modulate the bioavailability of IGFs is via phosphorylation. Dephosphorylated IGFBP1 enhances the effect of IGF-I on DNA synthesis, while the phosphorylated form is inhibitory. This is believed to be caused by phosphorylated IGFBP1 having a higher affinity for IGF-I than the non-phosphorylated form (Frost *et al.* 1991; Jones *et al.* 1991).

IGFBP-3 can also be phosphorylated, but its affinity for IGFs in its two phosphorylation states has not yet been compared.

1.2.9: Structure/function studies of IGFs

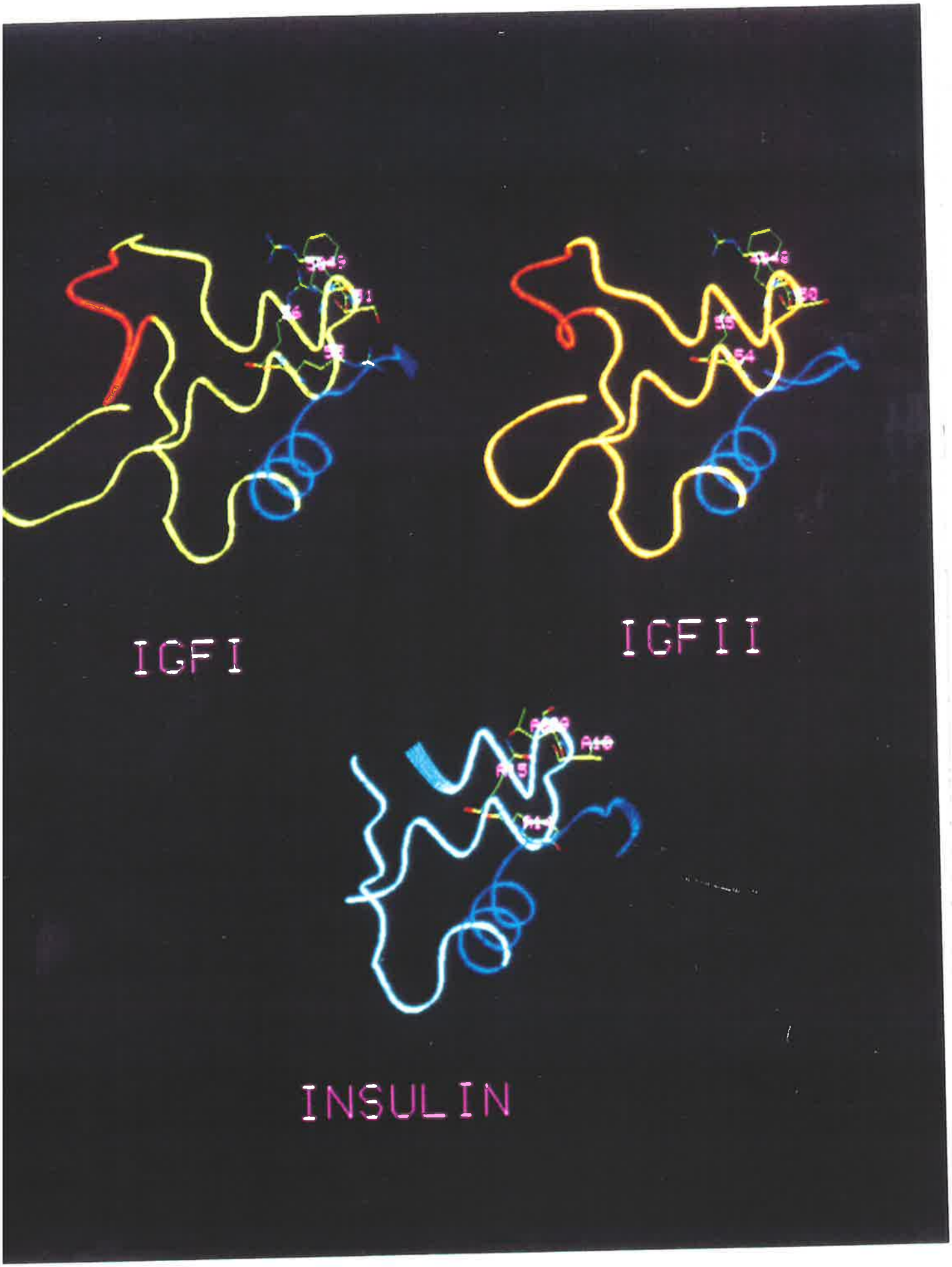
A great deal of research has been devoted to understanding the relationship of the IGF protein structure to function. In particular, the research has been directed at attempting to elucidate which regions of the molecule, and hence which amino acid residues, are important for interactions with insulin and IGF receptors, and with IGFBPs. The advent of recombinant DNA technologies has greatly assisted this goal, enabling IGF molecules with limited amino acid modifications to be rapidly generated. Thus, Tyr^{24} in IGF-I has been demonstrated to be crucial for high affinity binding to the type-1 IGF receptor (Cascieri *et al.* 1988a; Bayne *et al.* 1988). In addition, the residues Tyr^{31} and Tyr^{60}, as well as the C-peptide, are also thought to be involved with this interaction (Bayne *et al.* 1988, 1989; Maly & Luthi, 1988). Moreover, Tyr^{27} in IGF-II, in the analogous position to Tyr^{24} in IGF-I, has been found to be involved in binding of IGF-II to the type-1 IGF receptor (Burgisser *et al.* 1991; Roth *et al.* 1991). On the other hand, the residues Tyr^{60} (Bayne *et al.* 1988) and Phe^{48} (Burgisser *et al.* 1991) in IGF-II, and the residues Phe^{49}, Arg^{50}, Ser^{51}, Arg^{55}, Arg^{56} (Cascieri *et al.* 1989) in IGF-I, have been implicated in maintaining binding to the type-2 IGF receptor. Modifications of amino acids in the D-peptide of IGF-II have also been reported to alter interactions with IGF receptors (Roth *et al*, 1991; Oh *et al.* 1991). Extrapolation of structure/function relationships in insulin suggest that the residues Arg^{21}, Gly^{22}, Phe^{23}, Tyr^{24}, Phe^{25}, Gly^{42}, Ile^{43}, Val^{44}, Glu^{46}, Tyr^{60} and Ala^{62} in IGF-I may be important for binding to the insulin receptor (Cooke *et al.* 1991) (Fig. 1.4).

Evidence that the N-terminal region of IGFs are important for binding to IGFBPs has come from two directions. Firstly, the naturally occurring variant des(1-3)IGF-I has a greatly reduced affinity for IGFBPs (Szabo *et al.* 1988; Forbes *et al.* 1988). Secondly, either chemically synthesised or recombinantly-

FIGURE 1.4: Type-2 IGF receptor binding domains in the IGF-I, IGF-II and insulin structures. Three dimensional models of IGF-I, IGF-II and insulin were generated using the Insight II program (Biosym Technologies Inc., San Diego, CA, U.S.A., 1990) with coordinates obtained from the Protein data bank (Brookhaven National Laboratory, Upton, NY, U.S.A.). The models highlight the similarity in tertiary structure shared between the three proteins. The domains are shown in different colours as follows:

A domain, IGF-I	green
A domain, IGF-II	yellow
A domain, insulin	pale blue
B domain	blue
C domain	red

Residues of IGF-I involved in type-2 receptor binding (determined by site-directed mutagenesis) are highlighted. They are Phe^{49}, Arg^{50}, Ser^{51}, Arg^{55} and Arg^{56} of the A domain (Bayne *et al.* 1988). The corresponding residues of IGF-II (Phe^{48}, Arg^{49}, Ser^{50}, Ala^{54} and Lys^{55}) and insulin (Thr^{A8}, Ser^{A9}, Ile^{A10}, Tyr^{A14} and Gln^{A15}) are also indicated. Mutation of the B and C domains of IGF-I has also determined residues important for binding to the type-1 IGF receptor (Cascieri *et al.* 1988a; Bayne *et al.* 1989).


IGFI
IGFII
INSULIN

derived proteins with analogous modifications to the N-terminus, also exhibit a decreased affinity for IGFBPs (Cascieri *et al.* 1988b; Bagley *et al.* 1989). Indeed, a crucial amino acid for interaction with IGFBPs appears to be Glu at position 3 in IGF-I. Cascieri *et al.* (1989) have also implicated residues $Phe^{49}Arg^{50}Ser^{51}$ of IGF-I in IGFBP interactions, while Moss *et al.* (1991) has shown by chemical modification that Tyr^{60} in IGF-II is also involved in IGFBP-binding (Fig. 1.5).

1.2.10: Tertiary structure of the IGFs

The first model of the tertiary structure of the IGFs was proposed by Blundell *et al.* (1983) and was derived from the known structure of insulin using the residue prediction method of Chou & Fausman (1974). Recent nuclear magnetic spectroscopic studies of IGF-I (Cooke *et al.* 1991) have largely supported the original model. Cooke *et al.* (1991) found that the regions homologous to insulin were well defined, while the remainder of the molecule exhibited greater disorder. They also determined that residues thought to be involved with binding to the type-1 IGF receptor overlap those important for binding to the insulin receptor. On the other hand, residues implicated in binding to the type-2 IGF receptor overlap those which interact with IGFBPs.

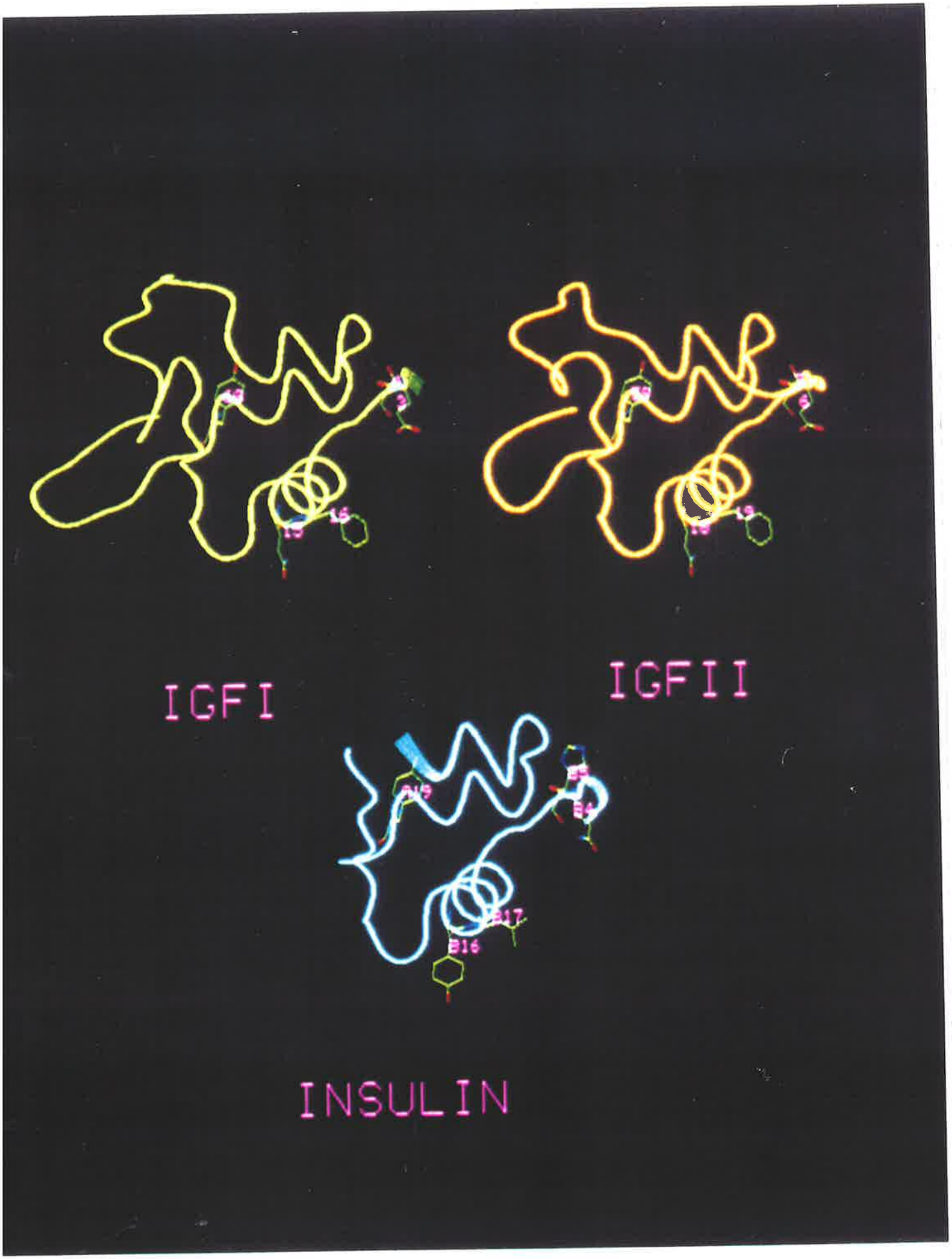
1.2.11: Biological effects of IGFs

1.2.11.1: In vitro studies of IGF actions

A number of studies have investigated the biological actions of IGFs *in vitro* using cultured cells and tissue explants. These studies have shown that IGFs act not only at an endocrine level but can also exert paracrine and autocrine actions (for review see Holly & Was, 1989).

The biological actions of IGFs can be broadly grouped into two classes, namely insulin-like effects and mitogenic effects. In general, the insulin-like actions elicited by IGFs are rapid, short-term effects and include classical insulin responses such as stimulation of glucose transport or enhanced lipid and glycogen synthesis.

FIGURE 1.5: Domains of IGF-I which interact with IGF-binding proteins. Three dimensional models of IGF-I, IGF-II and insulin were generated using the Insight II program (Biosym Technologies Inc., San Diego, CA, U.S.A., 1990) with coordinates obtained from the Protein data bank (Brookhaven National Laboratory, Upton, NY, U.S.A.). The models highlight the similarity in tertiary structure shared between the three proteins. Residues of Glu^{3}, Thr^{4}, Gln^{15}, Phe^{16} and Tyr^{60} of IGF-I are highlighted as they have been shown by site-directed mutagenesis or chemical modification to be involved in IGF-binding protein interactions (Cascieri *et al.* 1988b; Moss *et al.*, 1991). The corresponding residues of IGF-II (Glu^{6}, Thr^{7}, Gln^{18}, Phe^{19} and Tyr^{59}) and insulin (Gln^{B4}, His^{B5}, Tyr^{B16}, Leu^{B17} and Tyr^{A19}) are also indicated.


IGFI
IGFII
B19
B5
B17
B16
INSULIN

The non-insulin-like effects of IGFs are the proliferative and anabolic responses and include the stimulation of mitosis, cell differentiation and biosynthetic events such as DNA, RNA and protein synthesis. In addition, IGFs can inhibit protein breakdown and thus act as anabolic agents, improving the "food" conversion efficiency of the cell. These effects have been demonstrated in a diverse range of cell types such as muscle, cartilage and bone cells (Florini *et al.* 1986; Isaksson *et al.* 1991; Schmid *et al.* 1991). These longer-term effects are generally, mediated by the type-1 IGF receptor. IGFs have also been shown to be a differentiating factor for ovarian and testicular function (Adashi *et al.* 1984; Chatelain *et al.* 1987) and to induce erythropoiesis (Claustres *et al.* 1987; Congote & Esch, 1987), as well as to stimulate calcium influx (Kojima *et al.* 1988a,b), granulopoiesis (Merchav *et al.* 1988), chemotaxis (Grant *et al.* 1987), neurite outgrowth (Mill *et al.* 1985) and the expression of oncogenes (Ong *et al.* 1987). Furthermore, studies using pituitary cells have determined that IGFs inhibits GH release and hence regulate GH through a negative feedback mechanism (Morita *et al.* 1987). Clearly, the *in vitro* studies have demonstrated that the IGFs have a range of important biological actions which participate at many levels in growth and development. Indeed, it has been suggested that IGF-I is the "ultimate endocrine link in the chain of hormones regulating cell growth" (Spencer, 1985).

1.2.11.2: In vivo studies of IGF actions

The first studies investigating IGF actions *in vivo* were primarily based on the correlation of IGF serum levels with growth state. Thus it was ascertained that IGF-I levels are elevated in acromegaly (Clemmons *et al.* 1980) and in response to GH (Sara *et al.* 1980), and are low in hypopituitarism (Duaghaday & Trivedi, 1987). Similarly, IGF-I levels in humans were demonstrated to increase during childhood, rise during puberty, then to decrease thereafter (Hall *et al.* 1980). Moreover, IGF-I circulating IGF-I levels have been shown to be regulated diurnally and by nutrition (Clemmons *et al.* 1985; Philipps *et al.* 1989)

Information regarding the correlation of IGF-II serum levels with growth and development is scarce as specific IGF-II assays have only recently been developed.

The first studies in which IGFs were administered used preparations purified from serum. They demonstrated that IGF-I and IGF-II, though to a lesser extent, were able to stimulate the growth of hypophysectomized rats (Schoenle *et al.* 1985). More recently, the availability of large quantities of recombinant IGFs has enabled exogenous IGFs to be assessed in a range of models including hypophysectomized, diabetic and dexamethasone-treated rats, as well as in normal animals. Thus, exogenous IGF-I has been shown to cause acute insulin-like metabolic effects (Zapf *et al.* 1986), stimulate growth, reverse weight losses associated with catabolic states (Hizuka *et al.* 1986; Scheiwiller *et al.* 1986; Van Buul-Offers *et al.* 1988), stimulate renal function and kidney growth (Guler *et al.* 1989), stimulate erythropoiesis (Kurtz *et al.* 1988) and to enhance wound healing (Mueller *et al.* 1991). The results of the *in vivo* studies have been encouraging so that clinical trials assessing the effects of exogenous administration of IGFs in growth disorders and in a number of human disease states have commenced.

Transgenic animal models have also been useful in establishing the effects of IGFs *in vivo*. Transgenic mice overexpressing the IGF-I gene have increased weight compared to control littermates (Mathews *et al.* 1988). Similarly, studies of IGF-I transgenic mice crossed with dwarf transgenic mice lacking GH, have demonstrated that those offspring expressing IGF-I in the absence of GH grow larger than their GH-deficient littermates (Behringer *et al.* 1990). Moreover, the IGF-I expressing, GH-deficient offspring grow at rates similar to their non-transgenic siblings. Evidence of the importance of IGF-II in fetal growth and the role of genomic imprinting in regulating IGF-II gene expression has also been provided by transgenic mice studies. Thus De Chiara *et al.* (1990, 1991) found that mice that received a disrupted paternal IGF-II allele were markedly growth deficient, although they grew postnatally at a similar rate

to wild type animals. On the other hand, mice receiving a maternal disrupted IGF-II allele have normal sizes at birth.

1.3: NONMAMMALIAN IGFs

1.3.1: Early experiments

IGF activity was first identified in nonmammalian species by Shapiro & Pimstone (1977) who detected sulphation factor activity in birds, reptiles, amphibians and fish. Further evidence for the presence of IGFs in chickens was provided by Haselbacher *et al.* (1980) who found that chick embryo liver cells synthesised a somatomedin similar to insulin-like growth factor I. Wilson & Hintz (1982) also detected IGF activity in chicken serum using radioimmuno- and radioreceptor-assays. However, they were unable to detect IGF activity in the hagfish, carp, shark, frog or turtle. Likewise, Daughaday *et al.* (1985) used similar assays to measure IGF-I and IGF-II in non-mammalian sera. While IGF-I was found in chickens, turtles, frogs and fish, IGF-II was only detected in chicken and turtle sera. Zangger *et al.* (1987) also measured IGFs in sera from chickens and turtles, but not in frogs using a protein binding assay that involved a partially purified preparation of human IGFBP. Pancak-Roessler & Lee (1990), however, were able to detect IGF-I in frogs using a radioimmunoassay. Recently, Bautista *et al.* (1990) determined that both IGF-I and IGF-II were present in the skeletal tissues of the chicken, lizard, frog, trout and shark using immuno- and receptor-assays. While some of the results obtained from these studies are conflicting, taken together, they suggest that IGFs are indeed present in non-mammalian species, albeit at lower levels than found in mammals.

1.3.2: Sequences of IGF-I in nonmammalian vertebrates

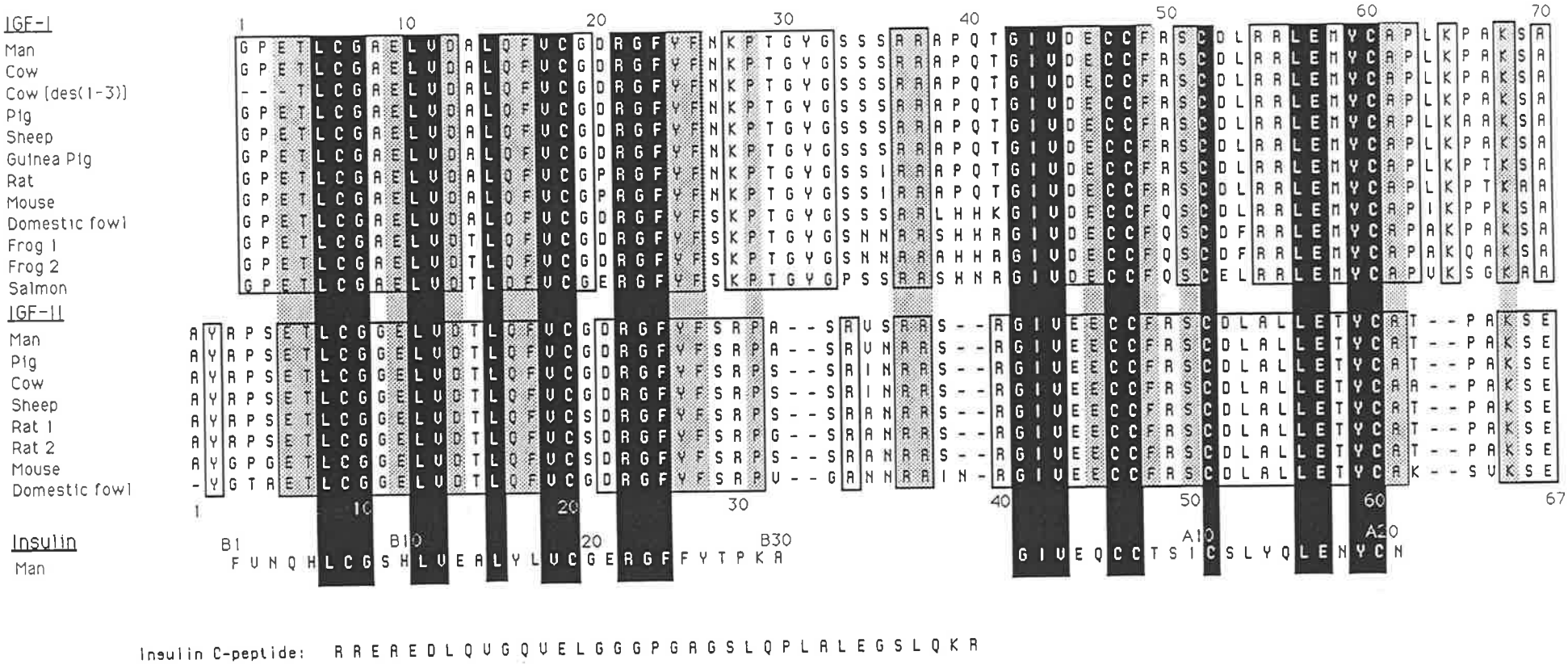
The first unequivocal evidence for the existence of IGFs in non-mammalian species was obtained when Dawe *et al.* (1988) purified chicken IGF-I and IGF-II from serum. However, the complete amino acid sequence of purified chicken IGF-I was not reported until 1990 (Ballard *et al.* 1990). In the meantime,

cDNAs for chicken IGF-I were isolated and characterized by Kajimoto & Rotwein (1989) and by Fawcett & Bulfield (1990). The protein sequences, whether determined by peptide sequencing or deduced from the cDNA, were identical and indicated that chicken IGF-I has eight amino acids which differ from human IGF-I. Moreover, amino acid changes occur in similar positions in cDNAs for the other non-mammalian IGFs which have been characterized, namely salmon (Cao *et al.* 1989) and frog IGF-I (Shuldiner *et al.* 1989; Kajimoto and Rotwein, 1990). The deduced amino acid sequences for salmon and the frog IGF-I have a similar degree of divergence from human IGF-I as chicken IGF-I, with 10 and 13 different amino acids respectively (Fig. 1.6). Interestingly, the frog species studied, *Xenopus*, with its tetraploid genome, has two very similar IGF-I peptides. Shuldiner *et al.* (1990) partially characterized the nucleotide sequences of the two genes and found there were six nucleotide substitutions, three of which were silent and three which resulted in conservative amino acid changes (Fig. 1.6). Except for these few changes, there is a high degree of identity in all the vertebrate IGF-Is, highlighting the fundamental importance of this protein in growth and development, and possible constraints on their structure due to the number of other proteins with which they must interact specifically.

1.3.3: Sequences of IGF-II in nonmammalian vertebrates

At the time I commenced my PhD, chicken IGF-II was the only non-mammalian IGF-II which had been characterized (Dawe *et al.* 1988; Kallincos *et al.* 1990). Peptide sequencing indicated that chicken IGF-II had a number of amino acids which differed from human IGF-II. These changes were found in the N-terminal, C-terminal and C-peptide regions of the protein. The subsequent publication of an antisense transcript to the chicken IGF-II gene by Taylor *et al.* (1991) confirmed most of the changes reported by Kallincos *et al.* (1990), with the exception that they identified an additional arginine residue at position 40. Subsequent re-examination of the original protein sequence data has indicated that the sequence for chicken IGF-II should in fact contain an additional arginine

FIGURE 1.6: Sequence comparison of vertebrate IGFs. Those residues conserved in IGF-I or in IGF-II are boxed, those residues conserved between IGF-I and IGF-II are shaded, while those residues also conserved in insulin are highlighted in black. Sequences for chicken, frog 1, frog 2 and salmon IGF-I were from Kajimoto & Rotwein (1989), Kajimoto & Rotwein (1990), Shuldiner *et al.* (1990) and Cao *et al.* (1989) respectively. The chicken IGF-II sequence is based on those reported by Kallincos *et. al.* (1990) and Taylor *et al.* (1991). The remaining sequences were obtained from the references described in Fig. 1.1.


IGF-I
Man
Cow
Cow (des(1-3))
Pig
Sheep
Guinea Pig
Rat
Mouse
Domestic fowl
Frog 1
Frog 2
Salmon
IGF-II
Man
Pig
Cow
Sheep
Rat 1
Rat 2
Mouse
Domestic fowl
Insulin
Man
Insulin C-peptide: R R E A E D L Q V G Q V E L G G G P G A G S L Q P L A L E G S L Q K R

residue at position 40 (Figs. 1.6 & 1.7)(*personal communication, G. Francis, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia*).

1.3.4: Evolution of IGFs

The recent isolation and characterization of a cDNA encoding IGF from Atlantic hagfish (Nagamatsu *et al.* 1991), a representative of the lowest vertebrate class, has raised a number of interesting questions as to when and how the IGFs evolved. The IGF cDNA from hagfish was of particular interest because firstly, it indicated that IGFs were indeed present in the earliest vertebrates, and secondly, the predicted sequence of the IGF was as similar to IGF-I as it was to IGF-II (Fig. 1.8). However, other factors suggested that hagfish IGF was in fact more similar to IGF-I than to IGF-II, including the findings that the predicted hagfish pre-pro IGF contained a characteristically long signal peptide and that hagfish IGF mRNA is only expressed in the liver of adult animals. This supports previous notions that the appearance of IGF-II is a more recent evolutionary event. As hagfish have previously been shown to possess a highly conserved insulin gene (Chan *et al.* 1981), the characterization of the hagfish IGF cDNA also supports the view that the IGF and insulin genes have separate gene lineages in vertebrates.

Studies comparing the sequences of vertebrate insulin and vertebrate IGF-I suggest that while both proteins appear to be evolving very slowly, insulin is diverging more rapidly than IGF-I. For example, salmon IGF-I is 90% identical to the A and B chains of human IGF-I, whereas salmon and human insulin share only 70% identity. Accordingly, Nagamatsu *et al.* (1991) were surprised that hagfish IGF did not show greater similarity to human IGF-I. To quantitate this difference, Nagamatsu *et al.* (1991) calculated the accumulated mutation rate for nonsynonomous codons in insulin and IGF-I from different species against their estimated evolutionary divergence time (Fig. 1.9). The results indicated that while the rate of sequence change versus divergence time is constant for rat,

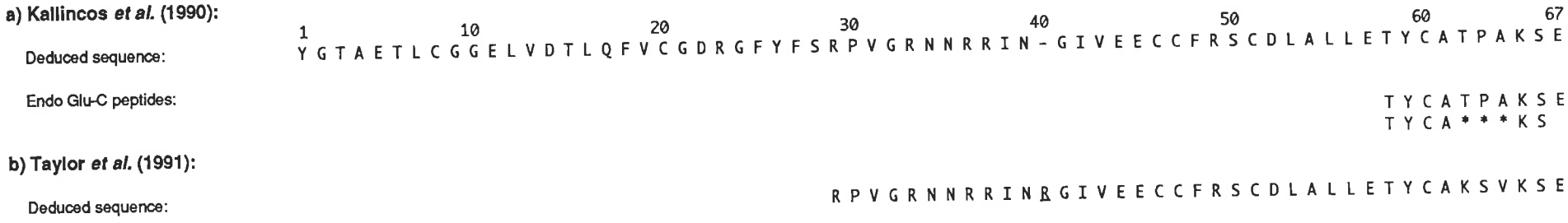


FIGURE 1.7: Comparison of the protein sequence for chicken IGF-II reported by a) Kallincos *et al.* (1990) with the amino acid sequence deduced from the antisense transcript to the chicken IGF-II gene reported by b) Taylor *et al.* (1991).

FIGURE 1.8: Sequence comparison of hagfish IGF, human IGF-I and human IGF-II. Those residues conserved in all three proteins are highlighted in black. Shaded boxes indicate either those residues conserved between hagfish IGF and IGF-I, or those conserved between hagfish IGF and IGF-II.

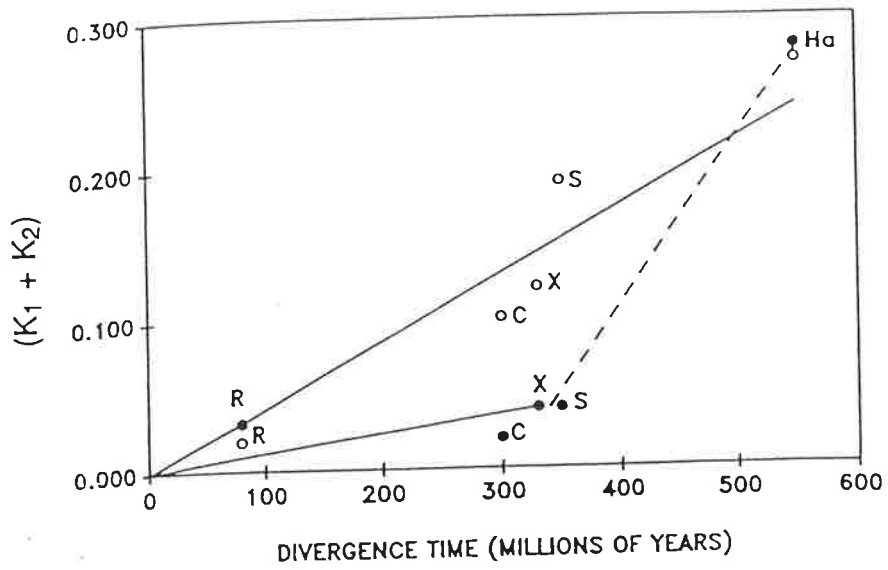


FIGURE 1.9: Plot of the evolutionary distance estimated by $K_1 + K_2$ against the divergence time (reproduced from Nagamatsu *et al.* 1991). The evolutionary distance of the first and second codon ($K_1 + K_2$) between human and different species in the A and B domains of insulin and IGF-I was calculated using the formula of Kimura (1983). Open and solid circles represent human and IGF-I respectively. The slope of the line shows the evolutionary rate. The references for nucleotide sequences of the human, rat (R), chicken (C), Xenopus laevis (X), salmon, (S) and hagfish (Ha) insulins and IGF-Is are described by Nagamatsu *et al.* (1991).

chicken, frog and salmon IGF-I sequences, the rate is much higher for hagfish IGF. Moreover, the rate of sequence change versus divergence time for insulin was 3 times higher than was found for IGF-I, yet was constant for all the species compared. One explanation for the apparent higher mutation rate between salmon and hagfish IGF, is that IGF-I has acquired additional structural constraints in the higher vertebrates.

The cloning of a cDNA for an insulin-like peptide from amphioxus has also been particularly intriguing (Chan *et al.* 1990). The deduced amino acid sequence from this clone indicated that the protein it encoded was as similar to insulin as it was to IGFs (Fig. 1.10). Since amphioxus are protochordates which are thought to be related to the invertebrate progenitor from which the vertebrates emerged, the insulin-like peptide may represent a transitional form connecting insulin and IGFs. Indeed, insulin-related peptides have been characterized in invertebrates and structural data is now available for insulin-related peptides in insects (bombyxins and locusta insulin-related peptides; Kawakami *et al.* 1989; Lagueux *et al.* 1990) and in molluscs (molluscan insulin-related peptides; Smit *et al.* 1988 & 1991). Taken together, these results suggest that IGF-like proteins developed before vertebrate development, and that IGF-I preceded IGF-II. Moreover, it is quite possible that the IGFs emerged after a insulin-type gene had evolved. However, further searches for IGF-like sequences in invertebrates need to be undertaken before this question can be answered.

1.3.5: Chicken IGF-I gene structure

To date, the only nonmammalian IGF gene to be characterized is that of chicken IGF-I (Fawcett & Bulfield, 1990; Kajimoto & Rotwein, 1991). The gene is similar to mammalian IGF-I genes in that it spans a large region of the genome (50 kb), in the location of its introns and in the structure of its exons. It differs from mammalian IGF-I genes, however, in that only two IGF-I transcripts (1.9 and 2.6 kb) are transcribed from the gene. This contrasts with the situation in mammals

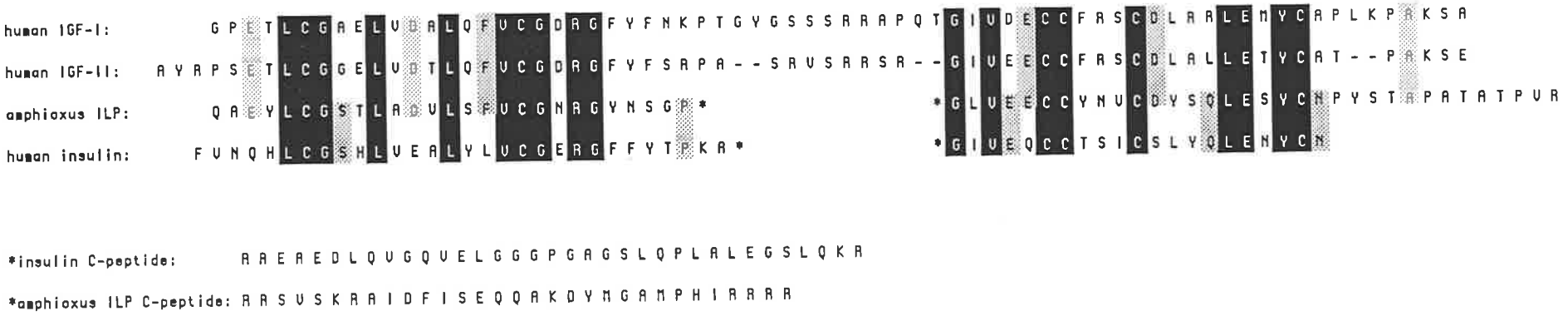


FIGURE 1.10: Sequence comparison of amphioxus insulin-like peptide (ILP), human insulin, human IGF-I and human IGF-II. Those residues conserved in all four proteins are highlighted in black. Shaded boxes indicate either those residues conserved between amphioxus ILP and insulin or those conserved between amphioxus ILP and the IGFs.

where alternative RNA splicing and multiple polyadenylation sites lead to the production of an array of mRNA transcripts. The chicken IGF-I gene consists of 4 exons, with exons 2 and 3 coding for the mature protein, while exons 1 and 4 encode N- and C-terminal extension peptides respectively, in addition to untranslated sequences. The two mRNA transcripts appear to arise due to the presence of two polyadenylation regions in exon 4. Analysis of the 5' end of the gene identified a 74-nucleotide region containing several transcription initiation sites which is highly conserved between the chicken and other mammalian IGF genes. Furthermore, the chicken IGF-I promoter was defined by constructing chimeras containing 5' sequences of the chicken IGF-I gene and the luciferase reporter gene. The chicken promoter was shown to enhance expression of luciferase in a human cell line which synthesizes IGF-I, thus suggesting that essential elements in the promoter may be conserved between chicken and human.

1.3.6: Expression of IGF genes in nonmammalian vertebrates

Compared to mammals, much less is known about the expression of genes in other vertebrates. Three major IGF-I mRNA transcripts of 1.6, 2.1 and 3.0 kb are detected in the liver of the frog. Moreover, IGF-I mRNAs are also detected in the lung, heart, kidney and peritoneal fat of adult frogs (Kajimoto & Rotwein, 1990). Although multiple IGF-I mRNA transcripts have also been detected in salmon liver, a 3.9 kb transcript predominates. Furthermore, GH regulation of hepatic IGF-I gene expression appears to be conserved in salmon (Cao *et al.* 1989). Nagamatsu *et al.* (1991) have examined the expression of the hagfish IGF gene using both Northern analysis and PCR amplification methods. In both instances, a single mRNA transcript of 4.2 kb was detected in hagfish liver tissue, while no IGF mRNA was detected in brain, heart, skeletal muscle or the islet organ.

Information regarding the expression of the chicken IGF-I gene is rather more extensive. Multiple IGF-I mRNA species in chicken liver were reported by

Fawcett & Bulfield (1990), whereas as mentioned earlier, Kajimoto and Rotwein (1991) detected a simpler transcript pattern. Serrano *et al.* (1990) and Kikuchi *et al.* (1991) have reported that the IGF-I gene is expressed early in organogenesis, before the appearance of GH, in developing chick embryos. In addition, both groups detected tissue specific developmental differences. Moreover, the liver does not appear to be the major source of IGF-I gene expression prenatally. By contrast, posthatch increases in GH secretion correlate with increases in liver IGF-I mRNA and serum IGF-I concentration, suggesting postnatal IGF-I expression in chicks may be partially GH-dependent.

The expression of the chicken IGF-II gene during chicken growth and development is yet to be comprehensively studied. Engstrom *et al.* (1987) have detected IGF-II mRNA in embryonic chick limb bud, eye and heart, while Taylor *et al.* (1991) have reported the presence of an array of both sense and antisense IGF-II transcripts in stage 22 and stage 36 chick embryos. The range of transcripts detected suggest that transcription from the chicken IGF-II gene appears to be as complex as is found in mammals. Indeed, the presence of multiple antisense transcripts suggests antisense transcription of the chicken IGF-II gene may be as complex as transcription of the coding strand. At the end of 1991 the function of the chicken IGF-II antisense transcripts was not known. They may however, regulate chicken IGF-II gene expression by hybridizing to IGF-II mRNAs (Minzuno *et al.* 1984).

1.3.7: IGF receptors in nonmammalian vertebrates

Classical type-1 IGF receptors have been characterized in the frog, (Hainaut *et al.* 1991; Scavo *et al.* 1991b) and in chickens (Scavo *et al.* 1991a). The IGF receptors isolated from frog oocytes were similar to mammalian type-1 IGF receptors, having an $\alpha 2\beta 2$ subunit structure and exhibiting intrinsic tyrosine kinase activity. However, two subtypes of the β subunit were identified, both of which appeared to be true type-1 IGF receptor β subunits. Two subtypes of the β subunit have also been found in fetal rat type-1 IGF receptors, one of which is

developmentally regulated and disappears in the adult. Whether either of the β subunits found in the type-1 IGF receptors from frog oocytes is also developmentally regulated has not been determined. Another explanation for the two subtypes may be that the frog species studied, *Xenopus*, being a tetraploid species, may have two non-allelic genes coding for the IGF receptor. As is found with mammalian type-1 IGF receptors, IGF-I exhibits significantly higher affinity for the frog IGF receptor, than is observed with insulin.

A number of groups have investigated the presence of IGF receptors in chickens. Duclos & Goddard (1990), Kallincos *et al.* (1990), Duclos *et al.* (1991) showed by affinity cross-linking experiments that a type-1 IGF receptor was present in chicken embryo fibroblasts, liver membranes and in muscle satellite cells respectively. Moreover, evidence suggests that the type-1 IGF receptor exclusively mediates the stimulation of DNA synthesis in chick muscle satellite cells by both insulin and IGF-I (Duclos *et al.* 1991). Scavo *et al.* (1991b) have demonstrated that type-1 IGF receptors are expressed early in chick development, with mRNA being detected as early as the blastoderm (day 0), during neurulation (day 1) and in early (days 2-3) and late (day 9) organogenesis. More recently, clones encoding the putative type-1 IGF receptor have been isolated. Preliminary DNA sequencing indicates greater than 90% homology to the human type-1 IGF receptor at the nucleotide level in the α subunit domain (Goddard & Boswell, 1991).

Zetterstrom *et al.* (1991) have also characterized an IGF-binding receptor isolated from toad retina. This particular receptor has proved to be particularly interesting since it appears to bind both IGF-I and insulin with equal affinity. The receptor has a disulphide-linked heterotetrameric structure with 105 kDa α subunits and 95 kDa β subunits and exhibits intrinsic tyrosine kinase activity. These findings are analogous to those reported by Stuart (1988) who found that liver membranes from a cartilaginous fish (stingray) possess a single binding site for insulin and IGF-I. Likewise, Shemer *et al.* (1986; 1988) demonstrated that both IGF-I and insulin stimulate autophosphorylation of insulin receptors from

reptile brain and liver. None of these studies however, has conclusively established whether a separate IGF receptor is present in these species. Thus it is difficult to know whether the receptors characterized actually do represent an ancestral form of IGF receptors. Given that a genuine type-1 IGF receptor is present in frog, it seems likely that classical type-1 IGF receptors will also be identified in these species.

There is no evidence for a type-2 IGF receptor in nonmammalian vertebrates. Indeed, affinity crosslinking studies using radiolabelled IGF-II in chicken tissues or cultured cells indicate that IGF-II binds to a receptor of the size expected for the type-1 IGF receptor (Duclos & Goddard, 1990; Kallincos *et al.* 1990; Duclos *et al.* 1991). Furthermore, both the chicken and frog mannose-6-phosphate cation-independent receptors have been purified and characterized, and both fail to bind IGF-II (Canfield & Kornfeld, 1989; Clairmont & Czech 1989). Thus the emergence of the specific IGF-II-binding type-2 IGF receptor appears to be a more recent evolutionary development, possibly co-evolving with mammals. The function of the type-2 IGF receptor and the significance of its recent evolution is yet to be resolved.

1.3.8: IGFBPs in nonmammalian vertebrates

While mammalian IGFBPs have been extensively studied, little is known about these proteins in nonmammalian species. Daughaday *et al.* (1985) investigated the presence of IGFBPs in chicken, turtle, trout and toad, while Drakenberg *et al.* (1989) studied IGFBP in fish. Both found IGF-binding activity in the nonmammalian sera studied, however, the binding appeared to be non-specific as it was not displaced by excess IGF. Bautista *et al.* (1990) were unable to demonstrate the presence of IGFBPs in the skeletal tissues of chicken, lizard, frog, trout and shark. Likewise, Reinecke *et al.* (1991) did not detect IGFBPs in the Atlantic hagfish. Nevertheless, studies by Armstrong *et al.* (1989), Lee *et al.* (1989) and Francis *et al.* (1990) have demonstrated that specific IGFBPs are indeed present in chicken serum, although there is disagreement as to the major

forms present. Thus Francis *et al.* (1990) detect one predominant IGFBP with a molecular mass of 55 kDa, while Armstrong *et al.* (1990) detect multimeric forms of IGFBPs with masses similar to those found in mammalian species. The reports are consistent, however, in that both found that up to 6% of the total serum IGF immunoreactivity is in an unbound state. This differs from the situation in mammals where little or no free IGF-I has been detected in serum (Daughaday *et al.* 1982). It has been proposed that the presence of free IGFs in chickens under normal physiological conditions may make the birds more tolerant than mammals to short-term insulin-like metabolic effects. Moreover, it has been demonstrated that chickens exhibit a high resistance to insulin, tolerating up to 1000 times the dose per kilogram of body weight that produces severe hypoglycaemia, convulsions and death in mammals (Chen *et al.* 1945).

1.3.9: Biological actions of IGFs in nonmammalian vertebrates

Given the scarcity of information on the primary structure and characterization of nonmammalian IGFs, IGFBPs and their IGF receptors, it is not surprising that little is known about the biological actions of nonmammalian IGFs. Most of the studies undertaken have investigated the roles of IGFs in either fish or in chickens. This may reflect the commercial importance of these nonmammalian species.

1.3.9.1: Biological actions of IGFs in fish

A number of studies have investigated the actions of GH in fish. Injections of human or bovine GH increase IGF-I mRNA expression and levels of circulating immunoreactive IGF-I, as well as stimulate growth. As in mammals, the growth-promoting actions of GH appear to be regulated by GH effects on hepatic IGF production (Funkenstein *et al.* 1989; Ng *et al.* 1991). Furthermore, IGF-I bioactivity measured by cartilage sulphation appears to be regulated by GH in the Japanese eel (Duan & Hirano 1990; Duan & Inui, 1990a,b) and in rainbow trout (Komourdjian & Idler, 1978). In addition, Gray & Kelly (1991) found that IGF-

I, but not GH, stimulated sulphate uptake in cultured oral cartilage from bony fish. Lindahl *et al.* (1985) determined that serum somatomedin levels peaked during salmon smoltification, a process in which GH is believed to be an important factor. IGF-I is also thought to have a role in osmoregulation in the trout (McCormick *et al.* 1991), again a process involving GH. Thus in fish it seems that IGF-I mediates the actions of GH in a number biological functions.

1.3.10: Biological actions of IGFs in chickens

1.3.10.1: Characterization of the biological actions of IGFs in chickens through in vitro studies

Compared to other nonmammalian species, the biological actions of IGFs in chickens have been more comprehensively studied. IGF-I has been demonstrated to stimulate proliferation and to increase glucose and amino acid uptake in chick embryo fibroblasts (Cynober *et al.* 1985). Balk *et al.* (1984) determined that IGFs, but not GH, are mitogenic for chicken heart mesenchymal cells and act synergistically with epidermal growth factor and fibroblast growth factor. Likewise, IGF-I, but not GH, has been shown to stimulate proliferation and proteoglycan synthesis of chicken chondrocytes (Rosselot *et al.* 1990). On the other hand, GH and IGF-I appear to cooperate in regulating collagen synthesis in chicken skin fibroblasts (Granot *et al.* 1991). Girabau *et al.* (1987) have demonstrated that IGF-I can stimulate embryonic growth and differentiation. Both IGF-I and IGF-II have been shown to preferentially enhance myoblast differentiation of primary cultures of chick embryonic cells (Schmid *et al.* 1983). Similarly, McFarland *et al.* (1991) demonstrated that IGFs stimulate differentiation of turkey embryonic myoblasts. IGFs also stimulate DNA synthesis in chicken muscle satellite cells (Duclos *et al.* (1991) and in adipocyte precursors (Butterwith & Goddard, 1991).

As is the case in mammals, GH stimulates the production of IGF-I by chicken hepatocytes (O'Neill *et al.* 1990). Moreover, Housten & O'Neill (1991) have determined that insulin and GH can act synergistically to stimulate IGF-I

production in these cells. There is also evidence for local (autocrine) production of IGFs by chicken cells. Haselbacher *et al.* (1980), Burch *et al.* (1986) and Balk *et al.* (1984) have shown that chicken embryonic liver cells, embryonic pelvic cartilage and heart mesenchymal cells respectively, produce endogenous IGF-I.

1.3.10.2: Characterization of the biological actions of IGFs in chickens through in vivo studies

A number of studies have used heterologous IGF-I radioimmunoassays in an attempt to correlate growth and development of chickens with serum IGF levels. Taken together the results suggest that IGF plasma concentrations vary with the physiological age and state of the bird. Serum IGF-I is measurable early in embryonic development (day 6), peaks at day 15, then declines, reaching a low concentration the day before hatching. Plasma IGF-I levels remain low during the first few weeks after hatching, then increase with age until plateauing at 4-6 weeks of age. Older birds (3-5 months) exhibit IGF-I levels similar to those observed immediately post-hatching (Huybrechts *et al.* 1985; Goddard *et al.* 1988; Johnson *et al.* 1990; McGuinness & Cogburn, 1990; Vasilatos-Younken & Scanes, 1991; Robcis *et al.* 1991).

Unlike the situation in mammals, there is no clear relationship between circulating IGF-I levels and growth rate. While there have been some suggestions of a positive correlation between growth and circulating IGF-I levels, others have reported a negative correlation (Huybrechts *et al.* 1985; Goddard *et al.* 1988; Lee *et al.* 1989; Johnson *et al.* 1990; Pym *et al.* 1991). Indeed, IGF-I levels are low during the first few weeks following hatching, yet this is the period when in terms of percentage of body weight, the most rapid gains occur. There have been few reports on chicken IGF-II plasma levels, however and there is no evidence for a correlation between circulating IGF-II levels and growth performance.

The relationship between plasma IGF-I and GH concentrations also remains unequivocal. Surprisingly, IGF-I levels in chickens increase as GH

secretion declines (Johnson *et al.* 1990). Nevertheless, circulating IGF-I levels in birds appears to be partially dependent on GH since hypophysectomy in chickens (Huybrechts *et al.* 1985) and in turkeys (McGuinness & Cogburn, 1990) significantly reduces IGF-I levels. Furthermore, in studies where exogenous GH was observed to increase growth, plasma concentrations of IGF-I were also elevated (Leung *et al.* 1986; Vasilatos-Younken *et al.* 1988; Buonomo & Baile, 1988). In studies where there was no effect with GH, IGF-I levels were not altered (Buonomo & Baile, 1986; Cogburn *et al.* 1989). In addition, a negative feedback relationship similar to that observed in mammals seems to exist between GH and IGF-I in chickens, since daily injections of IGF-I suppress circulating GH (Buonomo *et al.* 1987).

By the end of 1991 there were two reports on the effect of exogenous administration of IGF-I on growth and body composition of chickens. In the first, Spencer *et al.* (1990) found that human plasma-derived IGF-I administered *in ovo* did not have any significant effect on total body weight, bone length, organ weight or on muscle DNA, RNA or protein levels in hatched chicks. Likewise, the second study found that daily injections of IGF-I to young broiler chickens did not enhance growth performance or body composition (McGuinness & Cogburn, 1991). This result is not surprising given that rodent studies have shown that IGF-I needs to be infused for the peptide to be effective (Tomas *et al.* 1991).

1.3.10.3: Characterization of the biological actions of IGFs purified from chicken serum

In all of the studies described above, mammalian IGF preparations were used. There are a few reports, however, where chicken IGFs themselves have been characterized. Dawe *et al.* (1988) found that chicken IGF-I purified from serum had approximately half the efficacy of bovine IGF-I in stimulating protein synthesis in rat myoblasts, while Armstrong *et al.* (1990) found their chicken IGF-I preparation was equipotent with either human IGF-I or human IGF-II in stimulating DNA synthesis in chick embryo myoblasts. Dawe *et al.* (1988) also

determined that chicken IGF-II purified from serum was only half as potent as bovine IGF-II in the rat myoblast assay. On the other hand, Kallincos *et al.* (1990) found that chicken IGF-II was indistinguishable from ovine IGF-II in functional assays whether performed using cultured rat or chicken cells. In addition, studies undertaken by Dawe *et al.* (1988), Armstrong *et al.* (1990), and Ballard *et al.* (1990) have determined that chicken IGF-I is half as effective as bovine or human IGF-I in IGF-I radioimmunoassays employing mammalian antibodies and ligands, thus suggesting either that IGF-I levels in chicken serum may be higher than previously reported, or the chicken IGF-I preparations contained less peptide than calculated.

The only other study using serum-derived chicken IGF preparations is that reported by Francis *et al.* (1990) comparing plasma clearance and the association of chicken and human with IGFBPs. Interestingly, they found that while both human and chicken IGF-I injected into chickens rapidly associated with serum carrier proteins, human IGF-I was cleared from the circulation more rapidly than chicken IGF-I. Moreover, the half-lives for chicken IGF-I of 54 min and for human IGF-I of 33 min were significantly shorter than those reported for other species. This result suggests that it may be more appropriate to use chicken IGF-I rather than human IGF-I in studies assessing the effect of exogenous IGFs on chicken growth performance and body composition.

1.4: AIMS OF MY RESEARCH PROJECT

While our understanding of the roles of IGFs, IGF receptors and IGFBPs in mammalian growth and development is expanding rapidly, progress on the elucidation of the functions of IGFs in nonmammalian species is slower. This primarily seems to be due to the low amounts of pure nonmammalian IGFs available for testing. In order to address this situation I aimed to produce non-mammalian IGFs using recombinant DNA technologies.

I chose to produce recombinant chicken IGFs for two reasons. Firstly, I and others from this laboratory had previously reported the purification of IGF-I and IGF-II from chicken serum (Dawe *et al.* 1988; Ballard *et al.* 1990; Kallincos *et al.* 1990), and I wished to continue the *in vitro* characterization of these peptides. Secondly, as chickens are a commercially important nonmammalian species, the production of recombinant chicken IGFs would be useful tools in determining whether IGFs could be used to improve their growth performance. If IGFs are in fact involved in as many varied and important functions in chickens as has been found in mammals, the benefits to the poultry industry could be substantial.

While there were obvious practical reasons for attempting to produce chicken IGFs, I was also interested in characterizing their functions from an evolutionary perspective. Although there is considerable structural evidence that the IGFs share a long evolutionary history, little is known about the conservation of IGF function. Indeed, it is for this reason that I also chose to produce recombinant hagfish IGF, the most primitive vertebrate IGF identified thus far. As mentioned in the literature review, the deduced amino acid sequence for this protein as deduced from the cDNA, suggested that the protein was as similar to IGF-I as it was to IGF-II (Nagamatsu *et al.* 1991). Accordingly, I aimed to produce hagfish IGF by recombinant means and to determine whether the protein shares both IGF-I- and IGF-II-like functional characteristics.

Finally, I wished to establish whether the IGFBPs, like the IGFs, also had a long evolutionary history. At the time I commenced my PhD, the presence of

IGFBPs in nonmammalian species had only been unequivocally demonstrated in chickens.

CHAPTER 2

MATERIALS AND METHODS

2.1: MATERIALS

2.1.1: General chemicals and reagents

Activated charcoal, acrylamide, ampicillin, RIA grade bovine serum albumin (BSA), Coomassie Brilliant Blue (R_{250}), N-2-hydroxyethyl-piperazine-N'-ethane-sulfonic acid (Hepes), isopropyl-β-D-galactoside (IPTG), polyethylene glycol 6000, polyoxethylene-sorbitan monolaurate (Tween 20) and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Dissucinimidyl suberate was obtained from Pierce Pty. Ltd, Rockford, IL., U.S.A. while 2-hydroxyethyl disulphide was from Aldrich, Milwaukee, WIS, U.S.A. All other chemicals were analytical reagent grade.

2.1.2: Molecular biology reagents

The "Mutagene" *in vitro* mutagenesis kit was purchased from Bio-Rad, South Richmond, CA, U.S.A. Restriction endonucleases were either from New England Biolabs Inc. MA, U.S.A. or from Pharmacia-LKB Biotechnology, Uppsala Sweden. All other molecular biology materials, including dideoxynucleotide sequencing kits and synthetic oligonucleotides, were from Bresatec Ltd., Adelaide, SA, Australia.

2.1.3: Bacterial strains

E. coli JM101	[D(*lac, pro*) *supE, thi.* F' *tra*D36 *pro* AB, *lac* I^q *lac* ZDM15]. Provided by R. King, Department of Biochemistry, University of Adelaide, SA, Australia.
E. coli MV1190	[D(*lac,pro* AB), *thi*, *sup* E, (D*sr*1-*rec*A)306::Tn10(tet^r), [F': *tra* D36, *pro* AB, *lac* I^q ZDM15]. Supplied in "Mutagene" kit purchased from BioRad, South Richmond, CA, U.S.A.

E. coli CJ236 [*dut*-1, *ung*-1, *thi*-1, *rel* A-1; pCJ105 (Cm^r)]. Supplied in "Mutagene" kit purchased from BioRad, South Richmond, CA, U.S.A.

2.1.4: Bacterial culture media

Media were prepared using distilled water and then autoclaved prior to the addition of any antibiotics. Media plates were prepared by the inclusion of 1.2% agar per litre. L-broth contains 1% bacto-tryptone, 0.5% bacto-yeast extract and 0.17 mol NaCl/l. Minimal media contains 60 mmol K_2SO_4/l, 33 mmol KH_2PO_4/l, 1.7 mmol trisodium citrate/l, 7.6 mmol $(NH_4)SO_4$/l, 10 mmol glucose/l and 1.5 mmol thiamine/l.

2.1.5: Chromatography materials

Reverse phase high performance liquid chromatography (HPLC) columns were obtained from Waters, Sydney, NSW, Australia or Brownlee, Santa Clara, CA, U.S.A.. FPLC and general chromatography columns and apparatus were obtained from Pharmacia-LKB Pty. Ltd., North Ryde, NSW, Australia. Sephadex G-25M, Fast Flow DEAE-, Q- and S-Sepharose were also from Pharmacia-LKB. C18 Matrex silica was purchased from Amicon, Danvers, MA, U.S.A. HPLC grade acetonitrile and propan-1-ol were obtained from either Waters, Sydney, NSW, Australia or from Ajax Chemicals Pty. Ltd., Auburn, NSW, Australia. Trifluoroacetic acid (TFA) was purchased from Fluka Chemie, Buchs, Switzerland, while heptafluorobutyric acid (HFBA) was from Beckman Pty. Ltd., Palo Alto, CA, U.S.A. Water was purified by Milli-Q apparatus (Millipore, Sydney, NSW, Australia). Solvents were filtered through 0.22µm GV filters purchased from Millipore.

2.1.6: Reagents for cleaving fusion-proteins

Hydroxylamine-HCl was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., while recombinant H64A subtilisin BPN' (Carter *et al.* 1989) was generously provided by Genentech Inc., South San Francisco, CA, U.S.A.

2.1.7: Reference peptides and molecular weight markers

Reference recombinant human IGF-I and IGF-II were kindly provided by GroPep Pty. Ltd., Adelaide, SA, Australia. Human insulin was purchased from CSL-Novo Pty. Ltd., North Rocks, NSW, Australia. Low molecular weight and SDS-6H molecular weight markers were from Pharmacia-LKB Pty. Ltd., North Ryde, NSW, Australia and Sigma Chemical Co., St. Louis, MO, U.S.A. respectively, while ^{14}C-labelled rainbow markers for Western ligand blots were obtained from Amersham, North Ryde, NSW, Australia.

2.1.8: Radiochemicals

[α-^{32}P]dATP and [α-^{32}P]dCTP (3000 Ci/mmol) were purchased from Bresatec Ltd., Adelaide, SA, Australia. [^{125}I]-labelled recombinant human and chicken IGF-I and IGF-II with specific activities of 100-150 Ci/g, prepared by the chloramine T method (Van Obberghen-Schilling & Pouyssegur, 1983) were generously made available by S. Knowles, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia. [4,5-^{3}H]-leucine (specific activity 40-60 Ci/mmol) was obtained from Amersham, North Ryde, NSW, Australia.

2.1.9: Antibodies

A polyclonal antibody raised against recombinant human IGF-I (MAC 89/1) was kindly provided by Dr. P. Owens and M. Conlon, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia. A monoclonal antibody raised against recombinant rat IGF-II was obtained from Amano Pharmaceutical Co. Ltd., Nagoya, Japan. S. Knowles (Cooperative

Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia) generously provided "Flopsy", a polyclonal antibody raised against human Long [Arg3]-IGF-I.

2.1.10: Cell culture materials

Plasticware for routine assays was from Nunc, Roskilde, Denmark. All cells, except CHSE-214 were grown at 37°C in 5% CO_2 in Dulbecco's modified Eagle's medium (Gibco, Glen Waverly, Australia) containing 10% w/v fetal calf serum (Flow Laboratories, North Ryde, NSW, Australia) and 100 µg/ml streptomycin and 60 µg/ml penicillin (both from Glaxo, Boronia, VIC, Australia). CHSE-214 cells were grown at 21°C in 5% CO_2 in Eagles Minimal Essential Media with Earles' Balanced Salts (Gibco,Glen Waverly, Australia) containing fetal calf serum and antibiotics as described above. *Routine maintenance and subculturing of cell lines was carried out by I. Liepe and S. Tilley (Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia).*

2.1.11: Cell lines

L6 rat myoblasts (ATTC CRL 1458):

Purchased from American Type Tissue Culture Rockville, MD, U.S.A.

Chick embryo fibroblasts (CEF):

Prepared according to the method described by Rein & Reubin (1968)

Rat H35B hepatomas:

Provided by Dr. J. M. Gunn, Texas A & M University, College Station, TX, U.S.A.

Salmon embryo fibroblasts (CHSE-214; ATTC CRL 1681)

Purchased from American Type Tissue Culture Rockville, MD, U.S.A.

2.2: METHODS

2.2.1: Methods for molecular biology

2.2.1.1: Manipulation of DNA and recombinant organisms

All manipulations involving recombinant DNA or recombinant organisms were performed in accordance with the guidelines issued by the Genetic Manipulation Advisory Committee (Australia).

Unless otherwise stated, DNA was cloned, isolated and analyzed using standard procedures as described in "Molecular Cloning, A Laboratory Manual" by Sambrook *et al.* (1989). In particular, the following methods in Sambrook *et al.* (1989) were followed. Plasmid DNA was prepared using the lysis by alkali method described in section 1.29. Restriction endonuclease digestion and ligation of DNA was carried out as described in sections 5.28 and 1.68 respectively. Competent *E. coli* cells were prepared using the $CaCl_2$ method and transformed via heat shock following the protocols in sections 1.82 and 1.74. Single-stranded phagemid DNA was prepared using the methods in sections 4.47, 4.48 and 15.76. DNA was examined on agarose gels as described in section 6.9 and if required, isolated from the gels via electroelution into dialysis bags (section 6.28).

2.2.1.2: In vitro site-directed mutagenesis

Site-directed mutagenesis was performed on DNA fragments subcloned into the mutifunctional vector pTZ18 using the procedure described in the "Mutagene" kit BioRad, South Richmond, CA, U.S.A. This protocol is essentially the same as that described by Sambrook *et al.* (1989) in sections 15.51 and 15.74. The mutagenesis procedure involved the following steps (see Fig. 2.1):

1) The plasmid into which the changes were to be introduced was transformed into CJ236, an *E. coli* strain with a *dut ung* phenotype. The *dut* mutant phenotype is characterized by an inactive dUTPase, thus causing high intracellular levels of dUTP. The *ung* phenotype is characterized by an inactive uracil N-glycosylase, hence any uracil incorporated into the DNA remains. The

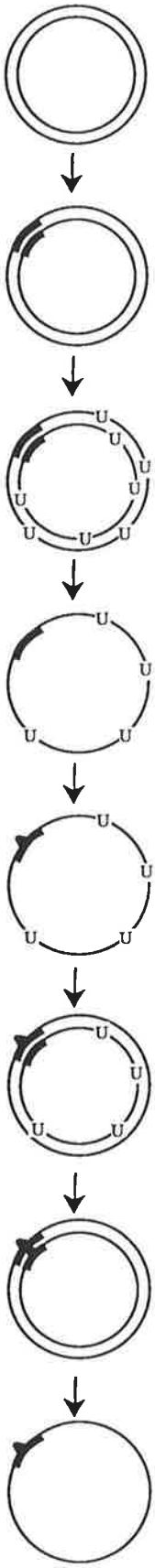


FIGURE 2.1: Schematic representation of the steps involved in performing *in vitro* site-directed mutagenesis.

combination of these two defective enzymes in this strain means that all DNA synthesized in it contains some uracil.

2) Single-stranded uracil-containing DNA was prepared from the transformed CJ236 as described by Sambrook *et al.* (1989) in section 15.76.

3) Purified oligonucleotide (3-50 pmol) was annealed to 0.3 pmol of the uracil-containing single-stranded DNA which was to be mutagenized by adding 1 µl of 10X Annealing Buffer (200 mmol Tris/l, 50 mmol $MgCl_2$/l and 500 mmol NaCl/l at pH 7.4), making the total volume up to 10 µl with water, vortexing the mixture and then placing the mix into a heating block at 70°C. The heating block was cooled slowly over 40 min to room temperature. Tubes were then left on ice for at least 30 min. The oligonucleotides were designed such that at least 12 codons of complementary DNA flanked the region where the changes were to be introduced.

4) Synthesis of the mutagenized strand was achieved by adding 1 µl of 10X Synthesis Buffer (5 mmol/l each of deoxynucleoside triphosphates, 10 mmol ATP/l, 100 mmol Tris pH 7.4/l, 50 mmol $MgCl_2$/l and 20 mmol DTT/l), 3 units of T4 DNA Ligase and 1 unit of T4 DNA Polymerase. Tubes were mixed, left on ice for 5 min, then for 5 min at 25°C and finally incubated at 37°C for 90 min. The synthesis reaction was stopped by freezing the tubes.

5) Five µl of the synthesis reaction mix containing double-stranded DNA, only one strand of which contains uracil, was transformed into *E. coli* MV1190. This strain has an active uracil N-glycosylase which selectively inactivates the uracil-containing parental strand.

6) Single-stranded DNA was prepared from single colonies of the transformed MV1190. Plasmids which had been successfully mutagenised were identified by DNA sequencing (Sanger *et al.* 1977).

2.2.1.3: Fermentation and isolation of inclusion bodies

E. coli JM101 [D*(lac, pro) supE, thi.* F' *tra*D36 *pro* AB, *lac* I^q D*lac* Z M15] cells transformed with expression plasmids were grown in a 15 litre fermenter

(Chemap, Volketswil, Switzerland) at 37°C, 55% pO_2, pH 7, with automated glucose feed, until the absorbance of the culture at 600 nm reached 50. Cells were then induced with 0.32 mmol IPTG/l and grown for a further 6 h. Inclusion bodies containing the expressed proteins were collected from the fermentation broth by homogenization and centrifugation (King *et al.* 1992). *Fermentations were performed by C. Senn, Bresatec Ltd, Adelaide, SA, Australia.*

2.2.2: Chromatographic methods

2.2.2.1: Solvents

Solvents were prepared in Milli-Q water then filtered through a 0.22 µm membrane prior to use. Solutions are described as volume/volume unless otherwise stated.

2.2.2.2: Reverse-phase HPLC

Analytical and small-scale preparative chromatography was performed on a chromatography system controlled by an ICI DP800 software package (ICI Pty. Ltd., Sydney, NSW, Australia). The chromatography system consisted of two Kortec K35D pumps (Kortec, Ermington, NSW, Australia), a dynamic mixer (Knaur, Berlin, Germany), a Kortec K95 variable wavelength detector and a ICI DP800 data interface module. Samples were loaded either via a Rheodyne model 7125 injector (Rheodyne Inc., Cotati, CA, U.S.A.) with a 1 ml loop or via the solvent A inlet line.

Preparative HPLC requiring flow rates of 25 ml/min was performed on a Waters Delta Prep 3000 chromatography system (Millipore-Waters, Milford, MA, U.S.A.).

2.2.2.3: Standard analytical HPLC

The recovery of peptide during the various stages of the production of the recombinant IGFs was monitored by analysis on a microbore C4 reverse-phase HPLC column (2.1 mm (diameter) x 100 mm, Brownlee Laboratories, Santa

Clara, CA, U.S.A.) employing a linear gradient from 15 to 50% acetonitrile over 35 min in the presence of 0.1% TFA and at a flow rate of 0.5 ml/min. Elution of protein was monitored by absorbance at 215 nm.

2.2.2.4: Estimation of protein amount by reverse-phase HPLC

Proteins were quantitated by calculating the area under their absorbance profile at 215 nm when eluted from the standard analytical HPLC described above. The area under the absorbance profile was converted to protein concentration using the calculated extinction coefficients (Buck *et al.* 1989; Gill & Von Hippel, 1989) outlined in Table 2.1.

2.2.3: Chemical analysis of proteins

2.2.3.1: N-terminal protein sequencing

N-terminal protein sequences were determined by Edman degradation using a gas-phase sequencer (model 470 A; Applied Biosystems, Foster City, CA, U.S.A.) and the method described by Hunkapillar *et al.* (1983). *Peptide sequencing was kindly performed by D. Turner, Department of Biochemistry, University of Adelaide, Adelaide, SA, Australia.*

2.2.3.2: Determination of molecular mass

Mass quantitation of proteins was determined by analysis on a VG Biotech Quattro mass spectrometer (VG Biotech Ltd., Altrincham, Cheshire, U.K.). *Electro-spray mass quantitation was performed by Dr. M. Sheil, Department of Chemistry, University of Wollongong, Wollongong, NSW, Australia.*

2.2.3.3: Electrophoretic analysis of proteins

Proteins were analysed on high-density polyacrylamide gels under both reducing and non-reducing conditions on Pharmacia's "PhastSystem" (Pharmacia-LKB Pty. Ltd., North Ryde, NSW, Australia) using the conditions and techniques suggested by the supplier.

Peptide	Extinction coefficient (mol^{-1} cm^{-1}, 215 nm)
hIGF-I	32.82
hIGF-II	31.70
Long [Arg^{3}]-hIGF-I	33.61
cIGF-I	33.70
cIGF-II	32.65
cIGF-II ($Thr^{62}Pro^{63}Ala^{64}$)	32.85
cIGF-II(-Arg^{40}, $Thr^{62}Pro^{63}Ala^{64}$)	33.16
Long [Arg^{3}]-cIGF-I	35.65
[Gly]-hagfish IGF	31.70

TABLE 2.1: Extinction coefficients used for estimation of protein. The extinction coefficients were calculated using the methods described by Buck *et al.* (1989) and Gill & Von Hippel (1989).

2.2.4: *In vitro* assays

2.2.4.1: Stimulation of protein synthesis in cell monolayers

Stimulation of protein synthesis in L6 rat myoblasts by growth factors was measured over an 18 h period using the method described by Francis *et al.* (1986). Confluent monolayers of L6 myoblasts in 24-place multiwells were incubated at 37°C for 2 h in 1 ml of Dulbecco's Modified Eagles Medium (DMEM). The medium in each well was replaced with 0.9 ml of DMEM containing 1 μCi/ml [4,5-^{3}H]-leucine together with 0.1 ml of sample. The samples to be tested had been dried under vacuum, resuspended in 0.01 mol HCl/l, then diluted into a solution of 0.01 mol potassium phosphate buffer/l (pH 7.4) containing 0.09% (w/v) NaCl and 0.1% (w/v) BSA. After labelling at 37°C for 18 h, the monolayers were washed twice with Hanks' salts (Gibco, Glen Waverly, NSW, Australia), twice with 5% (v/v) trichloroacetic acid over a 10-min period, and once with water before solubilizing in 0.5 mol/l NaOH containing 0.1% (v/v) Triton X-100. Stimulation of protein synthesis was measured as the increased incorporation of [^{3}H]-leucine into total cell protein during the 18 h incubation period above that which occurred in controls with no added peptides.

2.2.4.2: Measurement of intracellular protein breakdown in cell monolayers

The rates of intracellular protein breakdown in monolayers of rat L6 myoblasts and chick embryo fibroblasts were measured over a 4 h period following the procedure reported by Ballard *et al.* (1986). Briefly, confluent cell monolayers were prelabelled for 18 h in growth medium with [4,5-^{3}H]-leucine as the only source of leucine other than 5% (v/v) fetal bovine serum. After labelling, the cells were washed in DMEM, then chased for 2 h in DMEM containing 2 mmol leucine/l to allow unstable proteins to be degraded. Protein degradation was then determined as the percentage of labelled protein that is degraded to trichloroacetic acid-soluble radioactivity over a 4 h measurement period in DMEM containing 2 mmol leucine/l and in the absence of added peptides.

Breakdown rates were expressed as the per cent inhibition relative to the rate occurring in serum-free DMEM plus the sample diluent.

2.2.4.3: Binding to type-1 IGF receptors

Binding to the type-1 IGF receptor in cultured cells was determined using [^{125}I]-labelled recombinant human or chicken IGF-I according to the method described by Ross *et al.* (1989). Radiolabelled ligand was added in the presence of increasing concentrations of unlabelled peptides in a total volume of 0.5 ml Hepes' buffer (0.1 mol Hepes/l, 0.12 mol NaCl/l, 5 mmol KCl/l, 1.2 mmol $MgSO_4.7H_2O$/l and 8 mmol glucose/l at pH 7.6) containing 0.5 % (w/v) BSA to confluent cell monolayers in 24-place multiwells. IGF binding to type-1 receptors was measured at 4°C for 18 h in the rat L6 myoblast cell line and for 4 h in chick embryo fibroblast (CEF) cells. Following incubation, the cell monolayers were washed with Hanks' salts (Gibco, Glen Waverly, NSW, Australia) at 0°C to remove unbound radioligand. The cell monolayers were dissolved in 0.5 mol/l NaOH containing 0.1% (v/v) Triton X-100 and the cell-associated radioactivity determined. Binding was expressed as the percentage of bound radioactivity occurring in the absence of unlabelled IGF peptide.

2.2.4.4: Binding to type-2 IGF receptors

Binding to type-2 IGF receptors in sheep placental membranes was measured using radioiodinated hIGF-II and the method described by Anderson *et al* (1993). Fifty µg of microsomal protein was incubated with 20 000 cpm [^{125}I]-hIGF-II and unlabelled IGFs in 0.25 ml 0.1 mol Tris/l, pH 7.4 containing 0.01 mol $CaCl_2$/l and 0.5% BSA (w/v) for 18 h at 4°C. Subsequently, 1.0 ml of chilled 0.1 mol Tris/l, pH 7.4 containing 0.1 mol $CaCl_2$/l and 0.1% BSA (w/v) was added and mixed, and the bound radioactivity was pelleted by centrifugation at 3000 x g for 20 min at 4°C. The supernatant was aspirated and the radioactivity in the pellet was measured. Binding was expressed as the percentage of that bound in the absence of competing peptide.

2.2.4.5: Radioimmunoassays

Radioimmunoassays were carried out at 4°C for 18 h in a solution containing 0.25 ml 0.03 mol potassium phosphate/l, 0.2% (w/v) protamine sulphate, 0.2% (w/v) NaN_3 and 0.25% BSA at pH 7.5, antiserum, radiolabel (20 000 cpm) and competing peptides. The antibodies were precipitated with rabbit IgG and goat anti-rabbit gamma-globulin. After 30 min at 4°C, 1.5 ml of chilled 5% polyethylene glycol (6000) was added and the tubes centrifuged at 4000 x g before removal of the supernatants by aspiration. Radioactivity was measured in the pellets.

2.2.4.6: IGFBP competitive binding assay

The relative binding of recombinant IGFs to the IGFBPs present plasma was measured as described previously (Szabo *et al.* 1988). Briefly, 30-50 µl of plasma or 5-10 ng of purified IGFBPs was incubated with [^{125}I]-labelled human IGF-II in the presence or absence of competing unlabelled IGFs in assay buffer (10 mmol sodium phosphate/l, 150 mmol NaCl/l and 0.5% BSA (w/v) BSA, pH 7.4) at 4°C for 18 h. Free [^{125}I]-labelled hIGF-II was separated from IGFBP-bound label by incubating the reaction mixture with activated charcoal at 4°C for 30 min and then centrifuging the mixture. The radioactivity in the supernatant was determined and used to calculate the percentage of added radioactivity bound to IGFBPs in the supernatant. Binding was expressed as the percentage of [^{125}I]-labelled human IGF-II bound in the absence of added unlabelled peptide.

2.2.4.7: Interaction of labelled IGFs with plasma binding proteins after SDS-PAGE

Proteins in plasma were subjected to discontinuous electrophoresis (SDS-PAGE) using a 4% stacking gel and a 10% separating gel (Laemmli, 1970). Following electrophoresis, proteins were transferred to nitrocellulose sheets, then probed with [^{125}I]-labelled human or chicken IGF-I or IGF-II in the presence and absence of excess unlabelled IGFs using the procedure described

by Hossenlopp *et al* (1986). Molecular weights were assessed with ^{14}C-labelled Rainbow markers. Radioactivity bound to the nitrocellulose sheets was detected by either exposing the sheets to X-ray film, or by employing a phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.)

CHAPTER 3

PRODUCTION AND CHARACTERIZATION OF RECOMBINANT CHICKEN INSULIN-LIKE GROWTH FACTOR-I FROM *ESCHERICHIA COLI*

3.1: INTRODUCTION

This laboratory has previously reported the purification and amino acid sequence of the first non-mammalian IGF-I characterized, namely chicken IGF-I (cIGF-I) (Dawe *et al.* 1988: Ballard *et al.* 1990). Protein sequencing indicated that cIGF-I has eight amino acid differences compared with porcine, bovine or human IGF-I (hIGF-I). The sequences deduced from cDNA for cIGF-I by Kajimoto & Rotwein (1989) and Fawcett & Bulfield (1990) confirm the protein sequence. The amino acid changes occur in similar positions in cDNA clones for salmon (Cao *et al.* 1989) and frog (Shuldiner *et al.* 1989; Kajimoto & Rotwein, 1990) IGF-I. Except for these few changes, there is a high degree of identity between all vertebrate IGF-Is, suggesting the importance of this peptide for growth and development as well as possible structural constraints due to the number of proteins with which it must interact.

Although Dawe *et al.* (1988) have evaluated serum-derived cIGF-I in a limited range of *in vitro* test systems, they were not able to obtain sufficient quantities of the protein to permit a more thorough assessment of the *in vitro* effects and *in vivo* responses. In order to overcome this, I have developed a strategy to produce recombinant cIGF-I, and report here the production of the growth factor in *Escherichia coli (E. coli).* I also present evidence that recombinant cIGF-I has the same biological and receptor-binding activities as its human counterpart in cultured rat and chicken cells.

3.2: MATERIALS AND METHODS

3.2.1: Plasmid construction

The starting plasmid was the hIGF-I expression vector p[Met^{1}]pGH(1-46)-Val-Asn-hIGF-I described by King *et al.* (1992). These authors have further effected a deletion of the codons for 35 amino acids in this vector by site-directed *in-vitro* mutagenesis of the EcoRI-HindIII fragment after cloning it into M13mp8 by utilizing the oligonucleotide: 5'GCACAGGGTTTCCGGGCCGTTAACAAATAGGC

TGGACAAGGGCAT-3'. The resulting plasmid vector, p[Met^1]pGH(1-11)-Val-Asn-hIGF-I, contains a unique HpaI restriction endonuclease site (GTTAAC) coding for Val-Asn. The expression vector is derived from the parent plasmid pKT52 (Vize & Wells, 1987) and contains the *trc* promoter in conjunction with the *lac* operator. Thus, when propagated in *E. coli* strains which overproduce *lac* repressor, the addition of isopropyl-ß-D-galactoside (IPTG) induces high-level expression of hIGF-I as a fusion protein with a leader peptide containing the first 11 amino acids of methionyl porcine growth hormone ([Met^1]pGH(1-11)), followed by Val and Asn. This vector was kindly made available by the investigators.

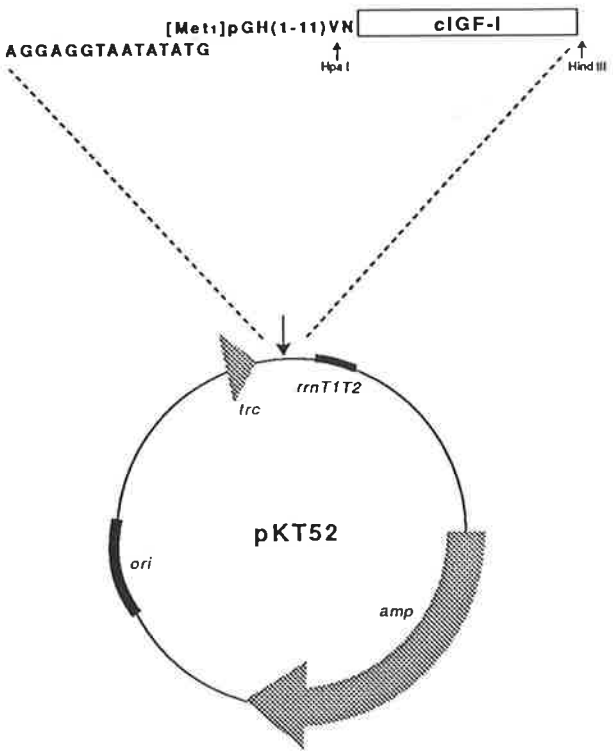
I subcloned the EcoRI-HindIII fragment of p[Met^1]pGH(1-11)-Val-Asn-hIGF-I into the multifunctional vector pTZ18 and selectively changed codons using site-directed mutagenesis following the procedure described in the "Mutagene" kit (see 2.1.2 and 2.2.1.2). Using several rounds of mutagenesis, the hIGF-I codons were replaced with codons which permit the expression of the amino acids that are different in cIGF-I. I employed the following synthetic oligonucleotides to direct these changes: Oligonucleotide 1: 5'-GGCTTCTACTTCTCTAAACCGACC GG-3'; Oligonucleotide 2: 5'-TACGGTTCTTCTTCTCGTCGTCTGCACCACAAAG GTATCGTTGACGAATGC-3'; Oligonucleotide 3: 5'-GAATGCTGCTTCCAGTCTT GCGACCTG-3'; Oligonucleotide 4: 5'-ATGTACTGCGCTCCGATCAAACCGCCG AAATCTGCTTGATGA-3'. Oligonucleotide 1 permitted the replacement of codons for Asn^{26} in hIGF-I with codons for Ser. The residues Ala-Pro-Gln-Thr at positions 38-41 in hIGF-I were replaced with codons for Leu-His-His-Lys, the residues found in the equivalent positions in cIGF-I, using oligonucleotide 2. Oligonucleotide 3 directed a change resulting in the substitution of codons for Gln in place of those for Arg at position 50. Finally, oligonucleotide 4 was employed to replace the codons for Leu^{64} and Ala^{67} in hIGF-I with codons for Ile and Pro respectively (Fig. 3.1a). Once all the required changes were introduced, the HpaI-HindIII fragment of the pTZ18 clone was ligated back into HpaI-HindIII-cut p[Met^1]pGH(1-11)-Val-Asn-hIGF-I to replace Asn-hIGF-I with Asn-cIGF-I,

FIGURE 3.1: a) Nucleotide and corresponding amino acid sequence of the Hpa1-HindIII sequence of the construct engineered for expression of chicken IGF-I. Shaded boxes indicate regions where changes were introduced to the DNA sequence of the starting vector by site-directed mutagenesis. The oligonucleotides employed to direct these changes are indicated (oligos 1-4). Amino acids are numbered and stop codons are marked by asterisks. b) Schematic representation of the construct engineered for expression of chicken IGF-I in *E. coli*. The expression plasmid, pKT52, contains an origin of replication (*ori*), a *trc* promoter, a transcription termination sequence (*rrnT1T2*) and a gene conferring ampicillin resistance (*amp*). The DNA sequence preceding Met has been modified to optimize ribosome binding (Vize & Wells, 1987).

a)

```
 HpaI  1                                                          19
    AsnGlyProGluThrLeuCysGlyAlaGluLeuValAspAlaLeuGlnPheValCysGly
 5'-AACGGCCCGGAAACCCTGTGCGGTGCTGAACTGGTTGACGCTCTGCAGTTCGTTTGCGCT
 3'-TTGCCGGGCCTTTGGGACACGCCACGACTTGACCAACTGCGAGACGTCAAGCAAACGCGA

                                                                  39
    AspArgGlyPheTyrPheSerLysProThrGlyTyrGlySerSerSerArgArgLeuHis
    GACCGTGGCTTCTACTTCTCTAAACCGACCGGTTACGGTTCTTCTTCTCGTCGTCTGCAC
        5'       oligo 1            3'
                                    5'_____________________oligo 2
    CTGGCACCGAAGATGAAGAGATTTGGCTGGCCAATGCCAAGAAGAAGAGCAGCAGACGTG

                                                                  59
    HisLysGlyIleValAspGluCysCysPheGlnSerCysAspLeuArgArgLeuGluMet
    CACAAAGGTATCGTTGACGAATGCTGCTTCCAGTCTTGCGACCTGCGTCGTCTGGAAATG
                    5'          oligo 3          3'          5'
    oligo 2_____________________3'
    GTGTTTCCATAGCAACTGCTTACGACGAAGGTCAGAACGCTGGACGCAGCAGACCTTTAC

                                    70
    TyrCysAlaProIleLysProProLysSerAla******   HindIII
    TACTGCGCTCCGATCAAACCGCCGAAATCTGCTTGATGATGCAAG-3'
    oligo 4                                  3'
    ATGACGCGAGGCTAGTTTGGCGGCTTTAGACGAACTACTACGTTC-5'
```

b)



creating p[Met^1]pGH(1-11)-Val-Asn-cIGF-I. The DNA sequence of the HpaI-HindIII cIGF-I fragment and a schematic representation of the final construct are shown in Fig. 3.1. This construct was then transformed into *E. coli* JM101 (lac I^q) at 37°C on minimal agar containing 29 µmol ampicillin/l.

3.2.2: Fermentation and isolation of inclusion bodies

E. coli JM101 cells transformed with p[Met^1]pGH(1-11)-Val-Asn-cIGF-I were grown in a 15 litre fermenter and induced with IPTG to produce inclusion bodies containing the cIGF-I fusion protein. The details of the fermentation and the subsequent isolation of the inclusion bodies from the fermentation broth are described in 2.2.1.3. *The fermentation was performed by C. Senn, Bresatec Ltd, Adelaide, SA, Australia.*

3.2.3: Fusion protein purification

Inclusion bodies (25 g) containing the cIGF-I fusion protein were solubilized by suspension in 250 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 0.5 mmol $ZnCl_2$/l and 20 mmol dithiothreitol (DTT)/l at pH 9.1. The dissolved, reduced inclusion bodies were desalted on a 100 mm (diameter) x 350 mm Sephadex G-25M column equilibrated with 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 0.5 mmol $ZnCl_2$/l and 1.6 mmol DTT/l at pH 9.1. The desalted, reduced inclusion bodies were loaded at 20 ml/min onto a 50 mm (diameter) x 200 mm column packed with 75ml Fast Flow DEAE Sepharose equilibrated with the same buffer as used for the desalting procedure. The cIGF-I fusion protein was eluted by washing the column at a flow rate of 20 ml/min with desalting buffer which had been adjusted to pH 6.5 with HCl.

The cIGF-I fusion proteins were refolded under oxidizing conditions by diluting the DEAE Sepharose eluate to a final concentration of 0.125 mg protein/ml in a solution containing 2 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 10 mmol EDTA/l, 0.4 mmol DTT/l and 1 mmol 2-hydroxyethyl disulphide/l at pH 8.5. The refolding reaction was monitored by analysis on the standard analytical

HPLC described in 2.2.2.3. Elution of protein was monitored by absorbance at 215 nm. Refolding was indicated by early elution of the protein compared with that observed when the reduced starting material was analyzed under identical conditions. Once refolding was complete, the reaction was stopped by acidification to pH 2.1 with HCl. The acidified pool containing the refolded clGF-I fusion proteins was then pumped at 20 ml/min on to a 50 mm (diameter) x 200 mm column packed with 25 g C18 Matrex silica previously equilibrated with 12% acetonitrile containing 0.1% TFA. The clGF-I fusion peptide was eluted by applying a linear gradient from 12 to 48% acetonitrile in 0.1% TFA over 16 min at a flow rate of 20 ml/min. The pool containing the clGF-I fusion protein was lyophilized before further processing.

3.2.4: Fusion protein cleavage

The generation of a fusion protein with Asn flanking Gly, the first residue of IGF-I, permits cleavage of the IGF-I from the leader peptide using hydroxylamine. Thus, lyophilized clGF-I fusion protein was cleaved at the unique Asn-Gly bond by incubation at 45°C for 14 h in a solution containing 2 mol hydroxylamine-HCl/l, 2 mol urea/l, 0.1 mol Tris/l, with the pH adjusted to 9.0 with LiOH and a final protein concentration of 0.5 mg/ml. The mixture was gently sparged with N_2 during the incubation to reduce oxidation of methionine residues during the incubation. The reaction was terminated by acidification to pH 2.1 with HCl. The conditions for the chemical cleavage reaction are based on those previously determined by Bornstein and Balian (1977) with the exception that urea solution instead of guanidine is used as the solvent.

3.2.5: Purification of the clGF-I peptide

The acidified, cleaved peptides were concentrated by chromatography on the C18 Matrex column described above. A single fraction containing the cleaved IGF was then pumped at 20 ml/min on to a 50 mm (diameter) x 200 mm column packed with 75 ml Fast Flow S Sepharose previously equilibrated with 1

mol acetic acid/l. The column was then washed with several volumes of 50 mmol ammonium acetate/l (pH 4.8), before eluting the cIGF-I with a gradient from 0 to 2 mol NaCl/l in 50 mmol ammonium acetate/l (pH 4.8), over 30 min, at a flow rate of 20 ml/min. The eluted IGF was detected by its absorbance at 280 nm and collected in a single fraction. This fraction was further purified by two reverse-phase HPLC steps on a Waters Delta Prep 3000 chromatography system employing a C4 PrepPak 500 cartridge (57 mm (diameter) x 300 mm). The first reverse-phase step utilized a linear gradient from 25 to 45% acetonitrile in 0.13% heptafluorobutyric acid over 100 min at a flow rate of 25 ml/min. The second HPLC step employed a gradient from 26 to 41% acetonitrile in 0.1% TFA over 150 min at a flow rate of 25 ml/min. Elution of protein in both purification steps was monitored by absorbance at 280 nm. The recovery of the cIGF-I at all stages during the purification was followed by analysis on the standard analytical HPLC earlier, by electrophoresis on high-density polyacrylamide gels using Pharmacia's "PhastSystem" (2.2.3.3), and by assessing fractions for their ability to stimulate protein synthesis in L6 rat myoblasts (2.2.4.1). Selected fractions were pooled, lyophilized, redissolved in 100 mmol acetic acid/l and desalted under sterile conditions on a Cellufine GH25(M) column (22 mm (diameter) x 250 mm) equilibrated with 100 mmol acetic acid/l.

The cIGF-I peptide was quantitated by calculating the area under the absorbance profile at 215 nm when eluted form a C4 microbore reverse-phase HPLC column using the standard analytical HPLC. The area under the absorbance profile was converted to protein concentration using the calculated extinction coefficient of 33.70 mol^{-1} $cm^{-1}$for recombinant cIGF-I (2.2.2.4).

N-terminal protein sequences and mass quantitation were determined as described in 2.2.3.1 and 2.2.3.2 respectively.

3.2.6: *In vitro* characterization of cIGF-I

In vitro characterization of the biological actions, receptor and IGFBP interactions of recombinant cIGF-I were determined using the biochemical assays described in 2.2.4.

3.2.7: Cross-linking experiments

Binding of radiolabelled cIGF-I to receptors in CEFs was carried out as described in 2.2.4.3 except cells were grown in 6-place multiwells and 8 x 10^5 cpm were added per well. Subsequently the cells were washed three times in Hepes binding buffer (0.1 mol Hepes/l, 0.12 mol NaCl/l, 5 mmol KCl/l, 1.2 mmol $MgSO_4.7H_2O$/l and 8 mmol glucose/l at pH 7.6) without BSA at 0°C, after which 0.5 ml of 0.5 mmol disuccinimidyl suberate/l in the same buffer was added and left on the cells for 20 min at 15°C. The cross-linking reaction was quenched with 1.5 ml of 0.1 mol Tris/l containing 1 mmol EDTA/l at pH 7.4, and the cells left for a further 20 min. The medium was aspirated and the cells were solubilized in 0.2 ml of 2% (w/v) SDS, 10% (w/v) glycerol, 100 mmol DTT/l, 0.1% Bromophenol Blue and 62.5 mmol Tris/l at pH 6.8. The solubilized cell mixtures were stored at -70°C until they were electrophoresed on 5% (w/v) SDS/polyacrylamide gels (Laemmli, 1970). Details of the straining, destaining, drying and autoradiography of the gels has been described previously (Ballard *et al.* 1986).

3.2.8: IGF-I radioimmunoassay

Immunoreactivity was measured by employing a polyclonal antibody raised in rabbits against recombinant hIGF-I (MAC 89/1) and the method described in 2.2.4.5. Recombinant hIGF-I was used as the radioligand and as a standard in this assay.

3.3: RESULTS

3.3.1: Fusion protein expression

E.coli JM101 cells transformed with p[Met^{1}]pGH(1-11)-Val-Asn-cIGF-I overproduced the cIGF-I fusion protein when induced with IPTG. SDS-polyacrylamide gel analysis of total cell protein following induction revealed a predominant band migrating with an approximate molecular mass of 9 kDa, the size expected for the cIGF-I fusion protein (Fig. 3.2, lane 4). Fusion-protein synthesis was completely dependent upon induction of the *trc* promoter as this band was not detected in uninduced cells (data not shown). A 15 litre fermentation of *E. coli* cells containing the expression vector for the cIGF-I fusion protein yielded 91 g wet weight of inclusion bodies.

3.3.2: Cleavage and purification of the cIGF-I fusion protein

Although King *et al.* (1992) have previously reported the processing of similar recombinant hIGF fusion peptides from *E. coli* inclusion bodies, a different strategy was required for the purification of the cIGF-I fusion proteins. N-terminal peptide sequencing revealed that when the cIGF-I fusion proteins were extracted from the inclusion bodies by the procedure described in King *et al.* (1992) a significant proportion (generally greater than 50%) was cleaved between Arg^{36} and Arg^{37}. This cleavage was presumably caused by bacterial proteinases. Inclusion of a DEAE anion-exchange chromatography step early in the purification substantially reduced this proteolysis.

Following anion-exchange chromatography, the fusion proteins were refolded using conditions previously determined as optimal for refolding other IGF fusion proteins (King *et al.* 1992). Refolding was indicated by early elution of the protein when analysed on a C4 HPLC column at 20 min (Fig. 3.3b) compared with reduced IGF fusion protein which eluted after 26 min (Fig. 3.3a). The refolded peptides were then concentrated on a C18 silica column by rapid elution, followed by lyophilization. Refolded fusion protein (730 mg) was recovered from the 25 g inclusion bodies that were processed. SDS-

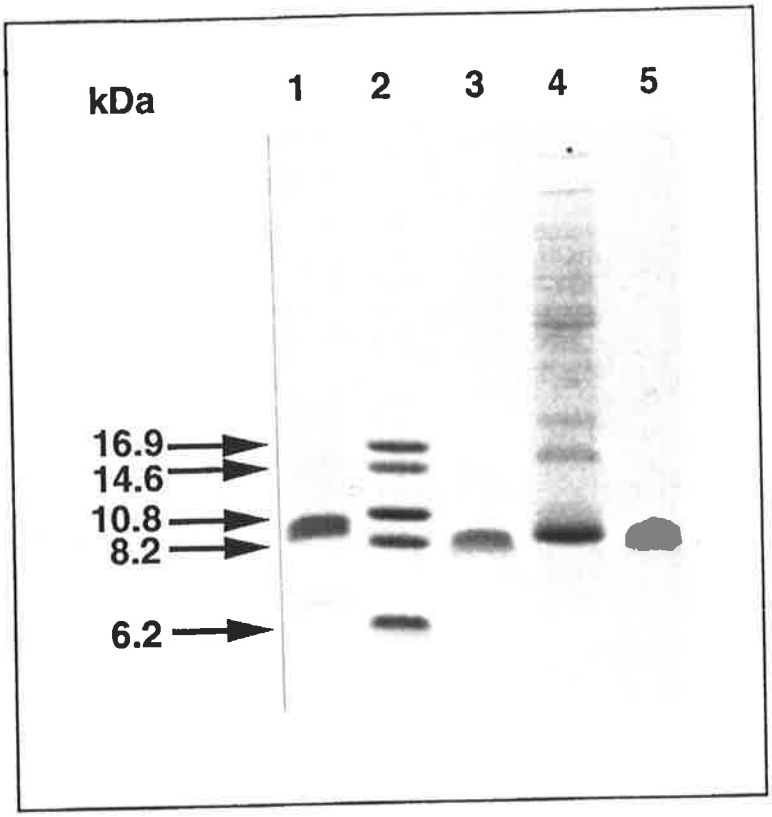


FIGURE 3.2: Polyacrylamide gel analysis of total cell protein from *E.coli* cells induced to express[Met1]-pGH(1-11)-Val-Asn-clGF-I (lane 4), partially pure chicken IGF-I (clGF-I) fusion protein (lane 1) and pure recombinant clGF-I (lane5). Reference recombinant human IGF-I and molecular mass markers are in lanes 3 and 2 respectively. Samples were electrophoresed on high-density SDS-polyacrylamide gels under reducing conditions.

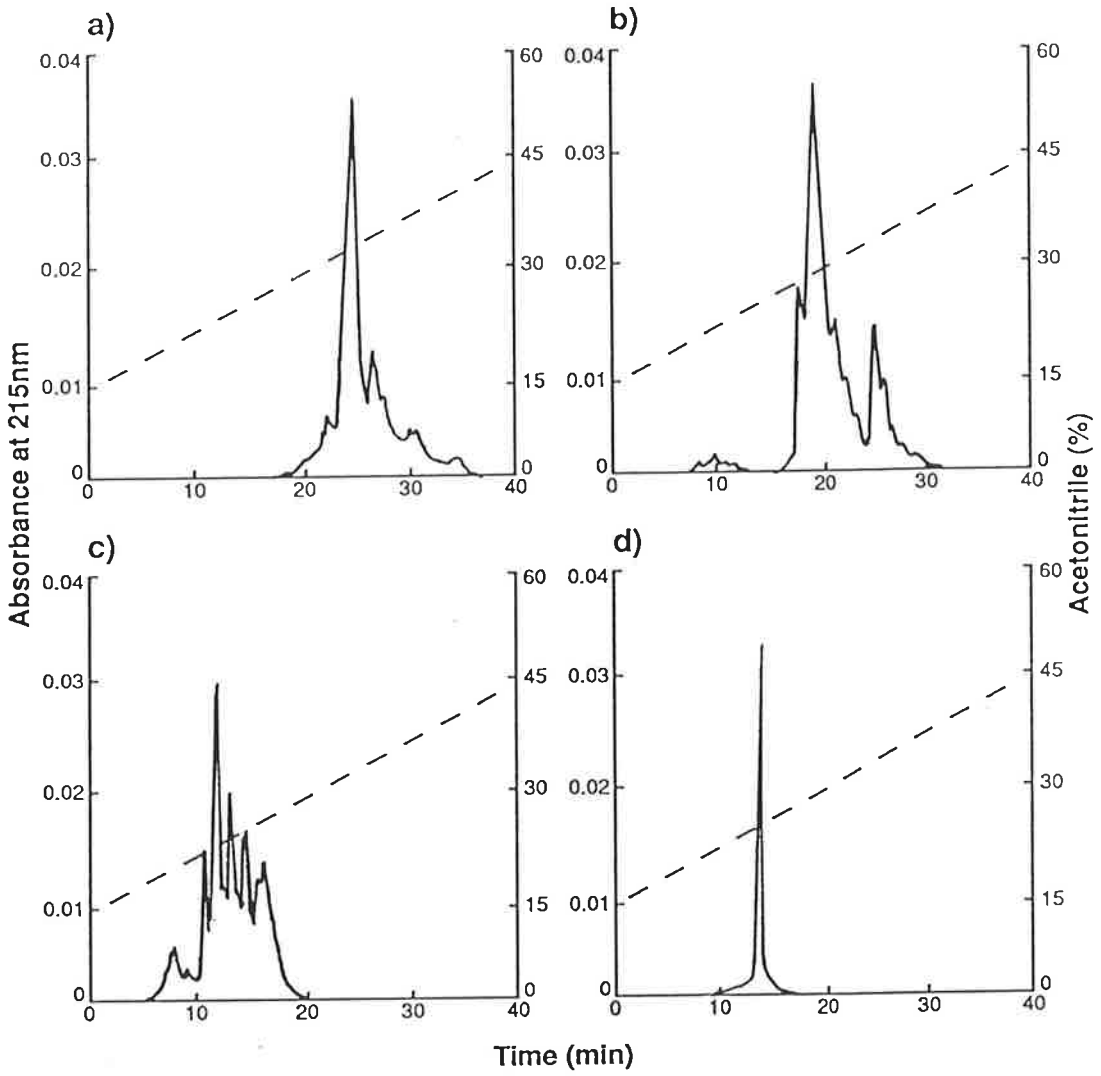


FIGURE 3.3: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC described in 2.2.2.3 after a) solubilization of [Met[1]]-pGH(1-11)-Val-Asn-cIGF-I inclusion bodies from *E. coli*, b) refolding of the reduced chicken IGF fusion protein, c) hydroxylamine cleavage of the chicken IGF-I fusion protein and d) final purification of chicken IGF-I. Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.

polyacrylamide gel analysis of the partially pure fusion protein under reducing conditions indicated that minimal cleavage of the fusion protein by bacterial proteinases had occurred (Fig. 3.2, lane 1).

Release of the required cIGF-I from the fusion protein was achieved using hydroxylamine. Analysis of the cleavage products on the standard analytical reverse-phase HPLC (2.2.2.3) revealed that a number of new protein species had been generated (Fig. 3.3c). The hydroxylamine-cleaved peptides were concentrated on a C18 silica column, subjected to a Fast Flow S Sepharose ion-exchange step and then further purified through two reverse-phase HPLC steps. The recombinant cIGF-I recovered from the final reverse-phase HPLC process eluted as a single peak from the standard analytical reverse-phase HPLC (Fig. 3.3d) and migrated as a single band at the expected size of approximately 8 kDa on a SDS-polyacrylamide gel run under reducing conditions (Fig. 3.2, lane 5). Recombinant hIGF-I migrated at a similar position (Fig. 3.2, lane 3).

N-terminal peptide sequencing of the recombinant cIGF-I for 57 cycles of Edman degradation indicated that the protein was at least 98% pure and there was no evidence that either incomplete hydrolysis by hydroxylamine or cleavage by bacterial proteinases had occurred. The protein sequence obtained here and in the analysis of the proteolytically cleaved peptide above confirmed that all the required changes had been correctly introduced into the hIGF DNA sequence, thus creating cIGF-I (Fig. 3.4). The yield from the hydroxylamine cleavage and subsequent purification steps was 8 mg pure cIGF-I from 125 mg refolded cIGF-I fusion protein. The low yield is largely due to the inefficiency of the hydroxylamine cleavage step.

3.3.3: Biological activity of recombinant cIGF-I

The biological activity of the recombinant cIGF-I was assessed by comparing the ability of the peptide to stimulate protein synthesis or inhibit protein degradation with that of recombinant hIGF-I and hIGF-II. The cIGF-I was as potent as recombinant hIGF-I in the protein synthesis assay in L6 rat

GPETL-GAELVDALQFV-GDRGFYFSKPTGYGSSSRRLHHKGIVDE--FQS-DLRRL

RLHHKGIVDE--FQS-DLRRLEMY-APIKPPKS

FIGURE 3.4: Sequences of chicken IGF-I obtained by N-terminal analysis of the complete protein (top), or the protein fragment generated by bacterial proteinases (bottom). The complete protein was purified using the procedure described in 3.2, whereas the protein fragment was purified using the procedure described by King et. al. (1992). The residues which were introduced into the DNA sequence by site-directed mutagenesis are underlined. Dashes represent residues not detected during sequencing.

myoblasts, with half-maximal responses at 22 and 20 ng/ml respectively. Recombinant hIGF-II was less potent, with half-maximal stimulation being observed at 150 ng/ml (Fig. 3.5). The similar biological activity of the human and chicken recombinant IGF-I was further confirmed by assessing their ability to inhibit protein degradation in L6 rat myoblasts. The half-maximal effects observed in this assay were at 0.48 ng/ml for cIGF-I and 0.50 ng/ml for hIGF-I. Again, recombinant hIGF-II was substantially less potent with a half-maximal effect at 13 ng/ml (Fig. 3.6a). Chicken and human IGF-I were also equipotent in their abilities to inhibit protein breakdown in CEF cells (Fig. 3.6b). However, the effects detected in these avian cells were smaller than those in rat myoblasts.

3.3.4: Receptor binding interactions of recombinant cIGF-I

The biological actions of IGF are generally considered to be mediated through interactions with the type-1 IGF receptor. In order to determine how the similarity of human and chicken IGF-I observed in the biological assays above is reflected by their interactions with IGF receptors, binding to the type-1 receptor in L6 rat myoblasts and CEF cells was assessed by measuring competition for [^{125}I]-labelled hIGF-I binding. Recombinant cIGF-I and hIGF-I displayed similar binding to the type-1 receptor in both L6 cells and in CEF cells. Half-maximal effects were observed at 1.7 and 2.2 ng/0.5 ml in L-6 rat myoblasts (Fig. 3.7a) and at 4.6 and 6.0 ng/0.5 ml in CEF cells (Fig. 3.7b) for cIGF-I and hIGF-I respectively. Recombinant hIGF-II exhibited a lower affinity (half-maximal competition at 6 ng/0.5 ml) for the type-1 receptor in L-6 cells, yet showed a slightly higher affinity (half-maximal competition at 3.5 ng/0.5 ml) relative to IGF-I for the receptor present in the CEF cells (Figs. 3.7). Competition for binding to the type-2 IGF receptor present on ovine placental microsomes by recombinant cIGF-I supported previous observations that IGF-I binds very weakly indeed to the type-2 receptor (Fig. 3.8).

Crosslinking of labelled cIGF-I to CEFs followed by SDS-electrophoresis under reducing conditions and autoradiography identified a protein band of

FIGURE 3.5: Effects of IGFs on protein synthesis in rat L6 myoblasts. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●) and human IGF-II (blue ▲). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

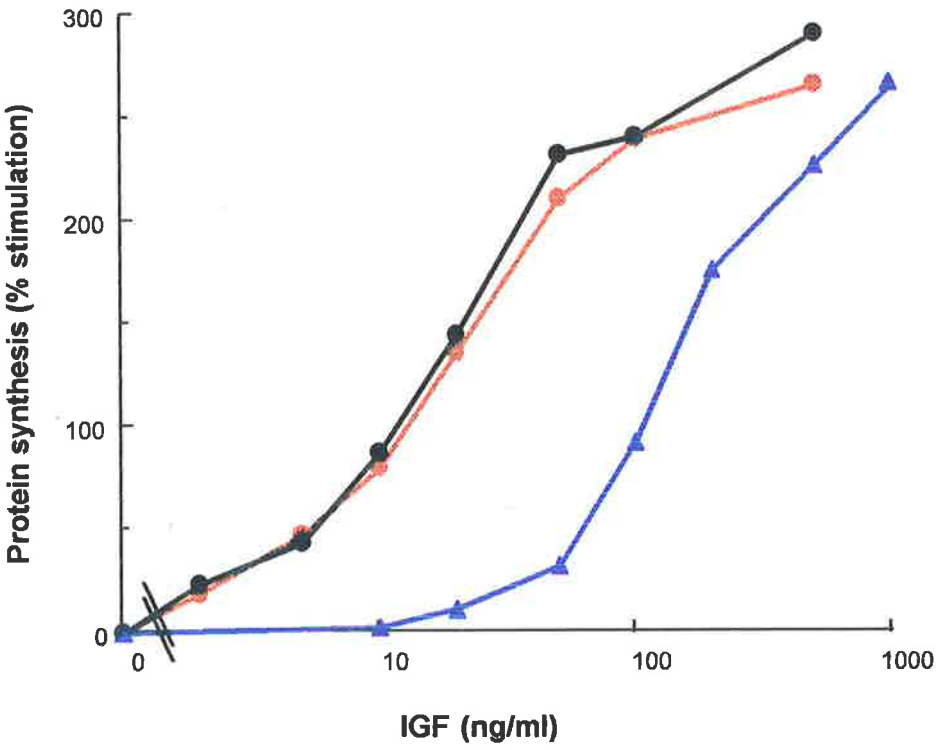
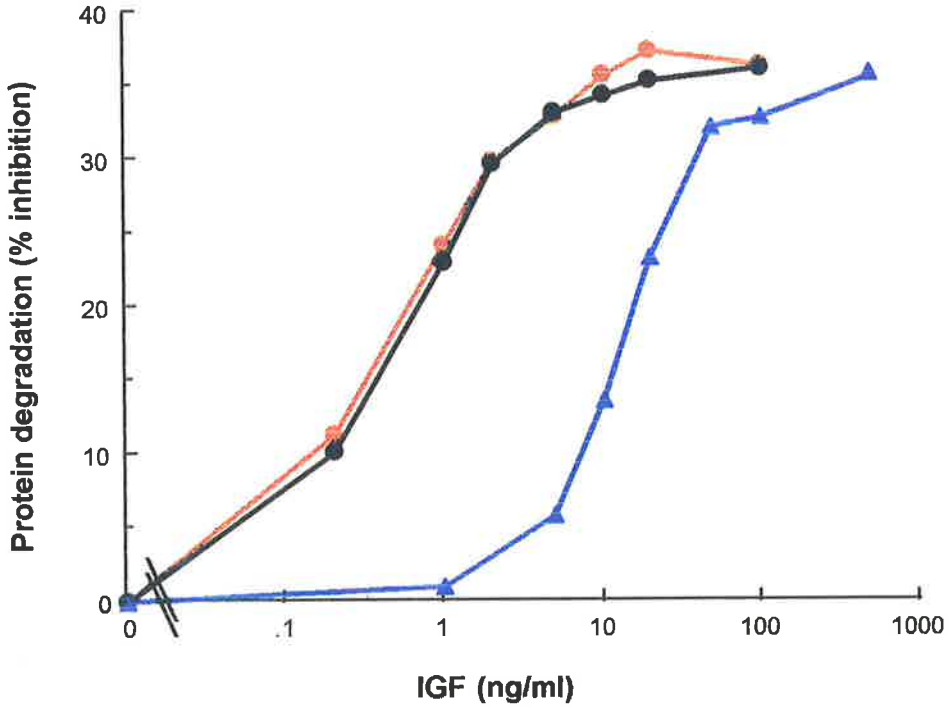

300
200
100
0
Protein synthesis (% stimulation)
0
10
100
1000
IGF (ng/ml)

FIGURE 3.6: Effects of IGFs on protein breakdown in a) rat L6 myoblasts and b) chick embryo fibroblasts. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●) and human IGF-II (blue ▲). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein degradation (% inhibition)
40
30
20
10
0
0
.1
1
10
100
1000
IGF (ng/ml)

b)

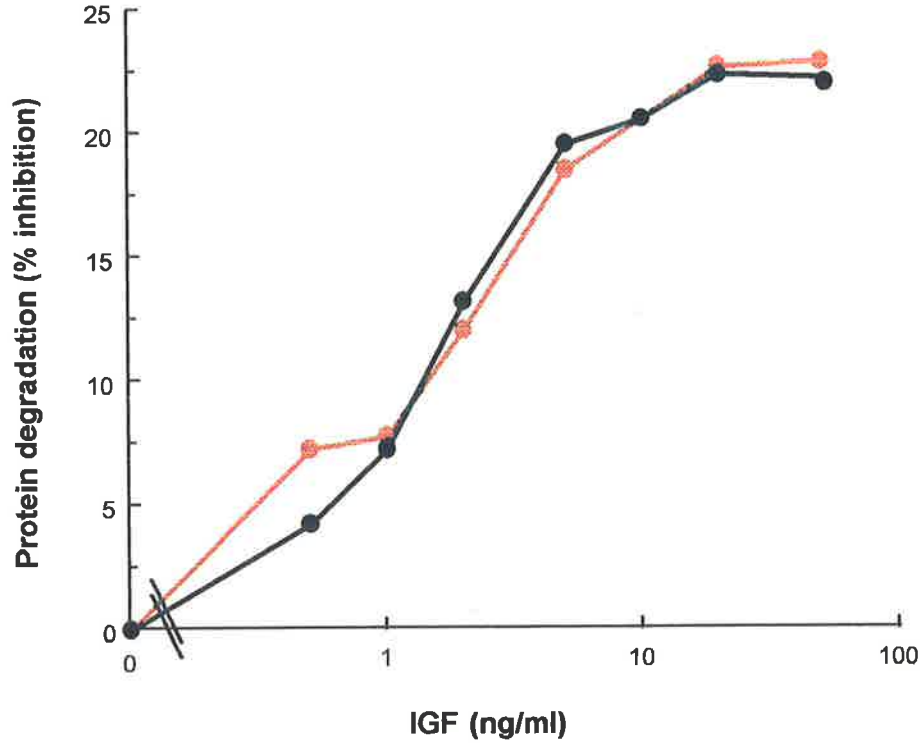
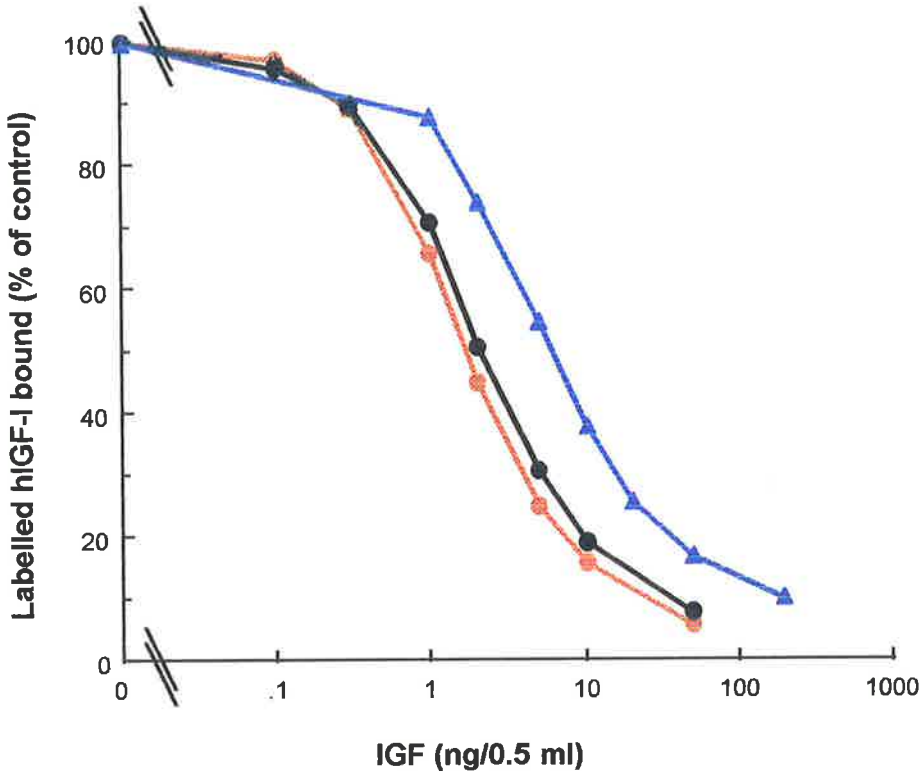

Protein degradation (% inhibition)
25
20
15
10
5
0
0
1
10
100
IGF (ng/ml)

FIGURE 3.7: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-I in a) rat L6 myoblasts and b) chick embryo fibroblasts. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●) and human IGF-II (blue ▲). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
.1
1
10
100
1000
IGF (ng/0.5 ml)

b)

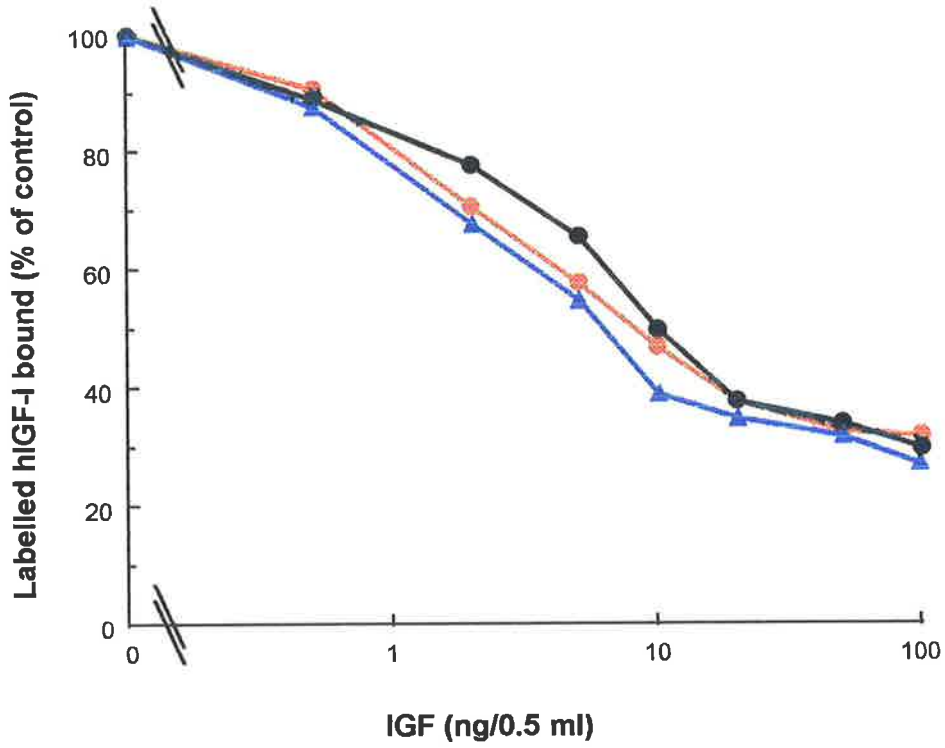
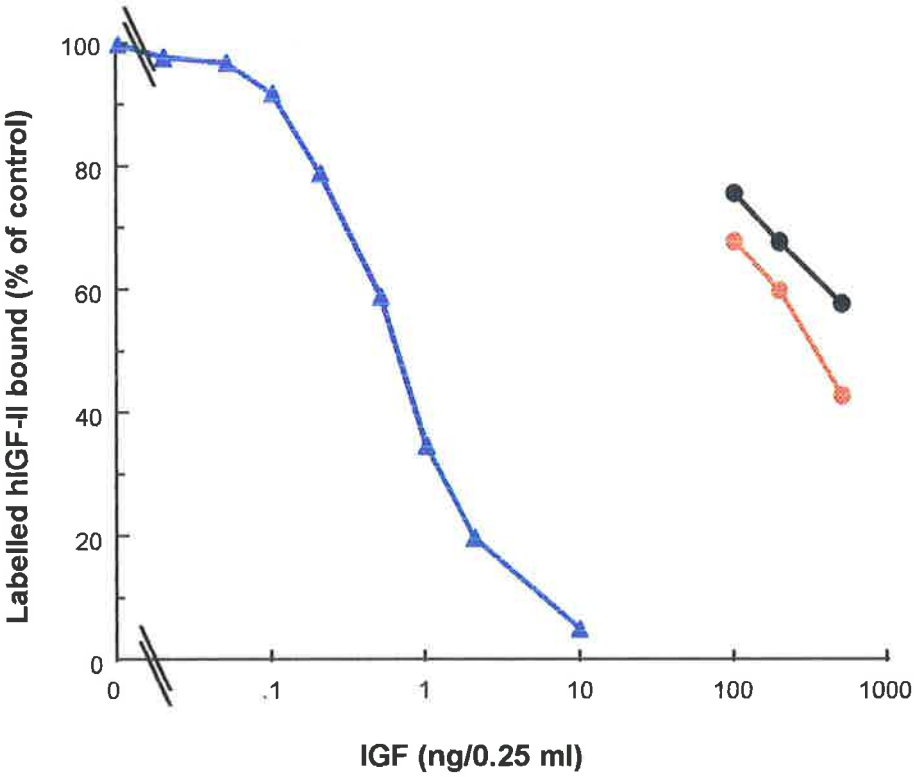

Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
1
10
100
IGF (ng/0.5 ml)

FIGURE 3.8: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to ovine placental membranes. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●) and human IGF-II (blue ▲). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
.1
1
10
100
1000
IGF (ng/0.25 ml)

approximately 130 kDa, consistent with the size expected for a type-1 IGF receptor (Fig. 3.9). Both unlabelled IGF-I (lanes 1-3) and unlabelled IGF-II (lanes 6-8) competed for [^{125}I]-cIGF-I binding to this protein. Insulin (20 µg/ml) was also able to compete for binding to the 130 kDa protein (lane 5).

3.3.5: Radioimmunoassay of cIGF-I

Chicken and human IGF-I cross-reactivities in an immunoassay were compared using a polyclonal antibody prepared against hIGF-I. Fifty per cent competition for binding occurred with 370 and 225 pg of recombinant cIGF-I and hIGF-I respectively, corresponding to a 1.6-fold difference in cross-reactivity (Fig. 3.10).

3.3.6: Binding protein interactions of recombinant cIGF-I

Labelled cIGF-I and hIGF-I were used as probes to detect IGF-binding proteins (IGFBP) in plasma by the ligand-blotting procedure (Fig. 3.11). Chicken IGF-I bound predominantly to IGFBP-3 in sheep and human plasma (Fig. 3.11, lanes 2 & 3) as indicated by the intense bands between approximately 35 and 50 kDa, although bands of smaller molecular mass were also observed. The most intense band in chicken plasma (Fig. 3.11, lane 1) was at 30 kDa, together with one at approximately 25 kDa and faint bands corresponding to IGFBP-3. The results obtained using labelled hIGF-I as a probe (Fig. 3.11, lanes 4-6) were very similar.

3.4: DISCUSSION

Although several research groups have produced recombinant hIGF-I, achieving high-level expression in both bacterial and yeast expression systems has been hampered by low translation levels, protein instability, bacterial proteinases or apparent toxic effects of the IGF-I itself (Bayne *et al.* 1988; Wong *et al.* 1988; Shuster *et al.* 1989). On the other hand, bacterial systems where the IGF-I has been expressed as a fusion protein have produced higher yields of the

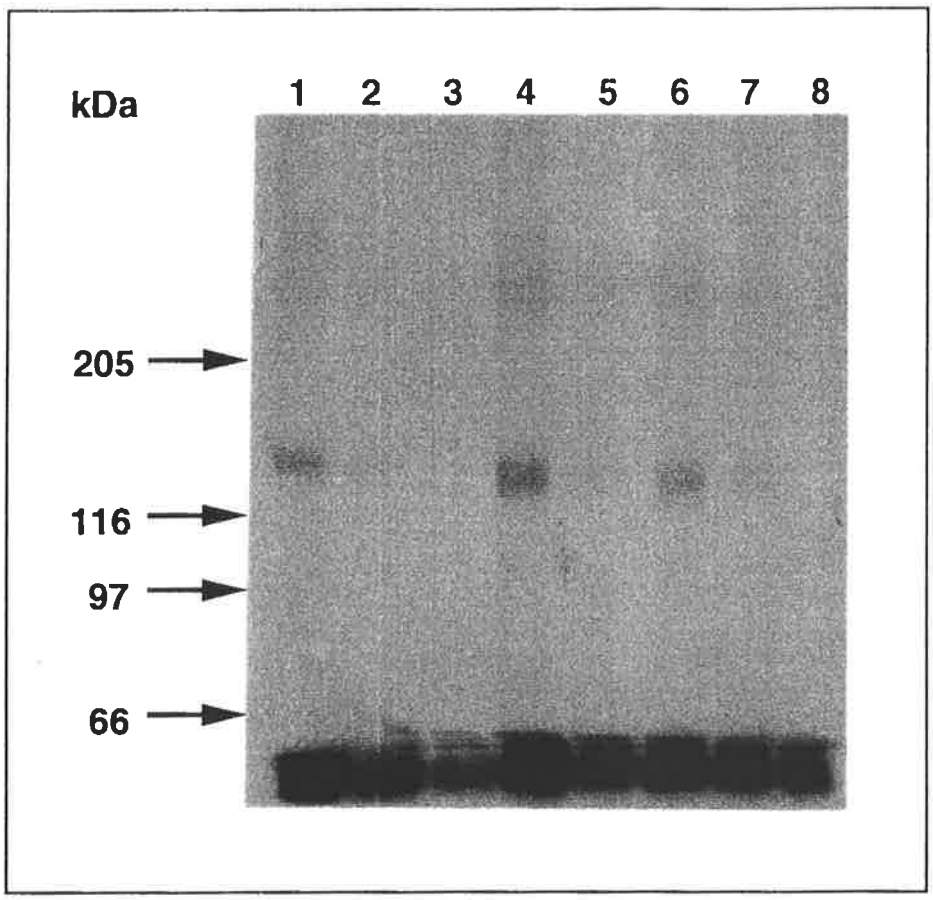
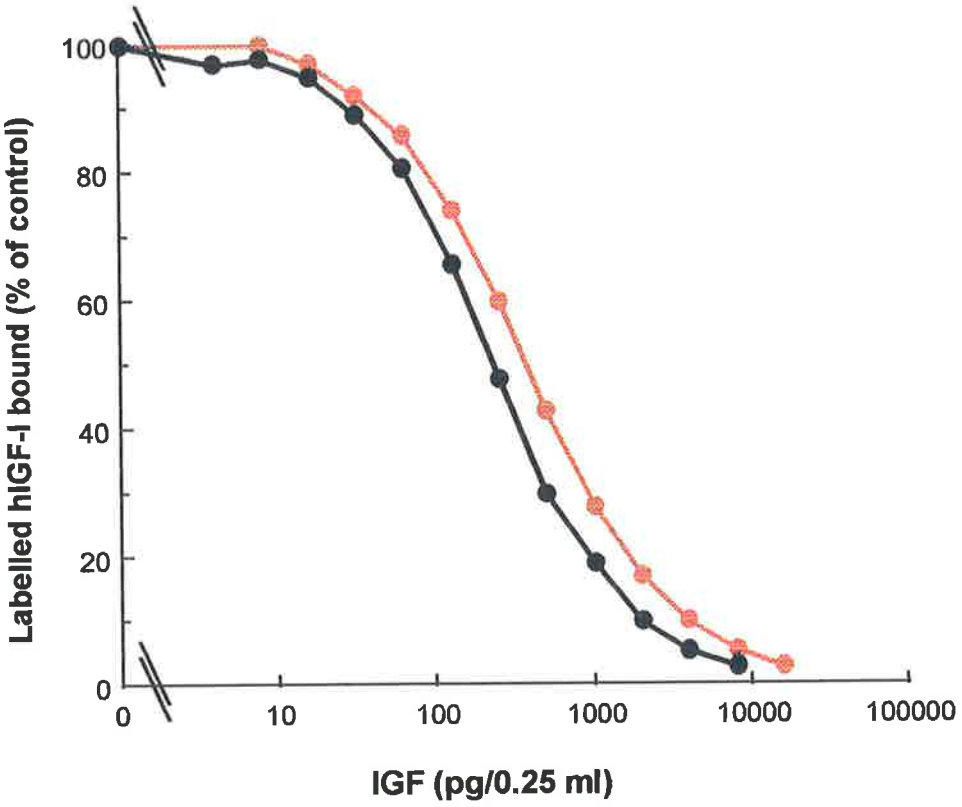


FIGURE 3.9: Phosphorimage of a dried gel after SDS-polyacrylamide gel electrophoresis under reducing conditions from cross-linking experiment with labelled chicken IGF-I in chick embryo fibroblasts. The concentrations of non-radioactive ligands were: lane 4, no addition; lanes 1, 10 ng chicken IGF-I (cIGF-I)/ml; lane 2, 100 ng cIGF-I/ml, lane 3, 1 µg cIGF-I/ml; lane 5, 20 µg insulin/ml; lane 6, 10 ng chicken IGF-II (cIGF-II)/ml; lane 7, 100 ng cIGF-II/ml; lane 8, 1 µg cIGF-II/ml.

FIGURE 3.10: Radioimmunoassay of IGFs using human IGF-I as radioligand and a polyclonal antiserum raised against recombinant human IGF-I. The proteins tested were human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
10
100
1000
10000
100000
IGF (pg/0.25 ml)

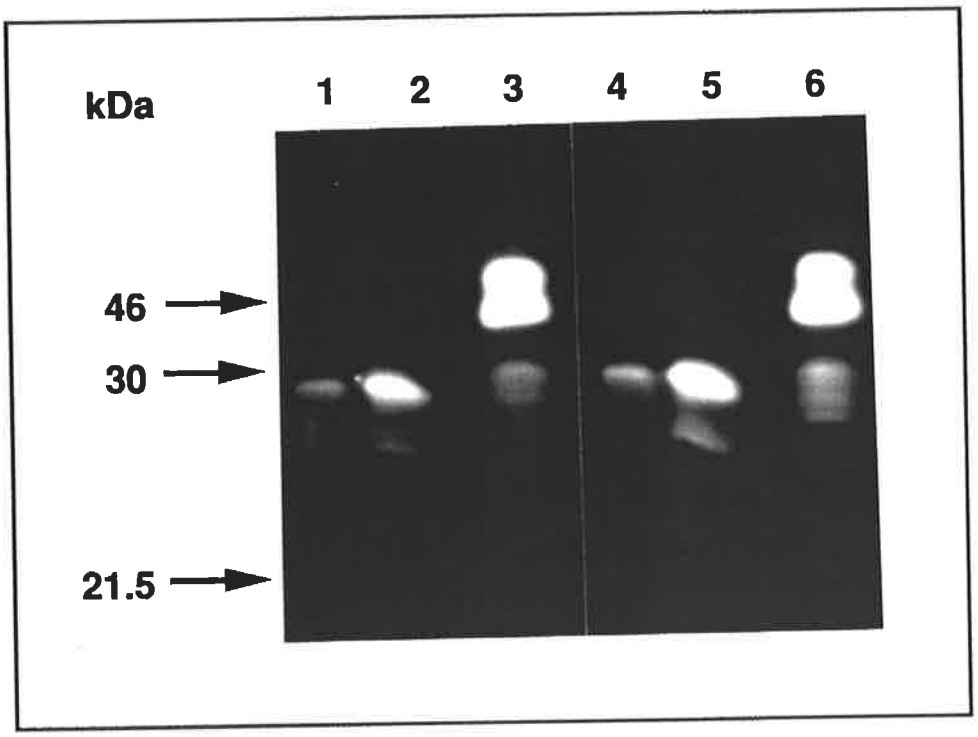


FIGURE 3.11: Ligand blotting of IGF-binding proteins in plasma using labelled chicken IGF-I (lanes 1-3) or labelled human IGF-I (lanes 4-6) as probes. The plasma samples tested were 10 µl from 7-week old broiler chickens (lanes 1 and 4), 1 µl from adult sheep (lanes 2 and 5) and 1 µl from adult human (lanes 3 and 6). Molecular mass markers are indicated. Plasmas were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-I and the probed nitrocellulose sheets exposed to X-ray film.

required peptide (Peters *et al.* 1985; Moks *et al.* 1987; Forsberg *et al.* 1990; King *et al.* 1992). In this chapter I have described the production and characterization of the first non-mammalian IGF. Production of cIGF-I by the recombinant gene-fusion approach described here offers the advantage of much higher yields than are obtained when purifying cIGF-I from serum. For example, only 30 µg of pure cIGF-I was purified from 30 litres of chicken serum (Ballard *et al.* 1990).

King *et al.* (1992) have previously utilized this same gene-fusion system to produce hIGF-I fusion proteins. However, the susceptibility of the cIGF-I fusion proteins to proteolysis by bacterial enzymes necessitated the development of a different downstream processing strategy. A similar proteolytic problem has been described for recombinant hIGF-II. Thus, Hammarberg *et al.* (1989) found that recombinant hIGF-II expressed in *E. coli* was cleaved between arginine residues 37 and 38. *E. coli* proteinase VII is a likely candidate for the proteolysis observed in both instances, as this proteinase is associated with the outer membrane and specifically cleaves between two basic amino acids (Sugimura & Higashi, 1988). Others in my laboratory have observed that hIGF-I fusion proteins are insensitive to proteolysis (*personal communication, G. Francis & S. Milner, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia*). This result is surprising, as hIGF-I also contains arginine residues at positions 36 and 37. Indeed, all IGF-I molecules characterized thus far contain two pairs of double arginines. Presumably additional determinants are required by the proteinase. Proteolysis of cIGF-I was reduced by processing the IGF fusion proteins through a DEAE anion exchange column early in the purification. As a further precaution against proteolysis, $ZnCl_2$ was included in the buffers in the early stages of the purification, since Sugimura & Higashi (1988) have shown that the activity of proteinase VII is inhibited by $ZnCl_2$. Development of a downstream processing procedure which reduces bacterial proteolysis has permitted the production of milligram quantities of recombinant cIGF-I and has provided an opportunity to investigate the roles of cIGF-I in the avian growth process.

We have previously reported that cIGF-I purified from chicken serum had about half the biological potency of porcine, bovine or human IGF-I (Dawe *et al.* 1988). I now show that recombinant cIGF-I is as potent as recombinant hIGF-I in both protein synthesis and protein degradation bioassays (Fig. 3.5 & 3.6). King *et al.* (1992) have established that the recombinant hIGF-I used as a reference in these assays has the same biological activity as bovine IGF-I purified from serum. The similarity of recombinant cIGF-I and recombinant hIGF-I in biological activity is mirrored by equal potency in receptor-binding studies (Fig. 3.7 & 3.8) and equivalent binding to the IGF-binding proteins in chicken, human and sheep plasma (Fig. 3.11). Moreover, the human and chicken IGF-I peptides were found to be equally potent in both mammalian and avian cell lines. In addition, crosslinking experiments using radiolabelled cIGF-I (Fig. 3.9) confirm previous results found when crosslinking mammalian IGF-I to these same cells (Kallincos *et al.* 1990; Duclos & Goddard, 1990.; Duclos *et al.* 1991). Thus, chickens appear to have a classical type-1 IGF receptor for which both IGF-I and IGF-II appear to have similar affinities. These results imply that the amino acid changes in cIGF-I do not modify its interactions with IGF receptors and binding proteins. In particular, the receptor-binding studies suggest that the cIGF-I amino acid substitution at position 26 (Ser for Asn), in the region associated with the binding domain for the type-1 receptor (Maly & Luthi, 1988), does not appear to be important for interactions with this receptor.

A number of studies have used radioimmunoassays for serum IGF-I to assess growth performance in chickens (Huybrechts *et al.* 1985; Vasilatos-Youncken *et al.* 1988; Ballard *et al.* 1990; McGuinness & Cogburn, 1990). One of the problems with this approach has been that heterologous IGF peptides have generally been used as reference standards, thus making it difficult to interpret the results in terms of absolute levels of cIGF-I. While there is a high degree of identity between the human and chicken IGF-I peptides, with only 8 differences out of 70 amino acid residues, there are sufficient changes to elicit a 1.6-fold difference in immunoreactivity, as detected by the radioimmunoassay utilized

here (Fig. 3.10). This result is similar to a previous finding that cIGF-I purified from chicken serum had a lower crossreactivity compared with reference hIGF-I (Dawe *et al.* 1988). Armstrong *et al.* (1990) have also reported that cIGF-I purified from serum had a 50% potency compared with hIGF-I in a radioimmunoassay using antiserum raised against hIGF-I. The availability of my recombinant cIGF-I has enabled the development of a specific radioimmunoassay using cIGF-I as antigen, radiolabelled tracer and standard for the first time (McMurtry *et al.* 1993).

Considerable research is required to determine whether IGF-I plays any roles unique to avian growth. Nevertheless, the production of recombinant cIGF-I by the fusion-protein strategy described in this chapter now provides an opportunity to use homologous peptide when studying the functions of IGF-I in the avian endocrine system.

CHAPTER 4

PRODUCTION AND CHARACTERIZATION OF RECOMBINANT CHICKEN INSULIN-LIKE GROWTH FACTOR-II FROM *ESCHERICHIA COLI*

4.1: INTRODUCTION

While much is known about the function of IGF-I and its role in promoting and maintaining mammalian post-natal growth, less is known about IGF-II and whether it exhibits any roles separate to IGF-I. However, recent research suggests that IGF-II may play an important part in mammalian fetal development (review, Daughaday & Rotwein, 1989). The role of IGF-II in nonmammalian growth and development is even less well defined.

Kallincos *et al.* (1990) reported the first nonmammalian IGF-II characterized, namely chicken IGF-II (cIGF-II). Protein sequencing of chicken IGF-II purified from serum indicated that it has amino acids which differ from those found in human or ovine IGF-II in the N-terminal and C-peptide regions of the molecule. Endoproteinase Glu-C digestion of purified cIGF-II revealed two C-terminal sequences, only one of which was homologous to human IGF-II. The deduced protein sequence from a cDNA for trout IGF-II also indicates that amino acids differ from hIGF-II in similar regions of the protein to those found in cIGF-II (Shamblott & Chen, 1992) (Fig. 4.1a & Fig. 4.2).

The cIGF-II purified from serum was indistinguishable from ovine IGF-II in potency whether the assays were performed in cultured rat or in chicken cells. However, as was the case with cIGF-I purified from serum, complete assessment of the *in vitro* and *in vivo* biological effects of the serum-derived cIGF-II was not possible as insufficient quantities of the growth factor were available for testing. Clearly, further investigation of the roles of cIGF-II in avian growth and development required a recombinant approach.

At the time I commenced this study there was no DNA sequence information available on cIGF-II to confirm the protein sequence reported by Kallincos *et. al.* (1990). Accordingly, I made recombinant cIGF-II based on the published protein sequence. The subsequent publication of an antisense transcript to the cIGF-II gene by Taylor *et al.* (1991) confirmed most of the changes identified by Kallincos *et al.* (1990), with the exception that they identified an additional arginine residue. Re-examination of the original protein

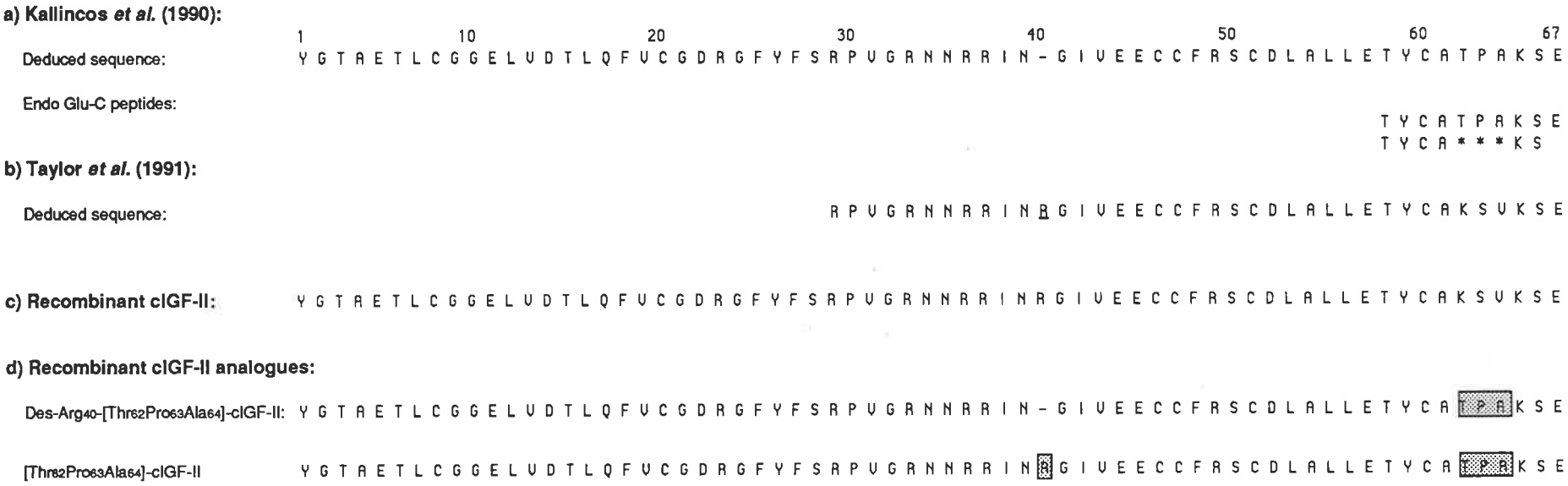


FIGURE 4.1: Amino acid sequences for chicken IGF-II (cIGF-II) reported by a) Kallincos *et al.* (1990) and b) Taylor *et al.* (1991). The amino acid sequence of the recombinant cIGF-II and the cIGF-II analogues I produced are shown in c) and d) respectively. Shaded boxes indicate the amino acid differences present in the analogues.

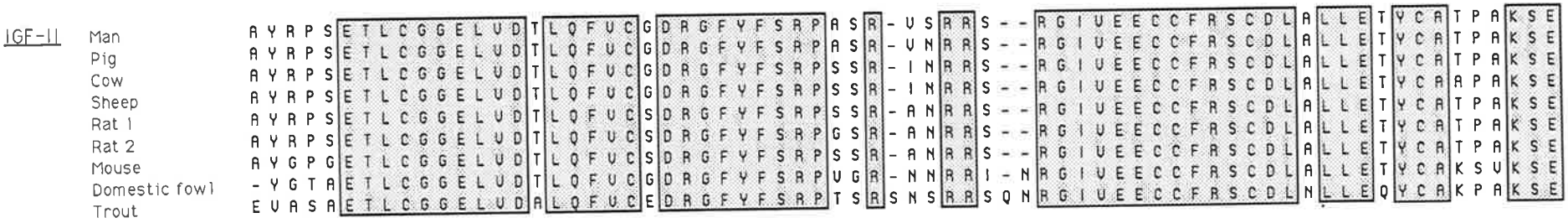


FIGURE 4.2: Sequence comparison of vertebrate IGF-IIs. Shaded boxes indicate those residues conserved. The references from which the sequences for the mammalian species were obtained are described in Figure 1.1. The sequence for domestic fowl is based on the sequences reported by Kallincos *et al.* (1990) and Taylor *et al.* (1991). The sequence for trout is from Shamblott & Chen (1992)

sequence data indicated that the sequence for cIGF-II should in fact contain an additional arginine residue at position 40. The antisense transcript also confirmed one of the two alternative C-terminal sequences reported by Kallincos *et al.* (1990) (Fig. 4.1b). Using this revised information I have produced what is now considered to be the correct recombinant cIGF-II protein (Fig. 4.1c). Furthermore, I have also produced an analogue of recombinant cIGF-II, containing the additional arginine, but with the alternative C-terminal sequence reported by Kallincos *et al.* (1990). I report in this chapter the production of recombinant cIGF-II and present evidence that it differs from its human counterpart in its receptor binding affinities. In addition I show that the two recombinant cIGF-II analogues (Fig. 4.1d) I produced were essentially similar to cIGF-II in their biological activities, receptor- and IGFBP-binding interactions.

4.2: MATERIALS AND METHODS

4.2.1: Construction of plasmids

The starting plasmid was the hIGF-II expression vector p[Met^{1}]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II described by Francis *et al* (1993). The HpaI-HindIII fragment of p[Met^{1}]-pGH(1-11)-Val-Asn-Phe-Ala-His-Tyr-IGF-II was subcloned into the multi-functional vector pTZ18. Using several rounds of site-directed mutagenesis (see 2.1.2 & 2.2.1.2), selected hIGF-II codons were relaced with codons which allow the expression of the amino acids that are different in cIGF-II. Oligonucleotide 5 (5'-AACTTCGCCCATTATTACGGTACCGCTGAAACC CTGTGCGGCGGG-3') permitted the replacement of the residues Ala-Tyr-Arg-Pro-Ser at positions 1-5 in hIGF-II with codons for Tyr-Gly-Thr-Ala. In addition, this oligonucleotide optimised the codons for Ala^{-3}, His^{-2}, Tyr^{-1} in the leader peptide, and Glu^{6} in cIGF-II, to complement the tRNA species found in *E. coli.* Oligonucleotide 6 (5'-TACTTCAGCCGTCCGGTTGGTCGTAACAACCGTCGTAT CAACCGTGGCATCGTTGAGGAG-3') directed a change resulting in the substitution of codons for Val-Gly-Arg-Asn-Asn-Arg-Arg-Ile-Asn in place of those for Ala-Ser-Arg-Val-Ser-Arg-Arg-Ser at positions 32-39 in hIGF-II. The residues

Thr-Pro-Ala at positions 62-64 in hIGF-II were replaced with codons for Lys-Ser-Val using oligonucleotide 7 (5'-ACTTACTGTGCTAAATCTGTTAAGTCCGAGTGA -3').

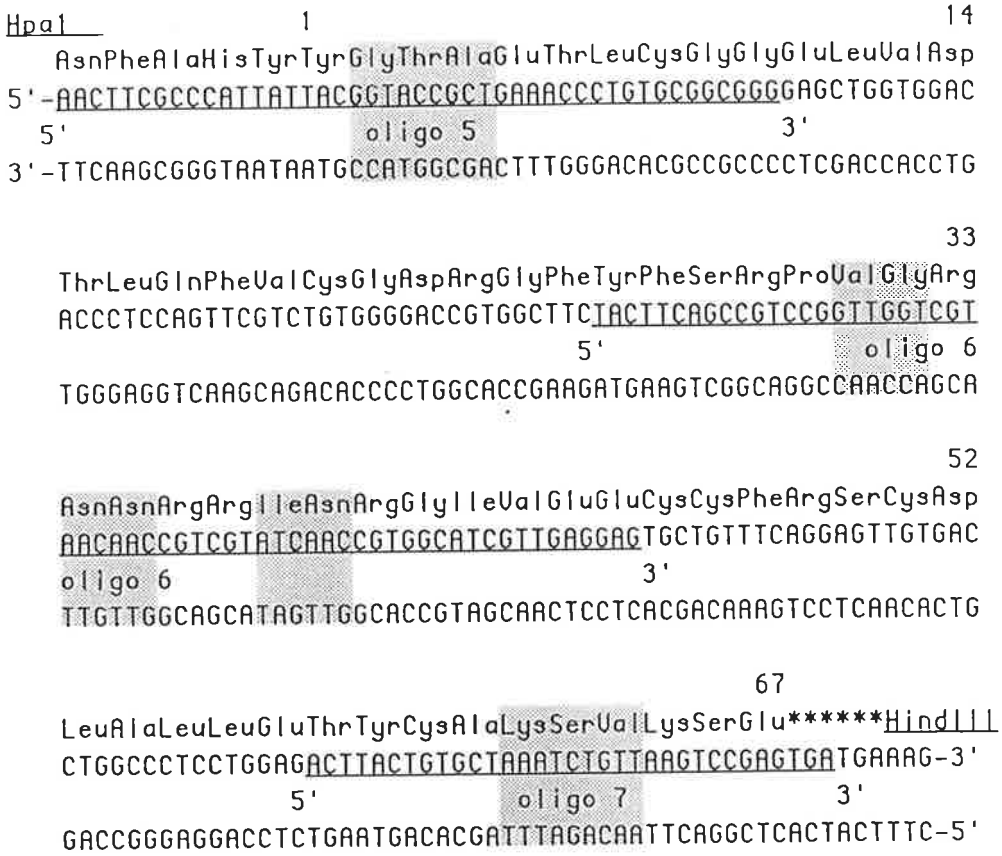
The DNA construct for the cIGF-II analogue, Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II was created by using oligonucleotide 5 described above along with oligonucleotide 8 (5'-TACTTCAGCCGTCCGGTTGGTCGTAACAACCGTCGTATC AACGGCATCGTTGAGGAG-3'). Oligonucleotide 8 directs the incorporation of codons for Val-Gly-Arg-Asn-Asn-Arg-Ile-Asn in place of those for Ala-Ser-Arg-Val-Ser-Arg-Arg-Ser at positions 32-39 in hIGF-II.

The DNA construct for the cIGF-II analogue with the alternative C-terminal sequence, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II was made as described above for cIGF-II, but without utilizing oligonucleotide 7. Figure 4.3 depicts the amino acid modifications introduced into the hIGF-II DNA to allow expression of cIGF-II and the cIGF-II analogues.

Once all the required changes had been introduced as confirmed by dideoxy-DNA sequencing, the HpaI-HindIII fragments of the pTZ clones were ligated into the HpaI-HindIII cut expression vector, p[Met^{1}]-pGH(1-46)-Val-Asn previously described by King *et al* (1992). Briefly, this expression vector permits high level expression of IGFs in *E. coli* as fusion proteins with a leader peptide containing the first 46 residues of methionyl porcine growth hormone ([Met^{1}]-pGH(1-46)), followed by Val and Asn. The DNA sequences of the HpaI-HindIII fragments for cIGF-II and the two analogues, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, along with a schematic representation of the expression vector containing cIGF-II, are shown in Fig. 4.3. The constructs were then transformed into *E. coli* JM101 (lac I^{q}) at 37°C on minimal agar containing 29 µmol ampicillin/l. Fermentation of the transformed *E. coli* and isolation of the inclusion bodies produced was carried out as previously described (2.2.1.3).

FIGURE 4.3: Nucleotide and corresponding amino acid sequence of the Hpa1-HindIII fragment of the constructs engineered for expression of a) chicken IGF-II (cIGF-II), b) Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and c) [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II. Shaded boxes indicate regions where changes were introduced to the DNA sequence of the starting vector by site-directed mutagenesis. The oligonucleotides employed to direct these changes are indicated (oligos 5-8). Amino acids are numbered and stop codons are marked by asterisks. d) Schematic representation of the construct engineered for expression of chicken IGF-II in *E. coli*. The expression plasmid, pKT52, contains an origin of replication (*ori*), a *trc* promoter, a transcription termination sequence (*rrnT1T2*) and a gene conferring ampicillin resistance (*amp*). The DNA sequence preceding Met has been modified to optimize ribosome binding (Vize & Wells, 1987).

a)


Hpa I
1
14
AsnPheAlaHisTyrTyrGlyThrAlaGluThrLeuCysGlyGlyGluLeuValAsp
5'-AACTTCGCCCATTATTACGGTACCGCTGAAACCCTGTGCGGCGGGGAGCTGGTGGAC
5'
oligo 5
3'
3'-TTCAAGCGGGTAATAATGCCATGGCGACTTTGGGACACGCCGCCCCTCGACCACCTG
33
ThrLeuGlnPheValCysGlyAspArgGlyPheTyrPheSerArgProValGlyArg
ACCCTCCAGTTCGTCTGTGGGGACCGTGGCTTCTACTTCAGCCGTCCGGTTGGTCGT
5'
oligo 6
TGGGAGGTCAAGCAGACACCCCTGGCACCGAAGATGAAGTCGGCAGGCCAACCAGCA
52
AsnAsnArgArgIleAsnArgGlyIleValGluGluCysCysPheArgSerCysAsp
AACAACCGTCGTATCAACCGTGGCATCGTTGAGGAGTGCTGTTTCAGGAGTTGTGAC
oligo 6
3'
TTGTTGGCAGCATAGTTGGCACCGTAGCAACTCCTCACGACAAAGTCCTCAACACTG
67
LeuAlaLeuLeuGluThrTyrCysAlaLysSerValLysSerGlu******HindIII
CTGGCCCTCCTGGAGACTTACTGTGCTAAATCTGTTAAGTCCGAGTGATGAAAG-3'
5'
oligo 7
3'
GACCGGGAGGACCTCTGAATGACACGATTTAGACAATTCAGGCTCACTACTTTC-5'

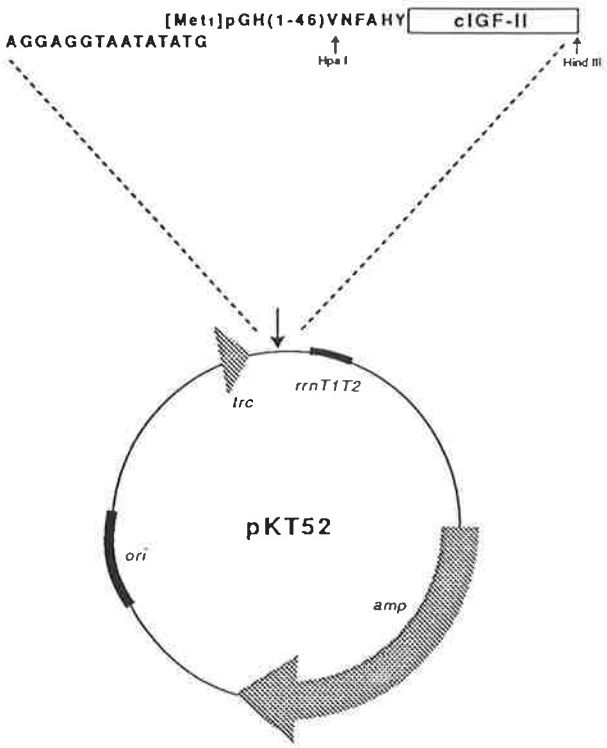
b)


Hpa I
1
14
AsnPheAlaHisTyrTyrGlyThrAlaGluThrLeuCysGlyGlyGluLeuValAsp
5'-AACTTCGCCCATTATTACGGTACCGCTGAAACCCTGTGCGGCGGGGAGCTGGTGGAC
5'
oligo 5
3'
3'-TTCAAGCGGGTAATAATGCCATGGCGACTTTGGGACACGCCGCCCCTCGACCACCTG
33
ThrLeuGlnPheValCysGlyAspArgGlyPheTyrPheSerArgProValGlyArg
ACCCTCCAGTTCGTCTGTGGGGACCGTGGCTTCTACTTCAGCCGTCCGGTTGGTCGT
5'
oligo 8
TGGGAGGTCAAGCAGACACCCCTGGCACCGAAGATGAAGTCGGCAGGCCAACCAGCA
52
AsnAsnArgArgIleAsnGlyIleValGluGluCysCysPheArgSerCysAspLeu
AACAACCGTCGTATCAACGGCATCGTTGAGGAGTGCTGTTTCAGGAGTTGTGACCTG
oligo 8
3'
TTGTTGGCAGCATAGTTGCCGTAGCAACTCCTCACGACAAAGTCCTCAACACTGGAC
66
AlaLeuLeuGluThrTyrCysAlaThrProAlaLysSerGlu******HindIII
GCCCTCCTGGAGACTTACTGTGCTACCCCCGCCAAGTCCGAGTGATGAAAG-3'
CGGGAGGACCTCTGAATGACACGATGGGGGCGGTTCAGGCTCACTACTTTC-5'

c)

```
Hpa1                1                                                  14
   AsnPheAlaHisTyrTyrGlyThrAlaGluThrLeuCysGlyGlyGluLeuValAsp
5'-AACTTCGCCCATTATTACGGTACCGCTGAAACCCTGTGCGGCGGGGAGCTGGTGGAC
  5'                    oligo 5                        3'
3'-TTCAAGCGGGTAATAATGCCATGGCGACTTTGGGACACGCCGCCCCTCGACCACCTG

                                                                   33
   ThrLeuGlnPheValCysGlyAspArgGlyPheTyrPheSerArgProValGlyArg
   ACCCTCCAGTTCGTCTGTGGGGACCGTGGCTTCTACTTCAGCCGTCCGGTTGGTCGT
                                    5'                 oligo 6
   TGGGAGGTCAAGCAGACACCCCTGGCACCGAAGATGAAGTCGGCAGGCCAACCAGCA

                                                                   52
   AsnAsnArgArgIleAsnArgGlyIleValGluGluCysCysPheArgSerCysAsp
   AACAACCGTCGTATCAACCGTGGCATCGTTGAGGAGTGCTGTTTCAGGAGTTGTGAC
   oligo 6                          3'
   TTGTTGGCAGCATAGTTGGCACCGTAGCAACTCCTCACGACAAAGTCCTCAACACTG

                                                          67
   LeuAlaLeuLeuGluThrTyrCysAlaThrProAlaLysSerGlu******HindIII
   CTGGCCCTCCTGGAGACTTACTGTGCTACCCCCGCCAAGTCCGAGTGATGAAAG-3'
   GACCGGGAGGACCTCTGAATGACACGATGGGGGCGGTTCAGGCTCACTACTTTC-5'
```

d)



4.2.2: Chicken IGF-II fusion protein purification

Inclusion bodies (20 g) containing the cIGF-II fusion protein were solubilized, reduced and desalted as described for cIGF-I fusion proteins in 3.2.3. The desalted, reduced cIGF-II fusion protein was then loaded at 20 ml/min onto a 50 mm (diameter) x 200 mm column packed with 100 ml Fast Flow Q Sepharose equilibrated with 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 0.5 mmol $ZnCl_2$/l and 1.6 mmol dithiothreitol/l at pH 9.1. The cIGF-II fusion protein was eluted by washing the column at a flow rate of 20 ml/min with the above buffer minus $ZnCl_2$ and with the pH adjusted to 6.5.

The cIGF-II fusion protein was refolded, concentrated on a C18 Matrex silica column and lyophilized as described in 3.2.3 with the exception that the final urea concentration in the refold pool was 6 mol/l.

4.2.3: Fusion protein cleavage and purification of the cIGF-II peptide

A portion of the lyophilized cIGF-II fusion protein (140 mg) was resuspended in 2 litres of 6 mol urea/l, 20 mmol Tris/l, 5 mmol $CaCl_2$/l and 0.2 mol NaCl/l at pH 8.5. The mixture was pre-warmed to 37°C before adding 7 mg of H64A subtilisin BPN' and incubating at 37°C 4.5 h. The digest was stopped by acidification to pH 2.8 with HCl. Under these conditions the subtilisin cleaves the fusion protein between Gln and His at positions 19-20 in [Met^1]-pGH(1-46) in the leader peptide, thus creating pGH(20-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II (Fig. 4.4). Progress of the digest was monitored by analysis on a C4 microbore reverse-phase HPLC column using the standard analytical HPLC described in 2.2.2.3. Digestion of the fusion protein was indicated by early elution of the protein compared with that observed with the starting material. The acidified digest pool was concentrated by chromatography on the C18 Matrex silica column and lyophilized as outlined above.

The lyophilized pGH(20-46)-Asn-Val-Phe-Ala-His-Tyr-cIGF-II (130 mg) was resuspended in 40 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 5 mmol EDTA/l and 20 mmol DTT/l at pH 9.1. The reduced protein was then refolded as

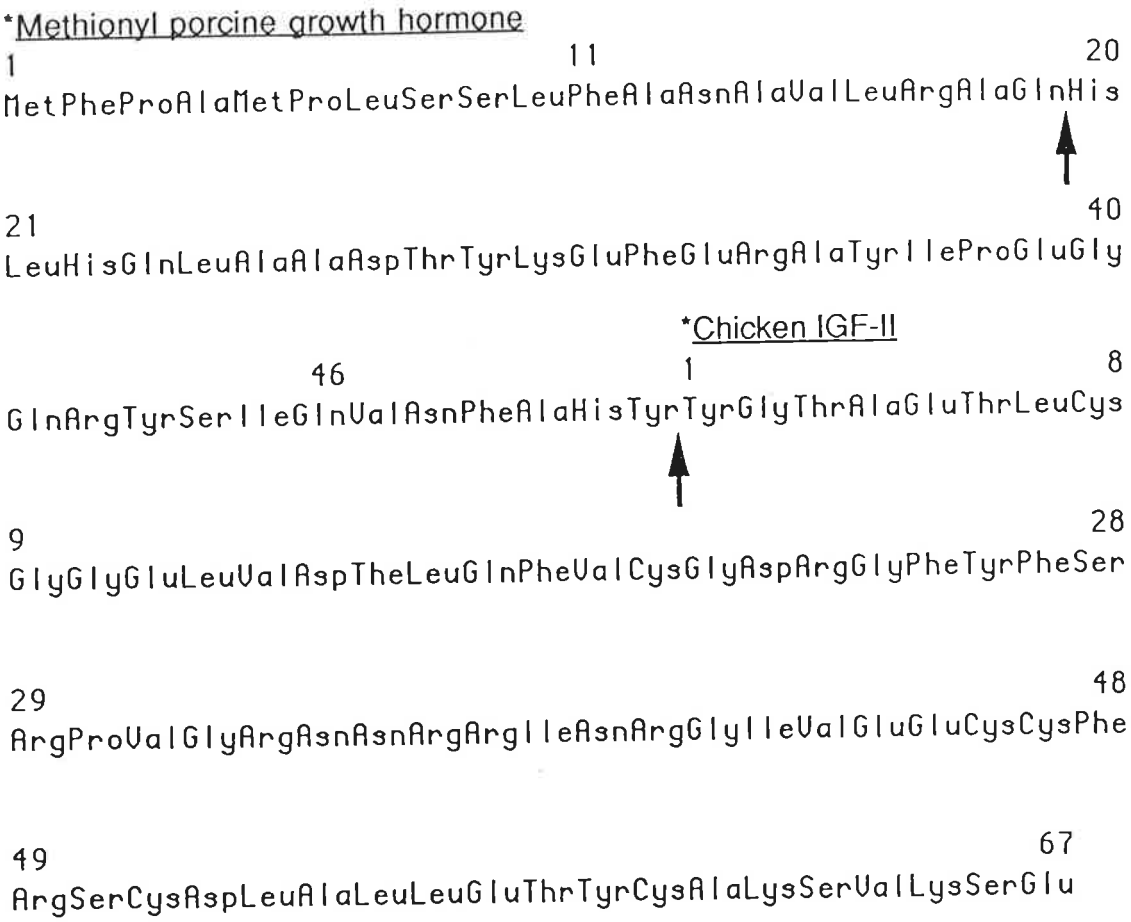


FIGURE 4.4: Sequence of the chicken IGF-II fusion protein. Amino acids are numbered. The beginning of methionyl porcine growth hormone and chicken IGF-II are indicated by asterisks. Arrows show sites cleaved by H64A subtilisin BPN'.

described earlier, but this time in a final urea concentration of 3 mol/l. The refold mixture was acidified to pH 2.8 with HCl, concentrated on a C18 Matrex silica column and lyophilized.

The lyophilized, refolded protein was resuspended in 100 ml 3 mol urea/l, 20 mmol Tris/l, 5 mmol $CaCl_2$/l and 0.2 mol NaCl/l at pH 8.5. The solution was prewarmed to 37°C before adding 4.5 mg H64A subtilisin BPN'. At t = 8 h and again at t = 22 h, a further 4.5 mg of subtilisin was added. Again, progress of the digest was monitored by analysis on a C4 microbore column using the conditions described for the standard analytical HPLC (2.2.2.3). The digest was stopped by acidification to pH 2.8 at t = 46 h and then concentrated by chromatography on the C18 Matrex silica column described above. In this second digest, the enzyme recognises the motif Phe-Ala-His-Tyr and cleaves on the carboxyl side of the Tyr, thus releasing the required clGF-II protein from the leader peptide (Carter *et al.* 1989).

A pool containing the clGF-II from the C18 Matrex chromatography was pumped at 20 ml/min on to a 50 mm (diameter) x 200 mm column packed with 75 ml Fast Flow S Sepharose previously equilibrated with 1 mol acetic acid/l. The column was then washed with 8 mol urea/l, 40 mmol glycine/l and 50 mmol ammonium acetate/l at pH 4.8, before eluting the clGF-II with 8 mol urea/l, 40 mmol glycine/l, 50 mmol ammonium acetate/l and 2 mol NaCl/l (pH 4.8) at a flow rate of 20 ml/min. A single fraction containing the clGF-II was further purified by three reverse-phase HPLC steps. The first HPLC step used a C4 PrepPak 500 cartridge (57 mm (diameter) x 300 mm) and employed a linear gradient from 15.2 to 49.6% acetonitrile in 0.1% TFA over 172 min at a flow rate of 25 ml/min. The second HPLC step utilized a C4 PrepPak column (25 mm (diameter) x 100 mm) and employed a gradient from 25 to 35% acetonitrile in 0.1% TFA over 200 min at a flow rate of 5 ml/min. The third and final HPLC step used the same column as described for the second HPLC, but this time employed a linear gradient from 25.6 to 31% acetonitrile in 0.1% TFA over 323 min at a flow rate of 5 ml/min. The recovery of clGF-II during the purification was monitored by

analysis on the standard analytical HPLC (2.2.2.3) and by electrophoresis on high density gels using Pharmacia's 'PhastSystem' (2.2.3.3).

The cIGF-II peptide was quantitated by calculating the area under their absorbance profile at 215 nm when analysed on the standard analytical HPLC (2.2.2.3). The area under the absorbance profile was converted to protein concentration using a calculated extinction coefficient of 32.65 mol^{-1} cm^{-1} for cIGF-II (2.2.2.4). The N-terminal protein sequence and mass quantitation were determined as described in 2.2.3.1 and 2.2.3.2 respectively.

4.2.5: Production of the cIGF-II analogues

Production and downstream processing of the two cIGF-II analogues, [$Thr^{62}Pro^{63}Ala^{64}$]-cIGF-II and Des-Arg^{40}-[$Thr^{62}Pro^{63}Ala^{64}$]-cIGF-II was carried as described for cIGF-II above. The purified peptides were quantitated as described in 2.2.2.4 using calculated extinction coefficients of 33.16 and 32.85 mol^{-1} cm^{-1} for Des-Arg^{40}-[$Thr^{62}Pro^{63}Ala^{64}$]-cIGF-II and [$Thr^{62}Pro^{63}Ala^{64}$]-cIGF-II respectively.

4.2.5: *In vitro* characterization of recombinant cIGF-II and the cIGF-II anaolgues

In vitro characterization of the biological actions, receptor and IGFBP interactions of recombinant cIGF-II was determined using the biochemical assays described in 2.2.4.

4.2.6: IGF-II Radioimmunoassay of recombinant cIGF-II

Immunoreactivity was measured by employing a monoclonal antibody raised against hIGF-II and the method described in 2.2.4.5. Recombinant hIGF-II was used as the radioligand and as a standard in this assay. *This assay was performed by Dr. K. Kita, visiting scientist, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia.*

4.3: RESULTS

4.3.1: Fusion protein expression

Initial attempts to express cIGF-II and the analogues in *E. coli* using the bacterial gene-fusion expression system described by Francis *et al.* (1993) for production of recombinant human IGF-II, namely as a fusion protein with a leader peptide containing the first 11 residues of methionyl growth hormone, followed by the amino acids Val-Asn-Phe-Ala-His-Tyr, were unsuccessful. However, high level expression of cIGF-II proteins in *E. coli* was obtained when they were expressed as fusion proteins containing a longer leader peptide ([Met^{1}]pGH(1-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II) (Fig. 4.5, lane 7). A 15 litre fermentation of *E. coli* JM101 cells transformed with p[Met1]pGH(1-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II yielded 120 g wet weight of inclusion bodies following induction with IPTG. Fermentation of *E. coli* cells transformed with constructs for the two analogues, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, yielded 100 and 115 g of inclusion bodies respectively.

4.3.2: Cleavage and purification of the cIGF-II fusion protein

Although Francis *et al.* (1993) have previously reported the processing of similar recombinant hIGF-II fusion peptides from *E. coli* inclusion bodies, it was necessary to develop a different downstream processing strategy for the purification of cIGF-II from these particular fusion proteins. In particular, expression of cIGF-II with the longer leader peptide necessitated two cleavage steps with H64A subtilisin-BPN'. The genetically engineered mutant serine protease we employed, H64A subtilisin-BPN', recognises the motif Phe-Ala-His-Tyr and cleaves on the C-terminal side of the tyrosine (Carter *et al.* 1989). Thus in this situation, the protease should release cIGF-II from the cIGF-II fusion protein, [Met^{1}]-pGH(1-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II. However, the protease can also readily cleave between Gln-His (Matthews & Wells, 1993). The [Met^{1}]-pGH(1-46) leader peptide in the fusion protein contains a Gln-His motif at positions 19-20, hence complicating the cleavage of cIGF-II from the

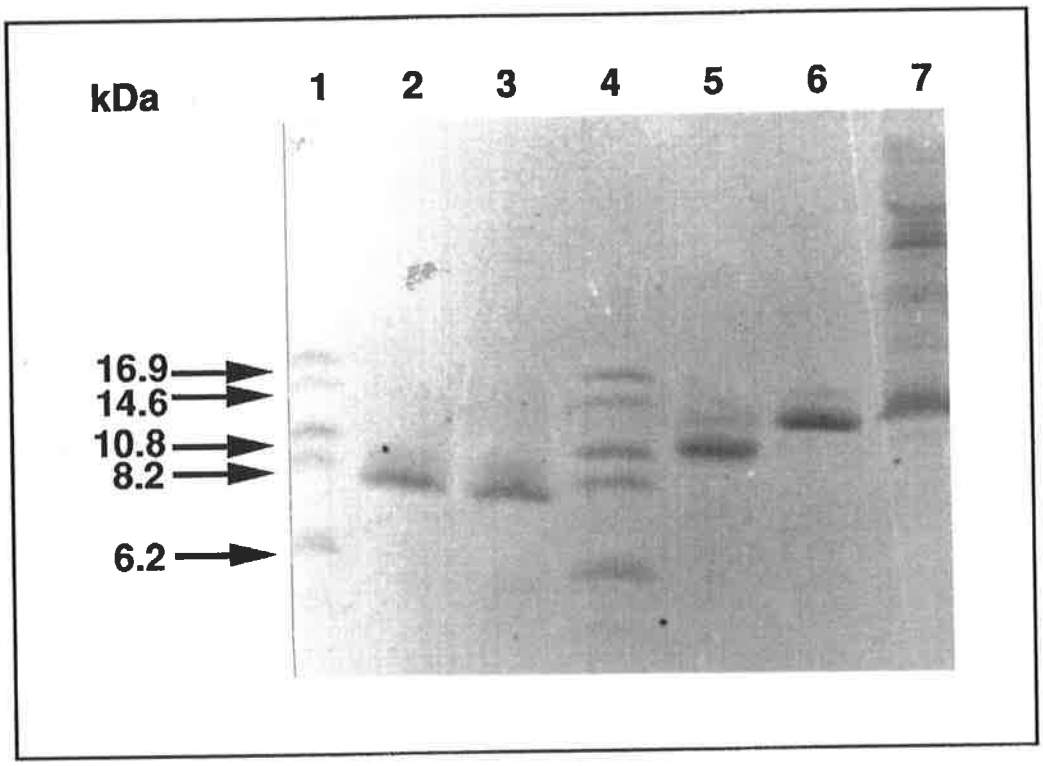


FIGURE 4.5: Polyacrylamide gel analysis of total cell protein from *E.coli* cells induced to express [Met1]-pGH(1-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II (lane 7), partially pure chicken IGF-II (cIGF-II) fusion protein (lane 6), the truncated fusion protein isolated following the first digestion with H64A subtilisin BPN' (lane 5) and pure recombinant cIGF-II (lane 3). Reference recombinant human IGF-II is in lane 2, while molecular mass markers are in lanes 1 and 4. Samples were electrophoresed on high-density SDS-polyacyrlamide gels under reducing conditions.

leader peptide (Fig. 4.4). While recovery from the first digest which created pGH(20-46)-Val-Asn-Phe-Ala-His-Tyr was greater than 90% (Fig. 4.6a & b), yield from the second subtilisin digest was extremely low with only 1.3 mg of correctly cleaved cIGF-II eventually being purified from the 130 mg of pGH(20-46)-Val-Asn-Phe-Ala-His-Tyr digested (Fig. 4.6 c & d). The recombinant cIGF-II recovered from the final reverse-phase HPLC step eluted as a single peak from the microbore C4 standard analytical HPLC (Fig. 4.6e) and migrated as a single band at the expected size of 7.5 kDa on a high density polyacrylamide gel run under reducing conditions (Fig. 4.5, lane 3). Recombinant hIGF-II migrated at a similar position (Fig. 4.5, lane 2).

N-terminal peptide sequencing of recombinant cIGF-II indicated that the protein had the correct amino-terminal sequence and was at least 96 % pure. Mass determination of the pure cIGF-II protein also confirmed that the correct cIGF-II protein had been produced as the only protein detected in the analytical spectroscopy had the expected mass of cIGF-II (7513 Da).

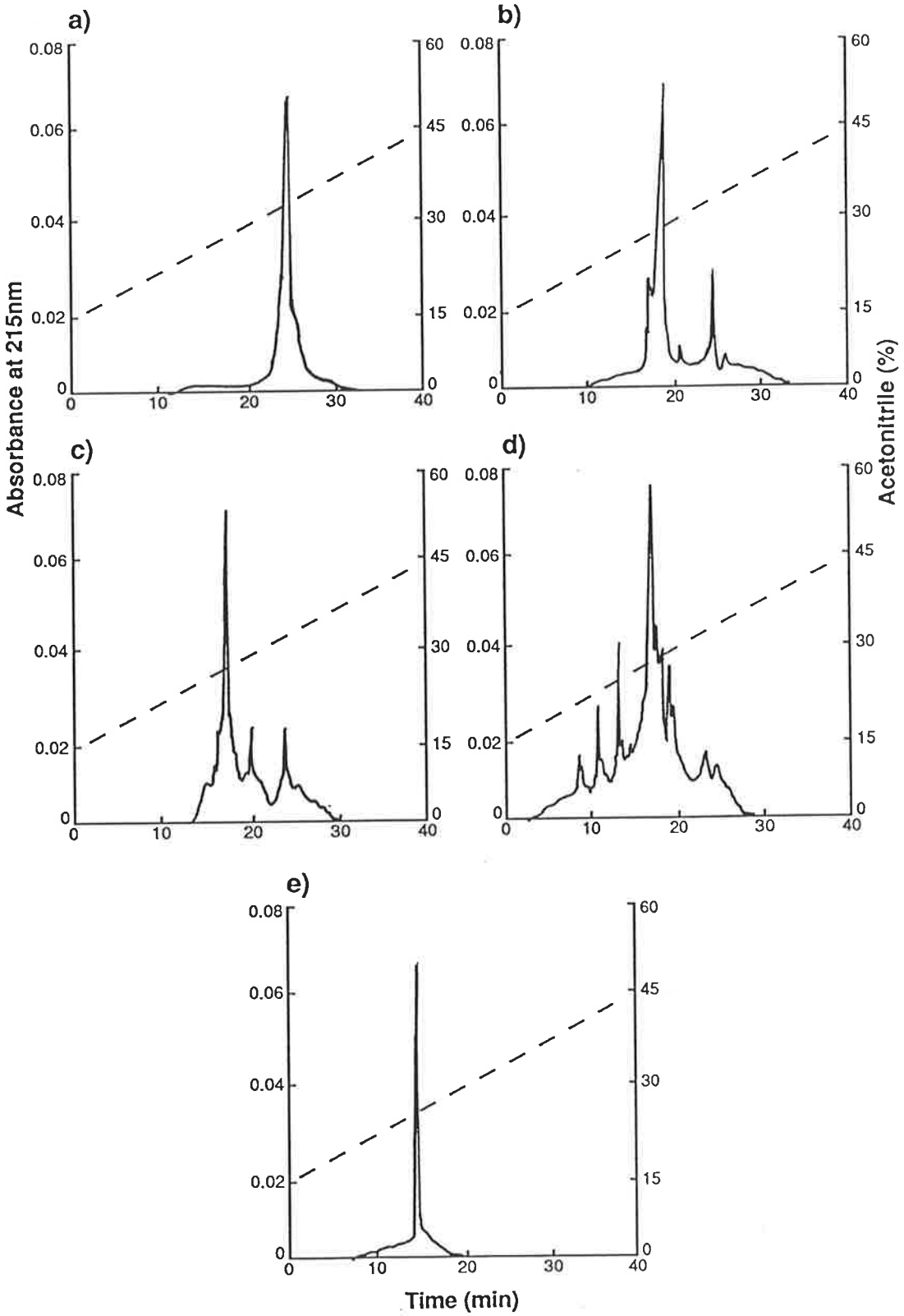
4.3.3: Purification and downstream processing of the cIGF-II analogues

The two analogues, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, were processed in a similar manner to that described for cIGF-II. As was the case for cIGF-II substantial losses of protein were incurred during the release of the required proteins from the expressed fusion-proteins via H64A subtilisin-BPN'. N-terminal protein sequencing, and mass spectroscopy confirmed that the correct cIGF-II analogues had been produced.

4.3.4: Receptor binding interactions of recombinant cIGF-II

The ability of recombinant cIGF-II to bind to IGF receptors was compared to its human counterpart, hIGF-II, in both chick embryo fibroblasts (CEF) and in L6 rat myoblasts. Binding to the type-1 IGF receptor in CEFs and in L6 rat myoblasts was assessed by measuring competition for [^{125}I]-labelled hIGF-I

FIGURE 4.6: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) a) before and b) after digestion of [Met^{1}]-pGH(1-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II with H64A subtilisin BPN'. Analysis of pGH(20-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II before and after digestion with H64A subtilisin BPN' is depicted in c) and d) respectively, while analysis after the final purification of chicken IGF-II is shown in e). Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.


a)
b)
c)
d)
e)
Absorbance at 215nm
Acetonitrile (%)
Time (min)
0.08
0.06
0.04
0.02
0
60
45
30
15
0
10
20
30
40

binding. While recombinant cIGF-II and hIGF-II displayed similar affinities for the type-1 IGF receptor in CEF cells, the two peptides differed in their affinities for the type-1 IGF receptor present in L6 rat myoblasts. Half-maximal effects were observed at 2.5 and 1.7 ng/0.5 ml in CEFs (Fig. 4.7a) and at 40 and 11 ng/0.5 ml in L6 rat myoblasts (Fig. 4.8a) for cIGF-II and hIGF-II respectively. Recombinant cIGF-I was slightly less effective than either chicken or human IGF-II in competing for binding to the type-1 IGF receptor in CEFs (half-maximal effect at 4.1 ng/0.5 ml, Fig 4.7a) but was more effective than either cIGF-II or hIGF-II at competing for binding to the type-1 IGF receptor in L6 rat myoblasts (half-maximal effect at 1.4 ng/0.5 ml, Fig. 4.8a). The binding results for all the recombinant peptides tested were the same in both cell lines regardless of whether human or chicken radiolabelled-IGFs were used in the studies (results not shown).

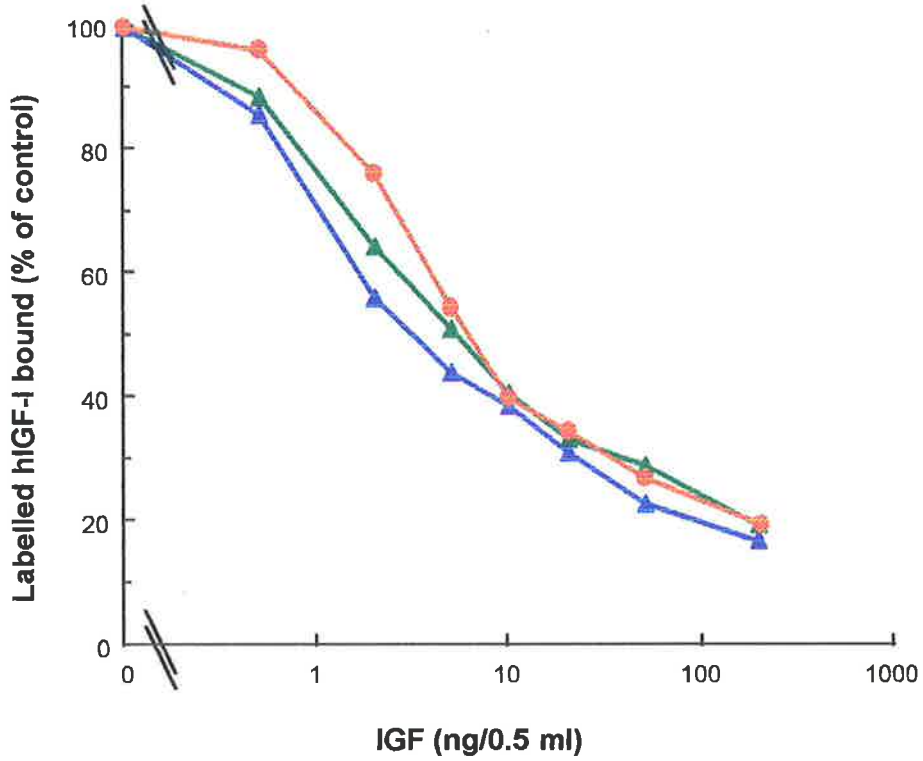
Both cIGF-II and hIGF-II exhibited similar affinities (half maximal competition at 1.6 ng and at 1.3 ng/0.35 ml respectively) for binding to the type-2 IGF receptor present on sheep placental membranes (Fig. 4.9).

4.3.5: Biological activity of recombinant cIGF-II

The type-1 IGF receptor is thought to mediate most of the biological actions of IGFs. Thus the ability of recombinant cIGF-II and hIGF-II to inhibit protein degradation in CEFs or to stimulate protein synthesis in L6 rat myoblasts mirrored the results observed in the experiments assessing their ability to bind to the type-1 IGF receptor in these two cell lines. Recombinant cIGF-II and hIGF-II were similar in their abilities to inhibit protein breakdown in CEFs with half-maximal effects at 1.4 and 0.8 ng/ml respectively (Fig. 4.7b). Again as expected from the type-1 IGF receptor binding results, recombinant cIGF-II was less potent than recombinant hIGF-II in stimulating protein synthesis in L6 rat myoblasts, with 3.7-fold more peptide required to achieve the same half-maximal response (Fig. 4.8b). Recombinant chicken IGF-I was similar to human and chicken IGF-II in its ability to inhibit protein breakdown in CEFs (half-maximal effect at 1.1 ng/ml). However, cIGF-I was more effective than either IGF-II in stimulating protein

FIGURE 4.7: Effects of IGFs on a) competition for binding of [^{125}I]-labelled human IGF-I and b) protein breakdown in chick embryo fibroblasts. The proteins tested were human IGF-II (blue ▲), chicken IGF-II (green ▲) and chicken IGF-I (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.5 ml)

b)

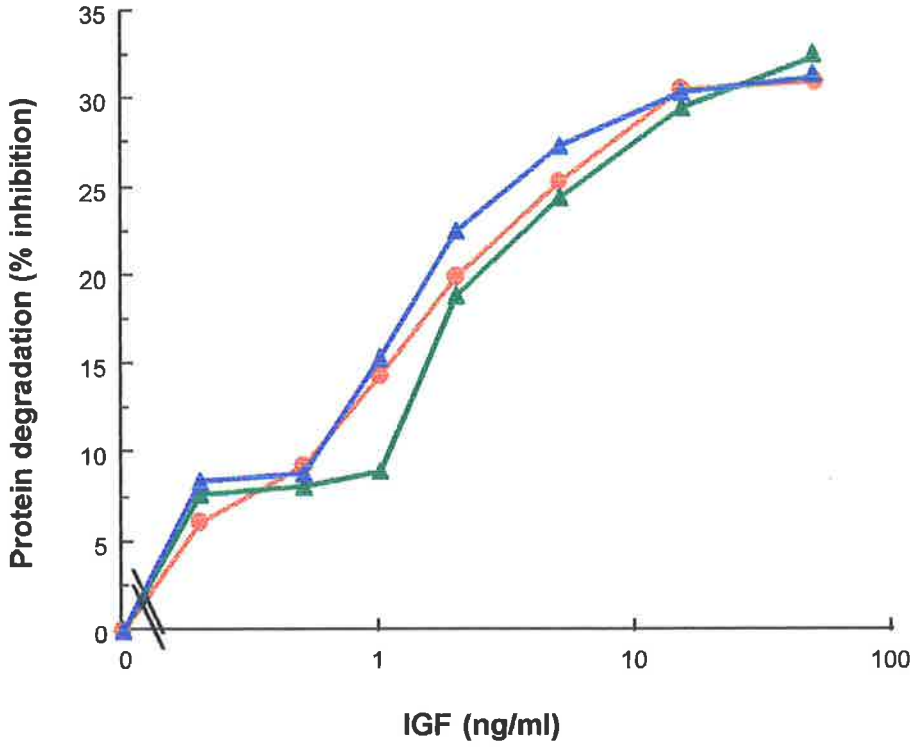
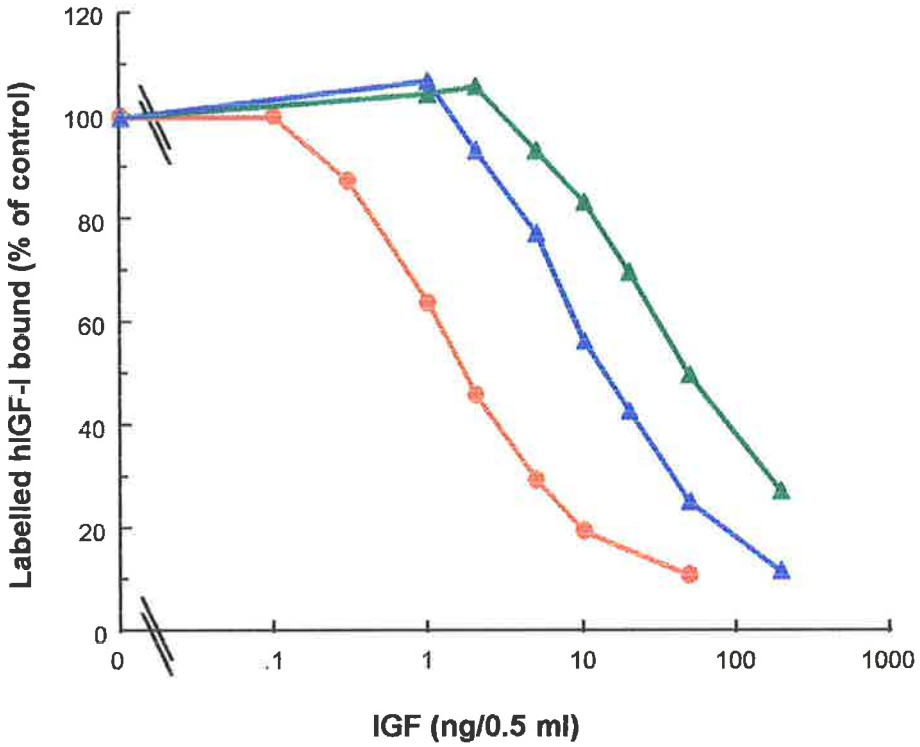

Protein degradation (% inhibition)
35
30
25
20
15
10
5
0
0
1
10
100
IGF (ng/ml)

FIGURE 4.8: Effects of IGFs on a) competition for binding of [^{125}I]-labelled human IGF-I and b) protein synthesis in rat L6 myoblasts. The proteins tested were human IGF-II (blue ▲), chicken IGF-II (green ▲) and chicken IGF-I (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-I bound (% of control)
120
100
80
60
40
20
0
0
.1
1
10
100
1000
IGF (ng/0.5 ml)

b)

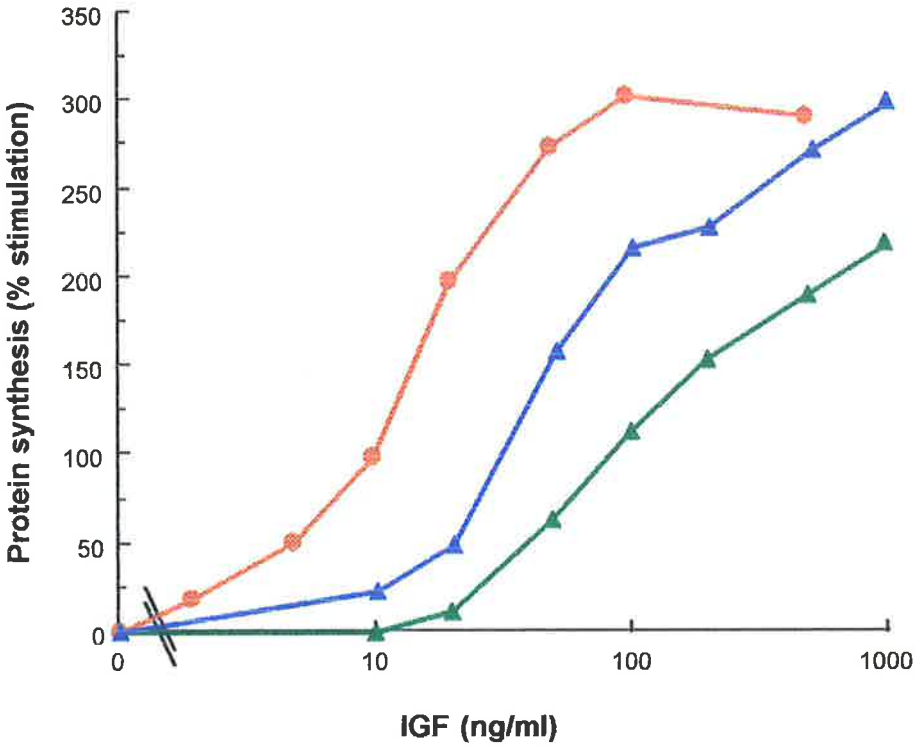
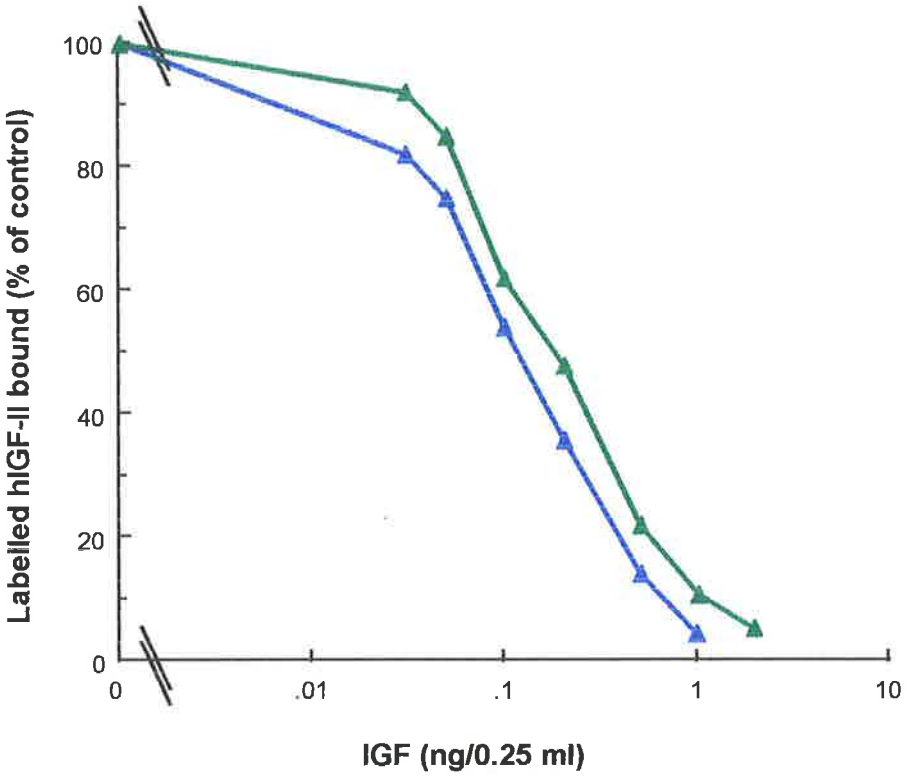

Protein synthesis (% stimulation)
350
300
250
200
150
100
50
0
0
10
100
1000
IGF (ng/ml)

FIGURE 4.9: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to ovine placental membranes. The proteins tested were human IGF-II (blue ▲) and chicken IGF-II (green ▲). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
.01
.1
1
10
IGF (ng/0.25 ml)

synthesis in L6 cells (half-maximal response at 13.5 ng/ml, Fig. 4.8b) hence reflecting the type-I IGF receptor interactions observed in this cell line.

4.3.6: IGF binding-protein interactions of recombinant cIGF-II

The *in vivo* and *in vitro* actions of IGFs are not only determined by their interactions with IGF receptors, but also by their interactions with IGFBPs. In order to determine whether the amino acid differences between cIGF-II and hIGF-II affect interactions with IGFBPs, labelled recombinant cIGF-II and hIGF-II were used as probes to detect IGFBPs in plasma following separation on polyacrylamide gels. Similar bands were detected in Western ligand blots of chicken or rat plasma regardless of whether the mammalian (lanes 1 & 2) or the avian (lanes 3 & 4) IGF-II probe was used (Fig. 4.10). However, additional bands (70-80 kDa) which are not observed in ligand blots on chicken plasma probed with [^{125}I]-labelled cIGF-I (Fig. 4.11a), were detected by both the chicken and human IGF-II probes. Radiolabelled human IGF-II, however, appeared to have a lower affinity than labelled chicken IGF-I for these protein species. Moreover, unlabelled cIGF-II was more effective than unlabelled cIGF-I at competing for [^{125}I]-cIGF-II binding to these IGF-II-binding proteins (Fig. 4.11b).

Binding of chicken and human IGF-II to the IGFBPs present in rat and chicken plasma was also assessed. Both recombinant chicken and human IGF-II were equipotent in their affinities for these IGFBPs (Fig. 4.12).

4.3.7: Immunoreactivity of recombinant cIGF-II

The cross-reactivities of cIGF-II and hIGF-II were compared in an immunoassay employing a monoclonal antibody raised against hIGF-II. Both peptides showed similar affinities for binding to the monoclonal antibody. Recombinant cIGF-I however, differed from its human equivalent, hIGF-I, as it exhibited little, if any affinity for the antibody (Fig. 4.13).

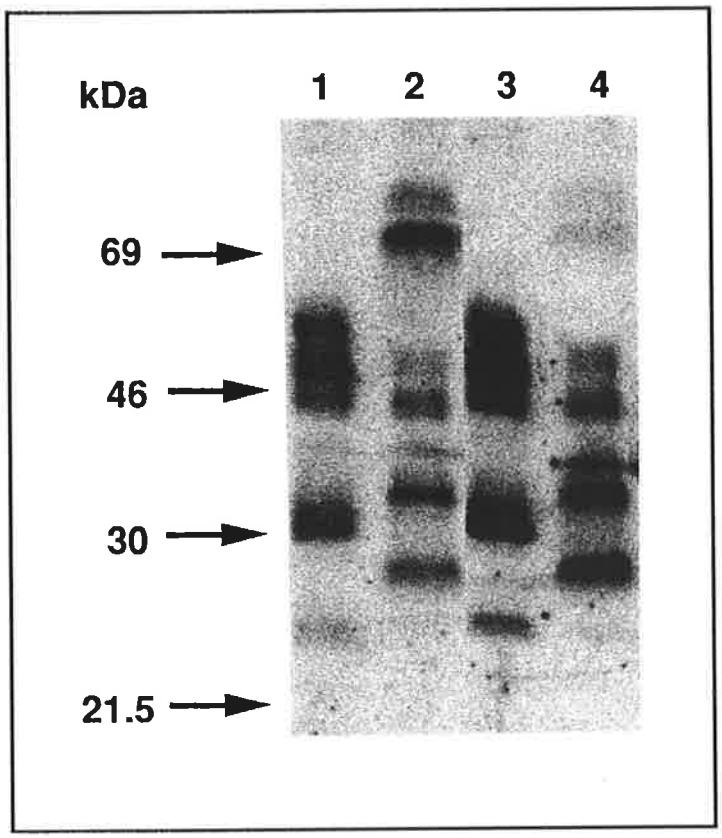
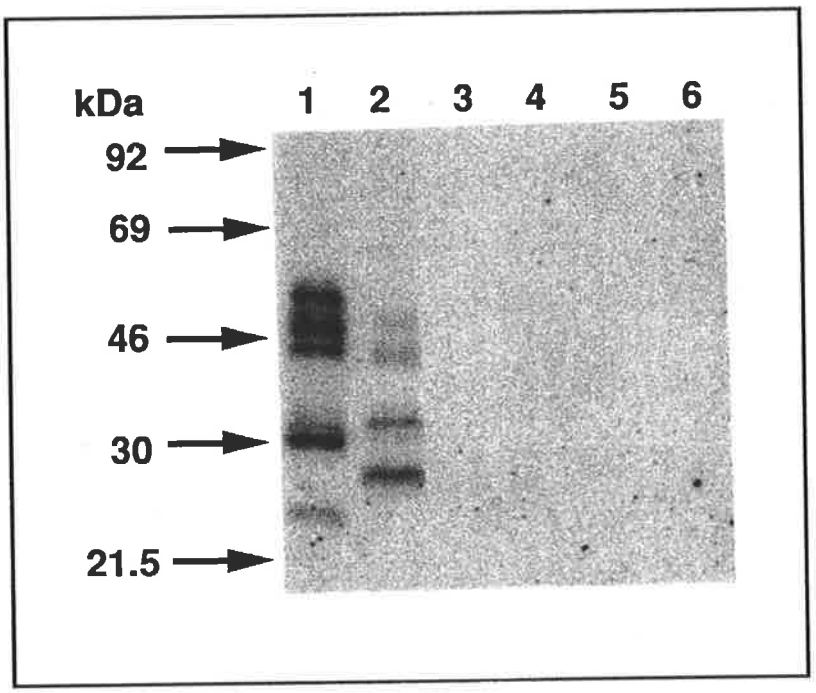


FIGURE 4.10: Ligand blotting of IGF-binding proteins in plasma using labelled chicken IGF-II (lanes 1 and 2) or labelled human IGF-II (lanes 3 and 4) as probes. The plasma samples tested were 1 µl from adult rat (lanes 1 and 3) and 10 µl from 7-week-old broiler chickens (lanes 2 and 4). Molecular mass markers are indicated. Plasmas were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-II and the probed nitrocellulose sheets exposed to phosphorimaging screens.

FIGURE 4.11: Ligand blotting of IGF-binding proteins in plasma using a) labelled chicken IGF-I or b) labelled chicken IGF-II as probes. The plasma samples tested were 1 µl from adult rat (lanes 1, 3 and 5) and 10 µl from 7-week-old broiler chickens (lanes 2, 4 and 6). Proteins in lanes 3 and 4 were incubated with labelled IGFs in the presence of 500 ng/ml chicken IGF-I, while proteins in lanes 5 and 6 were incubated with labelled IGFs in the presence of 500 ng/ml chicken IGF-II. Molecular mass markers are indicated. Plasmas were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGFs in the presence and absence of unlabelled IGFs, and the probed nitrocellulose sheets exposed to phosphorimaging screens.

a)



b)

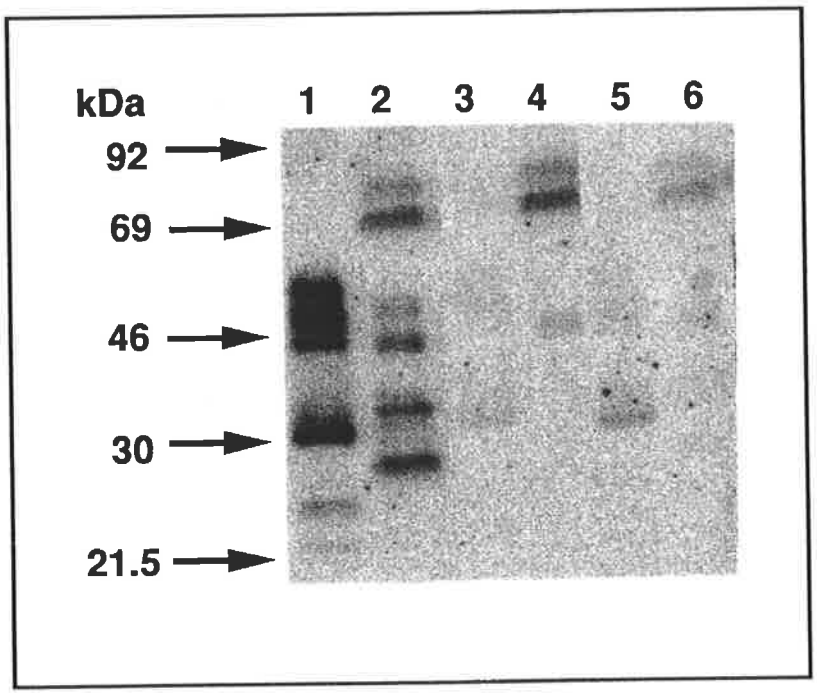
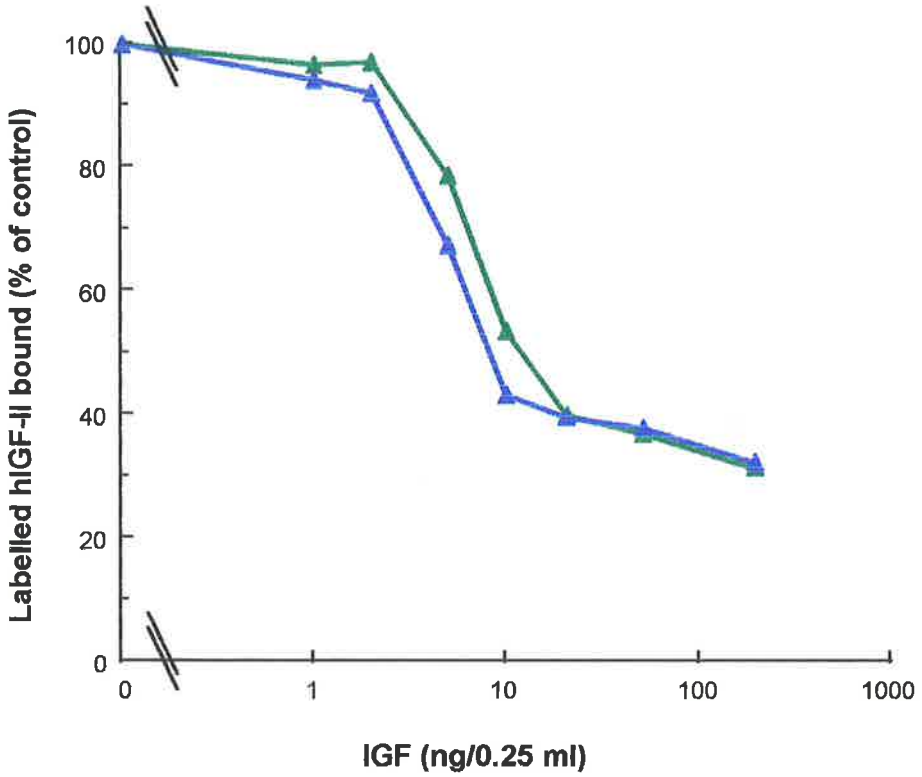


FIGURE 4.12: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to IGFBPs present in a) chicken and b) rat plasma. The proteins tested were human IGF-II (blue ▲) and chicken IGF-II (green ▲). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

b)

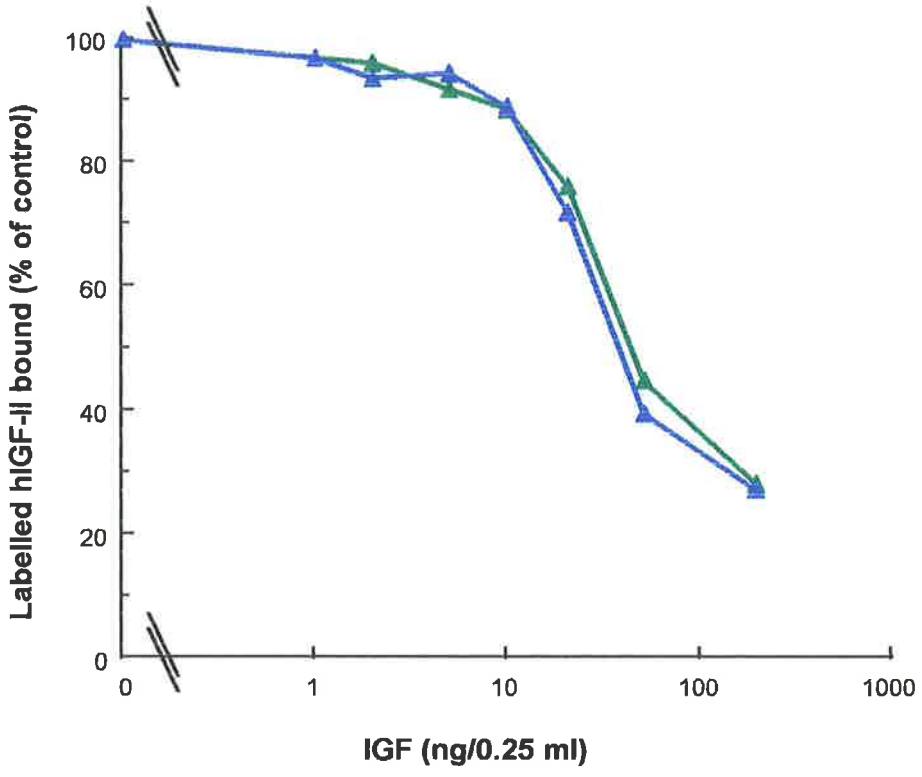
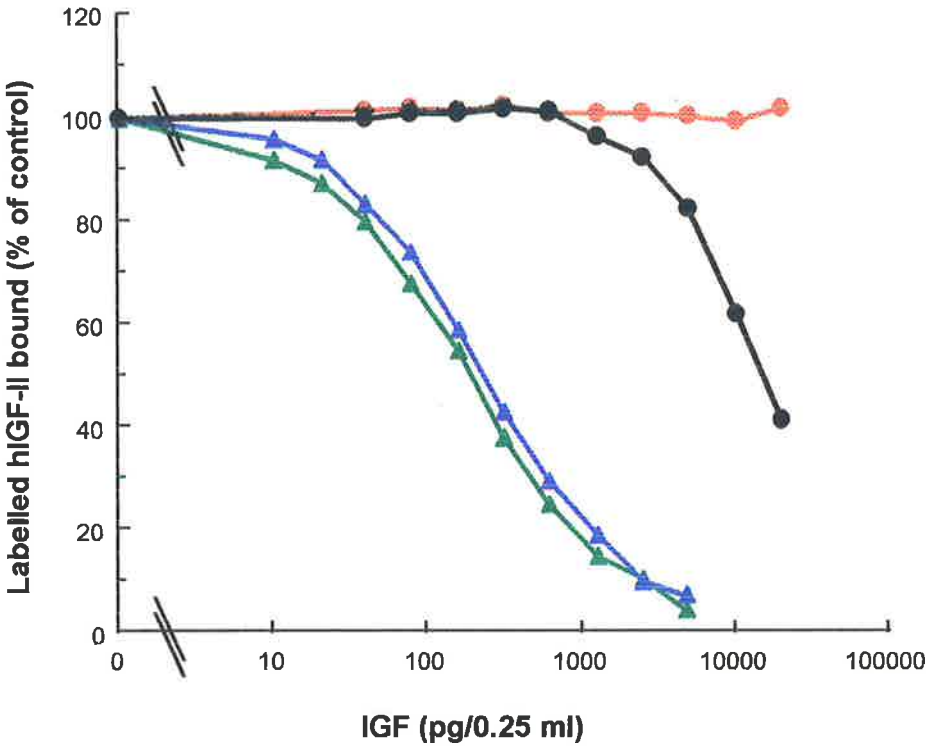

Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

FIGURE 4.13: Radioimmunoassay of IGFs using human IGF-II as radioligand and a monoclonal antiserum raised against recombinant human IGF-II. The proteins tested were human IGF-II (blue ▲), chicken IGF-II (green ▲), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled hIGF-II bound (% of control)
120
100
80
60
40
20
0
0
10
100
1000
10000
100000
IGF (pg/0.25 ml)

4.3.8: Biological activity, receptor-binding and IGFBP-binding interactions of the recombinant cIGF-II analogues

The two cIGF-II analogues, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, were compared to recombinant cIGF-II in a range of biochemical assays in order to determine whether any of the amino acid changes in the analogues significantly altered the structure and subsequent function of the proteins.

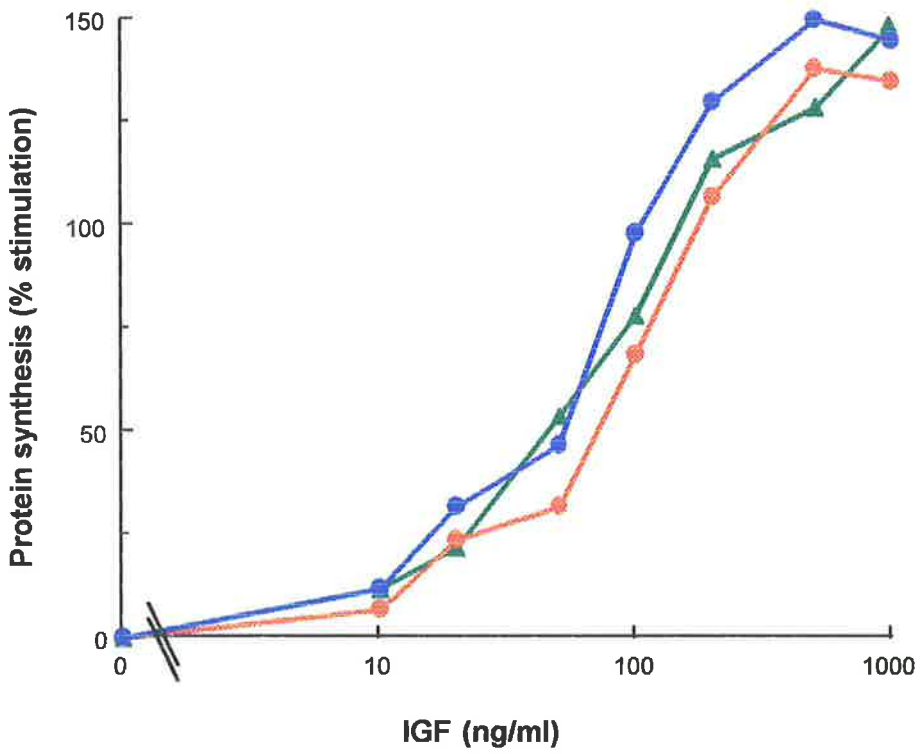
The three cIGF-II proteins were similar in their ability to stimulate protein synthesis in L6 rat myoblasts with half maximal effects observed at 80, 100 and 70 ng/ml for cIGF-II, [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II and Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II respectively (Fig. 4.14a). The abilities of the peptides to inhibit protein breakdown in CEFs were also similar with half maximal inhibition occurring at 1.5 ng/ml for cIGF-II and for [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, and at 1.8 ng/ml for Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (Fig. 4.15a). Furthermore, the relative affinities of the three cIGF-II peptides for binding to the IGF receptors present in CEFs and in L6 rat myoblasts were essentially similar (Fig. 4.14b & 4.15b). If anything, the cIGF-II analogue, Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, appeared to compete slightly more effectively for binding to the L6 type-1 IGF receptor than did the other two proteins (Fig. 14b). All three cIGF-II proteins were also equipotent at competing for binding to the type-2 IGF receptor on sheep placental microsomes (Fig. 4.16) and for binding to the IGFBPs present in rat and chicken plasma (Fig. 4.17).

4.4: DISCUSSION

In this chapter I have described the production and characterization of recombinant cIGF-II, as well as two analogues of cIGF-II. While the expression vector I utilized was able to produce high yields of inclusion bodies comparable to those reported by others using related gene-fusion systems (King *et al.* 1992; Francis *et al.* 1993), the subsequent downstream processing of the fusion-proteins was inefficient with low yields of the cIGF-II proteins ultimately produced.

FIGURE 4.14: Effects of IGFs on a) protein synthesis and b) competition for binding of [^{125}I]-labelled human IGF-I in rat L6 myoblasts. The proteins tested were chicken IGF-II (green ▲), Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (blue ●) and [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein synthesis (% stimulation)
150
100
50
0
0
10
100
1000
IGF (ng/ml)

b)

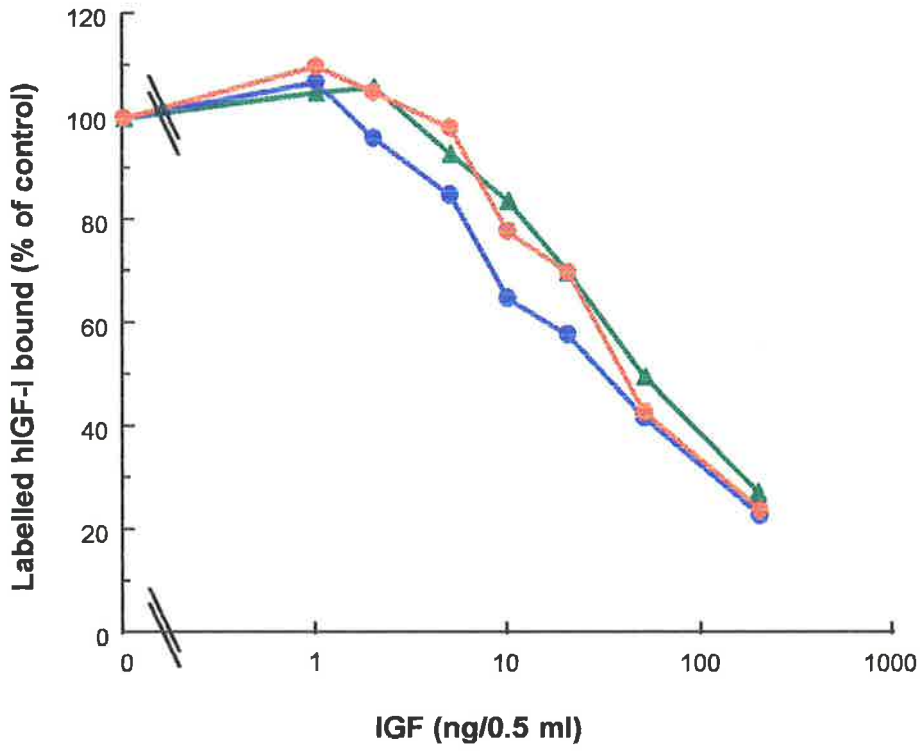
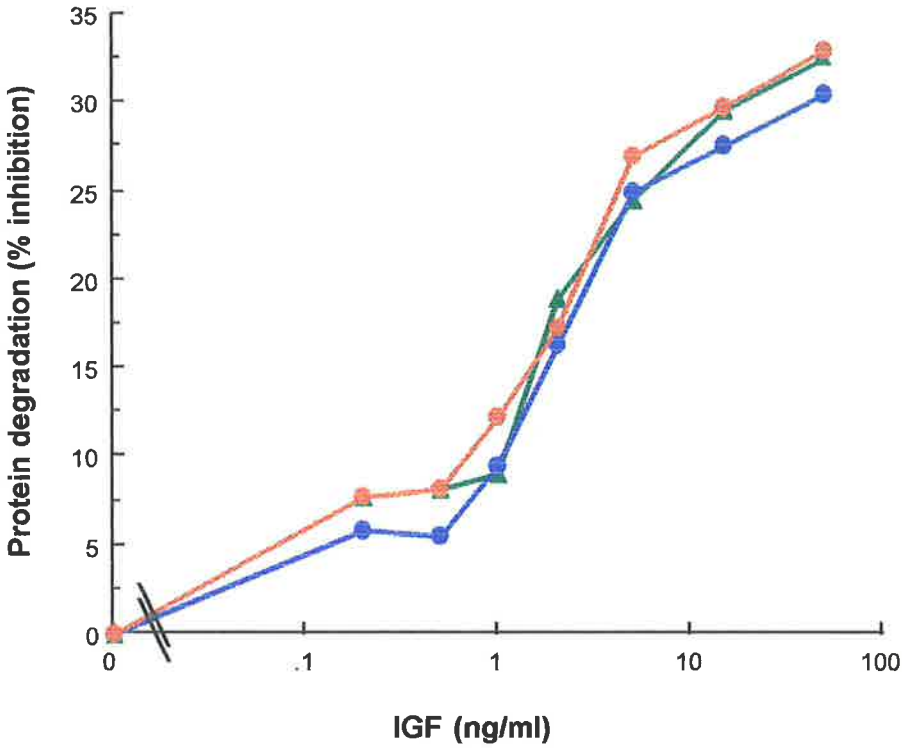

Labelled hIGF-I bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.5 ml)

FIGURE 4.15: Effects of IGFs on a) protein degradation and b) competition for binding of [^{125}I]-labelled human IGF-I in chick embryo fibroblasts. The proteins tested were chicken IGF-II (green ▲), Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (blue ●) and [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein degradation (% inhibition)
35
30
25
20
15
10
5
0
0
.1
1
10
100
IGF (ng/ml)

b)

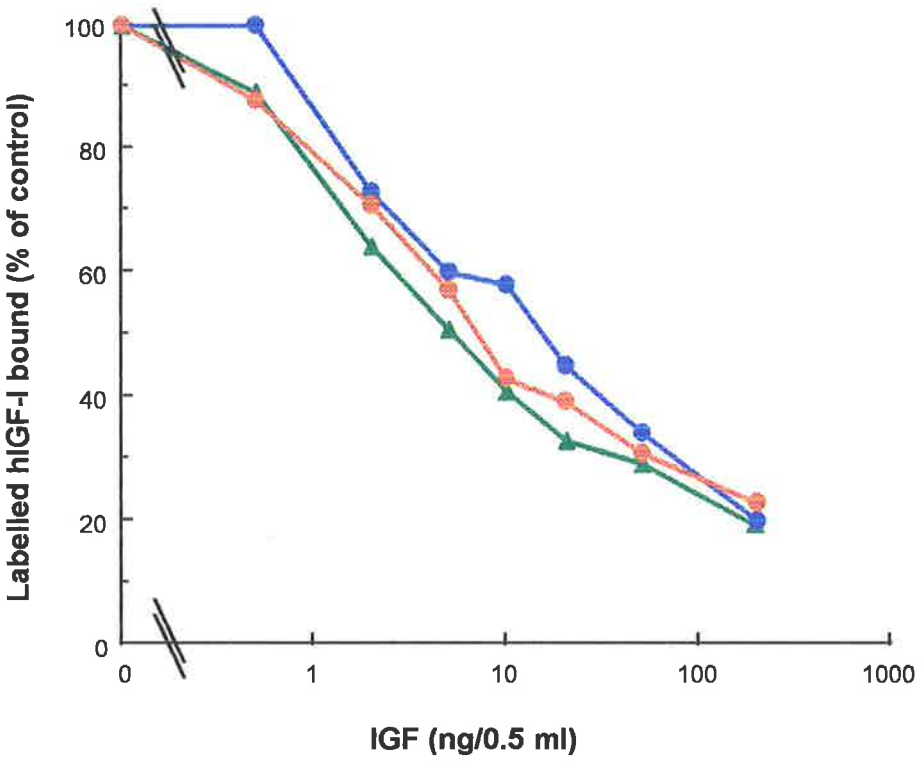

Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.5 ml)

FIGURE 4.16: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to ovine placental membranes. The proteins tested were chicken IGF-II (green ▲), Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (blue ●) and [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

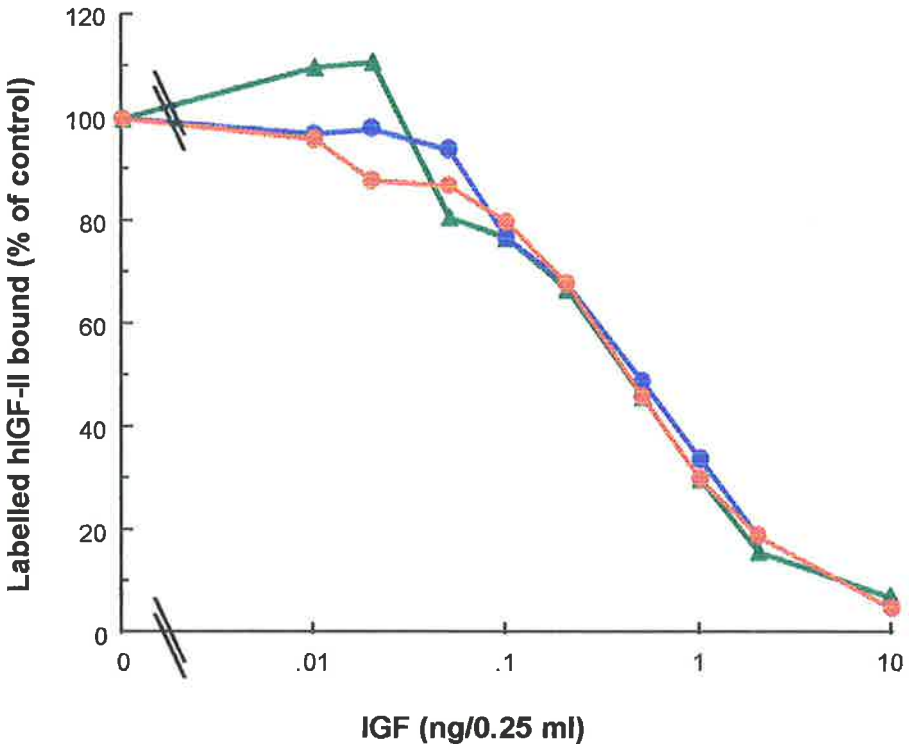
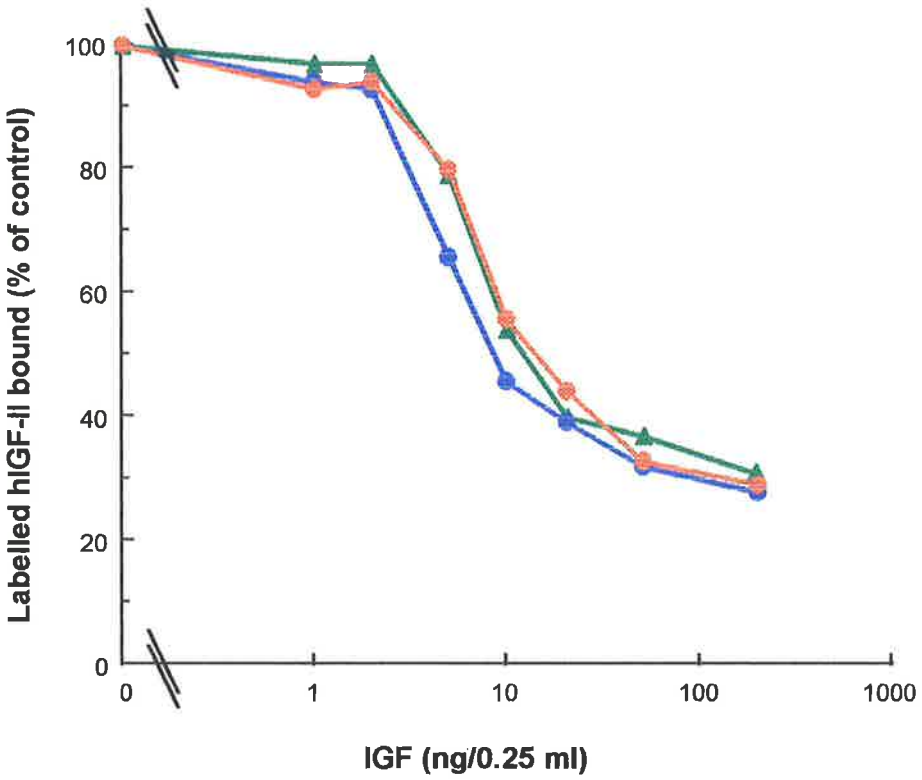
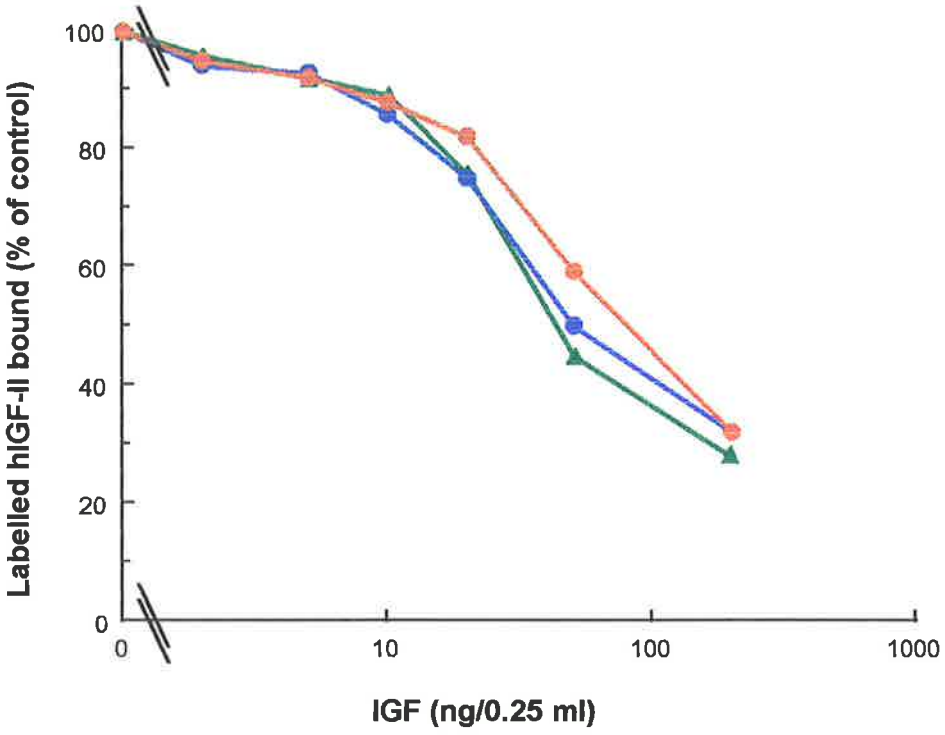

Labelled hIGF-II bound (% of control)
120
100
80
60
40
20
0
0
.01
.1
1
10
IGF (ng/0.25 ml)

FIGURE 4.17: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to IGFBPs present in a) chicken and b) rat plasma. The proteins tested were chicken IGF-II (green ▲), Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (blue ●) and [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

b)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
10
100
1000
IGF (ng/0.25 ml)

This was almost entirely due to losses incurred during the second subtilisin cleavage digest. Francis *et al.* (1993) have reported production of high yields of recombinant human IGF-II using subtilisin to release the required hIGF-II from a similar fusion protein. It would seem that additional determinants other than the H64A subtilisin-BPN' recognition motif, Phe-Ala-His-Tyr, are influencing the proteolytic activity of the enzyme. Human IGF-II contains an Ala at position 1, whereas cIGF-II has a Tyr. Thus H64A subtilisin-BPN' appears to be more efficient at cleaving on the carboxyl side of the Phe-Ala-His-Tyr motif when flanked by an Ala rather than a Tyr residue. The cleavage of the leader peptide between Gln and His identified in the first subtilisin digest also indicates that this genetically engineered protease is not as specific as first thought (Carter *et al.* 1989; Matthews & Wells, 1993). Clearly an alternative gene-fusion system needs to be developed before large yields of cIGF-II can be produced. A gene-fusion system similar to that which I have described but with a Met linking methionyl-porcine growth hormone to cIGF-II may be an alternative. As cIGF-II contains no Met residues, cIGF-II could be released from the leader peptide using cyanogen bromide. Indeed, a number of groups have used cyanogen bromide to release recombinant human IGF-II from fusion partners (Furman *et al.* 1987; Roth *et al.* 1991; Wadenstein *et al.* 1991; Marumoto *et al.* 1992). Nevertheless, the gene-fusion system described in this chapter has permitted milligram quantities of cIGF-II to be produced, hence has provided the first opportunity for a nonmammalian IGF-II to be thoroughly characterized.

Kallincos *et al.* (1990) previously reported that chicken IGF-II purified from serum was indistinguishable from ovine IGF-II in biological and receptor-binding assays in cultured rat and chicken cells. I now report that while recombinant cIGF-II and hIGF-II are identical in assays in CEFs (Fig. 4.7), they differ in their affinity for the rat L6 myoblast type-1 IGF receptor. This in turn provides an explanation for the lower potency of cIGF-II observed in the biological assay in these cells (Fig. 4.8). The difference in affinity for the mammalian type-1 IGF receptor is unlikely to be due to a difference in sequestering of the proteins by

the type-2 IGF receptor present in these cells, as both cIGF-II and hIGF-II are equipotent in competing for binding to the type-2 IGF receptor on ovine placental membranes (see Fig. 4.9). Moreover, the difference in affinities can not be explained by IGFBPs as the receptor binding assays are performed at 4°C, a temperature where no IGFBPs are produced by the cells. In addition, both human and chicken IGF-II have similar affinities for the IGFBPs present in rat and chicken plasma. It remains to be determined if the difference in binding to the CEF and L6 type-1 IGF receptors does in fact reflect a difference between mammalian and avian type-1 IGF receptors.

The receptor-binding and biological results I obtained with recombinant cIGF-II in rat L6 myoblasts differed from those reported by Kallincos *et al.* (1990). The difference can not be explained by the presence of two forms of cIGF-II with different C-terminal sequences in the serum-derived preparations as I have also produced recombinant cIGF-II with the alternative C-terminus. The two forms of cIGF-II, [$Lys^{62}Ser^{63}Val^{64}$]-cIGF-II and [$Thr^{62}Pro^{63}Ala^{64}$]-cIGF-II, were essentially similar in all the assays performed. Surprisingly, not even the loss of the charged residue Lys^{62} affected the action of the analogues. These results differ from experiments with human IGF-II mutants where deletion of residues 62-67 decreased receptor, but not IGFBP3 interactions (Roth *et al.* 1991). Oh *et al.* (1991) also found that deletion of the two C-terminal amino acids or acetylation of the ε-nitrogen of Lys^{65} in human IGF-II decreased affinity for the type-1 IGF receptor. Moreover, both modifications also decreased affinities for IGFBPs. Taken together, these findings suggest the amino acids at positions 65-67, but not at positions 62-64 in human IGF-II, may be important for IGFBP- and receptor-binding, an interpretation that is consistent with my findings. More recently, however, Bach *et al.* (1993) have reported that deletion of the D-domain of human IGF-II, appears actually, to enhance IGFBP interactions. Furthermore, Clemmons *et al.* (1992) have demonstrated that while the D-domain of human IGF-I is not important for interactions with IGFBP-1,-2 and -3, it appears to be

involved in interactions with IGFBP-4 and -5. Clearly, the importance of the D-domain for IGF function is far from resolved.

The second cIGF-II analogue I produced, Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, containing an amino acid deletion in the C-domain in addition to D-domain mutations, was also essentially similar to cIGF-II in all the experiments performed. To date, little is known about the importance of the amino acids in the C-domain region of IGF-IIs with respect to receptor and IGFBP-binding. A C-domain mutant of human IGF-II where Gln^{37}Gln^{38} were substituted for Arg^{37}Arg^{38}, has recently been reported to markedly alter binding to insulin receptors while having minimal impact on binding to both the type-1 and type-2 IGF receptors (Edwards & Bawden, 1992). However, the chicken IGF-II analogue I produced, Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II, containing an Arg deletion spatially close to those implicated in the insulin binding described above, does not exhibit altered affinity for the insulin receptor present on rat hepatocytes (*personal communication, G. Shooter, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia*).

Western-ligand blotting of chicken and rat plasma with radiolabelled cIGF-II and hIGF-II did not reveal any differences in the binding protein species detected (Fig. 4.10). However, the radiolabelled IGF-IIs, detected additional species in chicken plasma not found in Western-ligand blots probed with either radiolabelled hIGF-I (Fig. 3.11) or cIGF-I (Fig. 4.11). Furthermore, unlabelled cIGF-II competed more effectively than unlabelled cIGF-I for [^{125}I]-cIGF-II binding to the protein species represented by these bands. Schoen *et al.* (1992) and Duclos *et al.* (1992) have also detected a 70 kDa species when probing chicken plasma on Western-ligand blots with radiolabelled hIGF-II. Whether these bands represent an IGF-II-specific IGFBP or an avian form of a circulating type-2 IGF receptor remains to be elucidated.

While a number of studies have used radioimmunoassay for serum IGF-I to assess growth performance in chickens (Huybrechts *et al.* 1985; Vasilatos-Younken *et al.* 1988; Ballard *et al.* 1990; McGuinness & Cogburn, 1990), it has

not been possible to determine absolute levels as firstly, heterologous reference standards have been used, and secondly, there was no information regarding the cross-reactivity of cIGF-II in the IGF-I assays employed. The development of specific radioimmunoassays using the recombinant chicken peptides will overcome this problem, enabling absolute levels of chicken serum IGFs to be measured for the first time. This in turn will be useful in establishing whether serum IGF levels can be used as indicators or predictors of potential differences in growth and nutrient partitioning. The radioimmunoassay using a monoclonal antibody against hIGF-II described in this chapter may prove to be particularly useful, since cIGF-I, unlike hIGF-I, does not react with the antibody (Fig. 4.13). Sequence comparison of the human and chicken IGFs suggests that the monoclonal antibody may recognise Arg^{50} in IGF-I and Arg^{49} in IGF-II (Fig. 1.6). Chicken IGF-I has a Gln in the corresponding position hence may explain why it does not cross-react in this immunoassay. Moreover, computer models based on the hIGF-I NMR-derived structure, suggest that Arg^{50} of hIGF-I is exposed on the surface of the molecule, thus making it plausible that the monoclonal antibody was raised against this region.

The availability of recombinant cIGF-II, in addition to recombinant cIGF-I will facilitate investigations into the role of IGFs in chicken growth and development. This may prove to be particularly interesting since chickens, unlike mammals, do not have the specific type-2 IGF receptor (Canfield & Kornfeld, 1989; Duclos & Goddard 1990). Indeed, the use of avian, in addition to mammalian IGF peptides, may help in addressing the enigma of the role of the type-2 IGF receptor since the appearance of the type-2 IGF receptor, and its complex regulation via parental imprinting (Rappolee *et al.* 1992; Stoger *et al.* 1993, Rainer *et al.* 1993), appears to have co-evolved with mammals. Furthermore, the availability of recombinant cIGF-I and cIGF-II may be of benefit in identifying whether there are any features which are unique to the regulation and roles of IGFs in avian growth.

CHAPTER 5

PRODUCTION AND CHARACTERIZATION OF AN ANALOGUE OF RECOMBINANT CHICKEN INSULIN-LIKE GROWTH FACTOR-I WITH REDUCED AFFINITY FOR IGF-BINDING PROTEINS AND IGF RECEPTORS

5.1: INTRODUCTION

Others in this laboratory have previously reported the production of a novel recombinant human IGF-I fusion protein analogue with enhanced *in vitro* biological activity (Francis *et al.* 1992). The analogue, Long [Arg^3]-hIGF-I, has a 13 amino acid N-terminal amino acid extension peptide derived from methionyl porcine growth hormone. The increased biological potency of Long [Arg^3]-hIGF-I is not associated with an increase in affinity for the type-1 IGF receptor. Instead, the increased potency appears to be the result of decreased affinity for IGFBPs.

The expression system and downstream processing procedure developed by Francis *et al.*(1992) is efficient and thus has permitted gram quantities of the hIGF-I analogue, Long [Arg^3]-hIGF-I, to be produced. This in turn has allowed the *in vivo* effects of the analogue to be investigated. Thus, in vivo studies have demonstrated that the analogue has anabolic effects in normal (Ballard *et al.* 1993; Tomas *et al.* 1993a), dexamethasone-treated (Tomas *et al.* 1992; Read *et al.* 1992) and in diabetic rats (Tomas *et al.* 1993b).

Encouraged by the studies demonstrating enhanced potency of Long [Arg^3]-hIGF-I *in vitro* and *in vivo*, I have developed a gene-fusion strategy and downstream processing procedure to produce a similar recombinant chicken IGF-I analogue. The chicken IGF-I analogue I produced differs from the human analogue in that it has an eighteen rather than a thirteen amino acid N-terminal extension peptide. The additional residues in the leader peptide were engineered to include peptide recognition sequences for cleavage by hydroxylamine and by the genetically engineered mutant serine protease, H64A subtilisin BPN' (Carter *et al.* 1989). In this chapter I report the production of milligram quantities of Long [Arg^3]-cIGF-I and present evidence that the protein has similar biological, receptor- and IGFBP-binding activities to its human counterpart.

5.2: MATERIALS AND METHODS

5.2.1: Plasmid construction

The starting plasmid was the clGF-I expression vector p[Met^1]-pGH(1-11)-Val-Asn-clGF-I described in Chapter 3. The EcoRI-HindIII fragment of p[Met^1]-pGH(1-11)-Val-Asn-clGF-I was subcloned into the multi-functional vector pTZ18. Site-directed mutagenesis (see 2.1.2 & 2.2.1.2) using oligonucleotide 8 (5'-AGCCTATTTGTTAACGGCTTCGCCCATTATGGCCCGCGTACCCTGTGCGGT-3') permitted the replacement of codons for Glu at position 3 in clGF-I with codons for Arg. In addition, this oligonucleotide also inserted the residues Gly-Phe-Ala-His-Tyr at positions -5 to -1. The residues Gly-Phe-Ala-His-Tyr were included to facilitate downstream processing of the fusion protein if we subsequently wished to make [Arg^3]-clGF-I or an [Arg^3]-clGF-I analogue with a shorter leader peptide. Placing a Gly residue immediately after a Asn creates a hydroxylamine cleavage site, whereas the motif Phe-Ala-His-Tyr is the recognition sequence for cleavage by H64A subtilisin BPN'. Once the required changes had been introduced as confirmed by dideoxy-DNA sequencing (Sanger *et al.* 1977) the HpaI-HindIII fragment of the pTZ clone was ligated into a HpaI-HindIII expression vector, p[Met^1]-pGH(1-11)-Val-Asn described by King *et al* (1992). The DNA sequence of the EcoRI-HindIII fragment following mutagenesis and a schematic representation of the final construct, p[Met^1]-pGH(1-11)-Val-Asn-Gly-Phe-Ala-His-Tyr-[Arg^3]-clGF-I, are shown in Fig. 5.1. This construct was then transformed into *E. coli* JM101 (lac I^q) at 37°C on minimal agar containing 29 µmol ampicillin/l. Fermentation of the transformed *E. coli* and isolation of the inclusion bodies produced was carried out as previously described (2.2.1.3).

5.2.2: Fusion protein purification

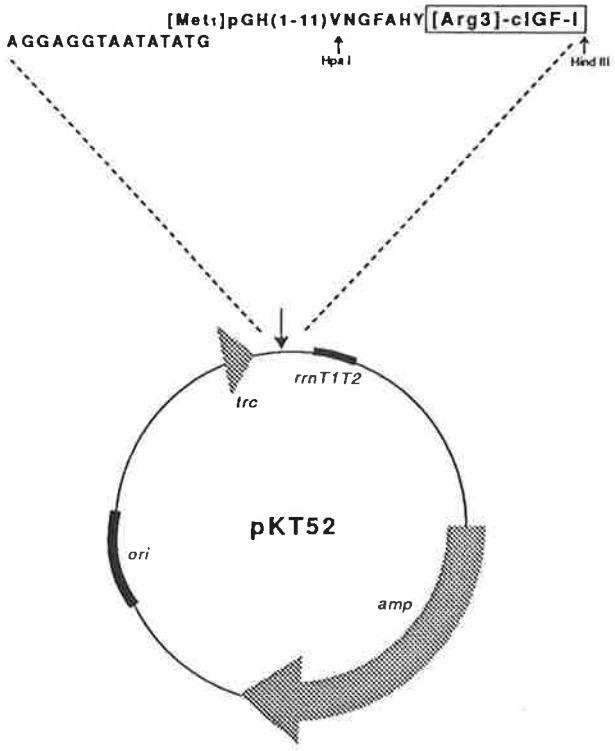
Inclusion bodies (25 g) containing the [Arg^3]-clGF-I fusion protein were solubilized, reduced, desalted and chromatographed on a Fast Flow Q Sepharose column as described for clGF-II fusion proteins in 4.2.2. The [Arg^3]-

FIGURE 5.1: a) Nucleotide and corresponding amino acid sequence of the Hpa1-HindIII fragment of the construct engineered for expression of chicken Long [Arg^3]-IGF-I. Shaded boxes indicate regions where changes were introduced to the DNA sequence of the starting vector by site-directed mutagenesis. The oligonucleotide employed to direct these changes is indicated (oligo 9). Amino acids are numbered and stop codons are marked by asterisks. b) Schematic representation of the construct engineered for expression of chicken Long [Arg^3]-IGF-I in *E. coli*. The expression plasmid, pKT52, contains an origin of replication (*ori*), a *trc* promoter, a transcription termination sequence (*rrnT1T2*) and a gene conferring ampicillin resistance (*amp*). The DNA sequence preceding Met has been modified to optimize ribosome binding (Vize & Wells, 1987).

a)

```
          HpaI                    1                                  10
   SerLeuPheValAsnGlyPheAlaHisTyrGlyProArgThrLeuCysGlyAlaGluLeu
5'-AGCCTATTTGTTAACGGCTTCGCCCATTATGGCCCGCGTACCCTGTGCGGTGCTGAACTG
 5'                    oligo 9                                  3'
3'-TCGGATAAACAATTGCCGAAGCGGGTAATACCGGGCGCATGGGACACGCCACGACTTGAC

                                                                30
   ValAspAlaLeuGlnPheValCysGlyAspArgGlyPheTyrPheSerLysProThrGly
   GTTGACGCTCTGCAGTTCGTTTGCGCTGACCGTGGCTTCTACTTCTCTAAACCGACCGGT
   CAACTGCGAGACGTCAAGCAAACGCGACTGGCACCGAAGATGAAGAGATTTGGCTGGCCA

                                                                50
   TyrGlySerSerSerArgArgLeuHisHisLysGlyIleValAspGluCysCysPheGln
   TACGGTTCTTCTTCTCGTCGTCTGCACCACAAAGGTATCGTTGACGAATGCTGCTTCCAG
   ATGCCAAGAAGAAGAGCAGCAGACGTGGTGTTTCCATAGCAACTGCTTACGACGAAGGTC

                                                                70
   SerCysAspLeuArgArgLeuGluMetTyrCysAlaProIleLysProProLysSerAla
   TCTTGCGACCTGCGTCGTCTGGAAATGTACTGCGCTCCGATCAAACCGCCGAAATCTGCT
   AGAACGCTGGACGCAGCAGACCTTTACATGACGCGAGGCTAGTTTGGCGGCTTTAGACGA

   ******   HindIII
   TGATGATGCAAG-3'
   ACTACTACGTTC-5'
```

b)



cIGF-I fusion protein eluted from the Fast Flow Q Sepharose column was then concentrated on a C18 Matrex silica column and lyophilized as described in 3.2.3.

A portion of the lyophilized fusion protein (350 mg) was resuspended in 180 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 10 mmol EDTA/l, 20 mmol DTT/l at pH 9.1. Analysis of the resuspended protein on the standard analytical HPLC (2.2.2.3) after 30 min indicated that the fusion proteins were fully reduced. The [Arg^3]-cIGF-I fusion proteins were then refolded under oxidizing conditions by diluting the resuspended protein to a final protein concentration of 0.125 mg protein/ml in a solution containing 2 mol urea/l, 40 mmol glycine/l, 0.1 mol/Tris/l, 10 mmol EDTA/l, 0.4 mmol DTT/l and 1 mmol 2-hydroxyethyl disulphide/l at pH 9.1. Refolding was indicated by elution of the protein earlier than the reduced starting material when analyzed on the standard analytical HPLC (Fig. 5.2a & b).

The refold mixture was acidified to pH 2.8 with HCl and then chromatographed on a Fast Flow S Sepharose Column as described in 3.2. A single fraction containing the [Arg^3]-cIGF-I fusion protein was further purified by three reverse-phase HPLC steps on a C4 PrepPak 500 cartridge (57 mm (diameter) x 300 mm). The first reverse-phase HPLC utilized a linear gradient from 25.6 to 54.4% (v/v) acetonitrile in 0.1% TFA over 144 min at a flow rate of 25 ml/min. The second HPLC step employed a gradient from 29.6 to 52% propan-1-ol (v/v) in 0.13% HFBA over 224 min at a flow rate of 25 ml/min. The third and final HPLC step eluted the [Arg^3]-cIGF-I fusion protein from the column using a linear gradient from 30.4 to 40% acetonitrile (v/v) in 0.1% TFA over 192 min at a flow rate of 25 ml/min. The recovery of the [Arg^3]-cIGF-I fusion protein during the purification was monitored by analysis on the standard analytical HPLC (2.2.2.3), by electrophoresis on high density gels using Pharmacia's 'PhastSystem' (2.2.3.3), and by assessing fractions for their ability to stimulate protein synthesis in L6 rat myoblasts (2.2.4.1).

The [Arg^3]-cIGF-I fusion peptide was quantitated by calculating the area under their absorbance profile at 215 nm when analysed on the standard

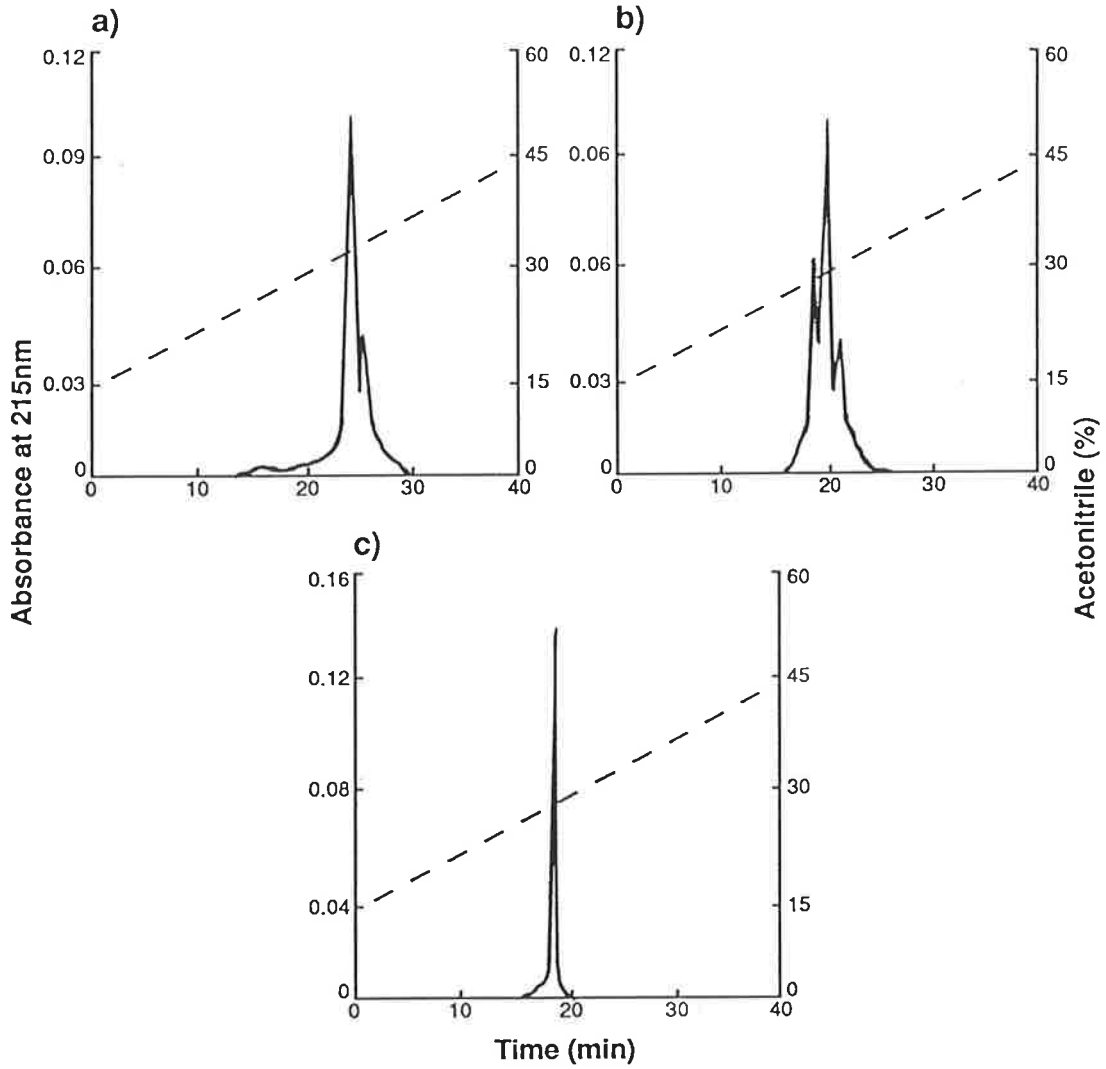


FIGURE 5.2: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) a) before and b) after refolding of chicken Long [Arg^3]-IGF-I. Analysis after the final purification of chicken Long [Arg^3]-IGF-I is shown in c). Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.

analytical HPLC (2.2.2.3). The area under the absorbance profile was converted to protein concentration using a calculated extinction coefficient of 35.65 mol^{-1} cm^{-1} (2.2.2.4). The N-terminal protein sequence and mass quantitation were determined as described in 2.2.3.1 and 2.2.3.2 respectively.

5.2.3: *In vitro* characterization of [Arg^3]-cIGF-I fusion protein

In vitro characterization of the biological actions, receptor and IGFBP interactions of recombinant [Arg^3]-cIGF-I fusion protein was determined using the biochemical assays described in 2.2.4.

5.2.4: Radioimmunoassay

Immunoreactivity was measured by employing a polyclonal antibody raised against Long [Arg^3]-hIGF-I and the method described by in 2.2.4.5. Recombinant chicken IGF-I was used as the radioligand in this assay. *This assay was performed by Dr. K. Kita, visiting scientist, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia.*

5.3: RESULTS

5.3.1: Expression and downstream processing of the fusion protein.

A recombinant analogue of cIGF-I with an 18 amino acid N-terminal extension as well as a substitution of Arg for Glu at position 3 in cIGF-I has been expressed in *E. coli*. The protein, [Met^1]pGH(1-11)-Val-Asn-Gly-Phe-Ala-His-Tyr-[Arg^3]-cIGF-I, will subsequently be referred to as Long [Arg^3]-cIGF-I. The N-terminal extension peptide contains the first 11 residues of methionyl porcine growth hormone followed by a short peptide sequence that includes recognition sequences for cleavage by hydroxylamine (Asn-Gly) and by H64A subtilisin-BPN' (Phe-Ala-His-Tyr) (Fig. 5.3). A 15 litre fermentation of *E. coli* JM101 cells transformed with the expression vector coding for Long [Arg^3]-cIGF-I yielded 100g wet weight of inclusion bodies following induction with IPTG.

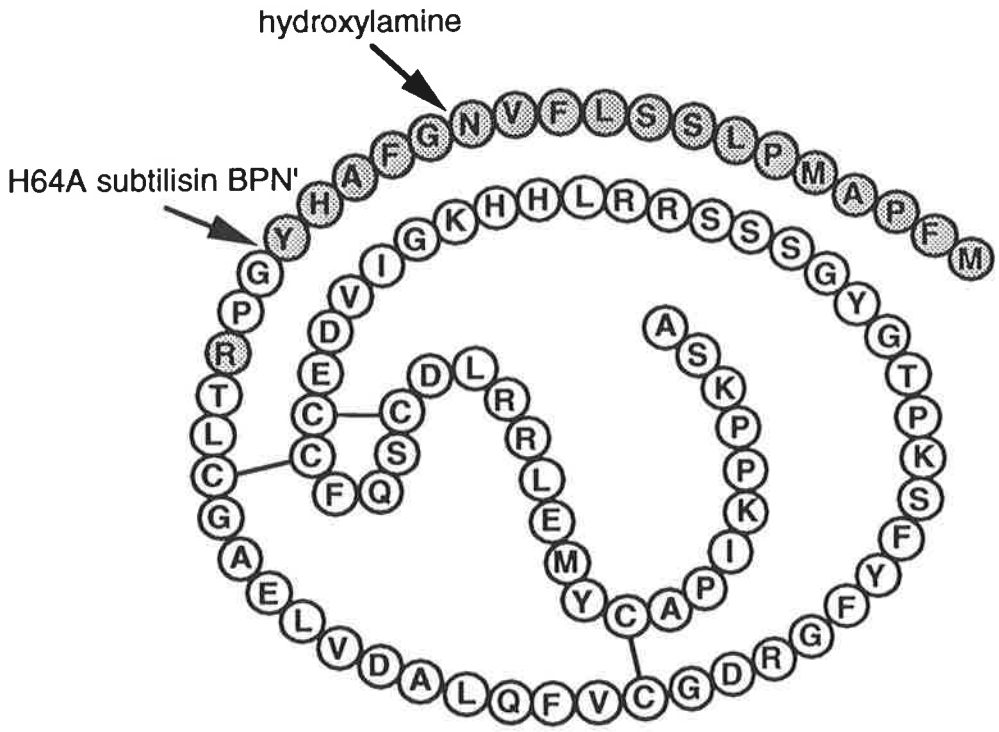


FIGURE 5.3: Schematic representation of recombinant chicken Long [Arg^3]-IGF-I. The cleavage sites for hydroxylamine and H64A subtilisin BPN' are indicated.

Francis *et al.* (1992) have previously reported the downstream processing of a similar recombinant human fusion protein analogue, Long [Arg^3]-hIGF-I. The purification strategy described here for Long [Arg^3]-cIGF-I is similar except for the inclusion of a anion exchange chromatography step as well as an additional reverse-phase HPLC separation. The anion exchange chromatography step was included to minimise proteolysis by bacterial proteases. Yields from the dissolution, reduction, desalting, and anion-exchange chromatography of 25 g of inclusion bodies were high, with 1.24 g of total protein being recovered. The subsequent refolding and reverse-phase chromatography on 350 mg of this protein yielded 80 mg of pure Long[Arg^3]-cIGF-I. The recombinant cIGF-I fusion protein eluted as a single peak on the standard analytical HPLC (Fig. 5.2c) and migrated as a single band at the expected size of 10 kDa on a SDS-polyacrylamide gel run under reducing conditions (Fig. 5.4, lane 4). N-terminal protein sequencing indicated that the protein had the correct amino-terminal sequence and was at least 97% pure. Electrospray mass spectrometry gave the expected mass of 9777 Da (Fig. 5.5).

5.3.2: Biological activity of recombinant Long [Arg^3]-cIGF-I

Long [Arg^3]-cIGF-I was compared with reference recombinant cIGF-I, hIGF-I and Long [Arg^3]-hIGF-I in a range of biochemical assays. Long [Arg^3]-hIGF-I is a related fusion-protein analogue similar to Long [Arg^3]-cIGF-I. It differs from the chicken analogue I produced in that it has a 13 amino acid N-terminal extension peptide rather than a 18 amino acid extension. The N-terminal extension of the human fusion-protein analogue contains the first 11 amino acid residues of methionyl porcine growth hormone, followed by the residues Val and Asn (Francis *et al.* 1992).

The biological actions of the chicken fusion-protein analogue were assessed in L6 rat myoblasts and in CEFs. Both the human and chicken fusion-protein analogues were equivalent in their ability to stimulate protein synthesis (half-maximal effects at 13 and 11 ng/ml respectively) in cultured L6 cells.

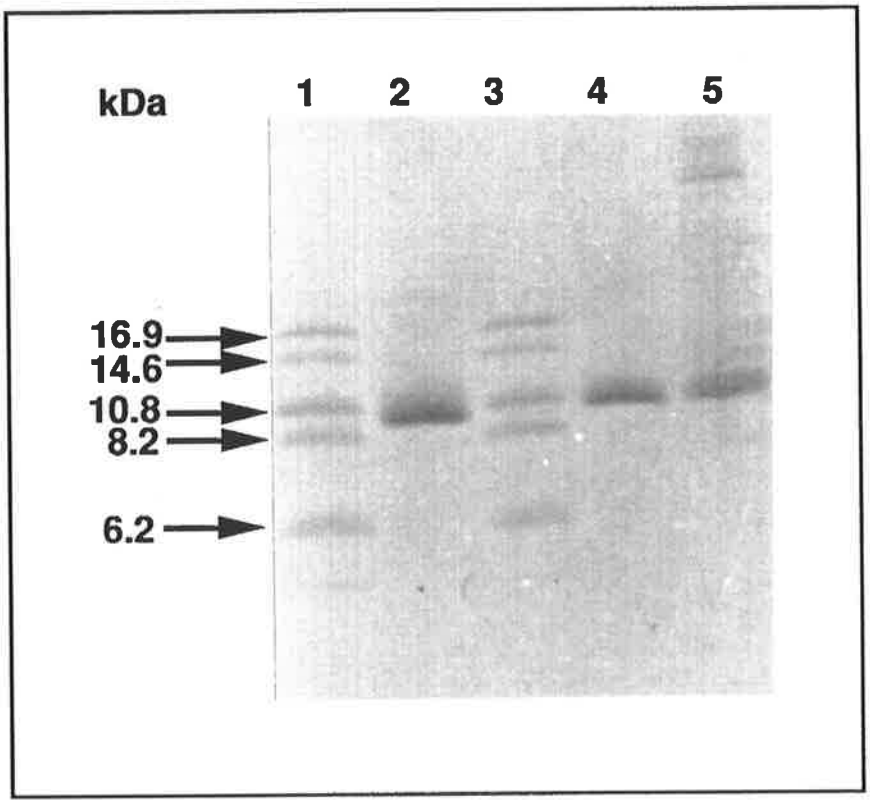


FIGURE 5.4: Polyacrylamide gel analysis of total cell protein from *E.coli* cells induced to express [Met1]-pGH(1-11)-Val-Asn-Gly-Phe-Ala-His-Tyr-[Arg3]-clGF-I (lane 5) and pure recombinant Long [Arg3]-clGF-I (lane 4). Reference recombinant human Long [Arg3]-IGF-I is in lane 2, while molecular mass markers are in lanes 1 and 3. Samples were electrophoresed on high-density SDS-polyacryl-amide gels under reducing conditions.

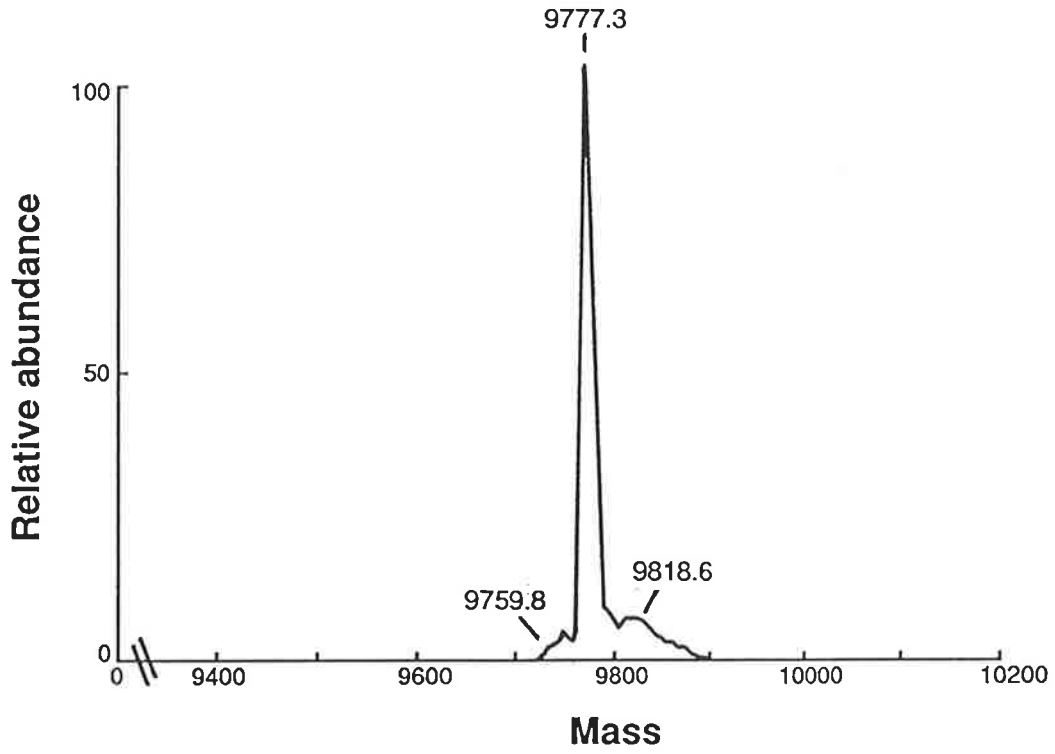


FIGURE 5.5: Electrospray mass analysis of chicken Long [Arg^3]-c-IGF. Numbers indicate the masses of the peaks.

Furthermore, both analogues were more potent than either cIGF-I or hIGF-I which exhibited half-maximal effects at 4.7 and 3.2 ng/ml respectively (Fig. 5.6a). The chicken fusion protein analogue however, was less effective than cIGF-I or hIGF-I in biological assays assessing the ability of the peptides to inhibit protein breakdown in CEFs. Whereas cIGF-I and hIGF-I exhibited half-maximal effects at 1.1 and 0.9 ng/ml respectively, 4.7 ng/ml of Long [Arg^3]-cIGF-I was required to achieve the same level of inhibition of protein breakdown (Fig. 5.7a). The human analogue was not compared in this assay.

5.3.3: Receptor-binding interactions of recombinant Long [Arg^3]-cIGF-I

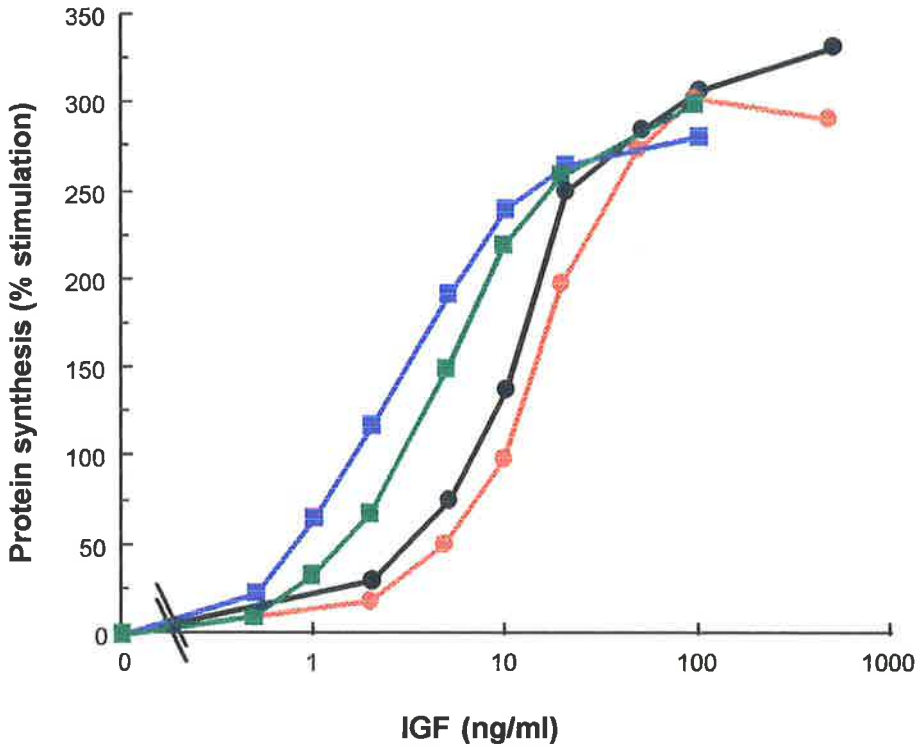
The IGF receptor-binding interactions of the chicken fusion-protein analogue were similar to its human counterpart in both chicken and rat cells. Thus Long [Arg^3]-cIGF-I and Long [Arg^3]-hIGF-I were both less effective (half-maximal effects at 4.8 and 5.0 ng/0.5 ml respectively) than either cIGF-I or hIGF-I (half-maximal effects at 1.1 and 1.2 ng/ml respectively) at competing for [^{125}I]-hIGF-I binding in L6 rat myoblasts. (Fig. 5.6b). The two fusion-protein analogues were also less effective than cIGF-I or hIGF-I at competing for binding of [^{125}I]-hIGF-I to the IGF receptor present in CEFs. Half-maximal effects were observed at 50, 48, 3.8 and 4.9 ng/0.5 ml for Long [Arg^3]-cIGF-I, Long [Arg^3]-hIGF-I, cIGF-I and hIGF-I respectively (Fig. 5.7b).

5.3.4: Binding protein interactions of recombinant Long [Arg^3]-cIGF-I

The biological actions of IGFs are determined by not only interactions with IGF receptors, but also by interactions with IGFBPs. Accordingly, the roles of IGFBPs in modulating the biological responses of the chicken fusion-protein analogue has been investigated. Binding to IGFBPs present in chicken and rat plasma (Fig. 5.8), as well as to purified ovine IGFBP3 and IGFBP4 (Fig. 5.9), has been assessed as indicated by competition for binding of [^{125}I]-hIGF-II. Both the human and chicken fusion-protein analogues exhibited little, if any, affinity for

FIGURE 5.6: Effects of IGFs on a) protein synthesis and b) competition for binding of [^{125}I]-labelled human IGF-I in rat L6 myoblasts. The proteins tested were human Long [Arg^3]-IGF-I (blue ■), chicken Long [Arg^3]-IGF-I (green ■), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein synthesis (% stimulation)
350
300
250
200
150
100
50
0
0
1
10
100
1000
IGF (ng/ml)

b)

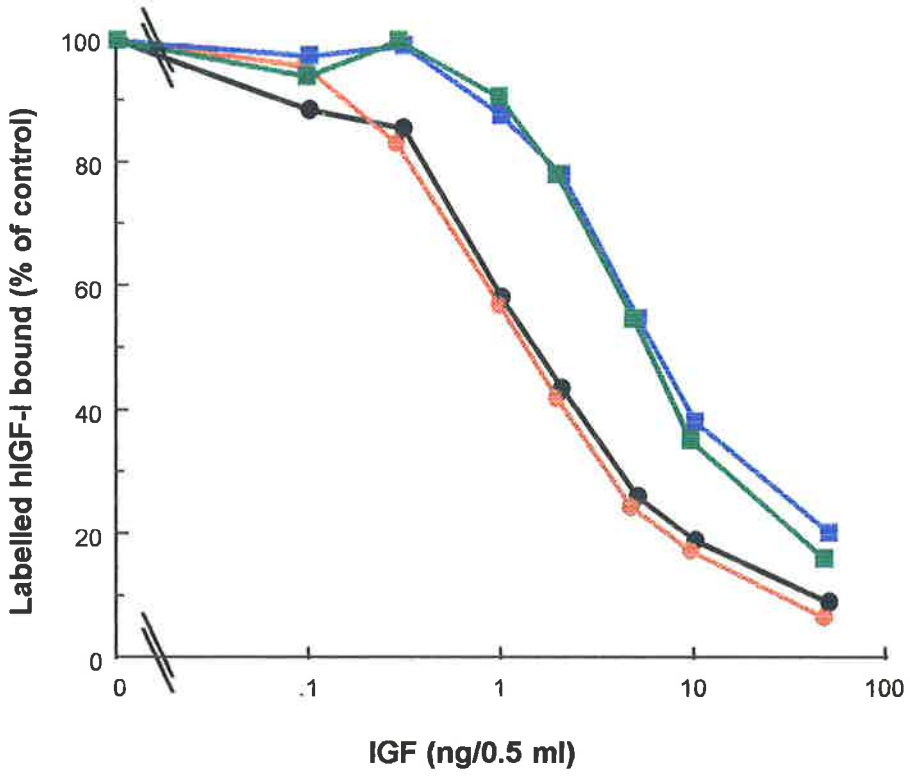
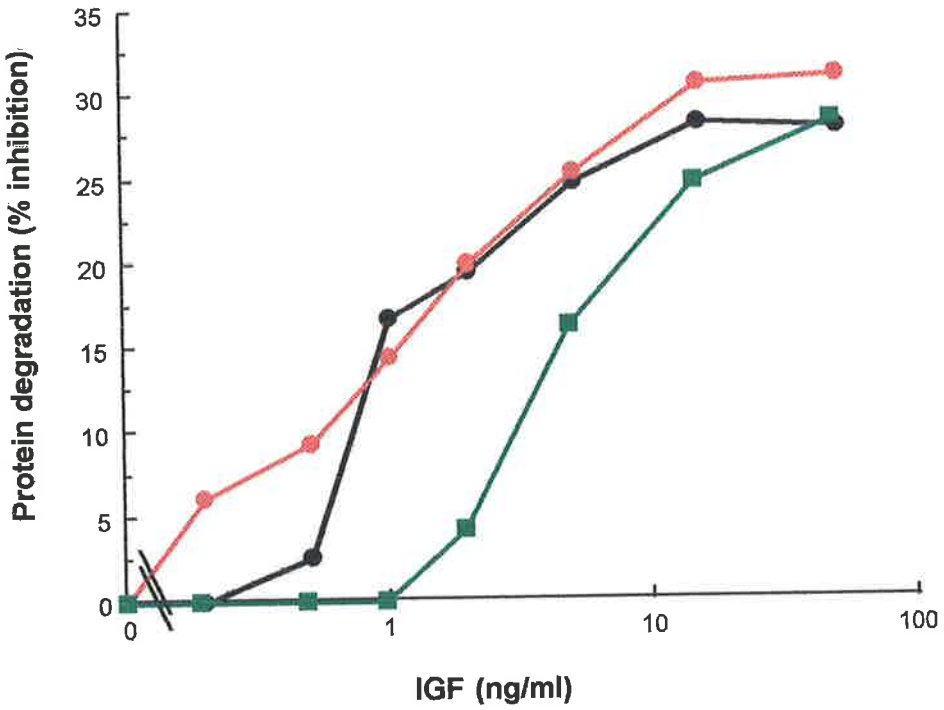

Labelled hIGF-I bound (% of control)
100
80
60
40
20
0
0
.1
1
10
100
IGF (ng/0.5 ml)

FIGURE 5.7: Effects of IGFs on a) protein breakdown and b) competition for binding of [^{125}I]-labelled human IGF-I in chick embryo fibroblasts. The proteins tested were human Long [Arg^3]-IGF-I (blue ■), chicken Long [Arg^3]-IGF-I (green ■), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein degradation (% inhibition)
35
30
25
20
15
10
5
0
0
1
10
100
IGF (ng/ml)

b)

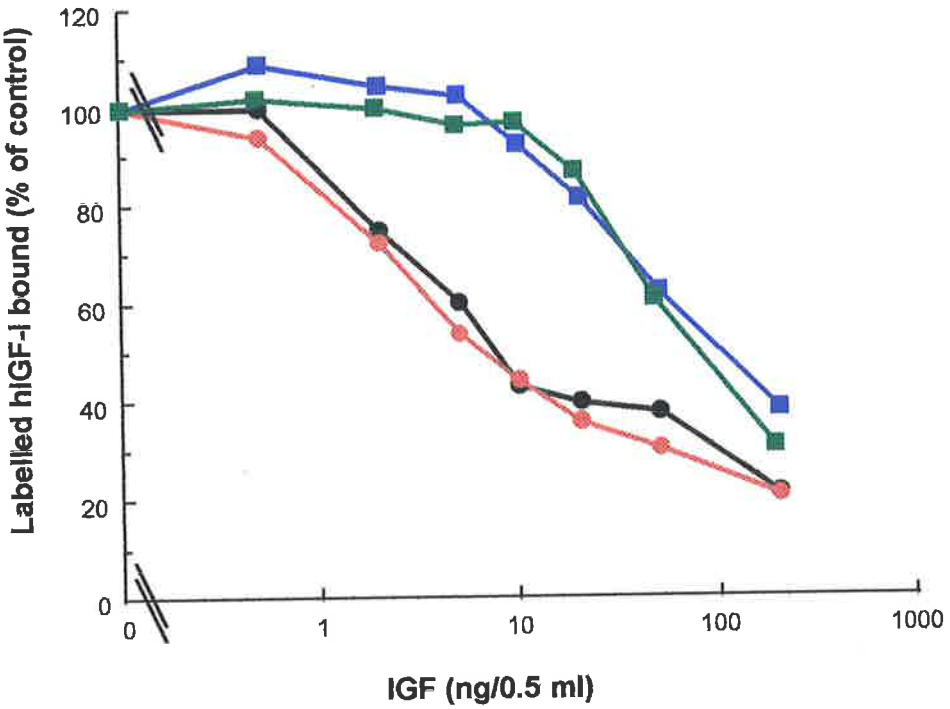
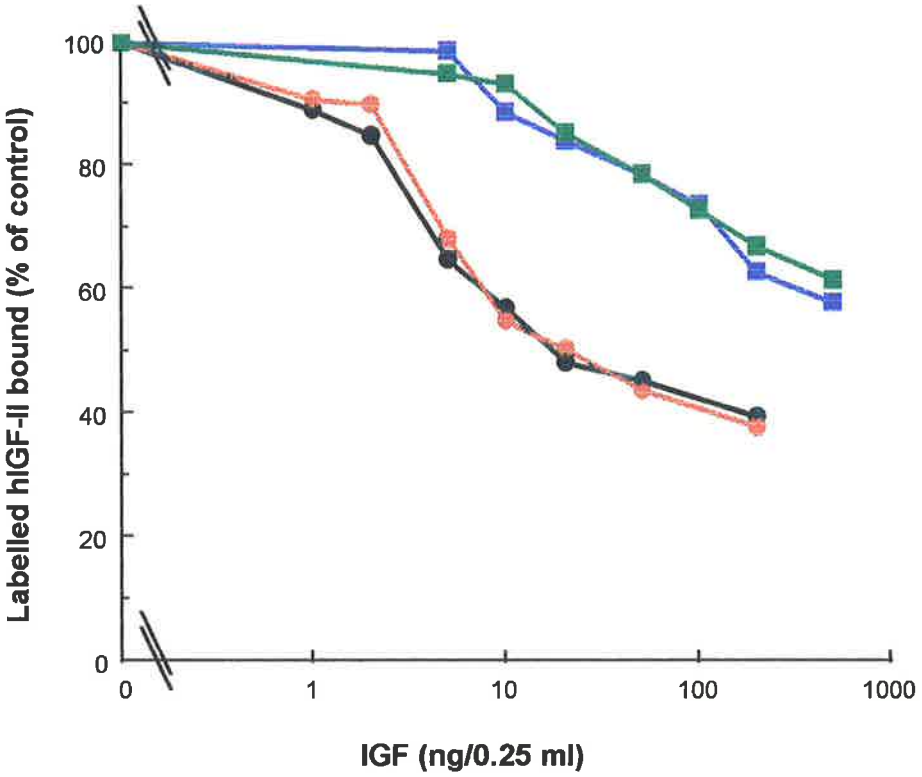

Labelled hIGF-I bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.5 ml)

FIGURE 5.8: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to IGFBPs in a) chicken and b) rat plasma. The proteins tested were human Long [Arg^3]-IGF-I (blue ■), chicken Long [Arg^3]-IGF-I (green ■), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

b)

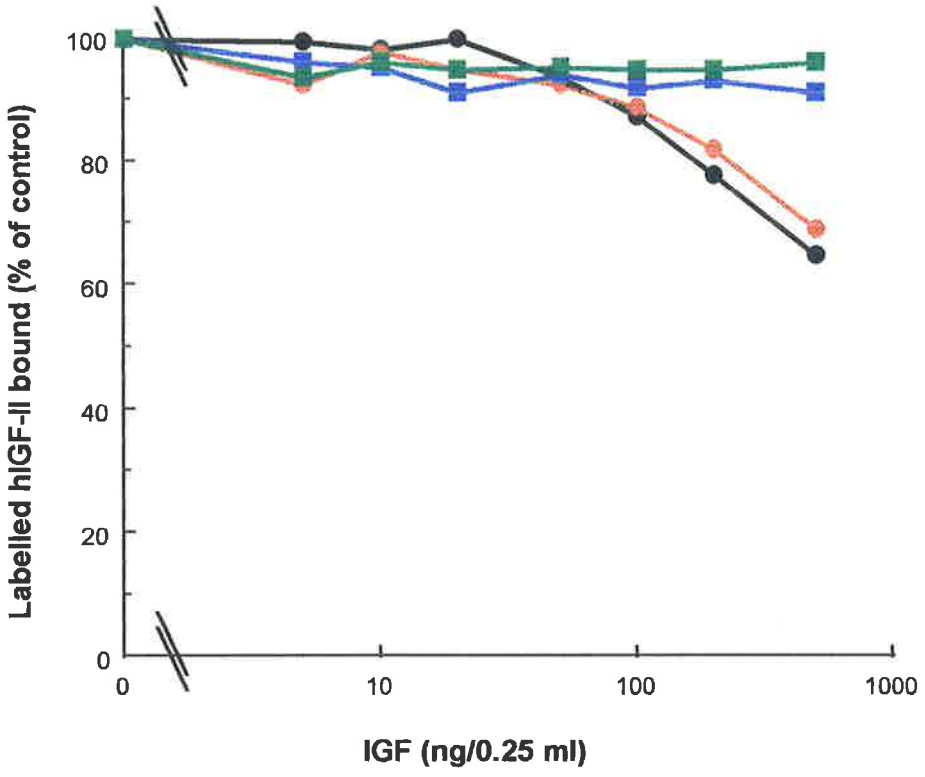
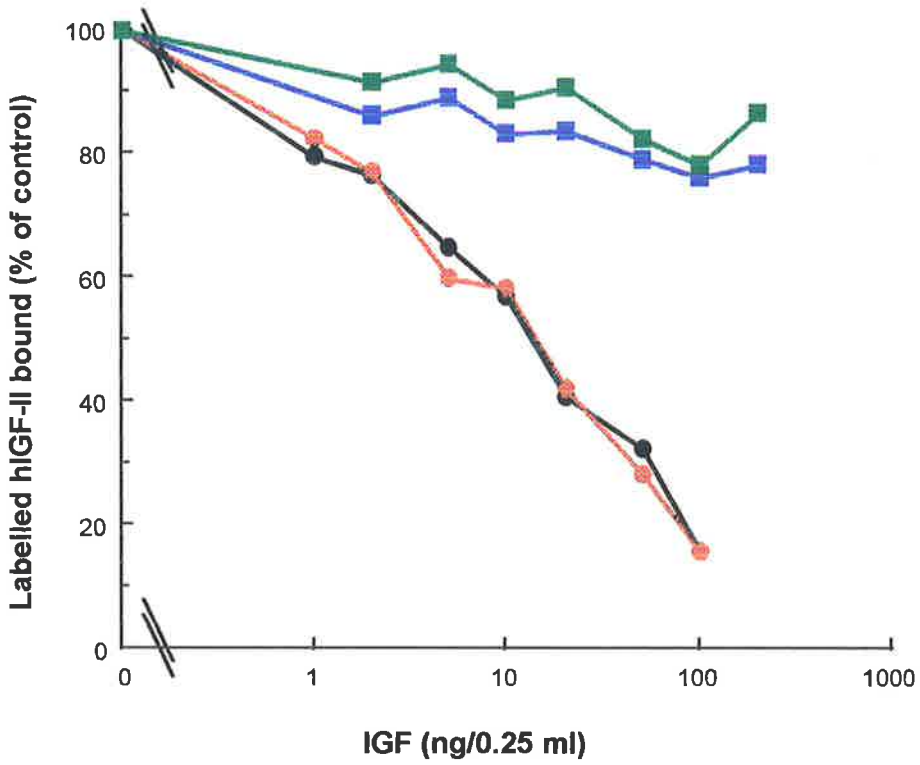
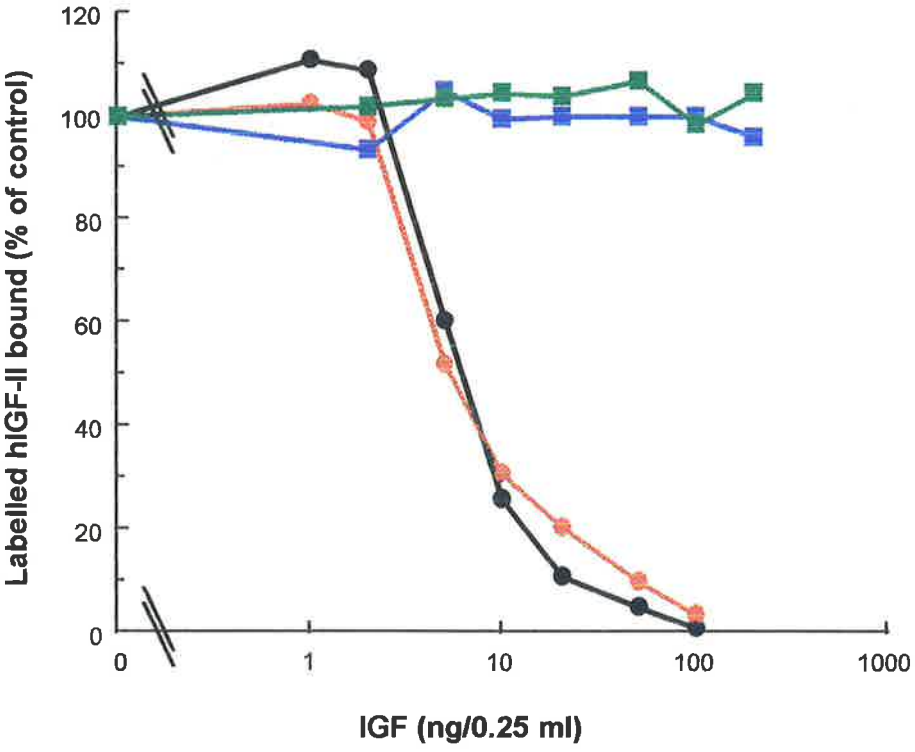

Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
10
100
1000
IGF (ng/0.25 ml)

FIGURE 5.9: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to a) purified ovine IGFBP3 and b) purified ovine IGFBP4. The proteins tested were human Long [Arg^3]-IGF-I (blue ■), chicken Long [Arg^3]-IGF-I (green ■), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

b)


Labelled hIGF-II bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

ovine IGFBP3 or IGFBP4, or for the total IGFBPs present in rat plasma. While the analogues did compete for binding to the IGFBPs present in chicken plasma they were less effective than either cIGF-I or hIGF-I (Fig. 5.8a).

5.3.5: Immunoreactivity of recombinant Long [Arg^3]-cIGF-I

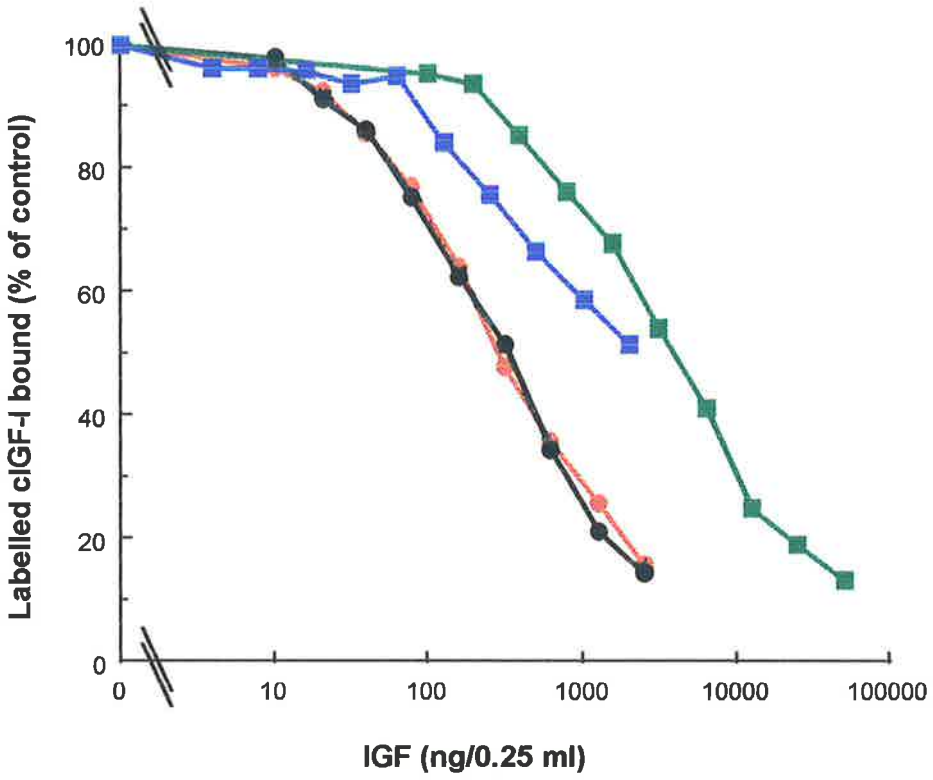
The cross-reactivity of the chicken fusion-protein analogue in a radioimmunoassay employing a polyclonal antibody raised against Long [Arg^3]-hIGF-I and labelled-cIGF-I as tracer, was compared to reference cIGF-I, hIGF-I and Long [Arg^3]-hIGF-I. Both cIGF-I and hIGF-I were equivalent at competing for binding to the antibody. However, the fusion protein analogues, Long [Arg^3]-cIGF-I and Long [Arg^3]-hIGF-I, differed 2-fold in their affinity for the antibody (Fig. 5.10). The difference in immunoreactivity is presumably caused by the five additional amino acid residues present in N-terminal extension of the chicken fusion-protein analogue.

5.4: DISCUSSION

In this chapter I describe the efficient production and subsequent *in vitro* characterization of a cIGF-I analogue. The protein, Long [Arg^3]-cIGF-I, has a 18 amino acid N-terminal extension peptide as well as a substitution of Arg for Glu at position 3 in cIGF-I. This particular analogue has been produced firstly because *in vitro* and *in vivo* studies with the human equivalent of this protein have shown that it is a more potent form of IGF-I and secondly, because high yields of the recombinant protein can be produced from *E. coli* inclusion bodies (Francis *et al.* 1992), hence making it a feasible growth factor for *in vivo* studies.

Several research groups have reported the production of IGFs from *E.coli.* but generally high-level expression has only been achieved when the IGFs are expressed as fusion-proteins (Peters *et al.* 1985; Moks *et al.* 1987; Forsberg *et al*, 1990; King *et al.* 1992). A fusion-protein strategy, however, creates additional problems. The extension peptides may hamper the solubility or refolding of the protein. Moreover, the expression of IGFs as fusion-proteins requires additional

FIGURE 5.10: Radioimmunoassay of IGFs using chicken IGF-I as radioligand and a polyclonal antiserum raised against recombinant human Long [Arg^3]-IGF-I. The proteins tested were human Long [Arg^3]-IGF-I (blue ■), chicken Long [Arg^3]-IGF-I (green ■), human IGF-I (black ●) and chicken IGF-I (red ●). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled cIGF-I bound (% of control)
100
80
60
40
20
0
0
10
100
1000
10000
100000
IGF (ng/0.25 ml)

downstream processing steps in order for the IGFs to be released and purified from the fusion protein. The approach reported by Francis *et al.* (1992), where the fusion-protein itself is more potent than hIGF-I, and consequently is the desired protein, has overcome some of these problems. Thus, Francis *et al.* (1992) report that the 13 amino acid N-terminal extension peptide in Long [Arg^3]-hIGF-I enhances expression in *E. coli.*, and subsequent refolding and solubility of the protein. Furthermore, production of this IGF-I analogue does not require a cleavage step to remove the leader peptide. Overall this leads to efficient production of the recombinant hIGF-I analogue and has permitted evaluation of this analogue as an anabolic growth factor in animal models of catabolic trauma (for example, Ballard *et al.* 1991; Tomas *et al.* 1991).

I have produced Long [Arg^3]-cIGF-I from *E. coli.* using a similar gene-fusion and downstream processing strategy. The cIGF-I analogue I produced differs from its human counterpart in that it has an 18, rather than a 13, amino acid N-terminal extension peptide (Fig. 5.3). The additional amino acids, Gly-Phe-Ala-His-Tyr, were incorporated to allow shortening of the leader peptide via hydroxylamine or release of [Arg^3]-cIGF-I from the fusion protein via H64A subtilisin BPN'. It has subsequently been established that H64A subtilisin BPN' shows markedly reduced catalysis rates when either the first (P1') or second (P2') amino acids following the cleavage site is a Pro residue (Carter 1990). As P2' in this fusion protein is Pro it is unlikely that H64A subtilisin BPN' would release [Arg^3]-cIGF-I from the leader peptide (Fig. 5.3). Nevertheless, the 18 amino acid extension peptide did not appear to hamper efficient production of the chicken analogue as the overall yield was comparable to that found for the human fusion-protein analogue (Francis *et al.* 1992).

As was found with its human counterpart, Long [Arg^3]-cIGF-I also exhibits decreased affinity for IGF receptors (Fig. 5.6b & 5.7b) and for IGFBPs (Fig. 5.8 & 5.9) while exhibiting enhanced biological potency in L6 rat myoblasts (Fig. 5.6a). However, both analogues were less effective than either cIGF-I or hIGF-I at inhibiting protein degradation in chick embryo fibroblasts (Fig. 5.7b). The

difference in biological potency between the rat and avian cell lines is most likely caused by the difference in the concentrations of IGFBPs produced by the cells. Thus, the rat L6 myoblasts produce abundant IGFBPs whereas the chick embryo fibroblasts produce little, if any IGFBPs (Ross *et al.* 1989). Consequently, in L6 rat myoblasts, interactions with IGF receptors as well as with IGFBPs are influencing the biological actions. In CEFs, on the other hand, the biological outcome is primarily determined by interactions with IGF receptors. Previous studies (Ballard *et al.* 1987; Francis *et al.* 1988; Bagley *et al.* 1989; King *et al.* 1992) have determined that Glu at position 3 in IGF-I is crucial for interactions with IGFBPs, hence the substitution of Arg for Glu in Long [Arg^3]-cIGF-I reduces the affinity of the protein for IGFBPs. Francis *et al.* (1992) have also established that the N-terminal extension peptide itself has an effect on not only IGFBP interactions but also IGF type-1 receptors. Thus the biological actions of the chicken fusion-protein analogue observed in cultured cells is dependent on the relative affinities for the type-1 IGF receptor present and for the IGFBPs secreted by the cells.

The expression and downstream processing approach I have described in this chapter will be useful for the production of large quantities of Long [Arg^3]-cIGF-I that will allow *in vivo* evaluation of the potential of this growth factor as an anabolic agent in chickens. Whether the cIGF-I analogue will improve growth performance in chickens remains to be determined. The reduced affinity of the analogue for IGFBPs may in fact mean that Long [Arg^3]-cIGF-I administered exogenously to chickens will be rapidly cleared from the circulation. Equally, since chickens have much reduced concentrations of serum IGFBPs (Francis *et al.* 1990), any form of IGF administered exogenously may be cleared rapidly. Alternatively, the low concentration of serum IGFBPs in chickens may actually lead to a more effective delivery of the growth factor, since there are less IGFBPs to withhold the IGFs from the potential tissue sites of action. Yet another effect to be considered is the lower affinity of the chicken fusion-protein analogue for the type-1 IGF receptor. Whether higher doses of the analogue would need to be

administered to overcome the reduced affinity for the type-1 IGF receptor, and hence diminish the cost-effectiveness of administering this growth factor also remains to be determined. Regardless, recombinant Long [Arg^3]-cIGF-I, with its significantly reduced affinity for IGFBPs, will be a useful tool for examining the interactions of chicken IGFBPs with IGFs both *in vitro* and *in vivo*.

CHAPTER 6

PRODUCTION AND CHARACTERIZATION OF RECOMBINANT HAGFISH IGF

6.1: INTRODUCTION

The amino acid sequences deduced from cDNAs for IGF-I from the nonmammalian species, chicken (Kajimoto & Rotwein, 1989; Fawcett & Bulfield, 1990), frog (Kajimoto & Rotwein, 1990) and salmon (Cao *et al.*, 1989), reveal a striking conservation of structure with mammalian IGF-Is (Fig. 1.6). The recent cloning of a cDNA for IGF-II from trout (Shamblott & Chen, 1992) along with the protein sequence reported for chicken IGF-II (Kallincos *et al.* 1990) suggest that the IGF-IIs also share a long phylogenetic history. The similarities in primary, secondary and tertiary structure of not only the IGFs, but also of pro-insulin, has led to suggestions that these proteins originated from a common precursor. Moreover, this concept was supported by the cloning of cDNA for an insulin-like peptide from amphioxus (Chan *et al.* 1990), a protochordate thought to be a possible relative of the invertebrate progenitor from which the vertebrates emerged (Pough *et al.* 1989; Fig. 6.1). The deduced amino acid sequence from the amphioxus cDNA suggested that the protein was as similar to insulin as it was to IGFs (Fig.1.10). It may thus may represent a transitional form connecting insulin with the IGFs. However, evidence for the existence of IGFs in lower vertebrates has been conflicting (Furlanetto *et al.* 1977; Wilson & Hintz, 1982; Lindahl *et al.*, 1985; Daughaday *et al.*, 1985; Cao *et al.* 1989; Drakenberg *et al.* 1989; Funkenstein *et al.*, 1989). Nevertheless, the situation was resolved with the cloning of an IGF cDNA from the Agnanthan species, Atlantic hagfish, a representative of the lowest vertebrate class (Nagamatsu *et al.* 1991). The deduced amino acid sequence from the cDNA revealed a protein that was very different from insulin and as similar to IGF-I as it was to IGF-II (Fig. 1.8), hence demonstrating that the divergence of the IGF and insulin genes occurred prior to the separation of the Agnantha from the main line of vertebrate evolution approximately 550 million years ago (Fig. 6.1). Furthermore, the predicted amino acid sequence of this primitive IGF indicated that the organization and tertiary structure of of IGFs have been extensively conserved throughout vertebrate evolution.

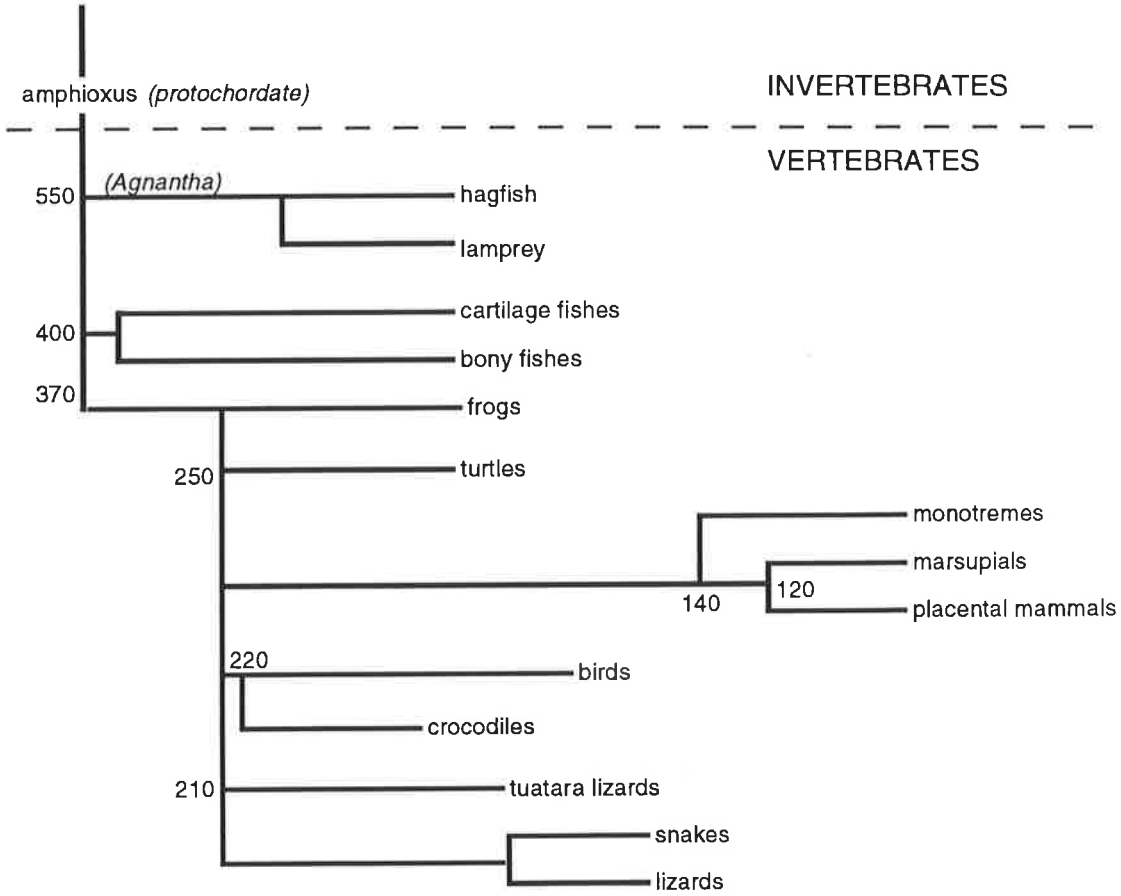


FIGURE 6.1: Simplified representation of the evolutionary relationships of the vertebrates discussed in this thesis. Numbers indicate the time (million of years) since the species diverged. (Based on information in Pough *et al.* 1989).

While there is structural evidence that the IGFs share a long evolutionary history, little is known about the conservation of IGF function. Chicken IGFs are the only nonmammalian IGFs to have been functionally characterized thus far (Dawe *et al.* 1988; Kallincos *et al.* 1990; Armstrong *et al.* 1990; Upton *et al.* 1992). In order to address this, I have made recombinant hagfish IGF and report in this chapter the production and characterization of this primitive vertebrate protein.

6.2: MATERIALS AND METHODS

6.2.1: Plasmid construction

Drs. S. Chan and D. Steiner, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA., generously provided the cDNA for hagfish IGF as reported in Nagamatsu *et al.* (1991). I subcloned the EcoR1-HindIII fragment of this cDNA into the multifunctional vector pTZ18, and using several rounds of site-directed mutagenesis (see 2.1.2 and 2.2.1.2), selectively optimized the codons for protein synthesis in *E.coli*. The oligonucleotides employed to direct these changes are depicted in Fig. 6.2. In addition to optimizing the hagfish IGF codons for protein synthesis in *E. coli*, Oligonucleotide 10 also introduced codons for the amino acids Val-Asn-Gly immediately upstream of the mature hagfish IGF protein. The codons for Val-Asn-Gly were included firstly because the DNA sequence for these residues includes a HpaI restriction endonuclease recognition site, and secondly, the amino acids Asn-Gly provide a specific and convenient hydroxylamine cleavage site. As depicted in Fig. 6.3, two mutagenic oligonucleotides were used at the same time to introduce changes to the hagfish cDNA. Once all the required changes had been introduced as confirmed by dideoxy DNA sequencing, the HpaI-HindIII fragment of the pTZ clone was ligated into the HpaI-HindIII cut p[Met^{1}]-pGH(1-46)-Val-Asn expression vector described in 4.2.1. This expression vector permits high level expression of IGFs in *E. coli* as fusion proteins containing the first 46 amino acids of methionyl porcine growth

Oligonucleotide 10:
5'-CTCACAAGGTGCACAGTTAACGGCCTGTCTGAAACACTTTGCGGTTCT-3'

Oligonucleotide 11:
5'-GAGGAGGGGAGCGCACCGTCGTTCTCGTGCTCGTAAAGGCATCGTTGAAG
AATGCTGCTTC-3'

Oligonucleotide 12:
5'-CTACCTGTCTGAAACCCTGTGCGGCTCTGAACTGGTTGACACCCTGCAGTT
TGTTTGT-3'

Oligonucleotide 13:
5'-ATGCTGCTTCAAAGGCTGCTCTCTGCGTCTGCTGGAAATGTAC-3'

Oligonucleotide 14:
5'-TGACACCCTGCAGTTCGTTTGCGACGACCGTGGCTTCTTCTTCGTTCCACA
ACACGTACCT-3'

Oligonucleotide 15:
5'-AATGTACTGCGCTCGTCCGTCTAAAGCTGAACGTGACGTTGCTAGACCAA
GGCAGAGA-3'

Oligonucleotide 16:
5'-CTTCTTCTTCGTTCCGCAGCACGTCCGCCGCGTCGTGGCGCTCACCGTCG
TTCTCGT-3'

Oligonucleotide 17:
5'-GAACGTGACGTTGCTTGATGAAAGCTTAGACCAAGGCAGAGA-3'

FIGURE 6.2: Oligonucleotides employed to direct the nucleotide changes introduced into the hagfish IGF cDNA using *in vitro* site-directed mutagenesis.

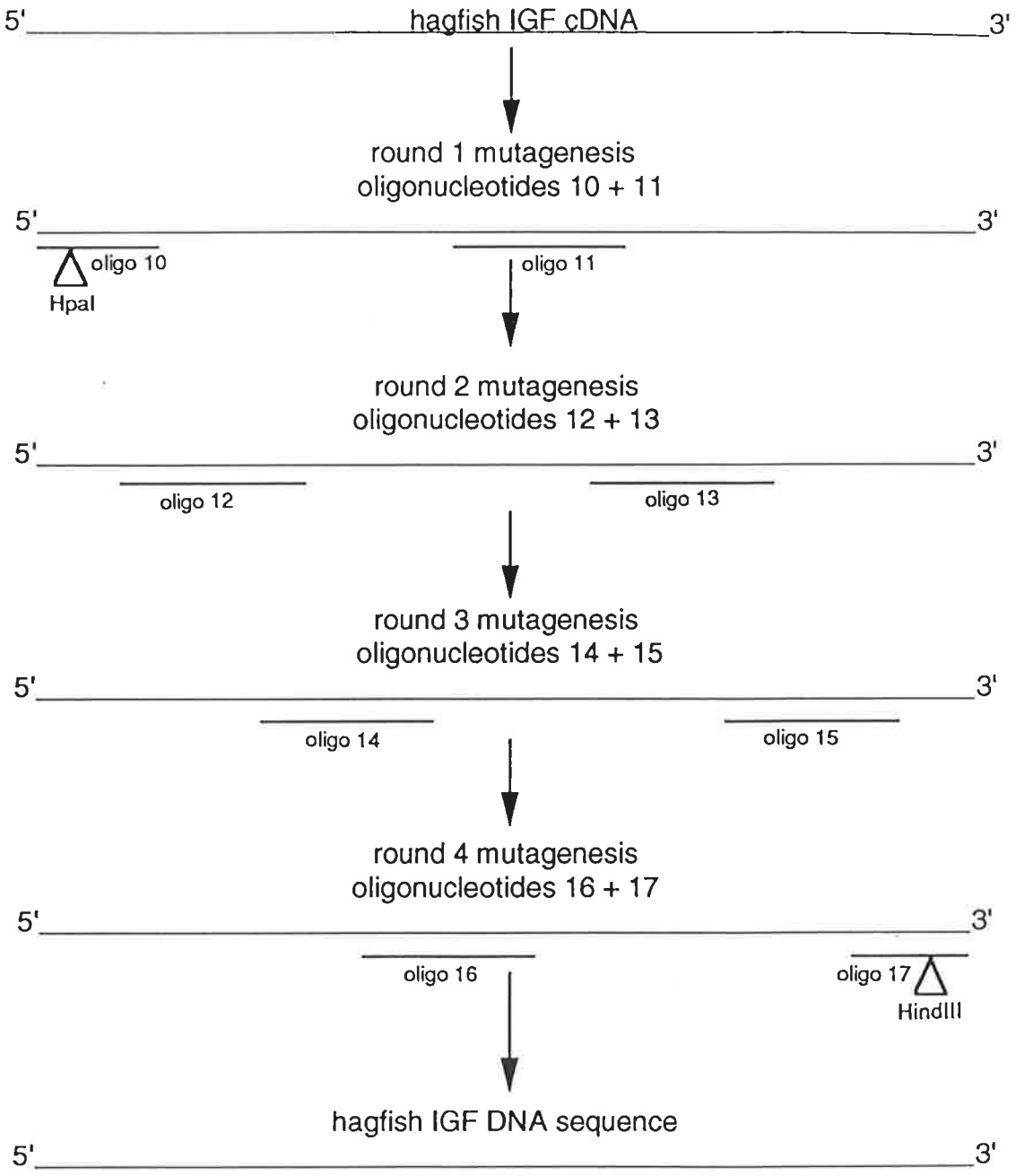


FIGURE 6.3: Nucleotide changes were introduced into the hagfish IGF cDNA using sequential rounds of mutagenesis. The order in which the mutagenic changes were introduced is schematically represented above.

hormone, followed by the residues Val and Asn. The DNA sequence of the HpaI-HindIII hagfish IGF fragment and a schematic representation of the final construct are shown in Fig. 6.4. This construct was then transformed into *E. coli* JM101 ($lacI^q$) at 37°C on minimal agar containing 29 µmol ampicillin/l. Fermentation of the transformed *E. coli* and isolation of the inclusion bodies produced was performed as described in 2.2.1.3.

6.2.2: Purification of the hagfish IGF fusion protein

Inclusion bodies (5 g) containing the hagfish IGF fusion protein were solubilized in 50 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 0.5 mmol $ZnCl_2$/l and 40 mmol DTT/l at pH 9.1. The reduced, solubilized inclusion bodies were filtered through a 0.45 µm HA filter (Millipore, Sydney, NSW, Australia), then desalted on a 50 mm (diameter) x 900 mm Fractogel TSK HW-55 F (Merck, Damstardt, FRG) column equilibrated with 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 0.5 mmol $ZnCl_2$/l and 1.6 mmol DTT/l at pH 9.1 at 1 ml/min. Fractions (20 ml) eluted from the column were analysed on high-density polyacrylamide gels using Pharmacia's "PhastSystem" (2.2.3.3) and those containing protein of the expected mass of 14.5 kDa for the hagfish IGF fusion protein were pooled. The pooled fractions were refolded under oxidizing conditions by dilution to a final concentration of 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 10 mmol EDTA/l, 0.4 mmol DTT/l and 1 mmol 2-hydroxyethyl disulphide/l at pH 9. The refolding reaction was monitored by analysis on the standard analytical HPLC described in 2.2.2.3. After 90 min the reaction was stopped by acidification to pH 2.1 with HCl. The acidified pool was concentrated on a C18 Matrex silica column and lyophilized as described in 3.2.3.

6.2.3: Cleavage of the fusion protein by H64A subtilisin BPN'

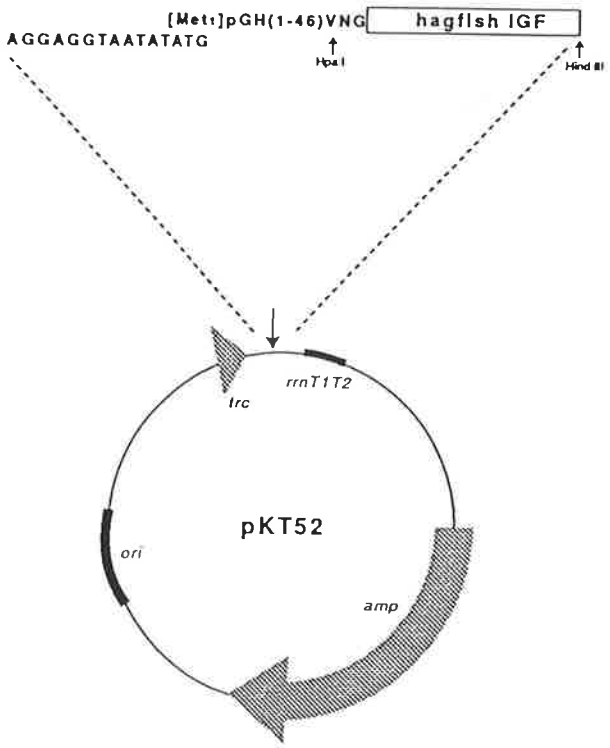
A portion of the lyophilized fusion protein (60 mg) was resuspended in 1 litre of 8 mol urea/l, 20 mmol Tris/l, 5 mmol $CaCl_2$/l and 0.2 mol NaCl/l at pH 8.5. The mixture was prewarmed to 37°C before adding 5.8 mg of H64A subtilisin

FIGURE 6.4: a) Nucleotide and corresponding amino acid sequence of the Hpa1-HindIII fragment of the construct engineered for expression of hagfish IGF. Underlined nucleotides indicate regions where changes were introduced to the DNA sequence of the starting vector by site-directed mutagenesis. Amino acids are numbered and stop codons are marked by asterisks. b) Schematic representation of the construct engineered for expression of hagfish IGF in *E. coli*. The expression plasmid, pKT52, contains an origin of replication (*ori*), a *trc* promoter, a transcription termination sequence (*rrnT1T2*) and a gene conferring ampicillin resistance (*amp*). The DNA sequence preceding Met has been modified to optimize ribosome binding (Vize & Wells, 1987).

a)

```
HpaI     1                                                        17
   AsnGlyLeuSerGluThrLeuCysGlySerGluLeuValAspThrLeuGlnPheVal
5'-AACGGCCTGTCTGAAACCCTGTGCGGCTCTGAACTGGTIGACACCCTGCAGTTCGTT
3'-TTGCCGGACAGATTTTGGGACACGCCGAGACTTGACAACCTGTGGGACGTCAAGCAA

                                                                 36
   CysAspAspArgGlyPhePhePheValProGlnHisValProProArgArgGlyAla
   TGCGACGACCGTGGCTTCTTCTTCGTICCGCAGCACGTICCGCCGCGICGIGGCGCI
   ACGCTGCTGGCACCGAAGAAGAAGCAAGGCGTCGTGCAAGGCGGCGCAGCACCGCGA

                                                                 55
   HisArgArgSerArgAlaArgLysGlyIleValGluGluCysCysPheLysGlyCys
   CACCGICGITCICGIGCTCGIAAAGGCATCGTIGAAGAATGCTGCTTCAAAGGCTGC
   GTGGCAGCAAGAGCACGAGCATTTCCGTAGCAACTTCTTACGACGAAGTTTCCGACG

                                                                 74
   SerLeuArgLeuLeuGluMetTyrCysAlaArgProSerLysAlaGluArgAspVal
   TCICTGCGICTGCTGGAAATGTACTGCGCTCGICCGTCIAAAGCIGAACGIGACGTT
   AGAGACGCAGACGACCTTTACATGACGCGAGCAGGCAGATTTCGACTTGCACTGCAA

    75
   Ala******HindIII
   GCTIGATGAAAG-3'
   CGAACTACTTTC-5'
```

b)



BPN' and incubating at 37°C for 4.5 h. DTT was then added to a final concentration of 20 mmol/l. After 60 min, the reduced digest was acidified to pH 2.8 with HCl. The subtilisin cleaves the fusion protein between Gln and His at positions 19-20 in [Met1]-pGH(1-46) in the leader peptide (see 4.3.2), thus creating a 12.1 kDa protein, pGH(20-46)-Val-Asn-Gly-Hagfish IGF (Fig. 6.5). Progress of the digest was followed by analysis using the standard analytical HPLC (2.2.2.3).

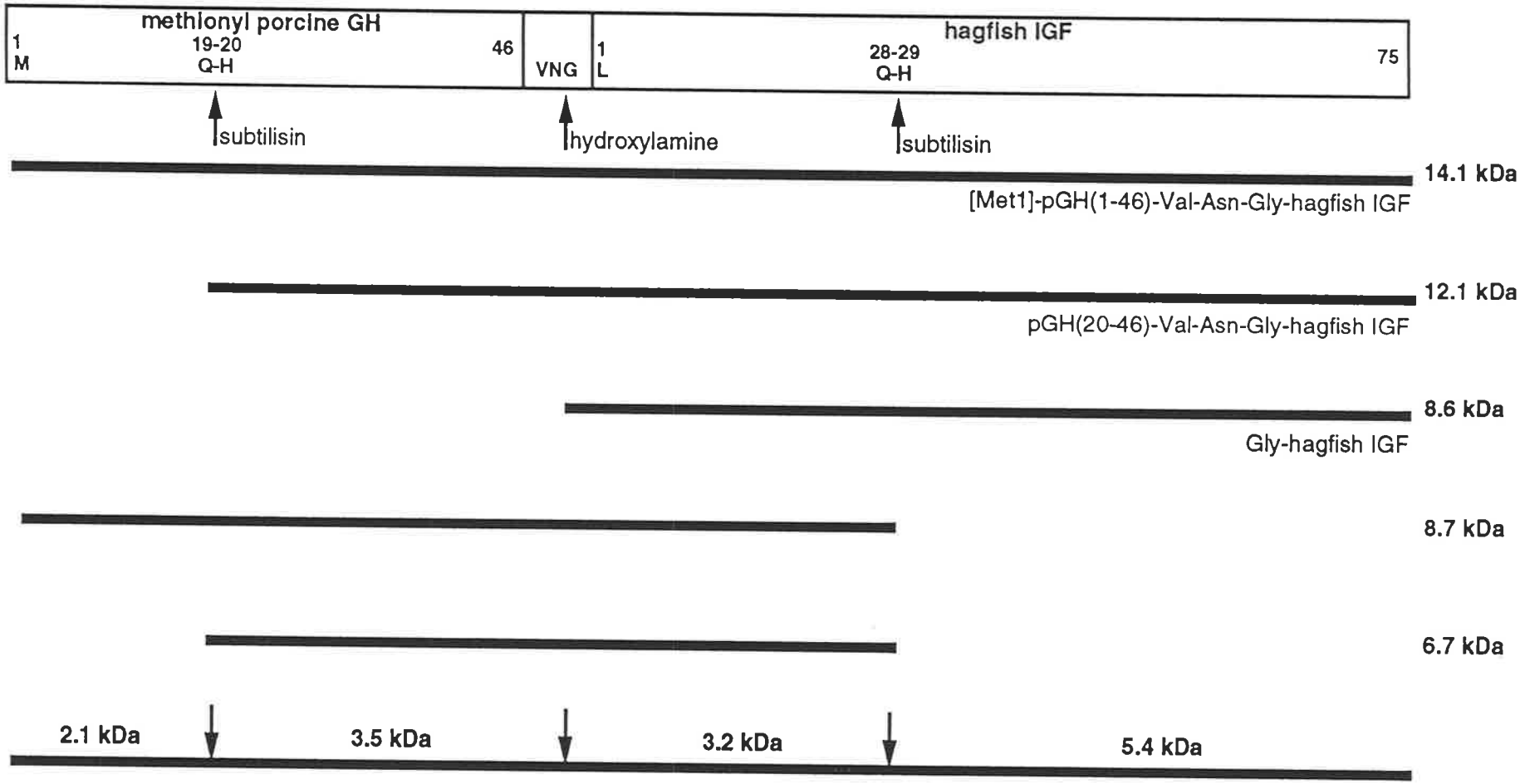
The acidified, reduced digest was purified by processing one third of the digest at a time on a C4 PrepPak column (25 mm (diameter) x 100 mm) utilizing a gradient from 29.6 to 50% acetonitrile in 0.1% TFA over 214 min, at a flow rate of 5 ml/min. Fractions (5 ml) eluted from the column were analyzed on high-density polyacrylamide gels and those containing protein with a molecular mass of approximately 12.1 kDa were pooled and lyophilized (Fig. 6.6, lane 4).

The lyophilized 12.1 kDa fusion protein (30 mg) was resuspended in 20 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 10 mmol EDTA and 20 mmol DTT at pH 9. The reduced 12.1 kDa fusion protein was then refolded in a final volume of 1 litre as described above. The refolded protein was concentrated on a C4 PrepPak column and lyophilized.

6.2.4: Cleavage of the 12.1 kDa fusion protein by hydroxylamine

The lyophilized, refolded 12.1 kDa fusion protein (18 mg) was cleaved at the unique Asn-Gly bond by incubation at 45°C for 13.5 h in 150 ml of a solution containing 2 mol hydroxylamine-HCl/l, 0.1 mol Tris/l, with the pH adjusted to 8.5 with LiOH. [Gly]-hagfish IGF is released from the fusion protein when hydroxylamine cleaves at the Asn-Gly bond (Fig. 6.5). The mixture was sparged with N_2 during the incubation to reduce oxidation of methionine residues. The reaction was terminated by acidification to pH 2.8 with HCl.

FIGURE 6.5: Schematic representation of the fragments generated following digestion of the hagfish IGF fusion protein with hydroxylamine and H64A subtilisin BPN'. Arrows indicate the cleavage sites. The single letter amino acid code is used within the box representing the intact fusion protein.


methionyl porcine GH
1
M
19-20
Q-H
46
VNG
hagfish IGF
1
L
28-29
Q-H
75
subtilisin
hydroxylamine
subtilisin
14.1 kDa
[Met1]-pGH(1-46)-Val-Asn-Gly-hagfish IGF
12.1 kDa
pGH(20-46)-Val-Asn-Gly-hagfish IGF
8.6 kDa
Gly-hagfish IGF
8.7 kDa
6.7 kDa
2.1 kDa
3.5 kDa
3.2 kDa
5.4 kDa

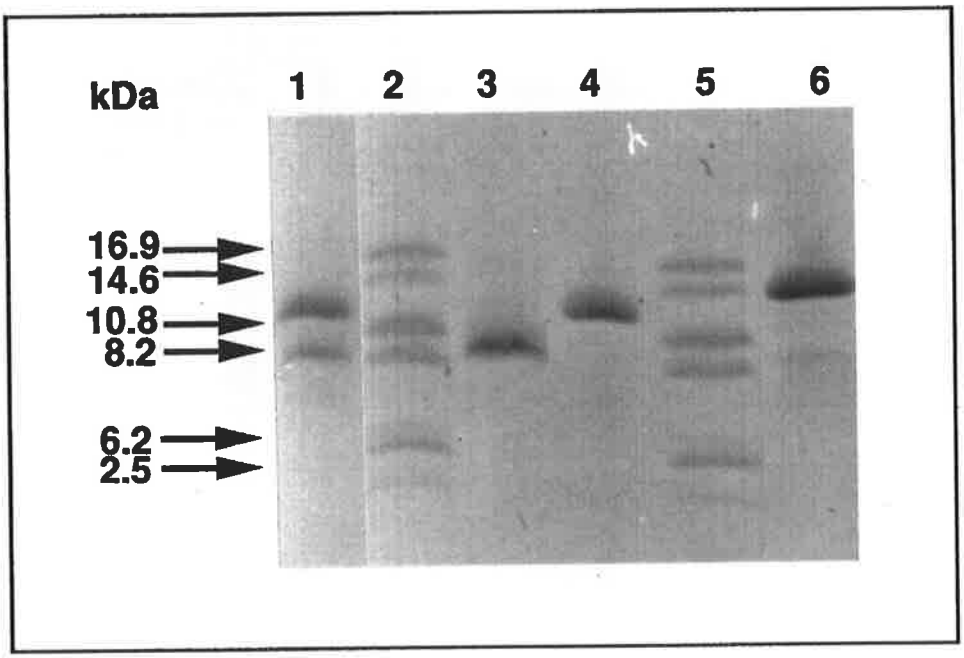


FIGURE 6.6: Polyacrylamide gel analysis of partially pure hagfish IGF fusion protein (lane 6), the 12.1 kDa fusion protein fragment isolated following digestion with H64A subtilisin BPN' (lane 4), the 12.1 kDa fragment digested with hydroxylamine (lane 1) and pure recombinant [Gly]-hagfish IGF (lane3). Molecular mass markers are in lanes 2 and 5. Samples were electrophoresed on high-density SDS-polyacrylamide gels under reducing conditions.

6.2.5: Purification of [Gly]-hagfish IGF

[Gly]-hagfish IGF was purified from the acidified digest using several reverse-phase chromatography steps. The first HPLC employed a C4 PrepPak column (25 mm (diameter) x 100 mm) and a gradient from 24.8 to 50% acetonitrile in 0.1% TFA over 252 min at a flow rate of 5 ml/min. Fractions (5 ml) containing the 8.6 kDa [Gly]-hagfish IGF protein (Fig. 6.5), as assessed by analysis on high density polyacrylamide gels, were pooled. Half of the pool was then processed in 8 separate batches on a C4 microbore HPLC column (2.1 mm (diameter) x 100 mm) employing a linear gradient from 28.2 to 30.7% propan-1-ol in 0.13% HFBA over 250 min at a flow rate of 0.5 ml/min. Fractions (0.25 ml) were pooled according to their ability to stimulate protein synthesis in rat myoblasts (2.2.4.1). The pooled fractions (500 µg) were concentrated on a C4 microbore HPLC column and lyophilized.

6.2.6: Refolding of [Gly]-hagfish IGF

The lyophilized [Gly]-hagfish IGF was resuspended in 2 ml 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l, 5 mmol EDTA/l and 16 mmol DTT/l at pH 9. The reduced proteins were then refolded under oxidizing conditions as described previously, but this time in a final urea concentration of 4 mol urea/l. After 90 min the mixture was acidified to pH 2.8 with HCl and the proteins separated on a reverse-phase HPLC employing a C18 NovaPak column (8 mm (diameter) x 100 mm) and a linear gradient from 24.8 to 40% acetonitrile in 0.1% TFA over 152 min. Fractions were pooled according to their ability to stimulate protein synthesis in L6 rat myoblasts (2.2.4.1).

6.2.7: Chemical analysis of [Gly]-hagfish IGF

The pooled fractions containing the peak of bioactivity were analysed on the standard analytical HPLC (2.2.2.3) and by electrophoresis on high density polyacrylamide gels (2.2.3.3). The [Gly]-hagfish IGF peptide present in the pooled fractions was quantitated by calculating the area under the absorbance

profile when eluted from the standard analytical HPLC. The area under the absorbance profile was converted to protein concentration using the calculated extinction coefficient of 31.70 mol^{-1} cm^{-1} for [Gly]-hagfish IGF (2.2.2.4). The N-terminal protein sequence and mass quantitation were determined as described in 2.2.3.1 and 2.2.3.2 respectively.

6.2.8: *In vitro* characterization of [Gly]-hagfish IGF

In vitro characterization of the biological and receptor interactions of [Gly]-hagfish IGF were determined using the biochemical assays detailed in 2.2.4.

6.3: RESULTS

6.3.1: Generation and expression of the hagfish fusion protein

Using the cDNA for hagfish IGF (Nagamatsu *et al.* 1991) as a template and employing site-directed mutagenesis, I optimized all of the codons for protein synthesis in *E. coli*. This approach was followed because most of the codons in the original hagfish cDNA are poorly expressed in *E. coli* (De Boar & Kastelein, 1986). Codons for the amino acids Val-Asn-Gly were also engineered to be immediately upstream of the mature hagfish IGF protein. The codons for Val-Asn were included because the DNA sequence for these residues code for a HpaI restriction endonuclease recognition site, thus facilitating manipulation of the DNA construct into the expression vector. Moreover, the amino acids Asn-Gly were included to provide a hydroxylamine cleavage site. Cleavage of the expressed fusion protein by hydroxylamine at this site allows recombinant [Gly]-hagfish IGF to be produced (Fig. 6.5).

Initial attempts to express hagfish IGF as a fusion protein with a shorter leader peptide, namely as a fusion protein with the first 11 amino acids of methionyl porcine growth hormone followed by the residues Val-Asn-Gly, were unsuccessful. However, as was found with chicken IGF-II (4.3.1), high level expression of hagfish IGF was achieved when it was expressed as a fusion protein with a longer leader peptide ([Met^1]pGH(1-46)-Val-Asn-Gly-hagfish IGF).

A fifteen litre fermentation of *E. coli* JM101 (lac I^q) cells transformed with p[Met^1]pGH(1-46)-Val-Asn-Gly-hagfish IGF yielded 130 g of inclusion bodies following induction with IPTG.

6.3.2: Downstream processing strategy

As I did not know whether hagfish IGF would in fact exhibit biological and receptor binding activities similar to what we have previously observed with avian and mammalian IGFs, an alternative strategy was required to monitor progress of the downstream processing. Thus, I decided to follow isolation of the required recombinant protein via analytical gel electrophoresis using high density SDS-polyacrylamide gels.

I initially attempted to release [Gly]-hagfish IGF from the fusion protein in a single cleavage reaction using hydroxylamine. However, little cleavage was observed after 15 h (Fig. 6.7). The second approach I followed was to first cleave the fusion protein with H64A subtilisin BPN' to generate the 12.1 kDa fragment, pGH(20-46)-Val-Asn-Gly-hagfish IGF, then to subsequently release [Gly]-hagfish IGF from this protein using hydroxylamine. However, as is depicted in Fig. 6.5, the downstream processing of the hagfish fusion protein was further complicated by the presence of a Gln-His motif within the hagfish protein itself. Hence, this approach required purification of the 12.1 kDa protein before commencing the second cleavage, to ensure that the correct 8.6 kDa [Gly]-hagfish IGF protein was ultimately purified. As can be seen in Fig. 6.5, failure to do this may lead to the co-purification of the 8.7 kDa fragment, [Met^1]pGH-(1-46)-Val-Asn-Gly-Hagfish IGF(1-28).

6.3.3: Downstream processing of the hagfish IGF fusion protein

While the conditions for dissolution and reduction of the inclusion bodies containing the hagfish IGF fusion proteins were similar to those I have described previously in this thesis, a different desalting procedure was adopted. The TSK chromatography step was included not only as a desalting procedure, but also

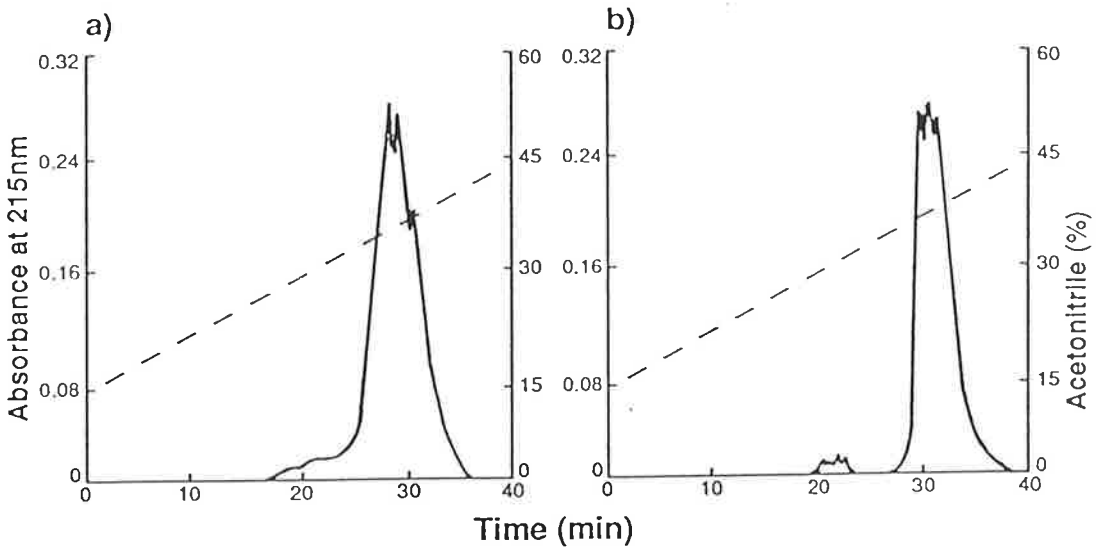


FIGURE 6.7: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) a) before and b) after digestion of [Met[1]]-pGH(1-46)-Val-Asn-Gly-hagfish IGF with hydroxylamine. Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.

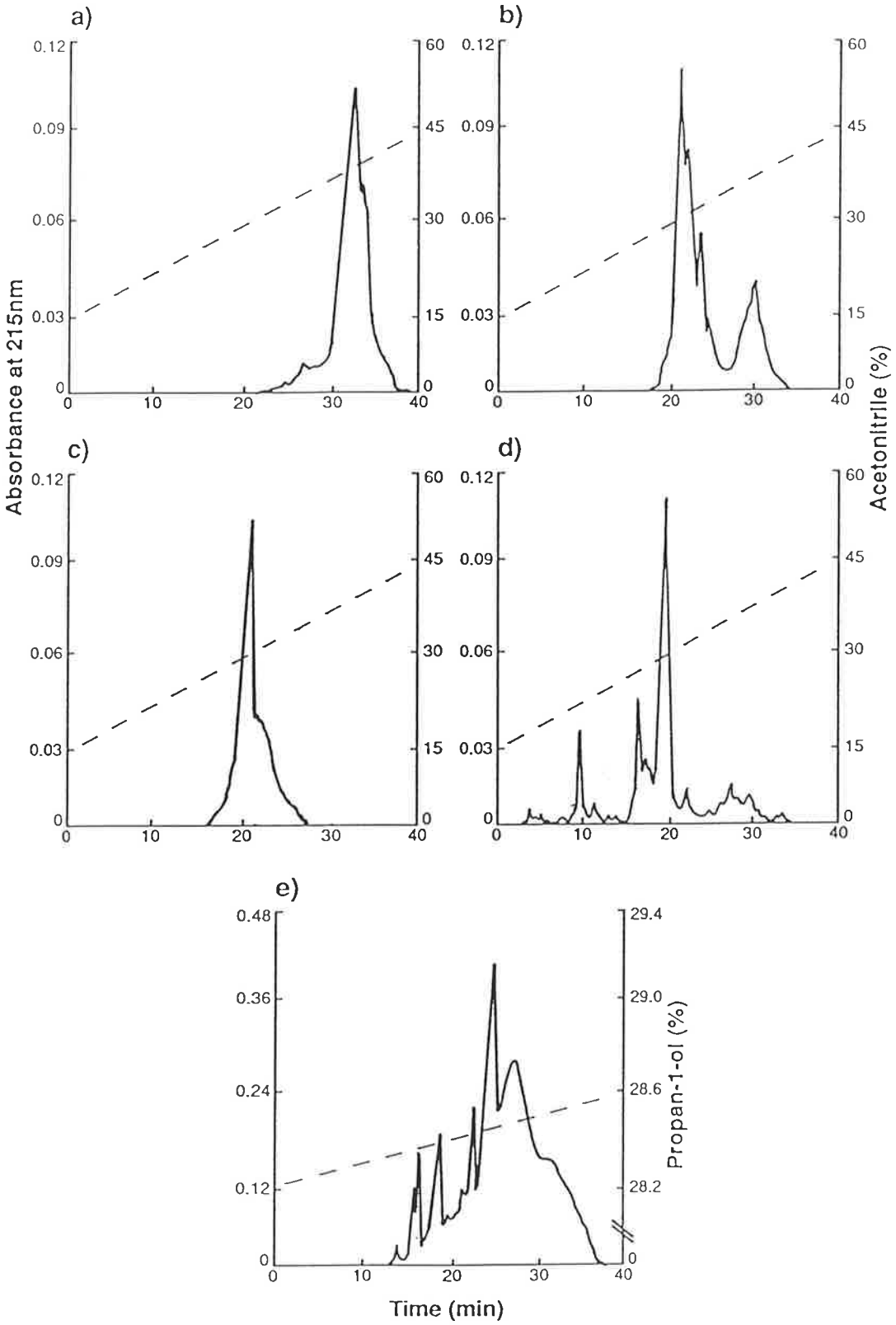
to remove any bacterial proteinases which may be present. In chapter 3 I suggested that *E. coli* proteinase VII may have caused the observed proteolysis. Since this proteinase has a molecular mass of 36 kDa (Sugimara & Nishihara, 1988) and the hagfish IGF fusion protein has a mass of 14.1 kDa, fractionation on the TSK column should separate the two. Indeed SDS-gel electrophoresis under reducing conditions on subsamples throughout the purification of [Gly]-hagfish IGF suggested that any proteolytic activity, if present, had been removed (Fig. 6.6).

Generation of the 12.1 kDa fragment, pGH(20-46)-Val-Asn-Gly-hagfish IGF, via cleavage with H64A subtilisin BPN' occurred rapidly, with approximately 90% of the starting substrate being digested after 4.5 h (Fig.6.8a & b). The subsequent chromatography steps effectively removed any uncleaved proteins, since there was no evidence of the 14.1 kDa starting substrate in the fractions eventually processed (Fig. 6.6, lane 4).

Cleavage of the 12.1 kDa fragment by hydroxylamine to release the required [Gly]-hagfish IGF was also successful (Fig. 6.8c & d). Analysis of the acidified digest on high density polyacrylamide gels indicated that at least 25% of the starting substrate was cleaved (Fig. 6.6, lane 1).

Separation of the 12.1 kDa fragment from the required 8.6 kDa [Gly]-hagfish IGF protein was more difficult. I was unable to separate the two protein species using a range of solvent systems including acetonitrile containing either 10 mmol triethylamine, 0.13% HFBA or 0.1% TFA. Furthermore, these solvent systems were unable to separate the two proteins whether they were in an oxidized or reduced state. Nor was it possible to separate the 12.1 kDa substrate from the 8.6 kDa cleavage product via chromatography on a Mono S HR5/5 (5mm (diameter) x 50 mm) ion-exchange column (Pharmacia-LKB, North Ryde, NSW, Australia) employing a linear gradient from 10 mmol to 1 mol ammonium acetate/l in 10% acetonitrile over 120 min at a flow rate of 1 ml/min. However, the two protein species were separated, as indicated by Phast gel analysis, when

FIGURE 6.8: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) a) before and b) after digestion of [Met^{1}]-pGH(1-46)-Val-Asn-Gly-hagfish IGF with H64A subtilisin BPN'. Analysis of pGH(20-46)-Val-Asn-Gly-hagfish IGF before and after digestion with hydroxylamine is depicted in c) and d) respectively, while the profile of the chromatography step employed to separate the proteins following digestion by hydroxylamine is shown in e). Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of either acetonitrile (dashed line) in 0.1% trifluoroacetic acid (a, b, c and d) or propan-1-ol in 0.13% heptafluorobutyric acid (e).


a)
b)
c)
d)
e)
Absorbance at 215nm
Acetonitrile (%)
Propan-1-ol (%)
Time (min)

chromatographed on a C4 microbore column (2.1 mm (diameter) x 100 mm) employing a shallow linear gradient of propan-1-ol in 0.13% HFBA (Fig. 6.8e).

Homogeneity of the pooled fractions was assessed by N-terminal protein sequencing, analytical SDS-polyacrylamide gel eletrophoresis (Fig. 6.6, lane 3) and mass spectroscopy (Fig. 6.9). Mass spectroscopy indicated that the majority of the protein present was hydroxamated.

6.3.4: Refolding of [Gly]-hagfish IGF

Prior to obtaining the required 8.6 kDa [Gly]-hagfish IGF, refolding of the proteins was performed in the presence of 8 mol urea/l. This concentration of urea was chosen to reduce losses due to insolubility of the proteins. However, as [Gly]-hagfish IGF was exhibiting little biological activity in rat myoblasts, I decided to attempt the refolding at a lower urea concentration, similar to what has previously been used for other recombinant IGF proteins. The refolding reaction was monitored by analysis on the standard analytical HPLC (2.2.2.3). Refolding was indicated by early elution of the protein compared with that observed when the reduced starting material was analyzed under identical conditions (Fig. 6.10). While the oxidized material did elute earlier than the reduced starting substrate, the protein eluted in a broader peak than I have observed with other recombinant IGFs. Nevertheless, the acidified mixture was chromatographed on a C18 Novapak (8 mm (diameter) x 100 mm) reverse-phase HPLC column employing a linear gradient from 24.8 to 40% acetonitrile in 0.1% TFA over 152 min and fractions were collected for assessment in the rat myoblast protein synthesis assay (Fig. 6.11a). The peak of biological activity coincided with the leading edge of the absorbance profile, suggesting that only a small proportion of the protein was correctly refolded. Analysis on the standard analytical HPLC indicated that the activity-containing fractions migrated as a single peak (Fig.6.11b). However, only 25 μg of protein was present. The biological activity and receptor-binding interactions of this protein pool was subsequently characterized. Protein sequencing gave the expected N-terminal sequence for

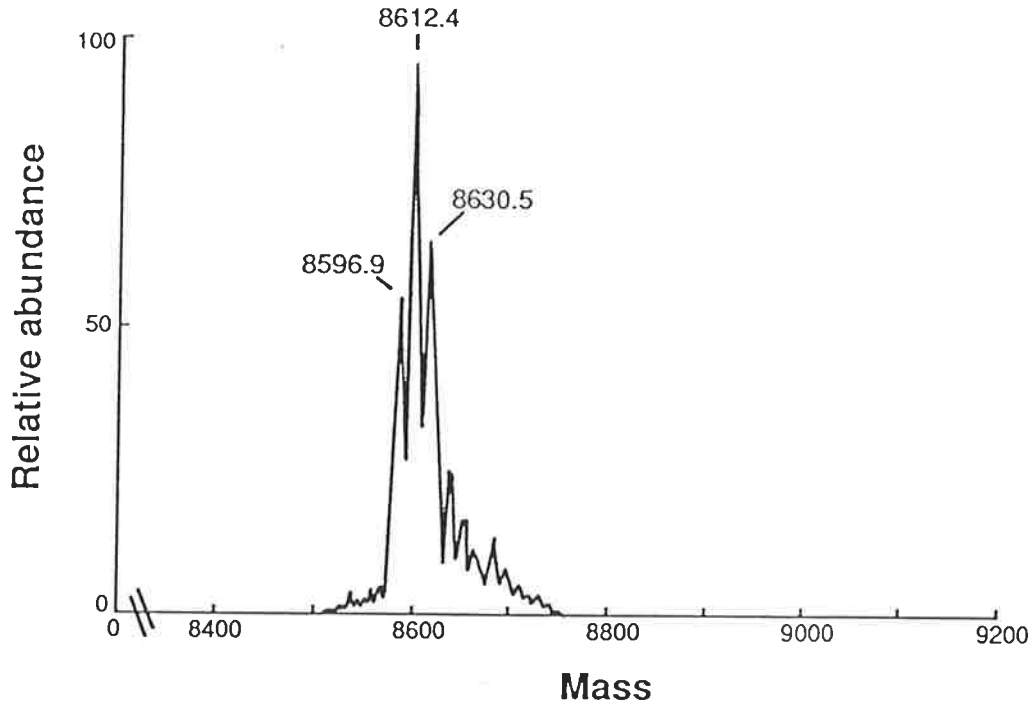


FIGURE 6.9: Electrospray mass analysis of [Gly]-hagfish IGF. Numbers indicate the masses of the peaks.

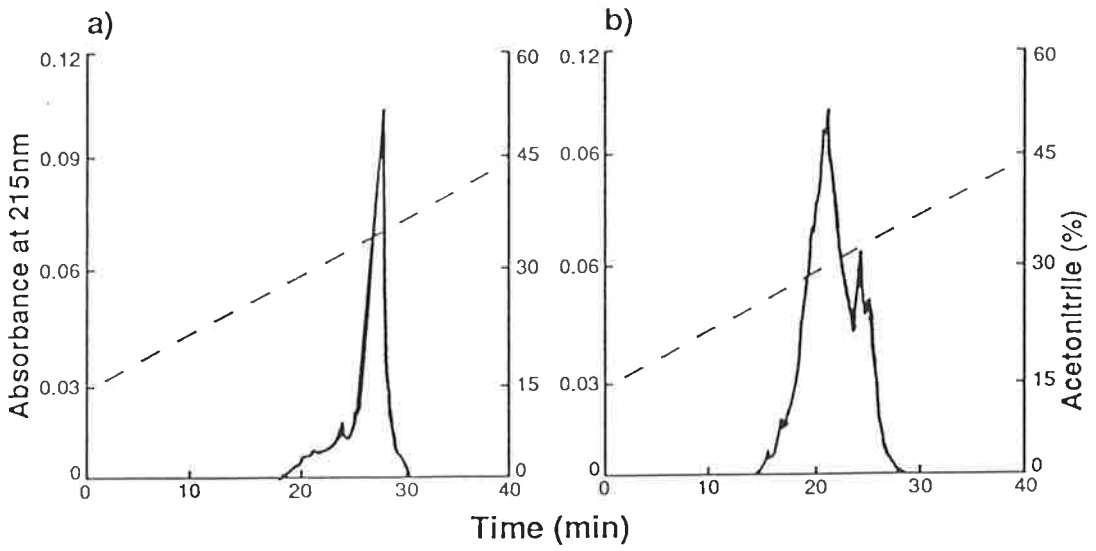
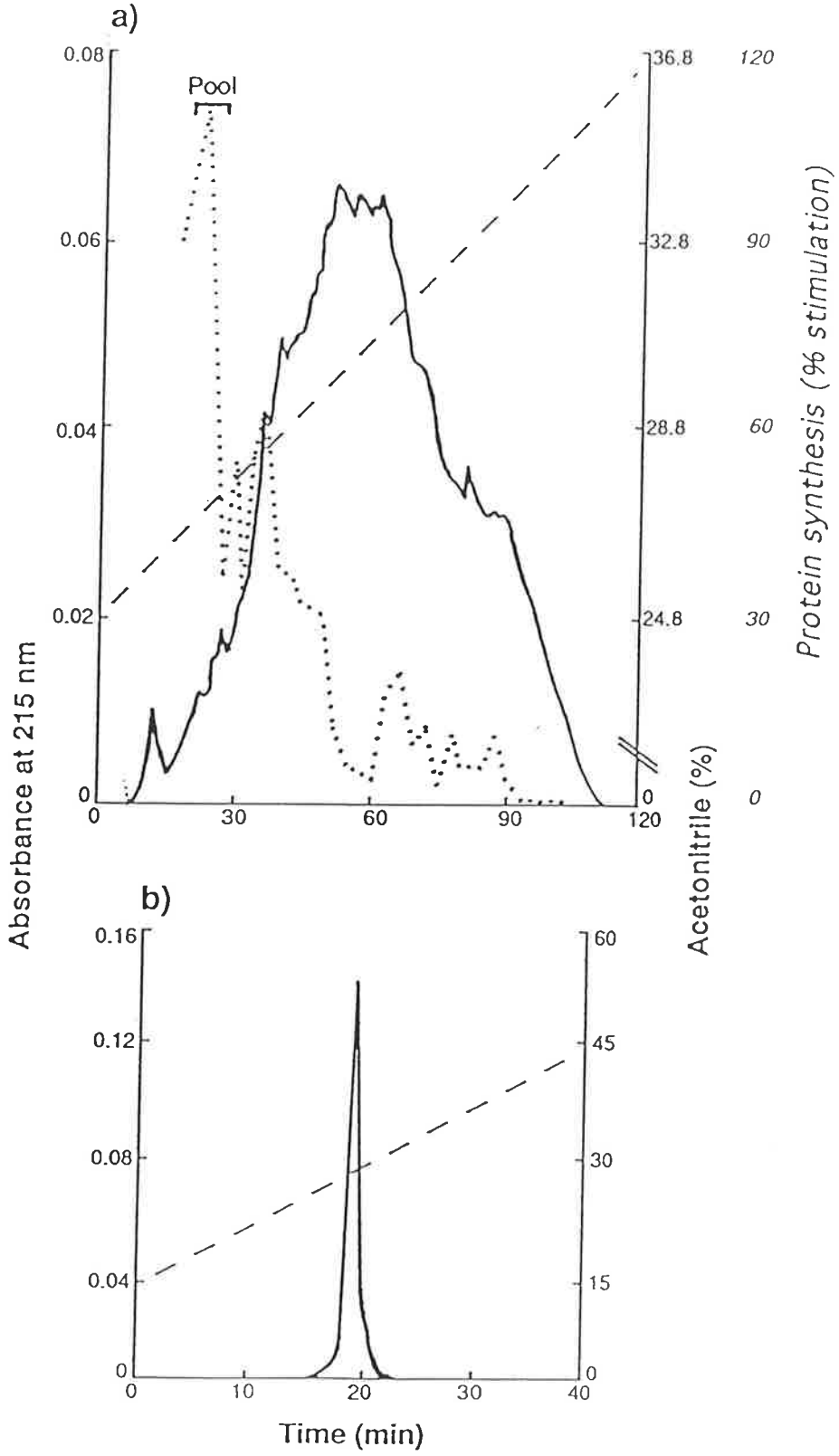


FIGURE 6.10: Downstream processing of recombinant proteins. Analysis on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) a) before and b) after refolding of [Gly]-hagfish IGF. Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.

FIGURE 6.11: Downstream processing of recombinant proteins. The profile of the chromatography step employed to separate refolded [Gly]-hagfish IGF is shown in a). The bioactivity of fractions (dotted line) and the fractions pooled for subsequent analysis are indicated. Analysis of the pooled fractions on a microbore C4 reverse-phase column using the standard analytical HPLC (described in 2.2.2.3) is depicted in b). Elution of protein, monitored as absorbance at 215 nm, was achieved by a gradient of acetonitrile (dashed line) in 0.1% trifluoroacetic acid.


a)
Pool
0.08
0.06
0.04
0.02
0
36.8
32.8
28.8
24.8
0
120
90
60
30
0
0
30
60
90
120
Absorbance at 215 nm
Acetonitrile (%)
Protein synthesis (% stimulation)
b)
0.16
0.12
0.08
0.04
0
60
45
30
15
0
0
10
20
30
40
Time (min)

[Gly]-hagfish IGF. Unfortunately I did not obtain sufficient material to allow a second mass analysis to be performed.

6.3.5: Alternative approaches to refolding [Gly]-hagfish IGF

The approach I used to refold [Gly]-hagfish IGF, namely, reoxidation by dithiothrietol, does not seem to be the most appropriate method for this protein. In order to try and address this problem I also investigated two other refolding procedures, namely glutathione reoxidation and air oxidation. Fractions containing proteins with little or no biological activity after the refolding procedure described above were concentrated by reverse-phase HPLC and lyophilized. The protein (400 μg) was then resuspended in 8 mol urea/l, 40 mmol glycine/l, 0.1 mol Tris/l and 1 mmol EDTA/l at pH 8.7. Half the protein solution was refolded using the glutathione reoxidation procedure described by Saxena & Wetlaufer (1970), whereas the remaining half was reduced by the addition of DTT to 16 mmol/l and then allowed to refold via air oxidation for 48 h (Ahmed *et al.* 1975). The refold mixtures were separated on the C18 Novapak HPLC column as described above and fractions collected for bioassay. Increased biological activity was not observed with either refolding procedure.

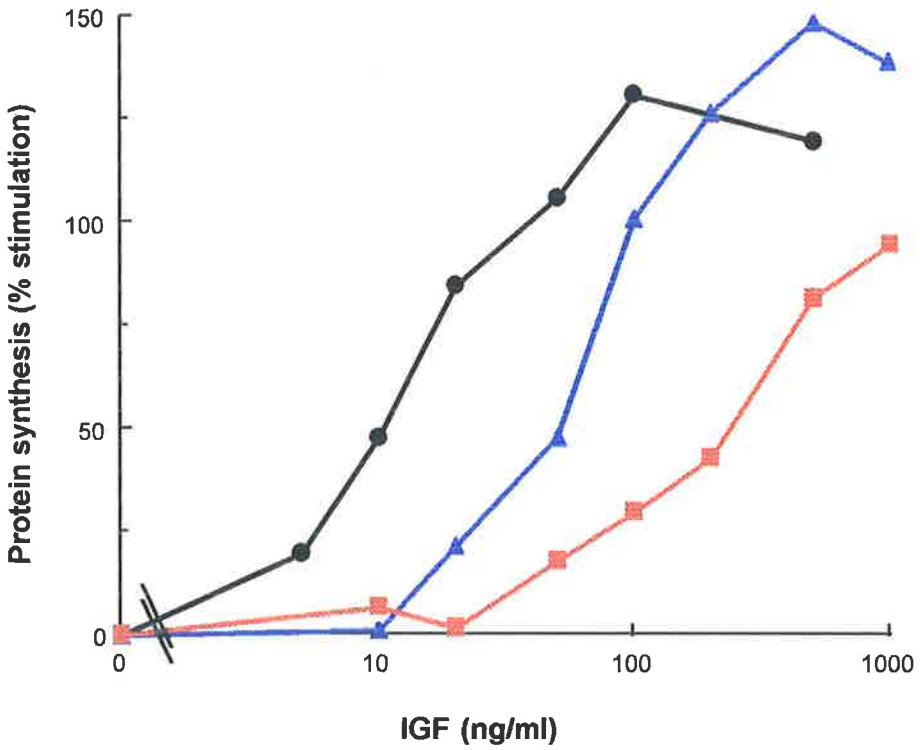
6.3.6: Biological activity of [Gly]-hagfish IGF-I

The biological activity of recombinant [Gly]-hagfish IGF was assessed by comparing the ability of the peptide to stimulate protein synthesis or inhibit protein degradation in cultured cells with that of recombinant human IGF-I (hIGF-I) and IGF-II (hIGF-II). Recombinant [Gly]-hagfish IGF was less effective than either human peptide at stimulating protein synthesis in rat L6 myoblasts. Half maximal effects were observed at 18, 68 and 380 ng/ml for hIGF-I, hIGF-II and [Gly]-hagfish IGF respectively (Fig.6.12a).

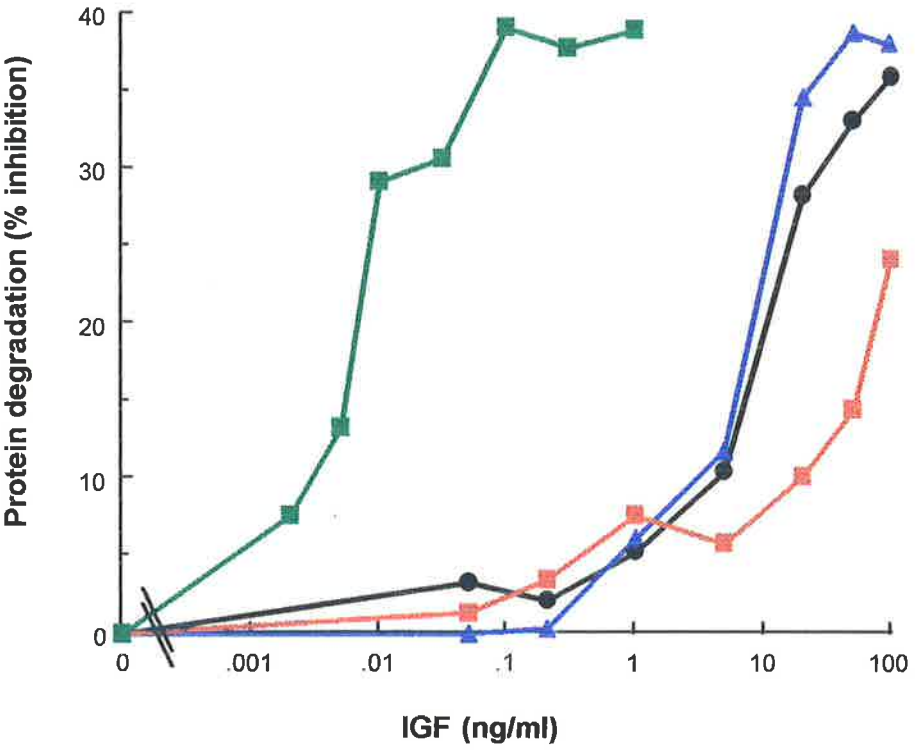
As [Gly]-hagfish IGF was significantly less effective at eliciting a biological response in L6 rat myoblasts, I decided to assess its biological activity in rat H35B hepatoma cells. Unlike L6 cells, H35B hepatomas possess abundant

FIGURE 6.12: Effects of IGFs on a) protein synthesis in rat L6 myoblasts and b) protein degradation in rat H35B hepatocytes. The proteins tested were human IGF-I (black ●), human IGF-II (blue ▲), [Gly]-hagfish IGF (red ■) and human insulin (green ■). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Protein synthesis (% stimulation)
150
100
50
0
0
10
100
1000
IGF (ng/ml)

b)


Protein degradation (% inhibition)
40
30
20
10
0
0
.001
.01
.1
1
10
100
IGF (ng/ml)

insulin receptors and few, if any, type-1 IGF receptors (Mottola & Czech, 1984). Again, however, [Gly]-hagfish IGF was substantially less potent than the human IGFs at inhibiting protein breakdown in these cells, with half-maximal effects observed at 11, 8.4 and 76 ng/ml for hIGF-I, hIGF-II and [Gly]-hagfish IGF respectively. Insulin was substantially more potent than any of the IGFs tested in H35B cells, with half-maximal effects observed at 0.0068 ng/ml (Fig. 6.12b).

6.3.7: Receptor binding interactions of [Gly]-hagfish IGF

In order to determine whether [Gly]-hagfish IGF exhibits IGF-I- or IGF-II-like receptor binding characteristics, its interactions were assessed in type-1 and type-2 IGF receptor binding assays. Affinity for binding to type-1 receptors in cultured cells was assessed by measuring competition for [^{125}I]-labelled hIGF-I binding. [Gly]-hagfish IGF exhibited a lower affinity than either hIGF-I or hIGF-II for the type-1 IGF receptors present in either rat L6 myoblasts (Fig. 6.13a) or in CHSE-214 salmon embryo fibroblasts (Fig. 6.13b). Half-maximal competition was observed at 1.3, 7.6 and 150 ng/0.5 ml in L6 rat myoblasts and at 4.7, 2.2 and 330 ng/0.25 ml in CHSE-214 salmon fibroblasts for hIGF-I, hIGF-II and [Gly]-hagfish IGF respectively.

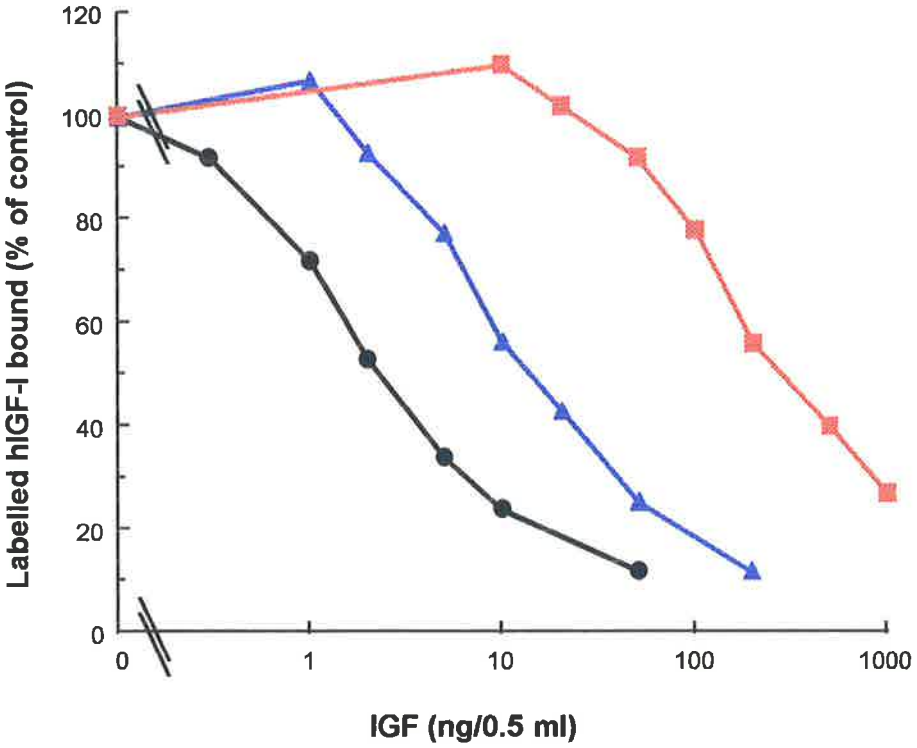
Affinity of [Gly]-hagfish IGF for binding to type-2 IGF receptors was assessed by measuring competition for binding of [^{125}I]-labelled hIGF-II to receptors on ovine placental membranes. [Gly]-hagfish IGF and hIGF-I exhibited similar affinities for binding to the type-2 receptor present on the membranes. However, the affinities of both peptides for binding to this receptor were significantly lower than observed with hIGF-II (Fig.6.14).

6.4: DISCUSSION

In this chapter I have described the production and preliminary characterization of recombinant [Gly]-hagfish IGF. I had originally intended to produce authentic "hagfish IGF" rather than "[Gly]-hagfish IGF" using a fusion protein expression system incorporating a H64A subtilisin BPN' cleavage site

FIGURE 6.13: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-I in a) rat L6 myoblasts and b) CHSE-214 salmon embryo fibroblasts embryo fibroblasts. The proteins tested were human IGF-I (black ●), human IGF-II (blue ▲) and [Gly]-hagfish IGF (red ■). Values are the means of triplicate determinations on three cultures at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-I bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.5 ml)

b)

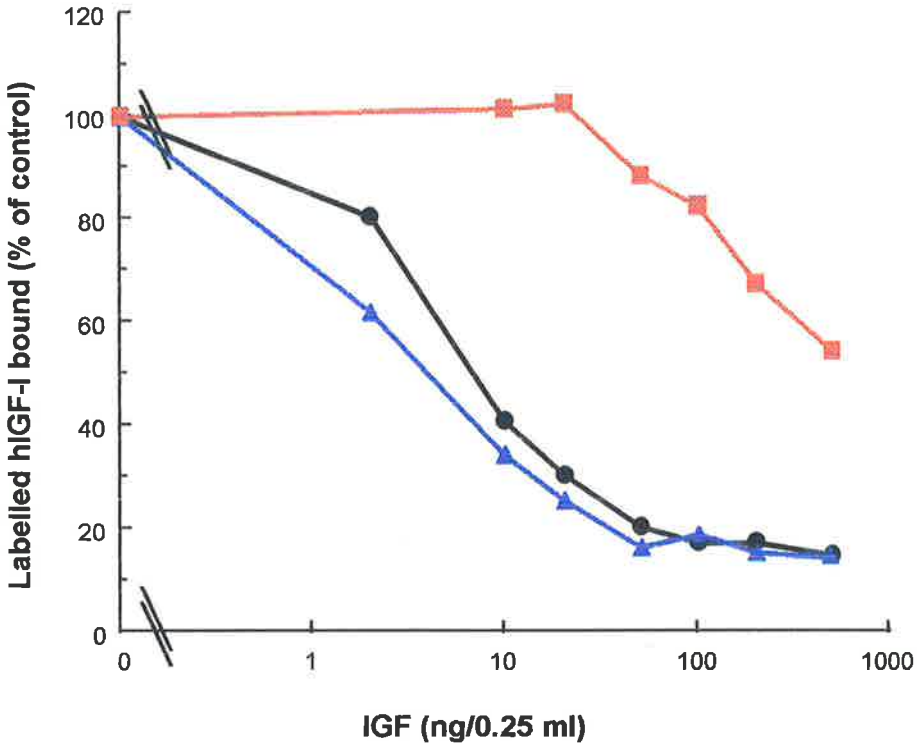
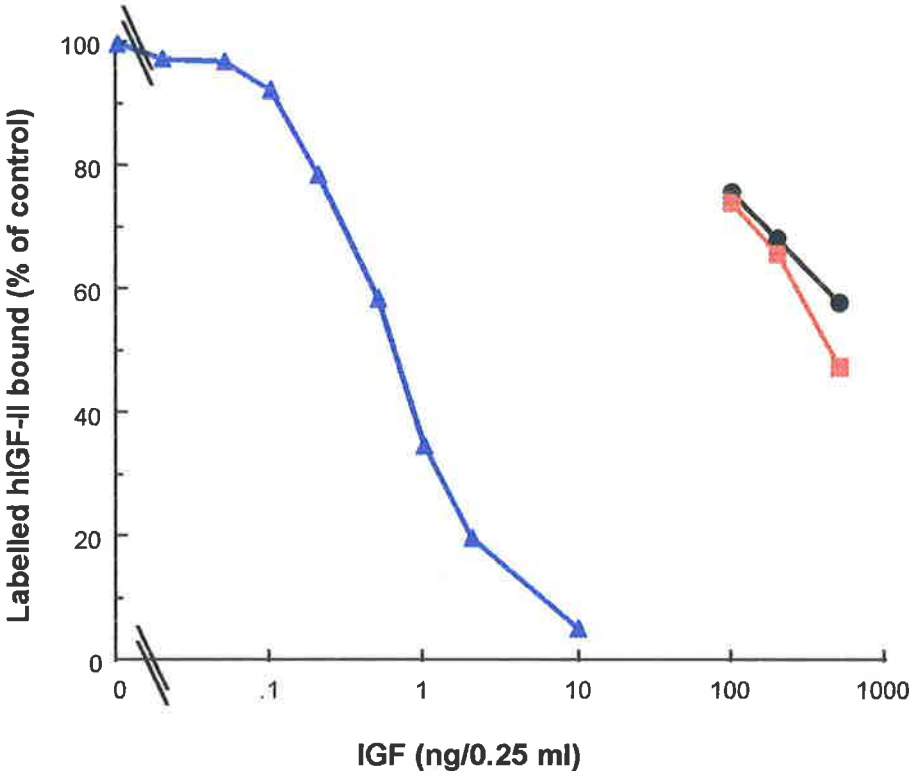

Labelled hIGF-I bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

FIGURE 6.14: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to ovine placental membranes. The proteins tested were human IGF-I (black ●), human IGF-II (blue ▲) and [Gly]-hagfish IGF (red ■). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
.1
1
10
100
1000
IGF (ng/0.25 ml)

(Phe-Ala-His-Tyr) similar to that described in chapter 4 for producing recombinant chicken IGF-II. However, the presence of a Gln-His motif within the hagfish IGF protein itself made this approach difficult since H64A subtilisin BPN' can also cleave at this site (Matthews & Wells, 1993).

Production of recombinant [Gly]-hagfish IGF has also been complicated by a number of other factors including the requirement of a longer leader peptide for fusion protein expression, reduced solubility of the protein, as well as problems in the refolding procedure. While I was able to address most of these problems by substantially modifying the downstream processing procedure, I was unable to successfully develop an appropriate refolding protocol. Thus, the low yield of biologically active recombinant [Gly]-hagfish IGF obtained was principally due to inefficient refolding.

I attempted to refold hagfish IGF using a number of different methods including air oxidation and glutathione reoxidation in addition to reoxidation by DTT. However, none of these approaches were particularly successful. Clearly an alternative approach needs to be adopted. Others in this laboratory are currently developing secretion systems for the expression of IGFs, as well as systems where chaperonins are co-expressed at the same time as the desired recombinant protein. These approaches may well prove to be more appropriate for the expression and production of recombinant hagfish IGF. Nevertheless, I was able to produce a small quantity of recombinant [Gly]-hagfish IGF which is biologically active, elutes as a single peak on an analytical HPLC and migrates as a single band on a SDS-polyacrylamide gel. Furthermore, N-terminal amino acid sequencing and mass spectrometry confirm that I have produced [Gly]-hagfish IGF.

Mass spectroscopy on the recombinant [Gly]-hagfish IGF I produced was reassuring on two accounts. Firstly, the analysis confirmed that all the required changes had been correctly introduced into the hagfish IGF cDNA. Secondly, the analysis indicated that there was no evidence of contamination by any other protein. However, the mass analysis did indicate that forms of [Gly]-hagfish IGF

with either 0, 1 or 2 side-chain derivatives were present. Approximately 50% and 25% of the total protein had molecular masses 15.6 and 33.6 Da higher respectively, than expected (Fig. 6.9). These masses strongly suggest that hydroxamation of Gln residues occurred during the hydroxylamine cleavage reaction (Bornstein & Balian, 1977). These side-chain derivatives are unlikely to affect the biological actions of the peptide since hydroxamate-containing human IGF-I has the same biological activity as human IGF-I (Canova-Davis *et. al.* 1992).

In vitro assessment of the recombinant protein in cultured cells indicates that [Gly]-hagfish IGF shares functional properties with IGFs. Thus [Gly]-hagfish IGF competes for binding to the type-1 IGF receptor present on both rat myoblasts and on salmon embryo fibroblasts, though with somewhat lower affinity than either IGF-I or IGF-II (Fig. 6.13). The amino acids Tyr^{24} and Tyr^{27} in mammalian IGF-I and IGF-II respectively, have been identified as being crucial for binding to mammalian type-1 IGF receptors (Burgisser *et al.* 1991). In this context hagfish IGF has a Phe in the corresponding position (Fig. 1.8). While Phe, being an aromatic residue, is structurally similar to Tyr, it may not be as well suited as Tyr for facilitating binding to the type-1 IGF receptor, and thus may explain why Gly-hagfish IGF has a lower affinity for this receptor. Indeed, insulin has a Phe residue in the analogous position (Fig. 1.1), and it too has a much lower affinity than the IGFs for binding to the type-1 IGF receptor. Furthermore, other residues including Tyr^{31} in hIGF-I, have also been identified as being important for type-1 receptor binding (Burgisser *et al.* 1991). Neither insulin nor hagfish IGF have a corresponding aromatic amino acid residue in this position.

Since the biological actions of IGFs are generally considered to be mediated through the type-1 IGF receptor, the result that [Gly]-hagfish IGF was also less effective than human IGF-I or IGF-II at stimulating protein synthesis in rat L6 rat myoblasts was not surprising (Fig. 6.12a). However, what is surprising, is that while 115-fold and 20-fold more [Gly]-hagfish IGF respectively was required to observe the same half-maximal binding to the type-1 receptor in L6

cells as was observed with human IGF-I and IGF-II (Fig. 6.13a), only 20-fold and 5-fold more peptide respectively was required to achieve the same half-maximal biological responses in these cells (Fig. 6.12a). This difference presumably can be explained by differences in the affinities for IGF binding proteins which are also present in the protein synthesis bioassay or by interactions with other as yet unidentified proteins.

In order to investigate whether the biological actions of [Gly]-hagfish IGF were in fact being mediated by the insulin receptor, I assessed the biological activity of the peptide in the rat H35B cell line (Fig. 6.12b). [Gly]-hagfish IGF was also less potent than human IGF-I, IGF-II or insulin at inhibiting protein breakdown in these cells. However, the effects it elicited were more similar to those observed with IGFs than those observed with insulin. Since this particular cell line has abundant insulin receptors and few, if any, type-1 IGF receptors (Mottola & Czech, 1984), this result suggests that [Gly]-hagfish IGF is functionally more closely related to the IGFs than to insulin.

Interpreting the results from the type-2 IGF competitive binding assays is more difficult. [Gly]-hagfish IGF was significantly less effective than human IGF-II at binding to the type-2 IGF receptor present on sheep placental membranes, yet was similar, if not more effective than human IGF-I (Fig. 6. 14). Thus, while [Gly]-hagfish IGF is markedly less effective than IGF-I at binding to the type-1 IGF receptor, it appears to have a similar affinity for binding to the type-2 IGF receptor. Hence at this stage, it seems likely that [Gly]-hagfish IGF is in fact, more similar to IGF-I than it is to IGF-II. However, these results need to be interpreted cautiously. Indeed, residues similar to those identified as being important in mammalian IGFs for binding to the type-2 IGF receptor are found in hagfish IGF. The amino acids $Phe^{52}Lys^{53}Gly^{54}$ in hagfish IGF may well be the equivalent of $Phe^{48}Arg^{49}Ser^{50}$ in mammalian IGF-II, and thus may also be important for type-2 IGF receptor binding. The residues $Ala^{54}Leu^{55}$ in human IGF-II have also been found to be important for type-2 receptor binding (Burgisser *et al.* 1991: Sakano *et al.* 1991). Hagfish IGF however, only has one of the equivalent residues.

Leucine at position 59 in hagfish IGF is in the analogous position to Leu^{55} in IGF-II. On the other hand, Arg^{58} in hagfish IGF, is identical to the equivalent amino acid found in IGF-I (see Fig. 1.8). Production of hagfish IGF mutants with $Phe^{52}Arg^{53}Ser^{54}$, Tyr^{24} or with Ala^{58}, may help to identify whether the binding results I obtained with [Gly]-hagfish IGF for the type-1 and type-2 IGF receptors, are caused by the difference in amino acids at these positions.

Whether I would have obtained different results in the functional characterization of the protein if I had actually produced "authentic" hagfish IGF rather than [Gly]-hagfish IGF is difficult to assess. However, there are two reasons why it is unlikely that the presence of the additional N-terminal glycine residue in [Gly]-hagfish IGF would markedly alter the functional properties of the IGF. Firstly, [Met]-human IGF-I exhibits the same biological, IGFBP- and receptor-interactions as purified ovine IGF-I (Hodgkinson *et al.* 1989). Secondly, glycine, being the smallest amino acid, is unlikely to significantly alter the sterical structure of IGF molecule itself, or to spatially hinder the interactions of the IGF with IGFBPs or receptors. Indeed, Francis *et al.* (1992) have found that the addition of 13 amino acid residues to the N-terminus of human IGF-I only causes a two-fold difference in potency compared with human IGF-I in myoblast protein synthesis and protein breakdown assays.

Although I encountered a number of difficulties in the downstream processing of recombinant [Gly]-hagfish IGF and consequently was only able to isolate a small quantity of biologically active peptide, the biochemical assays indicate that motifs important for functions associated with mammalian IGFs are also present in hagfish IGF. The results imply that these motifs evolved prior to when the Agnanthans diverged from the main line of vertebrate evolution. Accordingly, we now have functional as well as structural evidence that the IGFs have a long evolutionary history. Whether hagfish IGF is functionally more closely related to IGF-I than to IGF-II remains to be conclusively established. However, the results from the preliminary characterization of [Gly]-hagfish IGF which I report in this chapter suggest that hagfish IGF may in fact be more closely

related to IGF-I. These findings support observations by Nagamatsu *et al.* (1991) that the length of the signal peptide as deduced from the cDNA and the pattern of expression of the mRNA for hagfish IGF are characteristic of that observed for other vertebrate IGF-Is.

CHAPTER 7

EVOLUTION OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

7.1:INTRODUCTION

The bioavailability of IGFs in mammalian species is regulated by a family of IGF binding proteins (IGFBPs). While mammalian IGF binding proteins have been extensively examined, at the time I commenced this study little was known about these proteins in nonmammalian species. Daughaday *et al.* (1985) were the first to systematically investigate the presence of IGFBPs in nonmammalian sera. In their study, sera were acid-fractionated and the fractions subsequently assessed for IGF-II-binding activity. While IGF-binding activity could be detected in the nonmammalian sera tested (chicken, turtle, trout, toad), the activity appeared to be non-specific as it was not displaced by excess IGF-II. Likewise, a more recent study by Drakenberg *et al.* (1989) was not able to demonstrate specific IGF-binding activity in serum from bony fish. While IGF-binding activity was detected in acid chromatographed serum, again, the binding was not specific. However, both studies assessed the presence of IGFBPs after acid gel chromatography, hence any acid-labile IGF-specific binding proteins would not be detected. Bautista *et al.* (1990) were unable to demonstrate the presence of IGFBPs in the skeletal tissues of the chicken, lizard, frog, trout and shark. Similarly, Reinecke *et al.* (1991, 1992) were unable to detect IGFBPs in the Atlantic hagfish or in bony or cartilaginous fish.

The first unequivocal evidence for specific-IGFBPs in nonmammalian species occurred in 1989 when Armstrong *et al.* and Lee *et al.* (1989) demonstrated that specific IGFBPs were present in chicken serum. Subsequent studies by Francis *et al.* (1990) and by Pancak-Roessler & Lee (1990) were also able to show that IGFBPs were present in chickens and in toads respectively. Clearly, further investigations were required to understand the role of IGFBPs in nonmammalian species. In order to ascertain more about the origins and evolutionary relationships of IGFs and their binding proteins I commenced characterization of the IGFBPs in nonmammalian sera. I present here evidence that IGFBPs are, indeed, evolutionary-ancient proteins.

7.2: MATERIALS AND METHODS

7.2.1: Materials

Serum samples were generously provided by Drs. David Kennaway, Mark Adams, Chris Lee (University of Adelaide, Adelaide, SA, Australia), Max Cake (Murdoch University, Perth, WA, Australia) and Shu Chan (University of Chicago, Chicago, IL, U.S.A.). Blood was collected from the various vertebrate species, allowed to clot and the serum harvested following centrifugation. Sera were stored at -20°C and thawed immediately prior to analysis.

7.2.2: METHODS

7.2.2.1: Analytical gel filtration

In vitro radioligand binding and analytical gel filtration of serum samples was performed using either a Superose 12 size-exclusion column (1 cm x 30 cm; Pharmacia, Uppsala, Sweden) or a Sephadex G200 Superfine column (1.1 x 63 cm). Serum samples were eluted from the Superose 12 column in phosphate buffer (50 mmol NaH_2PO_4/l, 150 mmol NaCl/l, 0.02% (w/v) sodium azide, pH 7.2) at a flow rate of 0.5 ml/min or from the Sephadex G200 column in 50 mmol Tris/l, 150 mmol NaCl/l, pH 7.4 at a flow rate of 0.5 ml/min. Both columns were calibrated with protein markers of known molecular mass; human-γ-globulin (160 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and radiolabelled IGF-I (7.6 kDa). *Dr. S. Chan, Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, U.S.A., performed the chromatography of serum using the Sephadex G200 Superfine column.*

7.2.2.2: Analysis of IGFBPs using competitive binding assays and Western-ligand blotting

IGFBPs in plasma were identified using the ligand blotting procedure described in 2.2.4.7. Iodo-labelled hIGF-I, hIGF-II and cIGF-II were used as probes. IGFBP competitive binding assays were performed as described in

2.2.4.6. Fifty µl of chicken plasma and 30 µl of rat plasma were added to the tubes for the charcoal binding assays. The chicken blood was collected as a pool from 7 week old birds. The rat plasma was from a pool of blood collected from adult males.

7.3: RESULTS

7.3.1: Analysis of IGFBPs present in hagfish serum

As a first approach to identify whether IGFBPs were in fact present in agnanthan species, analytical gel filtration was employed to investigate the interaction of labelled IGFs with hagfish serum *in vitro*. Serum from the Pacific hagfish was pre-incubated with radiolabelled hIGF-I in the presence and absence of excess hIGF-I before separation under neutral conditions on a G-200 Sephadex column. The radiolabel associated predominantly with serum proteins with a molecular mass of approximately 60 kDa. The association of the radiolabel with the serum proteins appeared to be specific since excess hIGF-I competed for binding of the IGF tracer (Fig. 7.1). Ligand blotting using radiolabelled hIGF-I on pools of fractions eluted from the G200 column, confirmed that IGFBPs were present in the serum (Fig. 7.2). Two protein species with molecular masses of approximately 47 and 56 kDa were detected in both pool A (29-35 ml elution volume, lane 3) and pool B (36-45 ml elution volume, lane 4). Less of the higher molecular mass protein species was present in pool B. Ligand blotting of unfractionated Pacific hagfish serum revealed additional IGFBPs not present in either pool A or pool B (Fig. 7.2, lane 2). Ligand blotting of serum from the Atlantic hagfish (lane 5) revealed the presence of similar sized IGFBPs.

7.3.2: Analysis of IGFBPs present in lamprey serum

Gel filtration chromatography was used to investigate the IGF-binding characteristics of serum from two species of lamprey, *Petromyzon marinus* and *Geotria australis*. Serum samples were pre-incubated with radiolabelled IGFs

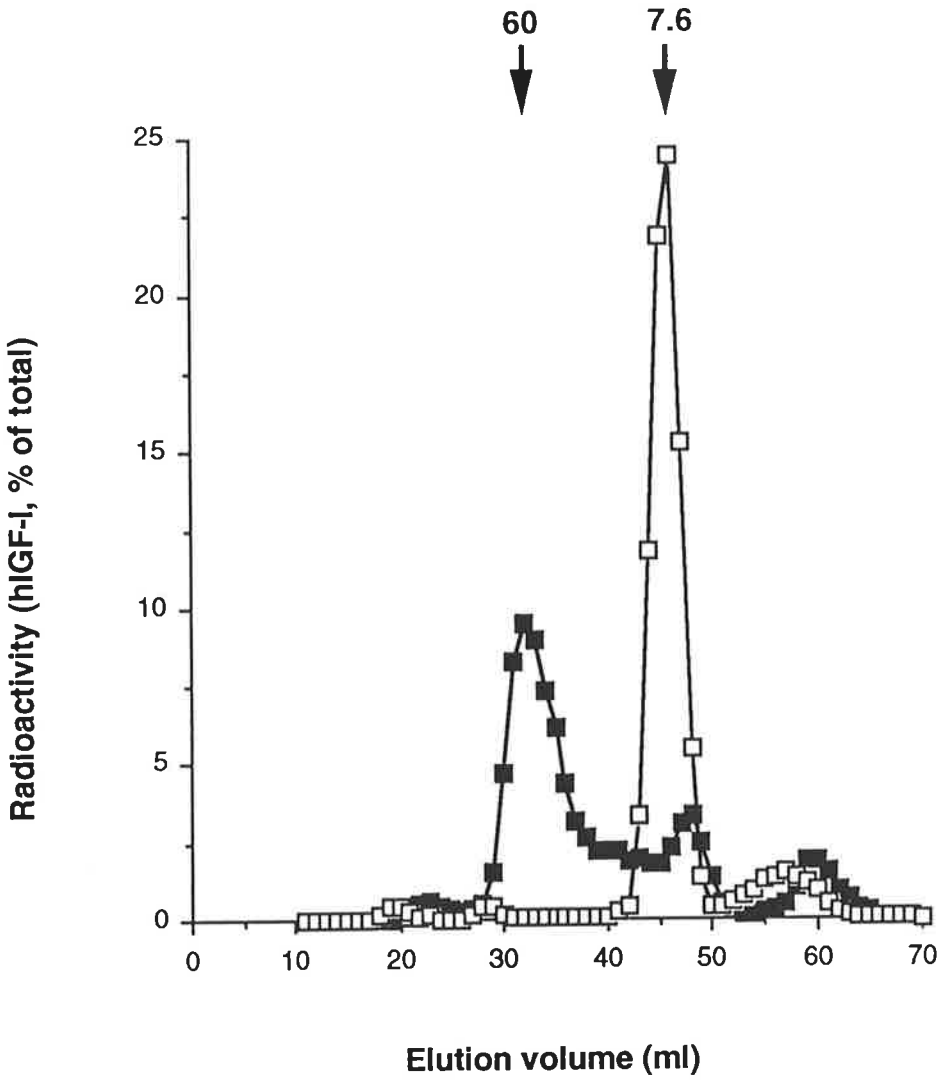


FIGURE 7.1: G-200 size-exclusion chromatography on 1 ml of Pacific hagfish serum following incubation with radiolabelled recombinant human IGF-I (■). The plots show the recovery of radioactivity in 1 ml fractions as a percentage of total radioactivity eluted. Serum pre-incubated with IGF-I tracer in the presence of excess human IGF-I was also analyzed (❑). The column was calibrated with protein markers of known molecular mass (see 7.2.2.1). Arrows indicate the deduced mass of the proteins complexed with the IGF-I tracer (60 kDa) and of the IGF-I tracer (7.6 kDa).

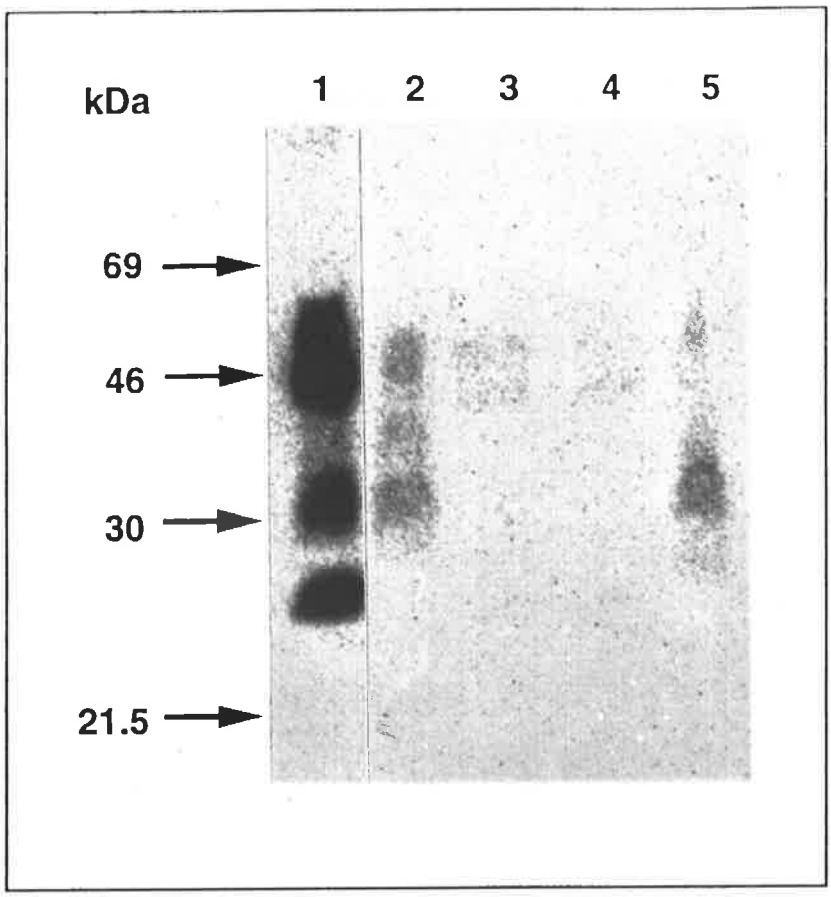


FIGURE 7.2: Ligand blotting of IGF-binding proteins in serum from two species of hagfish using labelled human IGF-I as a probe. Serum from the Pacific hagfish (20 µl, lane 2) and from the Atlantic hagfish (20 µl, lane 5) were tested. Proteins in fractions which were pooled following gel filtration chromatography of serum from the Pacific hagfish (7.3.1) were analyzed in lanes 3 (pool A) and 4 (pool B). Reference adult rat serum is in lane 1. Molecular mass markers are indicated. The samples analyzed were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-I and the probed nitrocellulose sheets exposed to phosphorimaging screens.

before separation under neutral conditions on a either a Sephadex G200 column (*Petromyzon marinus*) or a Superose 12 column (*Geotria australis*).

Iodo-labelled hIGF-I associated with proteins in *Petromyzon marinus* serum which eluted at 27 to 33 ml, corresponding to a size of approximately 75 kDa. Excess hIGF-I specifically competed with radiolabelled hIGF-I for binding to these protein species (Fig. 7.3). Ligand blotting using [^{125}I]-hIGF-I on pooled fractions (27-33 ml) (Fig. 7.4, lane 3) revealed IGFBPs similar in size to those detected in the fractionated hagfish serum. Ligand blotting of whole *Petromyzon marinus* serum revealed the presence of additional IGFBP species not present in the 27-33 ml pool (Fig. 7.4, lane 4).

Both labelled recombinant hIGF-I (Fig. 7.5a) and hIGF-II (Fig. 7.5b) associated with proteins in *Geotria australis* serum which eluted at 12-13 ml from the Superose 12 column. The addition of excess hIGF-I or hIGF-II to the incubations of the serum with either radiolabel specifically competed for binding of the IGF tracer to the proteins present. Ligand blotting using radiolabelled hIGF-I on fractions eluted from the Superose 12 column confirmed that IGFBPs were indeed present in those fractions which eluted at 12-13 ml. Moreover, the molecular mass of the IGFBPs in these fractions appeared to be approximately 50 kDa.

In order to compare the molecular mass of the serum IGFBPs in lamprey (*Geotria australis*) to those in chicken and rat, serum from these species were also chromatographed on a Superose 12 column, after pre-incubation with radiolabelled hIGF-I, in an identical manner to that described above for *Geotria australis* serum. The major sera IGFBPs in chicken and lamprey chromatographed at positions which coincide with molecular masses of 32 kDa and 48 kDa respectively, significantly lower than that found for rat serum (140 kDa) (Fig. 7.6).

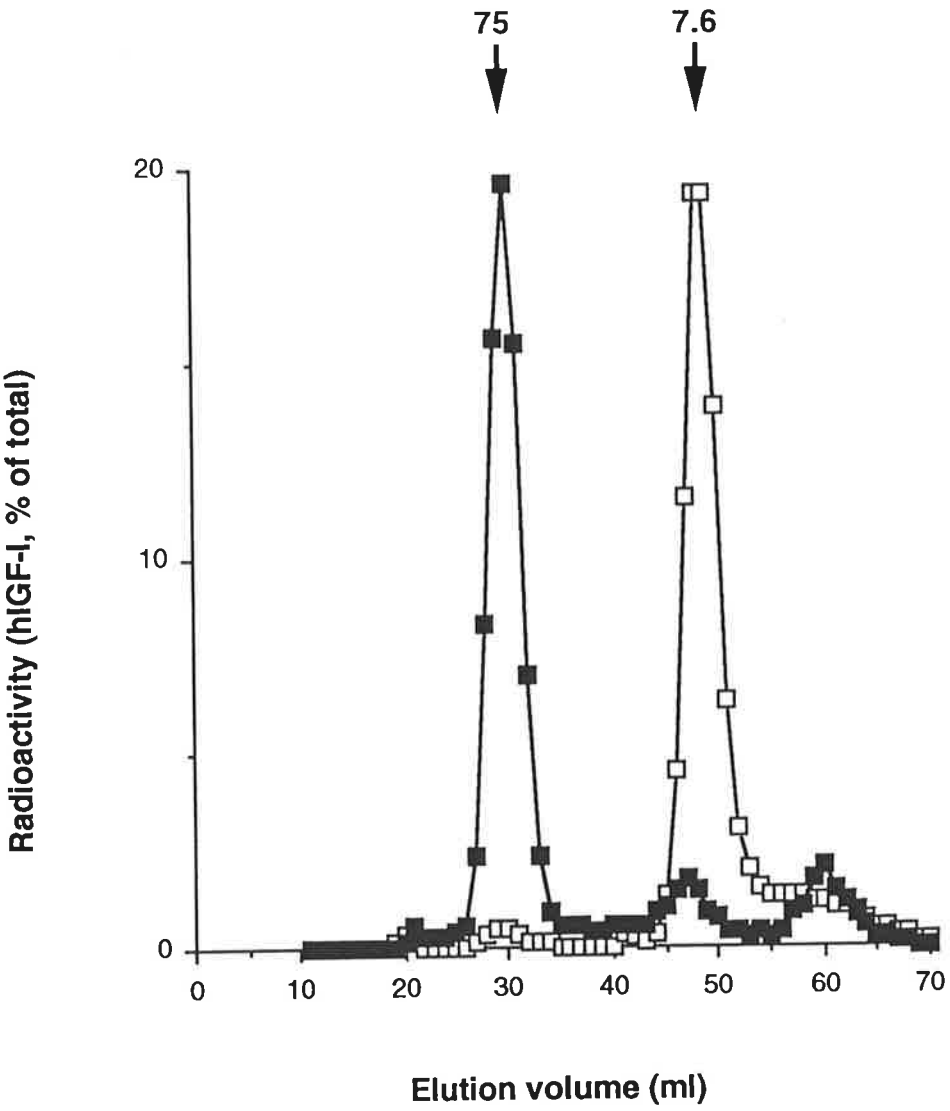


FIGURE 7.3: G-200 size-exclusion chromatography on 1 ml of lamprey (*Petromyzon marinus*) serum following incubation with radiolabelled recombinant human IGF-I (■). The plots show the recovery of radioactivity in 1 ml fractions as a percentage of total radioactivity eluted. Serum pre-incubated with IGF-I tracer in the presence of excess human IGF-I was also analyzed (❑). The column was calibrated with protein markers of known molecular mass (see 7.2.2.1). Arrows indicate the deduced mass of the proteins complexed with the IGF-I tracer (75 kDa) and of the IGF-I tracer (7.6 kDa).

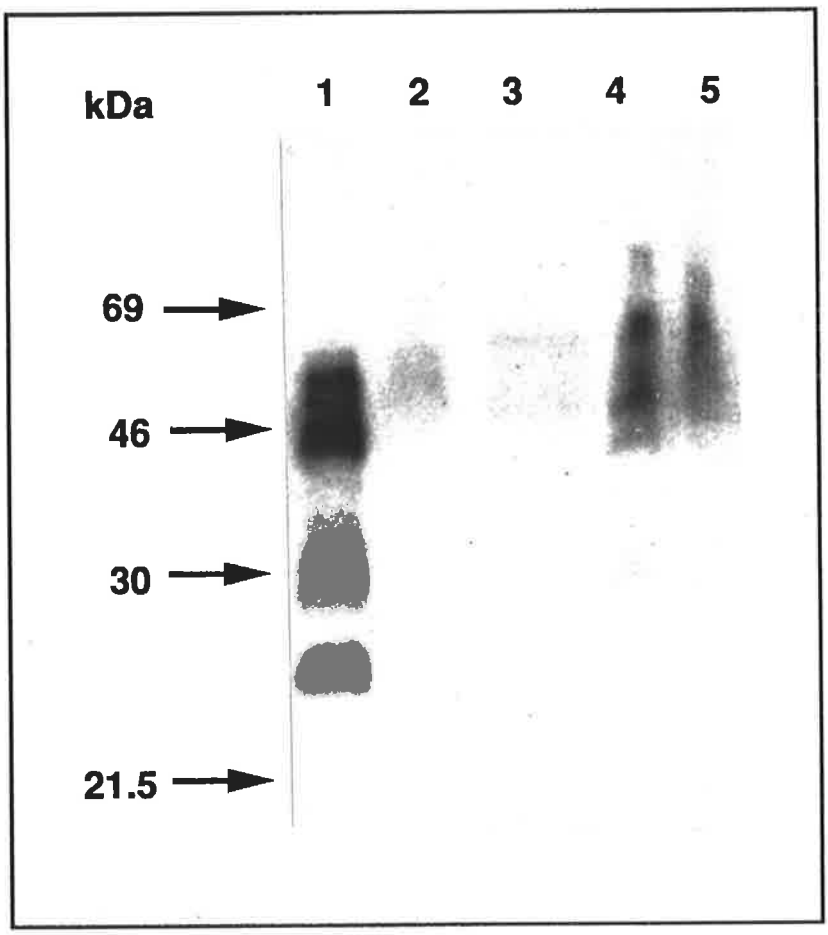
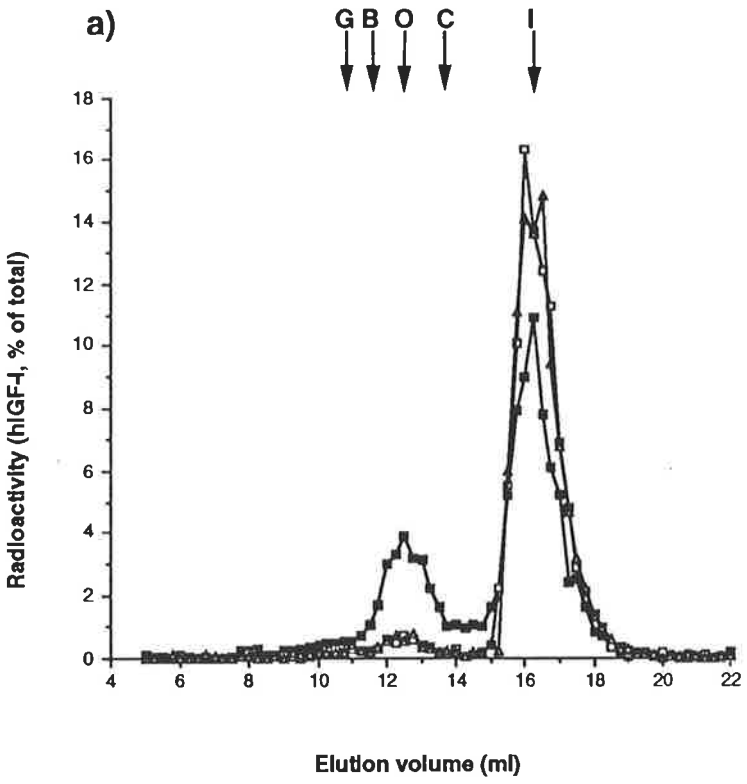
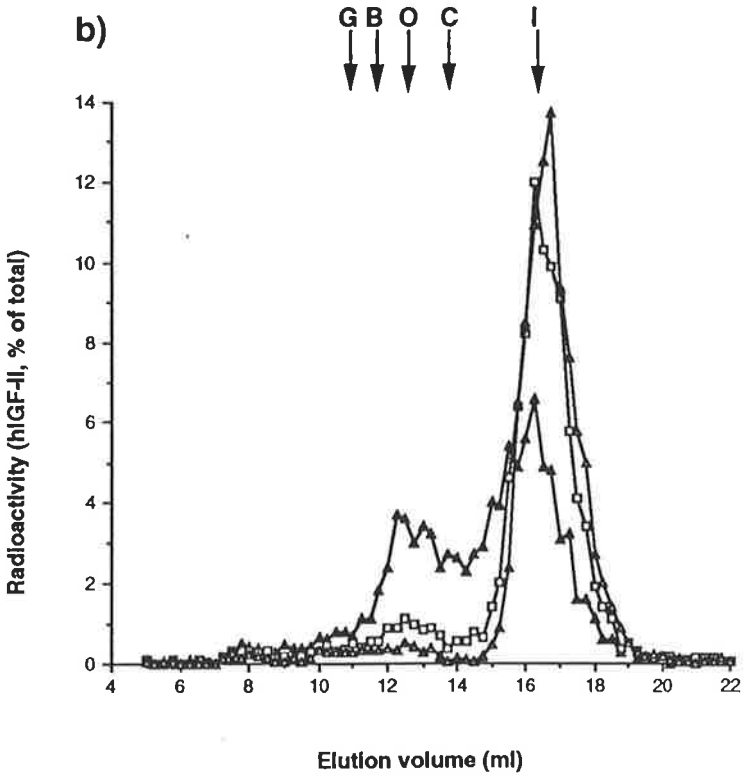


FIGURE 7.4: Ligand blotting of IGF-binding proteins in serum from two species of lamprey using labelled human IGF-I as a probe. The serum samples tested were 10 µl (lane 2) and 40 µl (lane 5) from adult *Geotris australis* , and 40 µl from adult *Petromyzon marinus* (lane 4). Proteins in fractions which were pooled following gel filtration chromatography of serum from *Petromyzon marinus* (7.3.2) were analyzed in lane 3. Reference adult rat serum is in lane 1. Molecular mass markers are indicated. The samples analyzed were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-I and the probed nitrocellulose sheets exposed to phosphorimaging screens.

FIGURE 7.5: Superose 12 size-exclusion chromatography on 50 µl of lamprey (*Geotria australis*) serum following incubation with radiolabelled a) recombinant human IGF-I (■) and b) radiolabelled recombinant human IGF-II (▲). The plots show the recovery of radioactivity in 0.25 ml fractions as a percentage of total radioactivity eluted. Serum pre-incubated with IGF-I tracer in the presence of excess human IGF-I (❑) or human IGF-II (Δ) were also analyzed. The elution positions of human-γ-globulin (G), 160 kDa; bovine serum albumin (B), 66 kDa; ovalbumin (O), 45 kDa; carbonic anhydrase (C), 29 kDa and radiolabelled IGF-I (I) are shown.


a)
G B O C I
Radioactivity (hIGF-I, % of total)
Elution volume (ml)


b)
G B O C I
Radioactivity (hIGF-II, % of total)
Elution volume (ml)

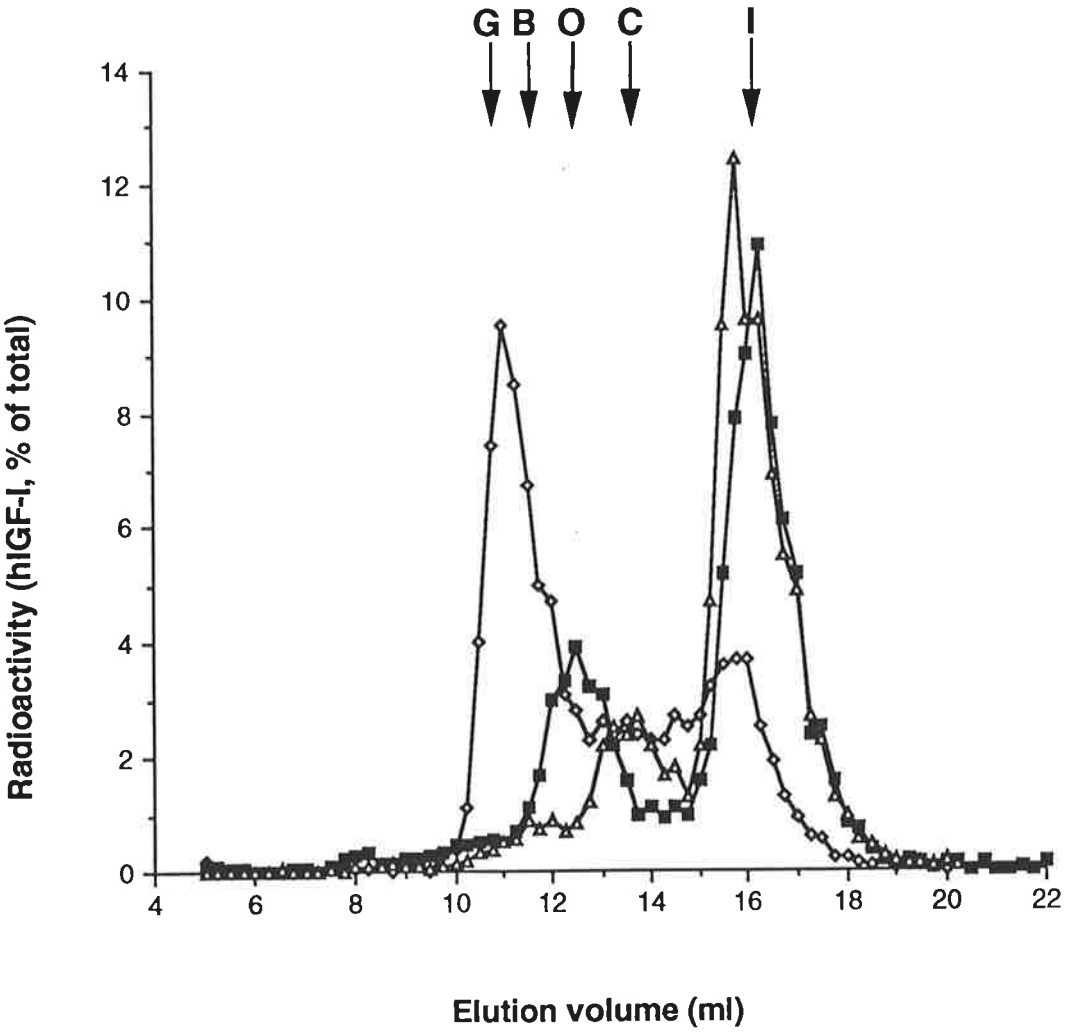


FIGURE 7.6: Superose 12 size-exclusion chromatography on 50 µl of lamprey (*Geotria australis*, ■), chicken (Δ) or rat serum (◊). Values represent the percent of total radioactivity in each fraction. The elution positions of human-γ-globulin (G), 160 kDa; bovine serum albumin (B), 66 kDa; ovalbumin (O), 45 kDa; carbonic anhydrase (C), 29 kDa and radiolabelled IGF-I (I) are shown.

7.3.3: Ligand blotting of vertebrate sera

Ligand blotting using radiolabelled hIGF-I (Fig. 7.7) was used to characterize the binding proteins in sera from a wide range of vertebrate sera. Serum from adult (lane 5) and ammocoete (lane 6) lamprey (*Geotria australis*) were analysed and both samples revealed a dominant IGFBP species with a molecular mass somewhat larger (50 kDa) than rat IGFBP3 (lane 1). Two smaller molecular mass proteins were also detected (32 kDa and 28 kDa), but were lower in abundance.

We also analysed sera from two species of turtles, *Emydura macquarii* (lane 3) and *Chelodina longicollis* (lane 4). The IGFBP patterns detected in these species were similar to that found in the chicken (lane 2). The dominant IGFBP in sera from these species appears to have a molecular mass slightly larger than the bands in lane 1 representing rat IGFBP1/ IGFBP2. An IGFBP with a size similar to rat IGFBP3 and one with a molecular mass intermediate between rat IGFBP4 and IGFBP1 (27 kDa) were also observed.

Serum from a monotreme (echidna) and a marsupial (sminthopsis) were also subjected to analysis using the ligand blotting procedure. Serum from echidna (lane 7) and sminthopsis (lane 8) shared similar IGFBP profiles, with a predominant IGFBP at approximately 36 kDa.

Serum from a range of reptiles were also analysed on ligand blots. IGFBPs were detected in serum from tiger snake (lane 9), sleepy lizard (lane 10), tuatara lizard (lane 11) and crocodile (lane 12). A wide range of IGFBPs were detected in these species, only some of which appear to have molecular masses similar to IGFBPs usually observed in mammals.

Ligand blotting of serum from rat (lane 1), sminthopsis (lane 3), echidna (lane 4), turtle (*Chelodina longicollis*, lane 5), lamprey (ammocoete, *Geotria australis*, lane 6), crocodile (lane 7) and tuatara lizard (lane 8) using radiolabelled hIGF-II (Fig. 7.8) revealed IGFBPs with similar molecular masses to those detected by radiolabelled hIGF-I. Chicken serum (lane 2) differed however,

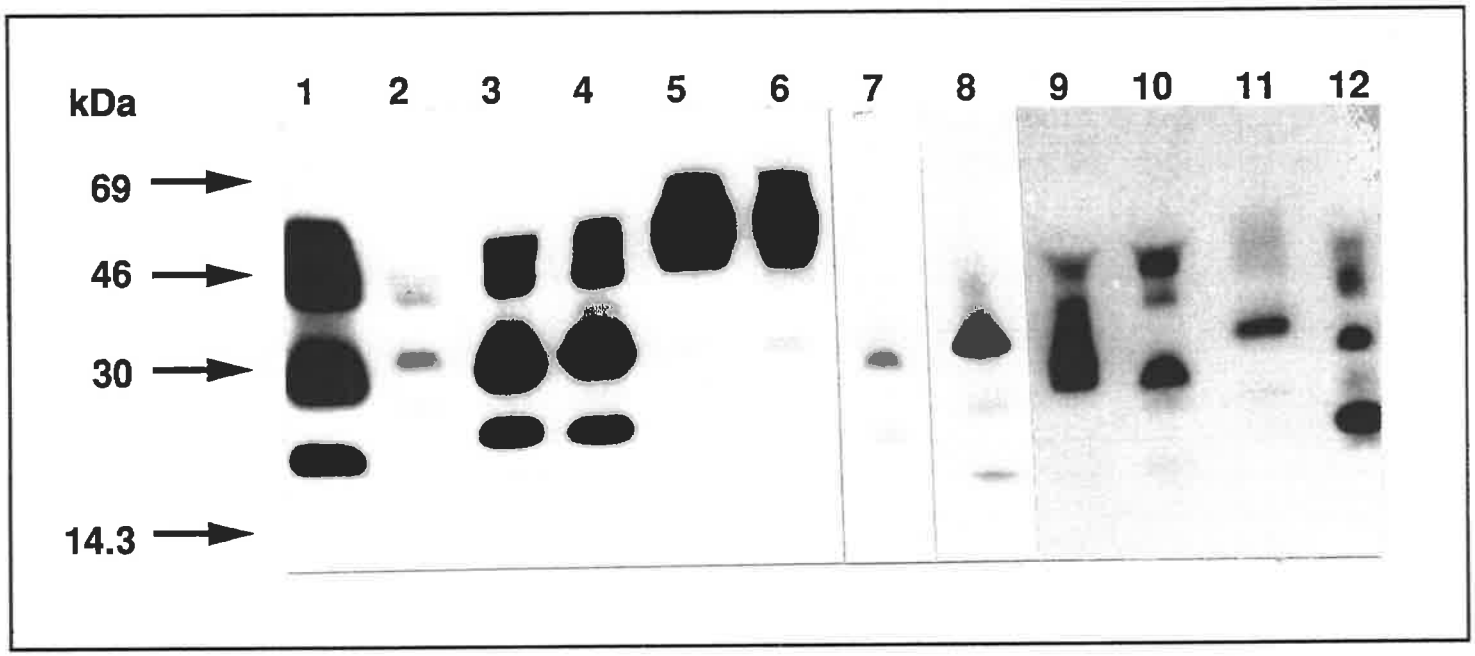


FIGURE 7.7: Ligand blotting of serum IGF-binding proteins using labelled human IGF-I as a probe. Serum from rat (1 µl, lane 1), chicken (10 µl, lane 2), turtle (*Emydura macquarii*, 10 µl, lane 3), turtle (*Chelodina longicollis*, 10 µl, lane 4), lamprey (adult, 10 µl, lane 5), lamprey (amocoete, 10 µl, lane 6), echidna (5 µl, lane 7), sminthopsis (2 µl, lane 8), tiger snake (10 µl, lane 9), sleepy lizard (10 µl, lane 10), tuatara lizard (10 µl, lane 11) and crocodile (10 µl, lane 12) were analyzed. Lanes 1, 3, 4, 5 and 6 were autoradiographed for 7 days; lane 2 for 14 days; and lanes 7, 9, 10, 11 and 12 for 10 days. Molecular mass markers are indicated. The samples analyzed were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-I and the probed nitrocellulose sheets exposed to X-ray film.

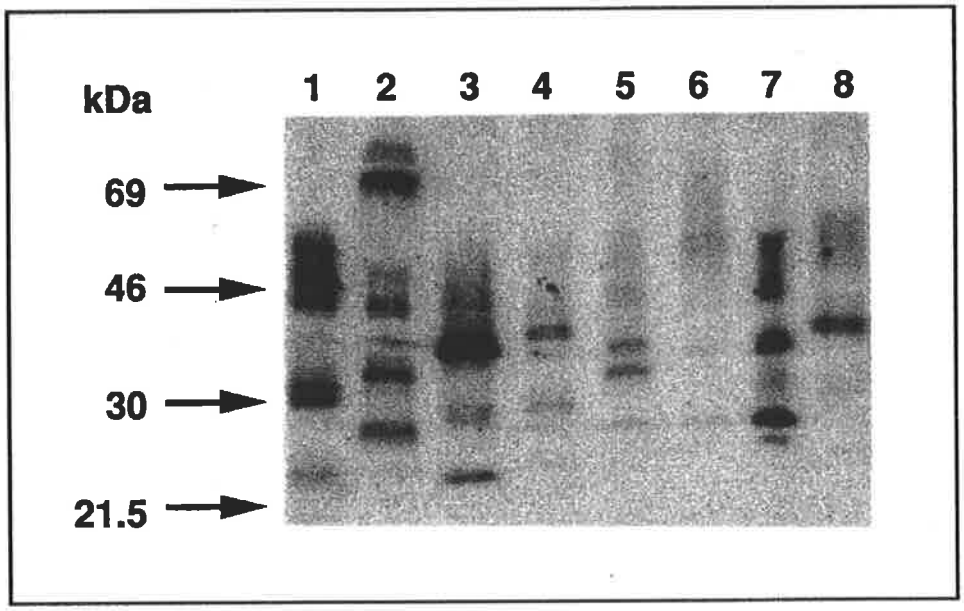


FIGURE 7.8: Ligand blotting of serum IGF-binding proteins using labelled human IGF-II as a probe. Serum from rat (1 µl, lane 1), chicken (15 µl, lane 2), sminthopsis (1 µl, lane 3), echidna (5 µl, lane 4), turtle (*Emydura macquarii*, 5 µl, lane 5), lamprey (amocoete, 8 µl, lane 6), crocodile (10 µl, lane 7) and tuatara lizard (10 µl, lane 8) were analyzed. Molecular mass markers are indicated. The samples analyzed were dissociated, subjected to polyacrylamide gel electrophoresis in 10% polyacrylamide gels and the proteins in the gels transferred to nitrocellulose sheets. These were then incubated with labelled IGF-II and the probed nitrocellulose sheets exposed to phosphorimaging screens for 36 h.

as additional bands (70-80 kDa) which are not observed in ligand blots of chicken sera probed with labelled hIGF-I, were detected.

7.3.4: Binding characteristics of human and chicken IGF-I and IGF-II

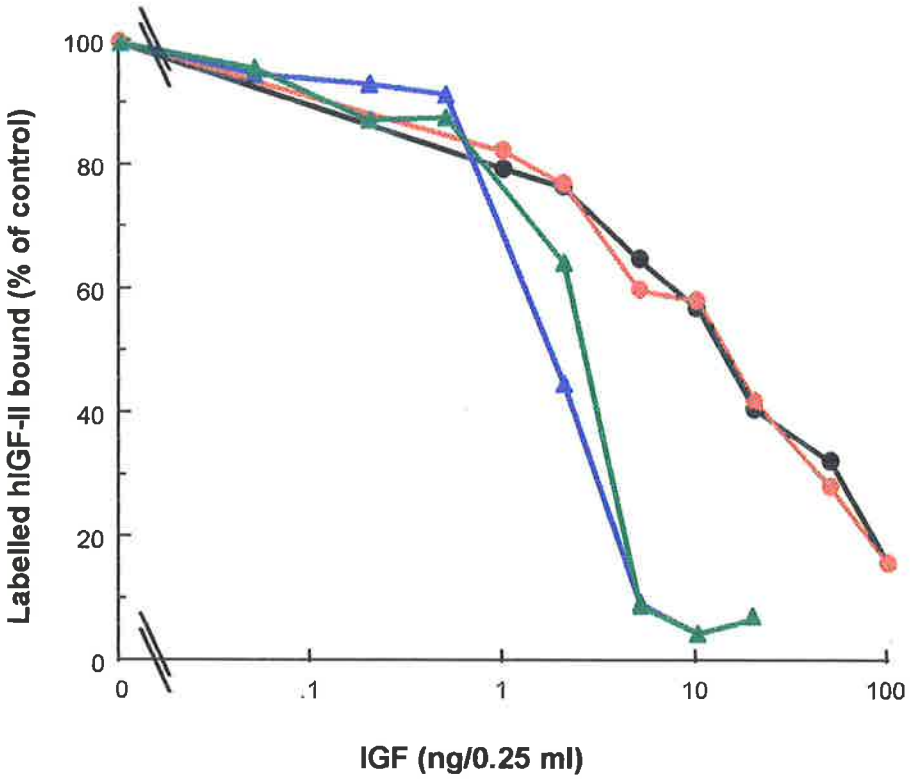
The IGFBP-binding characteristics of recombinant chicken IGF-I and IGF-II have been compared with human IGF-I and IGF-II in charcoal binding assays. I have determined that recombinant human and chicken IGF-II are equipotent in competing for binding of radiolabelled-human IGF-II to purified ovine IGFBP-3 (Fig. 7.9a), ovine IGFBP-4 (Fig. 7.9b) and to the binding proteins present in chicken (Fig. 7.10a) and rat plasma (Fig. 7.10b). Likewise, recombinant human and chicken IGF-I also appear to exhibit similar affinities for these proteins in charcoal binding assays (Fig. 7.9 & 7.10).

7.4: DISCUSSION

This study investigated the characteristics of IGFBPs in four Agnanthans, the Pacific and Atlantic hagfish, and the two lamprey species, *Petromyzon marinus* and *Geotria australis*. I have demonstrated using *in vitro* radioligand binding with subsequent analytical gel filtration, and with ligand blots, that serum from agnanthan species contain proteins which associate specifically with IGFs (Fig. 7.1, 7.2, 7.3, 7.4 & 7.5). Moreover, recombinant human IGF-I and -II bound to proteins in *Geotria australis*, inferring that the IGFBPs in this species contain motifs which recognise both IGF-I and IGF-II (Fig. 7.5, 7.7 & 7.8). As the agnanthans diverged from the main line of vertebrate evolution approximately 550 million years ago (Fig. 6.1), these results imply that IGFBPs, as well as IGFs (Nagamatsu *et al.* 1991) are indeed, very ancient proteins. The study by Reinecke *et al.* (1991) demonstrating that no IGFBPs were present in the Atlantic Hagfish is difficult to resolve since I detected IGFBPs in a ligand blot of serum from this species (Fig. 7.2, lane 5). Furthermore, recent studies have indicated that while serum IGFBP levels in the Atlantic hagfish appear to be lower than what is found in Pacific hagfish, specific IGFBPs are detected via both *in vitro*

FIGURE 7.9: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to a) purified ovine IGFBP3 and b) purified ovine IGFBP4. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●), human IGF-II (blue ▲), and chicken IGF-II (green ▲). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
.1
1
10
100
IGF (ng/0.25 ml)

b)

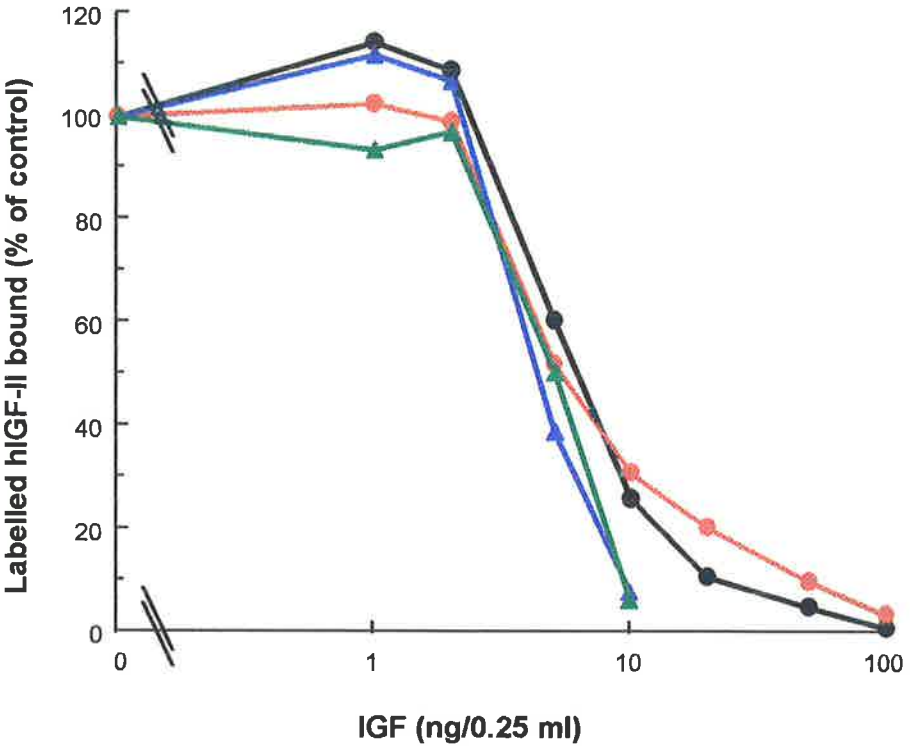
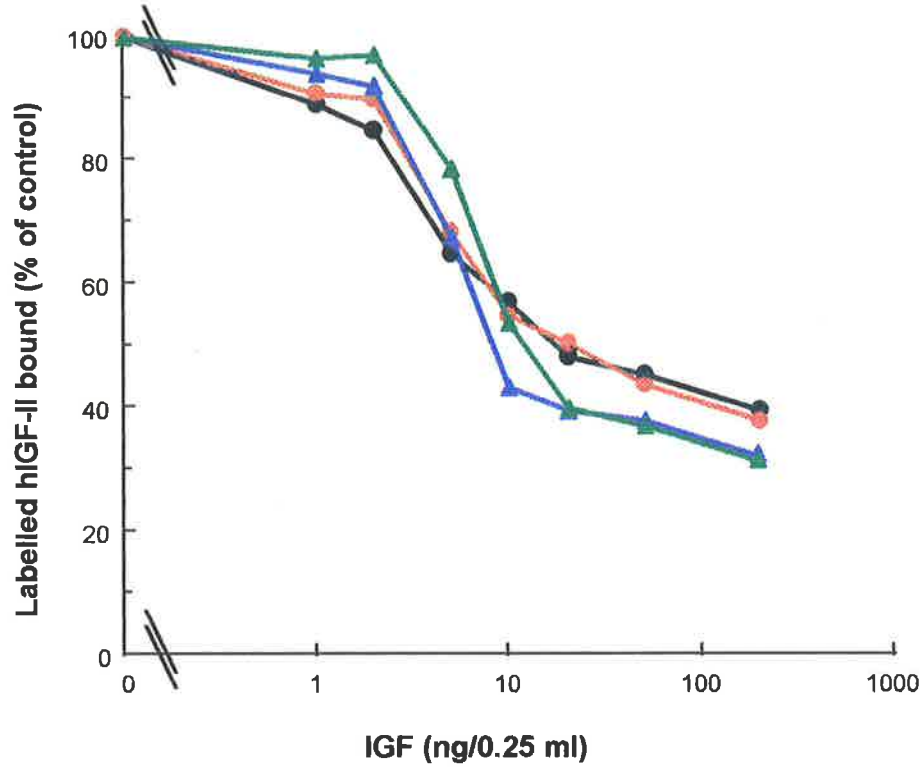
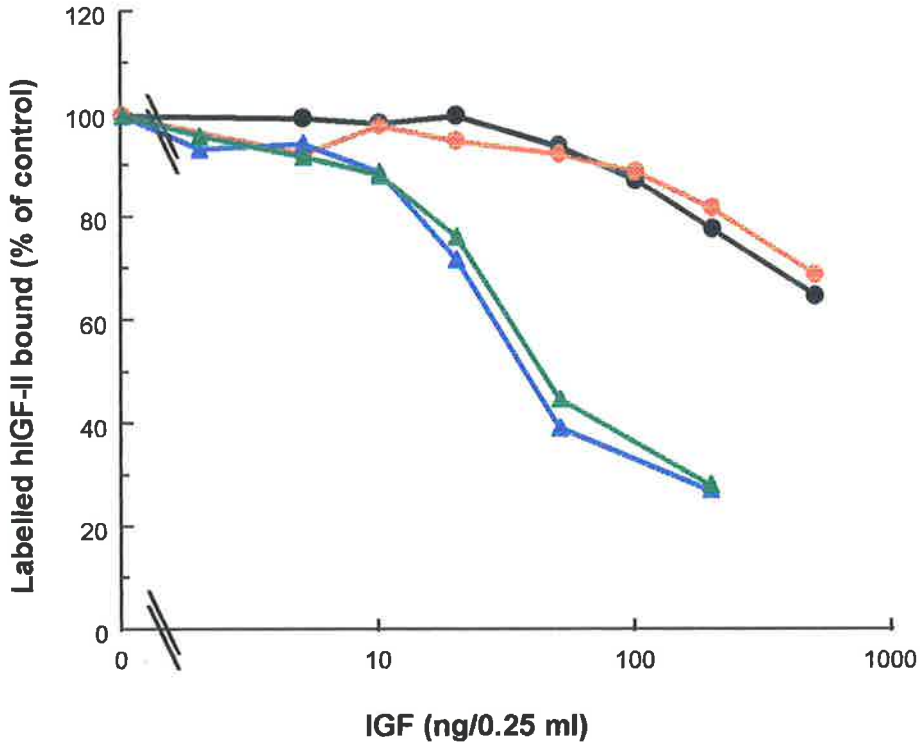

Labelled hIGF-II bound (% of control)
120
100
80
60
40
20
0
0
1
10
100
IGF (ng/0.25 ml)

FIGURE 7.10: Effects of IGFs on competition for binding of [^{125}I]-labelled human IGF-II to IGFBPs present in a) chicken and b) rat plasma. The proteins tested were human IGF-I (black ●), chicken IGF-I (red ●), human IGF-II (blue ▲), and chicken IGF-II (green ▲). Values are the means of triplicate determinations at each peptide concentration. The S.E.M. values were less than 5% for each peptide concentration.

a)


Labelled hIGF-II bound (% of control)
100
80
60
40
20
0
0
1
10
100
1000
IGF (ng/0.25 ml)

b)


Labelled hIGF-II bound (% of control)
120
100
80
60
40
20
0
0
10
100
1000
IGF (ng/0.25 ml)

labelling with subsequent analytical neutral gel chromatography and by ligand blotting (*personal communication, Dr. S. Chan, Department Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, USA*).

Size-exclusion chromatography under neutral conditions indicates that the molecular mass of the major IGFBP in serum from the three Agnanthan species I studied, is significantly smaller than the IGFBP3 ternary complex found in rat serum. The molecular masses of 62, 75 and 48 kDa for the major IGFBP in Pacific hagfish, *Petromyzon marinus* and *Geotria australis* respectively, as determined by size-exclusion chromatography, while not identical, are consistent with the molecular masses found by the ligand blotting procedure. Ligand blotting on sera from the three agnanthan species revealed that the predominant IGFBP species present had a molecular mass of approximately 50 kDa. These results are similar to that reported by Niu & Le Bail (1993) who found that ligand blotting of trout serum revealed a major protein species of approximately 42 kDa. Kelly *et al.* (1992) also detected IGFBPs of approximately 40-50 kDa in ligand blots of serum from four bony fish species. While Kelly *et al.* (1992) believe this IGFBP species is a good candidate for the fish version of mammalian IGFBP3, due to its size and the fact that it may be GH-regulated, no neutral gel chromatography studies on sera from bony fish have been undertaken thus far to ascertain if the IGFBP associates in a high molecular mass ternary complex. Moreover, Anderson *et al.* (1993) have found that the major IGFBP species detected in golden perch serum following *in vitro* radiolabelling and subsequent neutral analytical gel chromatography, is significantly smaller than the size found in rat serum. Superose 12 size-exclusion chromatography of *in vitro*-labelled chicken serum indicated that the major serum IGFBP in chickens was also smaller than that found in rat serum (Fig. 7.6), in agreement with a previous report by Francis *et al*, (1990) who compared chicken and human sera. Together these results suggest that the major serum IGFBPs in agnanthans, fish and chickens might not circulate in a ternary complex (an acid-labile subunit, IGFBP3 plus IGF) as is found in mammals (Baxter *et al.* 1989). Thus the association of

mammalian IGFBP3 with an acid-labile subunit may be a more recent evolutionary development and may serve a specific function in mammalian growth or development, similar to the way in which the IGF type-2 receptor appears to have evolved exclusively in mammals (Canfield & Kornfeld, 1989).

It should be noted that both Armstrong *et al.* (1989) and Lee *et al.* (1989) detected a high molecular mass IGF-binding species in chicken serum similar in size to that found in mammals. However, Armstrong *et al.* (1989) were unable to demonstrate specific binding as excess IGF-I did not compete for binding to this protein species. More recently, Morishita *et al.* (1993) have also demonstrated via *in vitro* labelling followed by analytical neutral gel chromatography that chicken serum contains a IGFBP with a higher molecular mass than what I observed. They too found that while this binding species appeared to be the predominant carrier of IGF in serum, labelled IGF was not readily displaced by the addition of excess IGF, suggesting that either the IGF/IGFBP complex is structurally stable, and/or the protein has more than one site for IGF binding. Resolution of these issues awaits the purification and characterization of the chicken serum IGFBPs.

The availability of recombinant chicken IGF-I and IGF-II has enabled comparison for the first time of the affinities of the peptides for IGFBPs with human IGF-I and IGF-II. The avian IGFs exhibited affinities similar to their human counterparts for purified ovine IGFBP3, IGFBP4 and for the IGFBPs present in rat and chicken plasma (Fig. 7.9 & 7.10). These results suggest that motifs in IGFs important for interacting with these IGFBPs had evolved prior to the divergence of avian and mammalian species. Of particular interest is the finding that the IGFBPs present in rat plasma have a higher affinity for IGF-II than for IGF-I, whereas the IGFBPs present in chicken plasma appear to have similar affinities for both IGF-I and IGF-II.

Ligand blotting of different nonmammalian sera has also provided insights into the evolution of IGFBPs. My results indicate that IGFBPs are widely distributed throughout nonmammalian vertebrates since IGFBPs were detected

in all samples analyzed (Fig. 7.7 & 7.8). Although a diverse range of IGFBP sizes were detected, further analysis is required before we can establish the relationship of these proteins to the IGFBPs found in mammals. Immunological characterization of the nonmammalian serum IGFBPs and cloning of their cDNAs will be required to relate the bands in the ligand blots to the IGFBPs found in mammals. Nevertheless, it is interesting to note that both adult and ammocoete lamprey serum share similar IGFBP patterns in the ligand blots, suggesting that the IGFBP serum profile appears to be conserved during development in lamprey. The detection of additional IGFBPs in ligand blots of chicken serum probed with radiolabelled IGF-II, but not found in blots probed with labelled IGF-I, has previously been discussed in Chapter 4. Duclos *et al.* (1992) and Schoen *et al.* (1992) also detect a 70 kDa protein species when probing chicken plasma on ligand blots with [^{125}I]-labelled hIGF-II. However, this differential detection between labelled IGF-II and labelled IGF-I appears to be a unique characteristic of chickens as no other vertebrate species in this study probed with radiolabelled hIGF-II gave a similar discriminating result. Whether the 70 kDa IGF-II-binding protein species is an IGF-II-specific chicken IGFBP or represents a precursor to the IGF-II-binding type-2 IGF receptor found in mammalian species, awaits further investigations.

Given that IGFs evolved very early in vertebrate evolution (Nagamatsu *et al.* 1991) it seems logical that the regulatory IGFBPs also share a long phylogenetic history. Presumably, both the IGFs and the binding proteins serve important functions in vertebrate growth and development. A cDNA encoding a hybrid IGF/insulin molecule has been cloned from the protochordate amphioxus (Chan *et al.* 1990) but it is not known whether IGFBPs are present in this organism. In addition, insulin-like receptors and insulin-like peptides have now been characterized in insects (Nishida *et al.* 1986; Laguex *et al. 1990*; Sevala *et al.* 1993; Gorczyca *et al.* 1993) and in molluscs (Smit *et al.* 1993), and it will be interesting to determine if proteins homologous to IGFBPs exist in non-

vertebrates. Recent reports detecting IGF-binding activity in plants suggest this will be the case (Komatsu & Hirano 1991; Barbashov *et al.* 1992).

CHAPTER 8

GENERAL DISCUSSION

8: GENERAL DISCUSSION

At the time I commenced research for this PhD thesis (March 1991) little was known about nonmammalian IGFs beyond the fact there was both structural and immunological evidence for their existence in lower vertebrates (see 1.3). In particular, knowledge about the functional properties of nonmammalian IGFs was scarce. Indeed, chicken IGFs were the only nonmammalian IGFs to have been functionally characterized (Dawe *et al.* 1988; Armstrong *et al.* 1990; Ballard *et al.* 1990; Kallincos *et al.* 1990). Even so, characterization of these IGFs was hindered by the small amount of protein available for testing. Information on the properties and functions of nonmammalian IGFBPs was even more limited. In fact, the few studies investigating the presence of IGFBPs in nonmammalian species gave conflicting evidence (see 1.3.8). Thus the goals of my PhD research were two-fold. Firstly, I aimed to produce nonmammalian IGFs using recombinant DNA technologies, hence allowing large amounts of non-mammalian IGFs to be available for functional characterization. Secondly, I wished to characterize their functions from an evolutionary perspective. Indeed, this is why I also investigated the presence of IGFBPs in nonmammalian vertebrates.

This laboratory had previously reported the purification of chicken IGF-I and IGF-II from serum (Dawe *et al.* 1988; Kallincos *et al.* 1990; Ballard *et al.* 1990). However, complete assessment of the *in vitro* and *in vivo* biological effects of the serum-derived IGFs was not possible as insufficient quantities of the growth factors were available for testing. In order to overcome this, I have produced recombinant chicken IGFs from *E. coli.*, thus allowing a more detailed assessment of nonmammalian IGFs for the first time. While the recombinant chicken IGFs were produced using gene-fusion systems similar those developed in this laboratory for the production of human IGFs, significant changes to the design of the fusion proteins and to the downstream processing procedures were required. For example, chicken IGF-I was susceptible to proteolytic cleavage by bacterial proteinases, a situation not observed during the

production of recombinant human IGF-I. Nevertheless, the addition of an anion exchange chromatography step, along with the inclusion of $ZnCl_2$ in the buffers in the early stages of the downstream processing procedure significantly reduced the proteolysis of chicken IGF-I.

Production of recombinant chicken IGF-II involved the development of changes not only to the downstream processing procedure, but also, to the design of the fusion protein itself. Expression of chicken IGF-II from the *E. coli* expression plasmid was only possible when the protein was expressed with a longer N-terminal extension peptide. Subsequent attempts to produce bovine IGFBP2 or human EGF using this same *E. coli* expression vector have also required the expression of the proteins with longer leader peptides (*personal communication, S. Aplin, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA 5000 Australia*). As described in chapter 4, expression of the chicken IGF-II with a longer N-terminal extension peptide, necessitated the inclusion of two cleavage steps during the isolation of the protein. While this in itself was not a major problem, it adds to the complexity of production of recombinant chicken IGF-II. What does appear to be a significant problem, however, is the low yield from the second cleavage step where cIGF-II is released from pGH(20-46)-Val-Asn-Phe-Ala-His-Tyr-cIGF-II using H64A subtilisin BPN'. Clearly, an alternative production system needs to be developed before large quantities of chicken IGF-II for *in vivo* studies can be produced. There are a number of ways to address this problem. As cIGF-II contains no Met residues, cIGF-II could be released from the leader peptide using cyanogen bromide and a methionine linker. Indeed, a number of groups have used cyanogen bromide to release recombinant human IGF-II from fusion partners (Furman *et al.* 1987; Roth *et al.* 1991; Wadenstein *et al.* 1991; Marumoto *et al.* 1992). However, considering the practical hazards associated with using cyanogen bromide, we would prefer to use an alternative cleavage system. Indeed, others in this laboratory are currently designing site-specific proteases which may prove to be more useful than H64A subtilisin BPN' for the production

of recombinant chicken IGF-II (*personal communication, S. Milner, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA 5000, Australia*).

The production of recombinant chicken IGF-I and IGF-II using the protocols outlined in this thesis has enabled the first systematic, functional characterization of nonmammalian IGFs *in vitro*. I have established that recombinant chicken IGF-I is in fact equipotent to human IGF-I in its biological, IGFBP- and receptor-binding interactions whether assessed in mammalian or avian test systems. *In vitro* analysis of recombinant chicken IGF-II on the other hand, has revealed differences between chicken IGF-II and its human counterpart. Recombinant chicken IGF-II has a lower affinity than human IGF-II for the type-1 IGF receptor present in rat myoblasts. This in turn appears to account for the lower biological activity of chicken IGF-II observed in these cells, since chicken and human IGF-II have similar affinities for mammalian IGFBPs and for the type-2 IGF receptor. The human and chicken IGF-II peptides are equipotent, however, in both biological and receptor binding studies in chick embryo fibroblasts, suggesting that there may be a difference between mammalian and avian type-1 IGF receptors. A partial cDNA sequence for the chicken type-1 receptor has recently been published and the deduced sequence reveals 96% identity with the human type-1 IGF receptor at the amino acid level (Armstrong & Hogg, 1992). However, the clone does not encode the N-terminal portion of the receptor where the IGF binding specificity is conferred. Complete characterization of the primary structure of the chicken type-1 IGF receptor may well confirm that there are differences between the mammalian and avian receptors.

In addition to producing and characterizing chicken IGF-II, I also made two recombinant chicken IGF-II analogues. As discussed in chapter 4, one of these analogues was produced by default because at the time I first commenced this project, there was no DNA information to confirm the chicken IGF-II protein sequence reported by Kallincos *et al.* (1990). Accordingly, I made recombinant chicken IGF-II based on the published protein sequence. Subsequently it was

established that chicken IGF-II should in fact contain an additional arginine residue at position 40. Taylor *et al.* (1991) also confirmed by DNA sequence information one of the two C-terminal sequences reported by Kallincos *et al.* (1990). Thus the first chicken IGF-II molecule I made was actually an analogue, that is, Des-Arg^{40}-[Thr^{62}Pro^{63}Ala^{64}]-cIGF-II. The second chicken IGF-II analogue I made included arginine at position 40, but was engineered to contain the alternative C-terminal sequence reported by Kallincos *et al.* (1990), viz [Thr^{62}Pro^{63}Ala^{64}]-cIGF-II. I have determined, however, that these two chicken IGF-II analogues exhibit no significant differences from chicken IGF-II in their biological activities and in their receptor and IGFBP interactions.

In addition to the two IGF analogues described above, I also produced a third analogue, Long [Arg^{3}]-cIGF-I. This particular analogue was produced because *in vitro* and *in vivo* studies with the human equivalent of this protein have shown that it is a more potent form of IGF-I. The increased potency is not associated with an increase in affinity for the type-1 IGF receptor, but is due to decreased affinity for IGFBPs (Francis *et al.* 1992). The recombinant chicken homologue I produced also exhibits decreased affinity for chicken, rat and sheep IGFBPs and enhanced biological activity in rat myoblasts. Moreover, the importance of IGFBPs in determining the biological outcome of IGFs in cultured cells was demonstrated by the fact that both the human and chicken fusion protein analogues were less effective than chicken IGF-I in inhibiting protein degradation in cultured chick embryo fibroblasts, a cell line which produces little, if any IGFBPs (Ross *et al.* 1989).

The availability of recombinant chicken IGF-I and IGF-II, as well as Long [Arg^{3}]-cIGF-I, will facilitate understanding of the actions of IGFs in chickens. The production of the recombinant peptides will permit not only a wide range of *in vitro* studies, but also experiments involving exogenous administration of chicken IGFs. There are two published studies in which human IGFs were administered to chickens *in vivo* (McGuiness & Cogburn, 1991; Huybrechts *et al.* 1992). In the study by McGuiness & Cogburn (1991), IGF-I was administered

intramuscularly and demonstrated no growth effects. On the other hand, Huybrechts *et al.* (1992) infused IGF-I using osmotic minipumps. They found that while administration of IGF-I did not increase weight gain or influence carcass composition, there was evidence of fat repartitioning and improved feed efficiency at the highest dose of IGF-I. However, both these studies may not reflect true physiological responses because of the use of human IGF-I. Thus Francis *et al.* (1990) have previously demonstrated that human IGF-I is cleared from the circulation more rapidly than serum-derived chicken IGF-I, suggesting that optimum metabolic effects are more likely to be observed with homologous IGF-I. Indeed, the first studies administering my recombinant chicken IGFs have recently commenced (*personal communication, Drs. F. Tomas and K. Kita (visiting scientist), Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA , Australia).* In particular we hope to use the information generated from the *in vitro* and *in vivo* studies to develop a form of chicken IGF which can be used to increase growth rates or food conversion efficiencies; reduce fat content in favour of muscle; or provide a treatment for disease states or for musculo-skeletal defects. The results from *in vivo* administration of chicken IGF-II may prove to be particularly interesting since chickens do not have the specific type-2 IGF receptor. Furthermore, there are no published reports on either the clearance of IGF-II in the circulation, or on the administration of IGF-II to chickens.

Another benefit which will arise out of the availability of recombinant chicken IGFs is that homologous radioimmunoassays for measuring serum IGF levels can be developed, thus enabling absolute levels of chicken serum IGFs to be measured for the first time. This in turn will be useful in establishing whether serum IGF levels can be used as indicators or predictors of potential differences in growth and nutrient partitioning. Accordingly, McMurtry *et al.* (1993) have developed a specific radioimmunoassay using my recombinant chicken IGF-I as antigen, radiolabelled tracer and standard.

Considerable research effort is still required to determine whether IGFs play any unique roles in avian growth, or indeed whether chicken IGFs, or

analogues of chicken IGFs can be used to improve growth performance of birds. However, the production of recombinant chicken IGFs now provides an opportunity to use homologous peptides when studying the functions of IGFs in the avian endocrine system. If IGFs are in fact involved in as many varied and important functions in chickens as has been found in mammals, the benefits to the poultry industry could be considerable. We recognise, however, that even if improved growth performance can be demonstrated with exogenous administration of chicken IGFs, alternative, cost-effective administration technologies need to be developed. To achieve this end we have commenced investigations examining the feasibility of administering chicken IGFs via *in ovo* and viral vector technologies. Moreover, the recent advances in avian transgenesis may make it plausible to manipulate the growth performance of birds by selectively controlling the expression of chicken IGF genes (Etches & Verrinder-Gibbins, 1993; Perry & Sang, 1993).

While there were obvious commercial reasons for producing recombinant chicken IGFs, I was also interested in characterizing their functions from an evolutionary perspective. The results I obtained suggest that motifs important for biological, as well as for receptor- and IGFBP-binding interactions have been conserved between human and chicken IGFs. Indeed, functional characterization of the recombinant [Gly]-hagfish IGF I produced also indicates that motifs associated with mammalian IGFs are present in hagfish IGF, suggesting that these motifs evolved prior to the Agnanthans diverging from the main line of vertebrate evolution 550 million years ago. Thus, we now have functional and as well as structural evidence that the IGFs share a long evolutionary history. While my results with [Gly]-hagfish IGF suggest that it seems likely that IGF-I evolved prior to IGF-II, this still remains to be unequivocally established.

Given that the IGFs evolved early in vertebrate evolution, my finding that the IGFBPs also share a long evolutionary history is not surprising. However, further characterization of nonmammalian IGFBPs is required before we can

determine whether counterparts for the six mammalian IGFBPs and the acid-labile subunit are in fact present in nonmammalian species. It is likely that the requirement for six IGFBP regulatory proteins in mammals is a more recent evolutionary event which has arisen due to the complexity of mammalian growth and development. Indeed, my results comparing the chicken and human IGF peptides *in vitro*, suggest that it is not so much the IGF molecules themselves which differ, but rather, the systems which regulate the bioavailability of the IGFs within mammalian and nonmammalian species. There are a number of factors that suggest this is the case, the most obvious being the presence of a type-2 IGF receptor in mammals, but not in chickens or in frogs (Canfield & Kornfeld, 1989; Clairmont & Czech, 1989). In addition, the presence in chicken serum of an IGF-II-specific binding protein which is not detected in rat serum; the parental imprinting of mammalian IGF-II and type-2 IGF receptor genes (Haig & Graham , 1991); and the diversity in the range of sizes of the predominant serum IGFBPs in mammalian and lower vertebrates, all point to there being significant differences between the regulatory systems for mammalian and nonmammalian IGFs. Clearly, much research remains to be undertaken and it is likely that even more differences will be found. However, the production of other non-mammalian IGFs in addition to those described in this thesis, should prove to be useful tools in characterizing and in attempting to gain some insight into the evolution of these regulatory mechanisms.

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