Structure-Function Studies of Insulin-like Growth Factor I

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ABSTRACT

The distinct physiological roles of IGF-I and insulin have been enhanced during evolution by variation in receptor tissue distribution and ligand binding specificity. Despite a large effort aimed at understanding the intrinsic signalling differences between the type 1 IGF receptor and the insulin receptor, it is still unclear how these receptors account for the different physiological roles of IGF-I and insulin. Better understanding of the molecular mechanism involved in receptor binding may clarify the interdependence of IGF-I and insulin actions.

Insulin-like growth factor-I (IGF-I) analogues were produced with the aim of identifying IGF-I residues that contribute to binding specificity for the IGF type 1 receptor and insulin receptor. Using a rational design approach, A- and B-domain amino acid positions were targeted and substituted with the primary aim of reducing cross-reactivity with the insulin receptor. Such analogues may be useful in distinguishing responses specifically due to the type 1 IGF receptor interaction. Receptor binding properties were compared using rat L6 myoblasts, soluble human IGF type 1 receptors and soluble human insulin receptor isoforms, HIR-A (-Ex11) and HIR-B (+Ex11). IGF-I analogues, [Leu^8] IGF-I and [Phe^{59}] IGF-I, were shown to be less insulin-like with respect to receptor binding. A 28- and 17-fold reduction in HIR-A binding affinity was observed for [Leu^8] IGF-I and [Phe^{59}] IGF-I respectively, albeit with an associated ~5-fold decrease in relative affinity for the soluble type 1 IGF receptor. In contrast, [Leu^{62}] IGF-I was shown to be 8-fold less potent than IGF-I in soluble IGF type 1 receptor binding and only showed a subtle decrease in HIR-A and HIR-B binding. The results presented in the thesis have identified a number of A- and B-domain positions in IGF-I that are important for maintaining high receptor binding affinity and receptor binding specificity. The substitutions made indicate that the receptor specificity of IGF-I evolved with a number of sequence changes within the B- and A-domains that collectively contribute to the observed receptor binding properties of IGF-I. This study supports the conclusion that the co-evolution of the IGF-I and insulin receptor/ligand systems has resulted in subtle structural differences in the A- and B-regions of each ligand important for defining receptor binding specificity.

As part of a broader study on the involvement of the B-domain in IGFBP interactions, the analogues [Leu^8] IGF-I and [Ser^{15}]IGF-I were assayed against IGFBPs 1-6. The analogue [Ser^{15}] IGF-I was shown to have similar binding affinities to native IGF-I for IGFBPs 1-6. The substitution of Ala^8 with Leu resulted in differential reductions in IGFBP binding affinities. Relative to IGF-I, the binding affinity of [Leu^8] IGF-I ranged from 3-fold to 77-fold lower for IGFBP-5 and IGFBP-2 respectively. These results suggest that the N-terminal region of the B-domain α-helix of IGF-I is important for conferring IGFBP specificity.

Numerous IGF-I analogue studies suggest that IGF-I induced biological response is directly related to type 1 IGF receptor binding affinity. The main exceptions have been IGF-I analogues with reduced binding affinity for the IGFBPs. The relatively high biological potencies of these analogues is believed to result from increased concentrations of free hormone available for receptor interaction. Comparisons for IGF-I analogues [Leu^8] IGF-I, [Phe^{59}] IGF-I, [Leu^{62}] IGF-I and [Ser^{15}, Leu^{62}] IGF-I shows that their biological potencies, as measured by the stimulation of protein synthesis in L6 myoblasts, does not correlate with receptor binding affinity and IGFBP binding affinity. The analogue [Phe^{59}] IGF-I provides the clearest example of this occurrence, as its affinity for IGFBPs secreted by L6 myoblasts was identical to native IGF-I. Despite a 12-fold decrease in affinity for the type 1 IGF receptor, [Phe^{59}] IGF-I was marginally more potent than native IGF-I in stimulating protein synthesis, as measured in the L6 myoblast cell line. These results suggest that factors other than receptor binding affinity may be important for determining the magnitude of the IGF-I mitogenic response. Indeed, insulin analogue studies demonstrate that the mitogenic response of the insulin receptor is related to the residence time of the ligand-receptor interaction rather than the receptor binding affinity. This study suggests that, like the insulin receptor, residence time rather than equilibrium binding affinity may be an important factor in determining the magnitude of the mitogenic response from the type 1 IGF receptor.

DECLARATION

The work presented in this thesis is the original work of the author, except where otherwise acknowledged. It has not been submitted, either in whole or part, for a degree at this or any other university.

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PUBLICATIONS

Shooter, G. K., Magee, B., Soos, M. A., Francis, G. L., Siddle, K. & Wallace, J. C. (1996). "Insulin-like growth factor (IGF)-I A- and B-domain analogues with altered type 1 IGF and insulin receptor binding specificities." *Journal of Molecular Endocrinology* **17** 237-246.

Magee, B. A., **Shooter, G. K**, Wallace, J. C. & Francis, G. L. (1999). Insulin-like growth factor I and its binding proteins: a study of the binding interface using B-domain analogues. *Biochemistry* **38,** 15863-15870.

Laajoki, L. G., Breton, E. L., **Shooter, G. K.**, Wallace, J. C., Francis, G. L., Carver, J. A. & Keniry, M. A. (1997). Secondary structure determination of ^{15}N-labelled human long-[Arg-3]-insulin-like growth factor I by multi-dimensional NMR spectroscopy. *FEBS Letters* **420** 97-102.

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ABBREVIATIONS

A	Alanine
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
APS	Ammonium persulphate
BSA	Bovine serum albumin
C	Cysteine
Cys	Cysteine
D	Aspartate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E	Glutamate
ED_{50}	Dose of a compound producing a half maximal response
EDTA	Ethylene diamine tetra-acetic acid
F	Phenylalanine
G	Glycine
Gln	Glutamine
Glu	Glutamate
Gly	Glycine
H	Histidine
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HIR	Human insulin receptor
His	Histidine
I	Isoleucine
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
Ile	Isoleucine
K	Lysine
L	Leucine
Leu	Leucine
Lys	Lysine
LB	Luria Berani medium

M	Methionine
Met	Methionine
mRNA	Messenger RNA
N	Asparagine
P	Proline
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
pGH	Pig growth hormone
Phe	Phenylalanine
PMSF	Phenylmethylsuphonylfluoride
Pro	Proline
Q	Glutamine
R	Arginine
RIA	Radio-immunoassay
RNA	Ribonucleic acid
RRA	Radio-receptor assay
RT	Retention Time
S	Serine
SDS	Sodium dodecyl sulphate
Ser	Serine
S.E.M.	Standard error of the mean
T	Threonine
TCA	Trichloroacetic acid
TEMED	N,N,N,N'-tetra-methylethylenediamine
TFA	Trifluoroacetic acid
Thr	Threonine
Tris	Tris (hydroxymethyl) aminomethane
Trp	Tryptophan
Tyr	Tyrosine
V	Valine
Val	Valine
v/v	Volume to volume
W	Tryptophan
WGA-Sepharose	Wheat germ agglutinin-Sepharose
w/v	Weight to volume
Y	Tyrosine

Chapter 1

Introduction

The insulin-like growth factors (IGFs) are a family of polypeptides which are structurally related to insulin. Despite the high degree of structural homology, IGFs and insulin have distinct physiological roles. In this chapter I will give an overview of IGF structure, IGF actions, the IGF binding proteins (IGFBPs) and IGF receptors. Studies aimed at mapping the regions and residues of IGF-I that are involved in the its various interactions will then be described in detail. Finally, I will address the aims of my PhD project. Results presented in Chapters 3 & 4 represent experimental work conducted between March 1992 and Aug 1995. This chapter will primarily encompass a review of literature available up to the end of 1992, however, where appropriate, more recent references have been included. Recent information relating to my project will also be included in the appropriate chapters.

1.1 The Insulin-like Growth Factors

1.1.1 Historical Identification of IGFs

IGF-I was first described by Salmon and Daughaday (1957) who observed that growth hormone injected into hypophysectomized rats stimulated sulfate incorporation into cartilage in rats *in vivo* but not when added to culture medium *in vitro*. The biological activities of this factor were later shown to include the stimulation of DNA synthesis (Daughaday & Reeder, 1966) and protein synthesis (Salmon *et al.*, 1970). The appearance of a 'sulphation factor' following growth hormone injection led to the somatomedin hypothesis that states growth hormone mediates a response by inducing a factor or somatomedin which circulates in the blood to target tissues (Daughaday *et al.*, 1972). During the period in which the biological activities of the sulphation factor were being characterized, parallel studies were investigating the serum factors with insulin-like activities. Froesch *et al.*, (1963) described a non-suppressible insulin-like activity (NSILA) in serum which acted like insulin in adipose tissues but could not be neutralized by anti-insulin antibodies. A third line of work by Dulak and

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Temin (1973) identified a peptide fraction from medium conditioned by buffalo rat liver cells which could stimulate the growth of cells dependent on serum. This factor was termed multiplication stimulating activity (MSA). Highly purified somatomedin and NSILA showed similar responses in stimulating sulphate incorporation into cartilage and glucose uptake into fat (Froesch *et al.*, 1976). This led to the conclusion that these two factors were identical or structurally related to insulin. NSILA was later purified and identified as two bioactive peptides that showed a high degree of homology with proinsulin and were designated IGF-I and IGF-II (Rinderknecht & Humbel, 1978a; 1978b). Somatomedin-C was later shown to be structurally identical to IGF-I (Klapper *et al.*, 1983), while MSA was found to be similar to IGF-II (Marquardt *et al.*, 1981).

1.1.2 Primary structure of IGFs

The two types of IGFs, IGF-I and IGF-II, are single chain polypeptides of 70 and 67 amino acids, respectively. Mammalian IGF-I amino acid sequences are highly conserved. The amino acid sequence of human (Rinderknecht & Humbel 1978a), bovine (Francis *et al.*, 1986; Honeggar & Humbel, 1986; Francis *et al.*, 1988), porcine (Francis *et al.*, 1989b) and guinea pig (Bell *et al.*, 1990) IGF-I are identical. Ovine (Francis *et al.*, 1989a), rat (Tamura *et al.*, 1989) and mouse (Bell *et al.*, 1986) IGF-I differ from these other mammalian IGF-I in 1, 3 and 4 residues respectively (Table 1.1). Similarly IGF-II amino acid sequences are highly conserved between mammals. Human (Rinderknecht & Humbel, 1978b) differs from bovine (Honeggar & Humbel, 1986) and porcine (Francis *et al.*, 1989b) IGF-II in 2 residues while ovine (Francis *et al.*, 1989) and rat (Dull *et al.*, 1984; Marquardt *et al.*, 1981) IGF-II differ from human IGF-II in 4 amino acid positions. Both IGF forms are structurally related with 70% sequence similarity. In contrast to the IGFs, there are substantial sequence alterations across mammalian insulins due to the highly divergent hystricomorph rodents (Horuk *et al.*, 1979; Baja *et al.*, 1986). Cross-species sequence homology for IGF-I, IGF-II and insulin is given in Figure 1.1.

IGFs have a domain structure that is analogous to proinsulin. This homology with proinsulin includes the conservation of six cysteine residues, which form identical disulphide bridge

arrangements. Amino acids 1-29 of IGF-I and 1-32 of IGF-II are homologous to the insulin B-chain with 11 residues conserved through each polypeptide class. Similarly, 10 amino acid positions are conserved between the insulin A-chain and the homologous regions 42-62 of IGF-I and 41-61 of IGF-II. These two IGF regions are termed the B- and A-domains. The twelve amino acid C-domain of IGF-I links these two domains. The IGF C-domain has no sequence similarities with the corresponding C-peptide of proinsulin. In addition, IGF-I has an eight amino acid C-terminus extension which has no equivalent in proinsulin, termed the D-domain. There are several distinctions between IGF-II and IGF-I sequences. In IGF-II, the C-domain and D-domain are shorter than IGF-I, consisting of eight and six amino acids respectively. Other notable differences from IGF-I include a longer N-terminal region in the B-domain and non-conservative variation in the C-terminal region of the A-domain. The structural domains and disulfide bond arrangements of the IGFs are schematically represented in Figure 1.2.

1.1.3 Tertiary Structure of IGFs

The first structural models of IGF-I and IGF-II were proposed by Blundell *et al*, (1978; 1983) based on conserved sequence regions between insulin and IGF-I and the crystal structure of molecule 2 of the insulin dimer (Dodson *et al.*, 1979). In these models, cysteine residues and other highly conserved residues, such as those that constitute the hydrophobic core, were assumed to occupy the same relative positions in the IGFs. Conservatively substituted residues in IGF were assigned similar positions to those in the insulin model. Structural assignment of the C- and D-domain which are lacking in insulin were determined using the residue prediction method of Chou & Fasman, (1974).

Nuclear magnetic resonance studies by Cooke *et al*, (1991) has subsequently generated a solution structure for IGF-I. This model confirmed many structural predictions proposed by Blundell *et al*, with regions homologous to insulin being well defined. IGF-I comprises 3 right handed α helices spanning residues 8-17, 44-49 and 54-59, with residues 3-6 adopting a β-strand-like confirmation. Regions including residues 23-26, 30-39 and 60-70 appear to be quite flexible. Two disulphide bridges between residues Cys[18] and Cys[61] and between Cys[47]

and Cys^{52} of IGF-I are buried in the hydrophobic core whereas the third bridge (Cys^6-Cys^{48}) is solvent exposed. This hydrophobic core consists of buried residues Leu^{10}, Ala^{13}, Leu^{14}, Val^{17}, Ile^{43}, Leu^{57} and Tyr^{60}. Another 13 peripheral residues having partially buried side chains reinforce the core. Based on the results from various structure-function studies, Cooke *et al*, (1991) proposed that residues involved with type 1 receptor binding overlap those involved with insulin receptor binding. In constrast, residues important for type 2 receptor binding overlap with those important for serum binding protein binding.

1.1.4 IGF-I and IGF-II Genes and mRNAs.

Jansen *et al*., (1983) were the first to isolate a cDNA clone for human IGF-I. They showed that IGF-I messenger RNA encodes a 130 amino acid precursor protein which is proteolytically cleaved at the C- and N-termini to form the native peptide. Subsequently two different mRNAs transcribed from the IGF-I gene have been identified and termed IGF-Ia and IGF-Ib (Rotwein *et al*., 1986). Multiple forms of mRNAs are generated in the rat from the IGF-I gene (Roberts *et al*., 1987). Northern analysis of tissues from human and rat have also shown the presence of many mRNA species. This heterogeneity is due to alternative splicing of the 5′ untranslated region and by multiple polyadenylations in exon 5. Human IGF-I is a product of a single gene localized on the long arm of chromosome 12 (Brissenden *et al*., 1984; Tricoli *et al*., 1984) and consists of five exons spanning greater than 90kb (Sussenbach, 1989; Rotwein *et al*., 1986). Exons 1 to 3 contain 5′ untranslated sequences and encode the signal peptide, the B-, C- and A-domains, as well as the first 16 amino acids of the E-domain. Exons 4 and 5 contain alternative C-terminal sequences for the E-domain. The two human IGF-I mRNAs are derived from exons 1,2,3 and 5 (IGF-Ia) or from exons 1,2,3 and 4 (IGF-Ib) and have C-terminal E-domains of 35 and 77 residues respectively.

The human IGF-II gene has been localized on the short arm of chromosome 11 and consists of 9 exons and at least 4 promoters spanning greater than 30 kb (Brissenden *et al*., 1984; Tricoli *et al*., 1984; Bell *et al*., 1985). Exons 1 to 6 contain a 5′ untranslated sequence with exons 7 to 9 encoding the precursor protein and a 3′ untranslated region. The 180 amino acid precursor form is cleaved to release mature IGF-II in an analogous process to IGF-I.

Heterogeneity identified with human and rat IGF-II mRNA is due to multiple promoter usage with additional variation in rat IGF-II mRNA due to alternate RNA processing and polyadenylation (Daughaday & Rotwein, 1989).

1.1.5 IGF-I and IGF-II Variants

Several variants of IGF-I and IGF-II have been identified. A biologically potent variant of IGF-I lacking the first three N-terminal amino acids has been isolated from bovine colostrum (Francis *et al.*, 1986; Francis *et al.*, 1988), human brain (Sara *et al.*, 1986; Carlsson-Skwirut *et al.*, 1986), porcine uterus (Ogasawara *et al.*, 1989) and human platelets (Karey *et al.*, 1989). Two IGF-II variants containing amino acid inserts at positions 29 and 33 with the latter having a 21 amino acid C-terminal extension, have been isolated from human plasma (Hampton *et al.*, 1989; Zumstein *et al.*, 1985). A large molecular weight form of IGF-II has also been identified in cerebral spinal fluid (Haselbacher & Humbel, 1982; Gowan *et al.*, 1987). As there is one IGF-II gene, it has been proposed that these variants are the result of allelic variation and alternative splicing. Six other IGF-I and IGF-II variants in human plasma have been identified by isoelectric focusing (Blum *et al.*, 1986).

1.1.6 Distribution and Regulation of IGFs

The IGFs were originally believed to act as endocrine hormones secreted by the liver and distributed to target tissues via the serum. The ubiquitous nature of IGF expression supports potential paracrine and autocrine actions in addition to this endocrine function. Many cell and tissue types produce IGFs with the liver being the primary source of circulating IGF-I (Daughaday & Rotwein, 1989). In rats, IGF-I mRNA and IGF peptide are present in most tissues while IGF-II mRNA is predominantly found in the brain, kidney, heart and uterus (Murphy *et al.*, 1987). IGF expression is not uniform within these tissues but produced within specific regions or cell types. For example, in the kidney IGF-I mRNA and IGF-I peptide are predominantly localized to the principal cells of the collecting duct (Bortz *et al.*, 1988). In the rat ovary IGF-I mRNA is primarily localized to the granulosa cells (Hernandez *et al.*, 1989; Oliver *et al.*, 1989) while IGF-II mRNA is produced exclusively by thecal-interstitial cells (Hernandez *et al.*, 1990).

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As previously described, multiple IGF mRNA transcipts are produced by multiple promoter usage and alternate mRNA processing. Transcript levels are regulated by hormonal, nutritional and developmental factors. The primary hormonal regulator of postnatal growth is growth hormone (GH). Circulating IGF-I levels correlate well with GH levels and growth rates and thus support the concept that the liver is the major source of circulating IGF-I (Rotwein, 1986; Rotwein *et al.*, 1987). Both IGF-I and GH levels are decreased or elevated in altered growth states such as Laron dwarfism (Daughaday & Trivedi, 1987) and acromegaly (Clemmons *et al.*, 1980). GH also stimulates the induction of IGF-I mRNA in hepatic cells across many species (Mathews *et al.*, 1986). In contrast to IGF-I, circulating IGF-II and hepatic IGF-II mRNA levels are not associated with GH status (Hynes *et al.*, 1987; Hall & Tally, 1989; Mesiano *et al.*, 1989). Thus the regulation of IGF-II expression appears to be GH-independent.

IGF gene expression is also regulated by a number of other endocrine factors. In particular, IGF-I levels increase in response to oestrogen, parathyroid hormone (PTH) and epidermal growth factor (EGF) while cortisol and interleukin-1 suppress IGF-I levels (Daughaday & Rotwein, 1989; Rotwein, 1991; Lin *et al.*, 1992). In contrast, the endocrine regulation of IGF-II expression is not well defined. Acute corticotropin (ACTH) stimulates IGF-II mRNA levels in human adrenal cells (Voutilainen & Miller 1987), while glucocorticoids suppress IGF-II mRNA in rat liver cells (Levinovitz & Norstedt 1989) and IGF-II peptide levels in cultured bone cells (Canalis *et al.*, 1991). Nutritional restrictions in rats reduce levels of both circulating IGF-I and IGF-I mRNA in various tissues (Emler & Schalch, 1987). A similar reduction in circulating IGF levels due to fasting also occurs in humans (Clemmons *et al.*, 1981; Merimee *et al.*, 1982). Other factors which regulate IGF-I gene expression include puberty (Zapf, 1981) and pregnancy (Bala *et al.*, 1981; Hall *et al.*, 1984) where there is an increase in IGF-I serum levels.

1.1.7 Biological Actions of IGFs

1.1.7.1 *In vitro* actions

The biological actions of IGFs include both insulin-like or metabolic effects and mitogenic effects. The *in vitro* metabolic effects of IGFs differ between cell lines but in general are rapid and short term. These responses include the classic insulin responses such as the stimulation of glucose uptake, lipid and glycogen synthesis (Froesch *et al.*, 1985). The longer term mitogenic effects of IGFs include the stimulation of protein and DNA synthesis, and the inhibition of protein degradation. These effects have been demonstrated in a range of cell lines such as chick embryo fibroblasts, rat L6 myoblasts (Ballard *et al.*, 1986) and bone cells (Froesch *et al.*, 1985). IGFs can also act as differentiation factors in neuron maturation (Pahlman *et al.*, 1991), erythropoiesis (Claustres *et al.*, 1987; Congote & Esch, 1987), osteoclast activation (Mochizuki *et al.*, 1992) and myoblast development to mature muscle cells (Florini & Magri, 1989). These *in vitro* studies also support IGFs acting at a paracrine and autocrine level (Holly & Wass, 1989).

1.1.7.2 *In vivo* actions

In vivo studies provide evidence in support of the endocrine action of IGF-I and the somatomedin hypothesis. The first studies on IGF administration in rats used peptide purified from serum. In hypophysectomized rats, IGF-I and to a lesser extent IGF-II, result in an increase in body weight (Schoenle *et al.*, 1985). The availability of large amounts of recombinant IGF-I has enabled studies on the anabolic and insulin-like effects of IGF administration. In normal rats and humans, bolus injection of hIGF-I causes a pronounced fall in blood glucose levels thus resulting in transient hypoglycaemia (Zapf *et al.*, 1986; Guler *et al.*, 1987). In contrast to insulin, IGF-I administration in non-diabetic rats has little effect on free fatty acid concentrations. Administration of IGF-I also stimulates organ weight gain, predominantly the spleen, kidneys and gut (Thissen *et al.*, 1991; Tomas *et al.*, 1992), and has positive effects on wound healing (Mueller *et al.*, 1991; Suh *et al.*, 1992) and recovery of renal function (Miller *et al.*, 1992).

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Transgenic mouse models have also been useful in accessing the *in vivo* effects of IGFs. Over expression of human IGF-I in transgenic mice results in an approximate 30% weight gain over control littermates (Mathews *et al.*, 1988). Although circulating IGF-I levels were elevated, both hepatic mouse mRNA levels and GH expression were decreased indicating a negative feedback on pituitary GH production by elevated IGF-I levels. Double transgenic mice lacking GH and overexpressing IGF-I grow larger than their GH-deficient littermates with the IGF-I transgene compensating almost precisely for the loss of GH (Behringer *et al.*, 1990). Transgenic mice have demonstrated the involvement of IGF-II in fetal growth and the role of genomic imprinting in regulating IGF-II gene expression. Mice with a disrupted paternal IGF-II allele have reduced birth weights, however, postnatal growth is comparable to the wild type mice. In contrast, disruption of the maternal IGF-II allele does not affect birth size (De Chiara *et al.*, 1990; 1991).

IGF-I

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                1         10        20        30        40        50        60      70
Human       G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Bovine      G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Porcine     G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Ovine       G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Guinea Pig  G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T G Y G S S S R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P A K S A
Rat         G P E T L C G A E L V D A L Q F V C G P R G F Y F N K P T G Y G S S I R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P T K S A
Mouse       G P E T L C G A E L V D A L Q F V C G P R G F Y F N K P T G Y G S S I R R A P Q T G I V D E C C F R S C D L R R L E M Y C A P L K P T K A A
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IGF-II

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             1           10        20        30          40        50        60      67
Human       A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P A - - S R V S R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Bovine      A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P S - - S R I N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Porcine     A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P A - - S R I N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Ovine       A Y R P S E T L C G G E L V D T L Q F V C G D R G F Y F S R P S - - S R I N R R S R - - G I V E E C C F R S C D L A L L E T Y C A A - - P A K S E
Rat 1       A Y R P S E T L C G G E L V D T L Q F V C S D R G F Y F S R P S - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Rat 2       A Y R P S E T L C G G E L V D T L Q F V C S D R G F Y F S R P G - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
Mouse       A Y G P G E T L C G G E L V D T L Q F V C S D R G F Y F S R P S - - S R A N R R S R - - G I V E E C C F R S C D L A L L E T Y C A T - - P A K S E
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Insulin

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            B1          B10       B20         B30          A1        A10         A21
Human       F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A         G I V E Q C C T S I C S L Y Q L E N Y C N
Bovine      F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A         G I V E Q C C A G V C S L Y Q L E N Y C N
Porcine     F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A         G I V E Q C C T S I C S L Y Q L E N Y C N
Rat         F V K Q H L C G S H L V E A L Y L V C G E R G F F Y T P M S         G I V D Q C C T S I C S L Y Q L E N Y C N
Guinea Pig  F V S R H L C G S N L V E T L Y S V C Q D D G F F Y I P K D         G I V D Q C C T G T C T R H Q L Q S Y C N
Casiragua   Y V G Q R L C G S Q L V D T L Y S V C K H R G F - Y R P S E         G I V D Q C C T N I C S R N Q L L T Y C N
Porcupine   F V N Q H L C G S H L V E A L Y L V C G N D G F F Y R P K A         G I V D Q C C T G V C S L Y Q L Q N Y C N
Coypu       Y V S Q R L C G S Q L V D T L Y S V C R H R G F - Y R P N D         G I V D Q C C T N I C S R R Q L M S Y C N D
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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

Figure 1.1 Sequence Comparison of insulin and the insulin-like growth factors. Residues conserved between IGF-I and IGF-II are shaded, while invariant residues in insulin and IGFs are highlighted in black. Conserved residues in each peptide class are boxed. The references for the sequence data are given in Table 1.1.

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Table 1.1 References for sequence data of various IGF-I, IGF-II and insulin peptides.
Sequence data and homology is shown in Figure 1.1.

IGF-I

Human	Rinderknecht & Humbel, 1978a
Bovine	Honeggar & Humbel, 1986
Porcine	Francis *et al.*, 1989b
Ovine	Francis *et al.*, 1989a
Guinea Pig	Bell *et al.*, 1990
Rat	Tamura *et al.*, 1989
Mouse	Bell *et al.*, 1986

IGF-II

Human	Rinderknecht & Humbel, 1978b
Bovine	Honeggar & Humbel, 1986
Porcine	Francis *et al.*, 1989b
Ovine	Francis *et al.*, 1989a
Rat 1	Dull *et al.*, 1984
Rat 2	Marquardt *et al.*, 1981
Mouse	Stempien *et al.*, 1986

Insulin

Human	Nicol & Smith. 1960
Bovine	Ryle *et al.*, 1955
Porcine	Brown *et al.*, 1955
Rat	Clark & Steiner 1969
Guinea Pig	Smith 1966
Casiragua	Horuk *et al.*, 1979
Porcupine	Horuk *et al.*, 1980
Coypu	Smith 1972
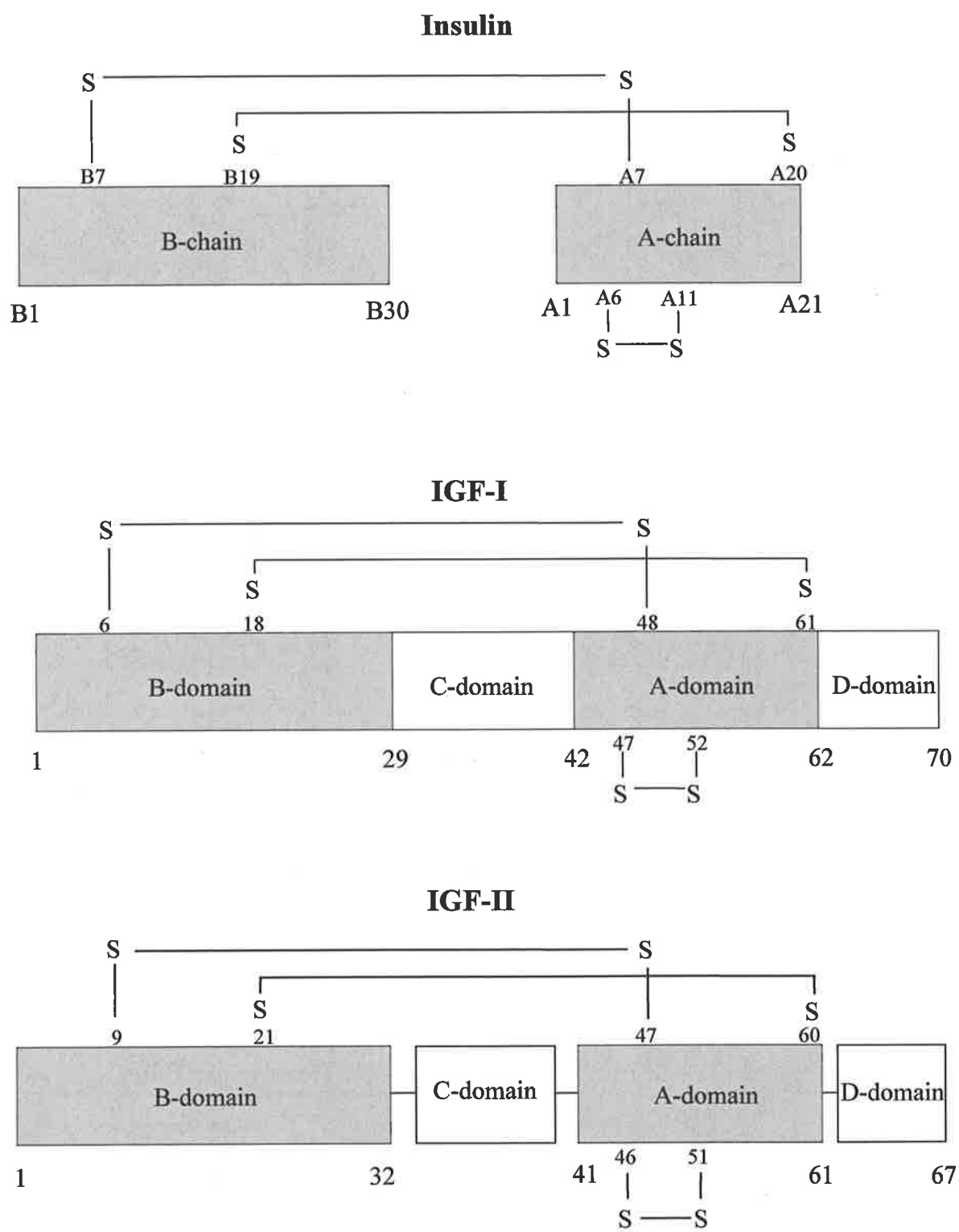
Figure 1.2 Domain structure of insulin, IGF-I and IGF-II. Conserved cysteine residues and disulphide bridge arrangements are also indicated.

1.2 IGF Binding Proteins (IGFBPs)

Characterization of serum IGF found that insulin-like activity existed as an acid-dissociable high molecular weight complex of 150 kDa. The high molecular weight binding activity was demonstrated to be specific for IGF suggesting the presence of IGF carrier proteins in serum (Zapf *et al.*, 1975; Hintz & Liu, 1977). Six IGFBPs designated IGFBP-1 through to IGFBP-6 have been identified and the primary sequences determined (references given in sections 1.2.2 to 1.2.7). IGFBPs have no sequence similarity with the IGF receptors and the IGFs demonstrate a higher binding affinity for the IGFBPs than that for the IGF receptors. Variation in binding affinity is described in Section 1.2.1-1.2.6 and is illustrated in Figure 1.3. Mature IGFBPs consist of 200 to 300 amino acids and share a high degree of sequence homology. The amino acid sequence is conserved at both the N- and C-terminal regions with a divergent central region. Most of the IGFBPs have 18 conserved cysteine residues, 12 in the N-terminal region and 6 in the C-terminal region, suggesting similarities in tertiary structure and IGF binding regions. However, human IGFBP-6 and rat IGFBP-6 lack 2 and 4 of the homologous cysteines respectively. Both human and rat IGFBP-4 also have 2 additional cysteine residues in the divergent central region. IGFBP-1 and -2 contain a C-terminal RGD sequence which is found in many extracellular proteins and is believed to be important for recognition of cell surface integrin receptors. The IGFBPs are glycosylated to varying degrees. The significance of this glycosylation is unclear although it may be important for IGFBP adherence to cell surfaces or the extracellular matrix. Additional studies suggest that IGFBP phosphorylation can alter the binding affinity for IGF-I (see section 1.2.7).

As mentioned above, the high degree of sequence conservation between of IGFBPs and in particular the conservation of the 18 cysteine residues suggests that IGFBPs share similar tertiary structure and have homologous IGF binding sites. Fragmented IGFBPs provide an indication of the region which is important for IGF association. An N-terminal truncated form of IGFBP-1, and C-terminal truncated forms of IGFBP-2 and IGFBP-3 have been reported to bind IGF (Huhtala *et al.*, 1986; Baxter & Skriver, 1993; Wang *et al.*, 1988; Zapf *et al.*, 1990).

These fragmented IGFBP forms suggest that the non-conserved central region of IGFBP is also involved in IGF binding.

Since the commencement of this thesis, there has been significant progress aimed at identifying IGFBP residues involved in IGF binding. In bovine IGFBP-2, Tyr^{60} and a 20 amino acid sequence commencing from Leu^{72} of human IGFBP-4 have been shown to contribute to IGF binding affinity (Hobba *et al.*, 1998; Qin *et al.*, 1998). The determination of a NMR structure for a IGFBP-5 fragment provides further evidence for the involvement of the amino-terminal region in IGF interactions by implicating the importance of residues Val^{49}, Tyr^{50}, Pro^{62}, and Lys^{68}-Leu^{74} of IGFBP-5 (Kalus *et al.*, 1998). Various deletion studies also indicate C-terminal residues of IGFBP-3 (Firth *et al.*, 1998), IGFBP-4 (Qin *et al.*, 1998) and IGFBP-5 (Andress *et al.*, 1993) contribute to high affinity IGF interactions. However, functional loss for IGFBP deletion mutants and fragments may be due to gross structural changes rather than indicative of specific interacting residues. Although these recent mutational studies have demonstrated the importance of the C- and N-terminal regions of IGFBPs in the association with IGFs, key binding residues remain unclear.

A number of major developments in the IGFBP field have occurred since the commencement of this thesis. In recent years it has become evident that IGFBPs are capable of biological actions that are independent of their abilities to bind IGFs (Baxter, 2000). Such type 1 IGF receptor independent actions may result from the association of IGFBPs with a variety of extracellular and cell surface molecules (Jones *et al.*, 1993; Oh *et al.*, 1993; Andress, 1995; Andress, 1998). The transport and localization of IGFBP-3 and −5 to the nucleus further supports the concept that these two IGFBPs have functions unrelated to direct IGF actions (Li *et al.*, 1997; Schedlich *et al.*, 1998). Recent studies have also identified a variety of IGFBP-related proteins that share structural and functional similarities to the IGFBPs. This has led to the proposal of an IGFBP superfamily that includes the low affinity IGFBP related proteins (Kim *et al.*, 1997; Hwa *et al.*, 2000).

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1.2.1 IGFBP-1

IGFBP-1 has been isolated from amniotic fluid (Chochinov *et al.*, 1977), the human hepatoma cell line HEP G2 (Moses *et al.*, 1983), and decidual stromal cells (Koistinen *et al.*, 1986). The primary structure of human IGFBP-1 (Brewer *et al.*, 1988; Lee *et al.*, 1988) and rat IGFBP-1 (Murphy *et al.*, 1990) determined from cDNA sequence analysis indicates 58% identity and a molecular weight of 25 kDa. IGF-I and IGF-II have similar affinities for IGFBP-I. The IGF-I binding affinity is 5-fold greater than what is observed for the type 1 IGF receptor at pH 7.0. Phosphorylated human IGFBP-1 has been shown to have a 6-fold higher affinity for IGF-I relative to the non-phosphorylated form (Jones *et al.*, 1991).

1.2.2 IGFBP-2

IGFBP-2 was first purified from rat BRL-3A liver cells (Moses *et al.*, 1979) and was subsequently shown to have limited N-terminal amino acid identity with IGFBP-1 (Mottola *et al.*, 1986). IGFBP-2 was later isolated from conditioned media of bovine kidney cells (Szabo *et al.*, 1988). The cDNAs for IGFBP-2 have been isolated and sequenced for rat (Brown *et al.*, 1989), human (Binkert *et al.*, 1989) and bovine (Upton *et al.*, 1990) indicating 85% sequence homology across these species. Expression of IGFBP-2 mRNA is elevated in fetal tissues and thus may be important in fetal development (Ooi *et al.*, 1990; Orlowski *et al.*, 1990). IGFBP-2 binds both IGF-I and IGF-II with higher affinity than IGFBP-1 and a 3-fold higher affinity for IGF-II relative to IGF-I has been reported.

1.2.3 IGFBP-3

IGFBP-3 is the most abundant form of IGFBP in serum existing primarily as a 150 kDa ternary complex (Baxter & Martin, 1989). The complex is comprised of IGFBP-3, IGF-I or -II and a 85 kDa acid labile subunit (ALS) (Martin & Baxter, 1986). The primary structure determined from cDNA sequence analysis for human (Wood *et al.*, 1988), rat (Albiston & Herrington, 1990) and bovine IGFBP-3 (Conover *et al.*, 1990) shows a high degree of sequence conservation between species. IGFBP-3 is secreted as a phosphoprotein with a molecular weight range of 45 to 53 kDa. The ALS is a core protein of approximately 90 kDa and is unstable at pH <5. Assembly of the 150 kDa ternary complex occurs in an ordered

fashion with ALS unable to bind free IGF or IGFBP-3 suggesting initial binding of IGF to IGFBP-3 followed by ALS association. IGFBP-3 has a very high affinity for both IGF-I and IGF-II.

1.2.4 IGFBP-4

IGFBP-4 has been isolated from human (Kiefer *et al.*, 1991a) and rat serum (Shimonaka *et al.* 1989), and from media conditioned by osteosarcoma cells (Mohan *et al.*, 1989). Sequence analysis of cDNA from human (La Tour *et al.*, 1990) and rat (Shimasaki *et al.*, 1990) indicate 92% identity to each other. IGFBP-4 is a 25 kDa protein, that also exists in a 29 kDa form due to a N-linked glycosylation. The binding properties are similar to IGFBP-2 and -3 with IGFBP-4 having high affinity for both IGF-I and IGF-II.

1.2.5 IGFBP-5

IGFBP-5 has been isolated from human bone (Bautista *et al.*, 1991) where it represents the major IGFBP and from serum (Kiefer *et al.*, 1991b). Human (Kiefer *et al.* 1991b) and rat (Shimasaki *et al.*, 1991a) cDNA sequences analysis indicates that the protein is approximately 29 kDa. Higher molecular weight glycosylated forms of IGFBP-5 have also been observed. Human and rat IGFBP-5 are 97% identical and have the highest affinity of all IGFBPs for the IGFs. IGFBP-5 binds IGF-II with greater affinity than IGF-I.

1.2.6 IGFBP-6

IGFBP-6 has been purified from serum (Zapf *et al.*, 1990), conditioned media of fibroblasts (Forbes *et al.*, 1990; Martin *et al.*, 1990), and osteosarcoma cells (Andress & Birnbaum, 1991). The cDNA sequences have been characterized in human (Kiefer *et al.*, 1991a) and rat (Shimasaki *et al.*, 1991b) and indicate the protein has a molecular weight of approximately 22 kDa. However, higher molecular weight forms of glycosylated IGFBP-6 have been reported. Human IGFBP-6 lacks the sixth and seventh cysteines, while rat IGFBP-6 lacks the third, fourth, sixth and seventh cysteines common to all other IGFBPs. The protein has a significantly higher affinity for IGF-II than IGF-I (Forbes *et al.*, 1990; Martin *et al.*, 1990).

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1.2.7 Regulation of IGF action by IGFBPs

The half-lives of circulating IGFs are dependent on their association with IGFBPs. When associated with the 150 kDa IGFBP-3 complex, the estimated 10 minute half-life of iodinated IGF-I is prolonged to 12-15 hours in humans (Guler *et al.*, 1989). IGF-I analogues with reduced affinity for the IGFBPs have also demonstrated the role of IGFBPs in increasing the half-life of IGF-I in various *in vivo* studies. The enhanced potency of des(1-3)IGF-I compared to IGF-I demonstrated in animal studies is thought to be in part the result of the increased clearance of des(1-3)IGF-I from blood to tissues (Gillespie *et al.*, 1990; Lemmey *et al.*, 1991; Tomas *et al.*, 1991). Indeed, radiolabeled des(1-3)IGF-I is more rapidly cleared than IGF-I or IGF-II in rats (Ballard *et al.*, 1991). Similarly, [Gln^3, Ala^4, Tyr^{15}, Leu^{16}] IGF-I incubated with IGFBP-3 and injected into rats has a 4-fold reduced serum half-life (Cascieri *et al.*, 1988a).

With the exception of the 150 kDa IGFBP-3 complex, IGFBPs can cross vascular barriers and may be important in determining IGF tissue distribution and localization. In the isolated perfused rat heart, IGFBP-1, -2, -3 and -4 whether alone or conjugated with IGF, can cross the capillary endothelium and preferentially localise to cardiac muscle (Bar *et al.*, 1990). IGFBP-1 and IGFBP-2 administered to rats rapidly leaves the circulation and equilibrates with extravascular compartments (Young *et al.*, 1992). This redistribution of circulating IGFBPs is believed to be important in modulating the transvascular transport of IGFs. IGFBPs have been shown to localize IGFs within different tissues and act as releasable pools for IGF storage. IGFBP-5 is predominantly found in bone tissue and has been proposed to localize IGF-II within bone (Bautista *et al.*, 1991). During bone remodelling, the matrix is digested to release IGF-II and IGFBP-5 which may subsequently be involved in the bone remodelling process (Bautista *et al.*, 1991).

IGFBPs have been demonstrated to inhibit and potentiate the biological actions of IGFs. Various *in vitro* studies have shown that IGFBPs inhibit the actions of IGF by sequestering free IGF and preventing its interaction with the type 1 IGF receptor. Ross *et al*, (1989) showed bovine IGFBP-2 inhibits the actions of IGF in chick embryo fibroblasts, but is less effective in inhibiting the action of IGF analogues with reduced binding affinities for the IGFBPs. Other cultured cell studies indicate

IGF analogues with reduced IGFBP affinity are more mitogenically potent than IGF-I as a result of free peptide being available for receptor interaction (Ballard *et al.*, 1987; Szabo *et al.*, 1988; Cascieri *et al.*, 1988b; Bagley *et al.*, 1989). Co-incubation of bovine fibroblasts with bovine IGFBP-3 and IGF-I inhibits the action of IGF-I while incubation with IGFBP-3 alone leads to cell surface association and has potentiating effects (Conover *et al.*, 1990). Conover *et al*, (1992) later showed this enhancement of IGF-I action is due to proteolysis of the cell associated IGFBP-3 to a form with low IGF affinity. Phosphorylation status of the IGFBPs may determine whether biological actions are inhibitory or potentiating. IGFBP-1 enhances the effects of IGF-I on DNA synthesis in the dephosphorylated state, and is inhibitory in the phosphorylated form. This modulation of IGF activity may be due to a higher IGF-I affinity for phosphorylated IGFBP-1 (Frost & Linda, 1991; Jones *et al.*, 1991).
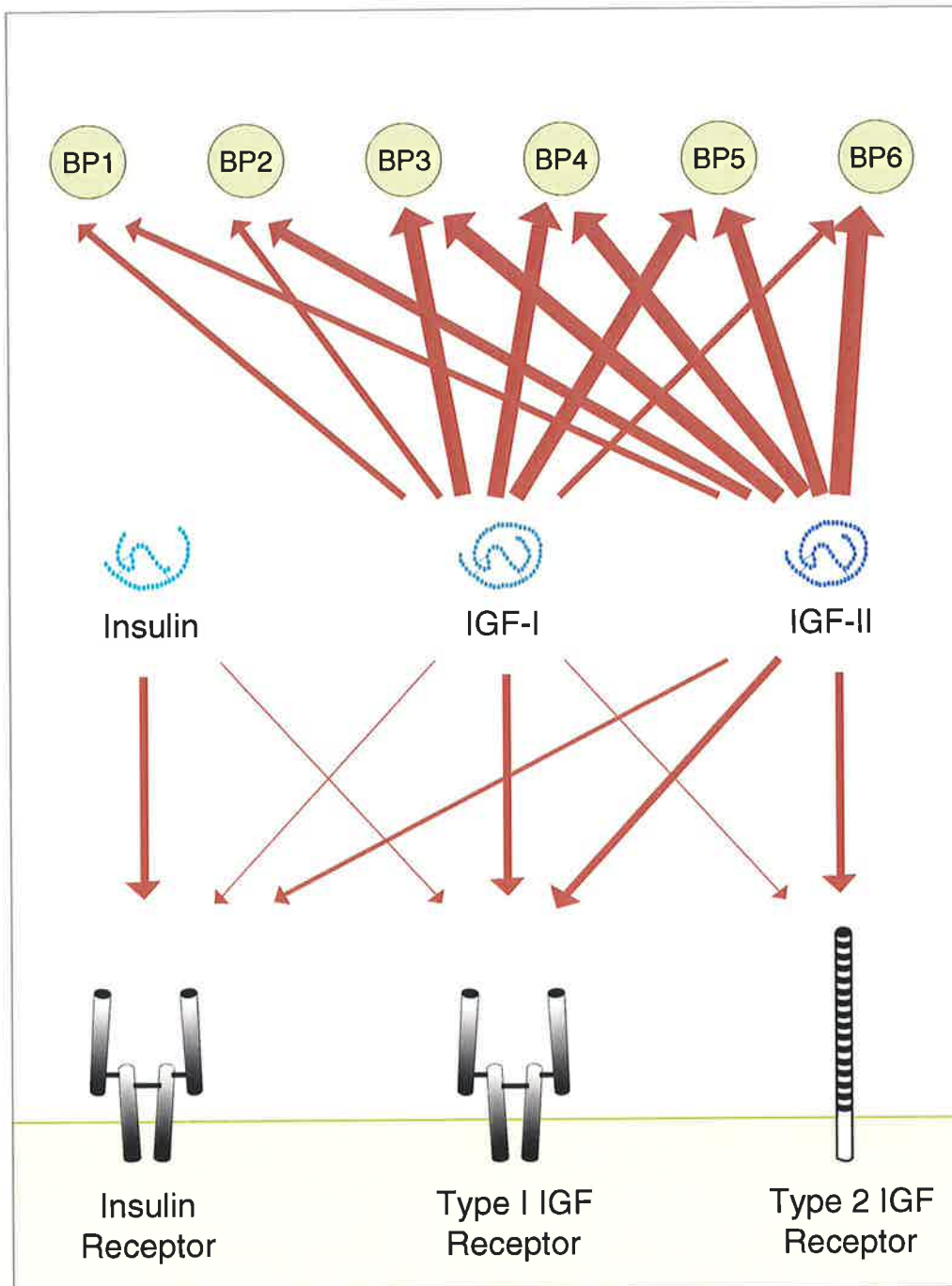
Figure 1.3 Schematic presentation of the interactions of IGF-I, IGF-II and insulin with the IGFBPs and receptors. Arrow width indicates relative binding affinity.

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1.3 Receptors

Affinity cross-linking techniques using radio-iodinated IGF-I (Kasuga *et al.*, 1981; Massague & Czech 1982) lead to the identification of two high affinity IGF receptor subtypes. The deduced amino acid sequence of the type 1 IGF receptor (Ullrich *et al.*, 1986) and insulin receptor (Ullrich *et al.*, 1985) from cDNA clones showed both receptors share a high degree of homology. In contrast, the type 2 IGF receptor is structurally unrelated to either the insulin or type 1 IGF receptors and is identical to the cation-independent mannose 6-phosphate receptor (Lobel *et al.*, 1987) (Figure 1.4).

1.3.1 Insulin and Type 1 IGF Receptors

1.3.1.1 Receptor Structure

The insulin and type 1 IGF receptors belong to the family of tyrosine kinase receptors which share structural and functional properties (Ullrich & Schlessinger, 1990; Ullrich *et al.*, 1986). Unlike other members of the tyrosine kinase family, insulin and type 1 IGF receptors have a disulphide linked dimeric structure. Each half consists of two disulphide-linked α- and β-subunits of approximately 135 kDa and 95 kDa respectively. The α-subunit is wholly extracellular and contains two homologous domains termed L1 and L2 that are separated by a cysteine rich domain. The β-subunit transverses the plasma membrane and contains the intracellular tyrosine kinase catalytic domain. The α- and β-subunits are synthesised as part of a single chain pro-receptor which undergoes several post-translational modifications including proteolytic cleavage, glycosylation, and fatty acid acylation (Figure 1.4). The structural detail of the insulin and IGF receptors has recently been refined by structural and homology analysis (Ward *et al.*, 2001).

1.3.1.2 Receptor Heterogeneity

Two isoforms of the insulin receptor exist due to alternative splicing of exon 11 in a tissue-specific manner (Seino & Bell, 1989; Moller *et al.*, 1989). Thus, alternate splicing of exon 11 leads to the presence or absence of 12 amino acids located close to the C-terminus of the α-subunit. The insulin receptor isoform containing the 12 amino acid insert has been shown to have a 2- to 3-fold reduced binding affinity (Mosthaf *et al.*, 1990) but increased tyrosine kinase activity (Kellerer *et al.*, 1992).

Two alternative type 1 IGF receptor transcripts have been identified resulting from alternative splicing at the 5' end of exon 14 (Yee *et al.*, 1989; Abbott *et al.*, 1992). The alternative splice isoform of the type 1 IGF receptor differs from the originally cloned sequence (Ullrich *et al.*, 1986) by the deletion of 3 nucleotides in the extracellular region of the β-subunit which results in substitution of an Arg residue for the two residues Thr-Gly.

Cells expressing both insulin and type 1 IGF receptors have been shown to have hybrid receptors consisting of αβ insulin receptor and αβ type 1 IGF receptor heterodimers (Soos & Siddle, 1989; Moxham *et al.*, 1989). Subsequently Soos *et al*, (1992) have shown purified hybrids bind insulin with a 10-fold lower affinity than the classical insulin receptors as measured by competition with ^{125}I-insulin. In contrast, ^{125}I-IGF competitive binding studies showed the affinity of the hybrid for IGF-I is similar to that of the type 1 IGF receptor. Thus, hybrid receptors are more likely to respond to IGF-I than insulin, however, their functional significance is still unclear.

1.3.1.3 Ligand Binding

The type 1 IGF receptors typically exhibit a higher binding affinity for IGF-I than IGF-II and have a weaker affinity for insulin. IGF-I cross-reacts with the insulin receptor with low affinity as does insulin with the type 1 IGF receptor (Massague & Czech, 1982) (Figure 1.3). The extracellular regions including the α-subunit and portions of the β-subunits display relatively low sequence homology (41-48%) and thus reflect differences in ligand binding specificity (Ullrich *et al.*, 1986). Studies using insulin receptor/type 1 IGF receptor chimeras in which homologous regions of both receptors have been interchanged have been used to map ligand binding regions. The ligand binding properties of such chimeras suggest that the N-terminal 68 amino acids and C-terminal 400 amino acids of the insulin receptor α-subunit are important for high affinity insulin binding (Kjeldsen *et al.*, 1991; Schumacher *et al.*, 1991). In contrast residues 223-274 within the cysteine rich domain of the type 1 IGF receptor have been shown to contribute to ligand binding specificity (Gustafson and Rutter, 1990; Kjeldsen *et al.*, 1991; Schumacher *et al.*, 1991). These studies suggest that the ligands interact over a large region of the α-subunits and that structural determinants involved in defining ligand binding specificity reside in different regions of the two receptors.

Since the commencement of this thesis there has been considerable progress in understanding the mechanisms involved in ligand binding to the insulin and type 1 IGF receptors. Recently, the crystal structure of the L1/cys-rich/L2 region of the type 1 IGF receptor has been determined (Garrett *et al.*, 1998). The structure indicates this extracellular portion adopts an extended biolobal structure with a central space sufficient for accommodating IGF-I or IGF-II. This large pocket includes regions of the type 1 IGF receptor that have been implicated in ligand binding through receptor chimera and mutational studies (Garrett *et al.*, 1998). De Meyts (1994) has proposed a two site binding model for insulin and IGF-I. This cross-linking model accounts for kinetic experiments that indicate two binding sites (low and high affinity) and negative cooperativity. In the high affinity state, insulin contacts distinct regions of the two monomers in the insulin receptor dimer. The observation of negative cooperativity can be explained if the receptor dimer has internal symmetry and ligand binding at the high affinity site induces a conformational change that precludes binding at the second site (De Meyts, 1994). Recent electron microscopy studies support the presence of internal symmetry in the insulin receptor (Luo *et al.*, 1999; Tulloch *et al.*, 1999). Furthermore, fluorescence spectroscopy suggests insulin binding induces conformational changes that move the insulin receptor dimeric units into closer proximity (Lee *et al.*, 1997).

1.3.1.4 Receptor Signalling

The greatest sequence similarity between the insulin and type 1 IGF receptors is in the tyrosine kinase domain of the β-subunit where there is 84 % amino acid identity (Ullrich *et al.*, 1986). These domains contain an ATP binding site comprising a common Gly-X-Gly-X-X-Gly sequence flanked by a C-terminal Lysine residue (Ullrich & Schlessinger, 1990). Within the tyrosine kinase domain there are three regions of sequence divergence which may determine tyrosine kinase substrate specificity (Ullrich *et al.*, 1986).

The autophosphorylation/kinase activity of the type 1 IGF receptor is likely to mediate its biological effects. In insulin studies, substitutions of the lysine residue within the tyrosine kinase domain of the insulin receptor abolishes insulin-stimulated autophosphorylation, kinase activity and the mediation of biological responses (McClain *et al.*, 1987; Chou *et al.*, 1987; Ebina *et al.*, 1987). Ligand binding to the α-subunit of the insulin receptor has been found to induce the autophosphorylation of three

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key tyrosine residues at positions 1158, 1162 and 1163 and the subsequent enhanced kinase activity of the receptor towards endogenous substrates (Tornqvist *et al.*, 1987; White *et al.*, 1988; Wilden *et al.*, 1992). Like the insulin receptor, the type 1 IGF receptor has been shown to possess ligand-stimulated tyrosine autophosphorylation and kinase activity (Jacobs *et al.*, 1983; Kadowaki *et al.*, 1987). More recent studies have better characterized the signalling pathways of the type 1 IGF and insulin receptors and are reviewed in detail by Butler *et al.* (1998) and Adams *et al.* (2000).

1.3.1.5 Accounting for the Distinct Biological Roles of Insulin and IGF-I

Despite structural similarities between the insulin and type 1 IGF receptors and their respective ligands, insulin and IGF-I have distinct physiological roles. As previously mentioned, IGF-I actions include short term insulin-like effects and mitogenic effects and is primarily involved in regulating growth and development. Insulin regulates rapid anabolic responses including glucose uptake into liver, muscle and fat cells. The distinct metabolic roles of IGF-I and insulin may have been enhanced during evolution at various levels including ligand binding specificity, differential receptor signalling and distribution, IGFBP interaction, and type 2 IGF receptor interaction (Adamo *et al.*, 1992; Siddle, 1992). The presence of the insulin and type 1 IGF receptors in most cell lines has complicated the assignment of responses specific to each receptor type. In addition, the possible formation of hybrid receptors further complicates the interpretation of these studies (Siddle, 1992).

At the commencement of this thesis, the insulin and IGF type 1 receptors were known to phosphorylate a similar set of cellular protein substrates, although the signal transduction pathways of the type 1 IGF receptor were less well defined. Both receptors stimulate the phosphorylation of pp185 (IRS-1), pp240 and phosphatidylinositol (PI) 3-kinase (Kadowaki *et al.*, 1987; Shemer *et al.*, 1987; Ruderman *et al.*, 1990) and it is unclear how the signalling capacities of the insulin and type 1 IGF receptors could account for the distinct physiological roles of IGF-I and insulin. Insulin has been shown to act through its own receptor to produce a mitogenic response in H35 cells (Massague *et al.*, 1982; Koontz, 1984), and F9 embryocarcinoma cells (Nagarajan & Anderson, 1982). Similarly, IGF-I can mediate metabolic responses similar to insulin in human muscle cells (Shimizu *et al.*, 1986) and 3T3-L1 adipocytes (Weiland *et al.*, 1991) through the type 1 IGF receptor. In order to accommodate the distinct roles of these two hormones, it has been speculated

that the insulin receptor and the type 1 IGF receptor utilize multiple divergent signalling pathways in which some signalling pathways are entirely separate while others are shared (Olefsky, 1990) (Figure 1.5). This model would thus predict that the type 1 IGF receptor may be more potent than the insulin receptor in mediating long term mitogenic responses such as the stimulation of DNA synthesis. Conversely, the insulin receptor may be more potent than the type 1 IGF receptor in stimulating the short-term metabolic responses such as glucose uptake.

Receptor chimeras, truncations and point mutations in the β-subunit have been used to investigate potential regions involved in divergent and convergent signalling. A chimeric receptor consisting of type 1 IGF receptor β-subunits is more effective in stimulating protein synthesis than the wild-type insulin receptor (Lammers et al., 1989). The C-terminal region of the β-subunit of the insulin receptor is highly conserved across species and shares limited homology with the type 1 IGF receptor (Ullrich et al., 1986). Truncation of the C-terminal 43 amino acids of the β-subunit of the insulin receptor results in a reduction of metabolic function (glucose transport and the stimulation of glycogen synthase) but augments mitogenic signalling (as measured by thymidine incorporation) when expressed in rat fibroblasts (McClain et al., 1988; Thies et al., 1989). Phenylalanine substitutions of C-terminal tyrosine residues have been shown to elicit similar results (Takata et al., 1991; Ando et al., 1992). These studies suggest that the C-terminal region of the insulin receptor is important for metabolic signalling and may account for a divergence in receptor signalling pathways. However, the 43 amino acid C-terminal truncated form of the insulin receptor when expressed in CHO cells was indistinguishable from the wild-type in mediating either a mitogenic or metabolic response (Myers et al., 1991). Furthermore, CHO cells expressing either human type 1 IGF receptor cDNA or insulin receptor cDNA were found to have similar potencies in stimulating autophosphorylation, phosphorylation of endogenous substrates, glucose uptake, glycogen synthesis, and DNA synthesis (Steele-Perkins et al., 1988; Roth et al., 1988). These results lead Roth et al, (1988) to propose that the different physiological roles of insulin and IGF-I are due to the pharmacodynamics of the two hormones and/or receptor distribution on different cell types, rather than intrinsic signalling differences.

Most recent signalling studies have identified adaptor proteins that differentially interact with the two receptors and link to multiple divergent signal pathways. The 14-3-3β and ζ phosphoserine-binding proteins have been shown to bind selectively to the type 1 IGF receptor in the yeast two-hybrid system (Furlanetto *et al.*, 1997). Multiple activities have been reported for 14-3-3 proteins including neurotransmitter biosynthesis, vesicular trafficking, cell-cycle progression and inhibition of apoptosis (Aitken *et al.*, 1995; Zha *et al.*, 1996). Another adaptor protein, Grb10, has been proposed to preferentially associate with the insulin receptor as compared with the type 1 IGF receptor in mouse fibroblasts (Laviola *et al.*, 1997). The biological role of Grb10 is unclear with over-expression of Grb10 showing both enhanced (Wang *et al.*, 1999) and inhibitory (Morrione *et al.*, 1997) mitogenic responses to IGF-I.

A monoclonal antibody (αIR-3) that specifically inhibits ligand binding to the type 1 IGF receptor (and not the insulin receptor) has been used to address the intrinsic metabolic and mitogenic responses of the insulin and type 1 IGF receptors. IGF-I stimulated thymidine incorporation in human fibroblasts was inhibited by αIR-3 indicating this response is primarily mediated through the type 1 IGF receptor (Chaiken *et al.*, 1986; Flier *et al.*, 1986). In lung fibroblast cell lines WI38 and HEL (Furlanetto *et al.*, 1987), and human fibroblasts (Van Wyk *et al.*, 1985), αIR-3 inhibits insulin-stimulated DNA synthesis suggesting that the mitogenic effect of insulin in these cell types is predominantly mediated through the type 1 IGF receptor. In contrast, insulin-stimulated DNA synthesis in fibroblast cell lines GM498 and HES, osteogenic sarcoma cell line M663 (Furlanetto et al., 1987), and human dermal fibroblasts (Flier *et al.*, 1986), was unaffected by αIR-3, indicating that insulin stimulates a mitogenic response predominantly through the insulin receptor in these cell types. As experimental conditions were consistent for all cell lines these results suggest receptor responses differ between cell types (Furlanetto *et al.*, 1987).

IGFBPs may contribute significantly to the physiological differences between IGFs and insulin. As previously mentioned, the IGFs are predominantly found in a 150 kDa complex with IGFBP-3 and the acid-labile subunit. This complex may prevent interaction with the relatively low affinity insulin receptors. As insulin does not bind the IGFBPs it may regulate hepatic glucose output directly by

binding hepatic insulin receptors. IGFBPs bind the IGFs with higher affinity than the type 1 and type 2 receptors and thus may regulate presentation of small, constant amounts of IGFs to the type 1 IGF receptor (Adamo *et al.*, 1992).

1.3.2 The Type 2 IGF Receptor

1.3.2.1 Structure and Ligand Binding

The type 2 IGF receptor is identical to the cation-independent mannose-6-phosphate receptor and is structurally unrelated to the IGF type 1 receptor and insulin receptor (Lobel *et al.*, 1987; Morgan *et al.*, 1987) (Figure 1.6). The type 2 IGF receptor consists of approximately 2400 amino acids with a large extracellular region consisting of 15 contiguous repeats containing a conserved pattern of hydrophobic and cysteine residues. A soluble form of the type 2 IGF receptor has been identified in human (Causin *et al.*, 1988), rat (Keiss *et al.*, 1987) and monkey serum (Gelato *et al.*, 1988). The physiological role of the soluble type 2 IGF receptor is unknown. Low serum levels suggest that it does not function as a major IGF-II binding protein and its affinity for mannose-6-phosphate-containing proteins indicates a possible role in regulating the activity of exogenous lysosomal enzymes (Moxham & Jacobs, 1992). IGF-II binds the type 2 IGF receptor with significantly greater affinity than IGF-I while the receptor does not bind insulin (Roth, 1988) (Figure 1.5). IGF-II can bind simultaneously to the type 2 IGF receptor with mannose-6-phosphate-containing ligands thus suggesting these two ligands have distinct binding sites (Braulke *et al.*, 1988).

1.3.2.2 Receptor Function

It is unclear whether the type 2 IGF receptor mediates any of the known biological actions of IGF-II. IGF-II mediated responses in H35 hepatoma (Mottola & Czech, 1984), L6 myoblasts (Hartmann *et al.*, 1992) and rat granulosa cells (Adashi *et al.*, 1990) are not affected by antibodies that block ligand binding to the type 2 IGF receptor. Furthermore, antibodies that block ligand binding to the type 1 IGF receptor inhibit IGF-II mediated responses (Conover *et al.*, 1986; Stracke *et al.*, 1989). These studies indicate that the effects of IGF-II are mediated through binding

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to the type 1 IGF and insulin receptors rather than type 2 IGF receptor. However, the type 2 IGF receptor has been shown to couple GTP-binding proteins (Nishimoto *et al.*, 1989). Furthermore IGF-II (and not IGF-I or insulin) has been shown to stimulate phospholipase C-mediated production of inositol trisphosphate and diacylglycerol in kidney membranes (Rogers & Hammerman, 1988). The type 2 IGF receptor may primarily function as a IGF-II sink, mediating a degradative pathway through receptor internalization (Oka *et al.*, 1985; Haig & Graham, 1991).
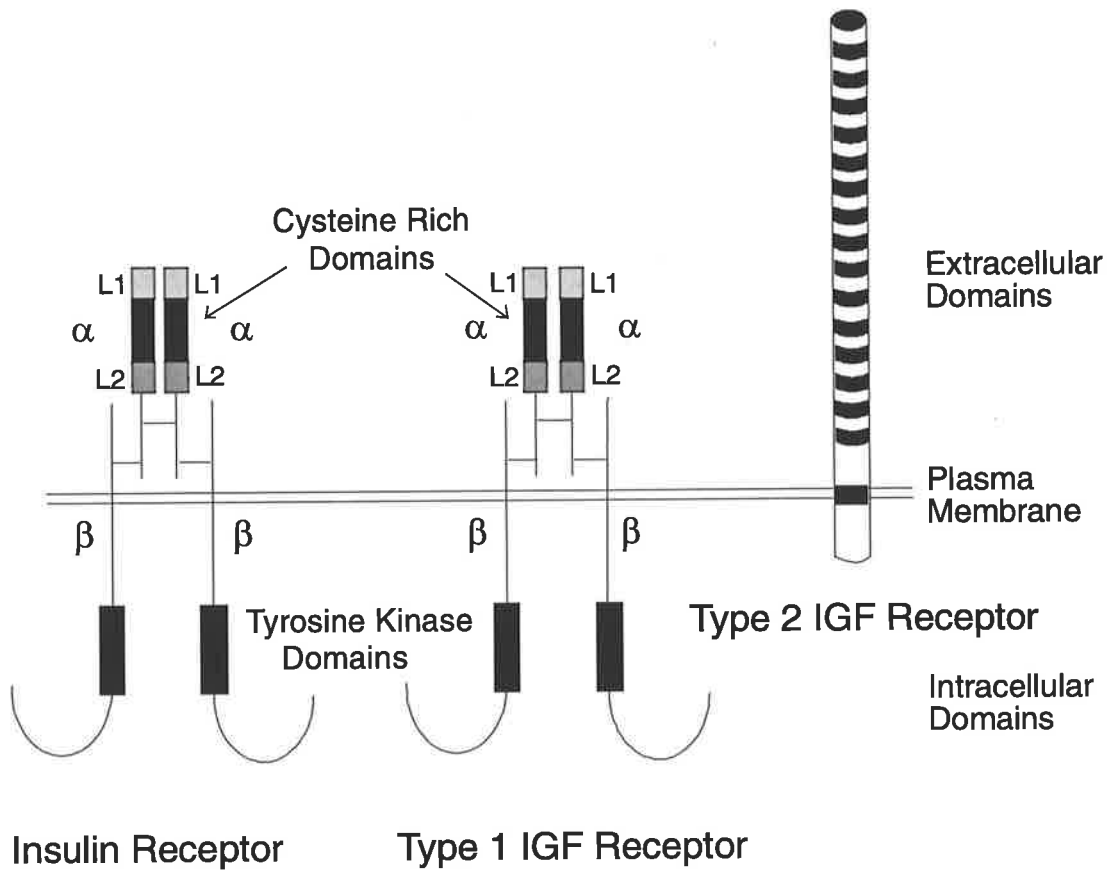
Figure 1.4 Schematic representation of the insulin, type 1 IGF and type 2 IGF receptors. The insulin and the type 1 IGF receptors are homologous with highly conserved cysteine rich and tyrosine kinase domains. The distinct type 2 IGF receptor consists of 15 extracellular repeat sequences. Disulphide bonds joining the α- and β-subunits of the insulin and type 1 IGF receptor are also shown.
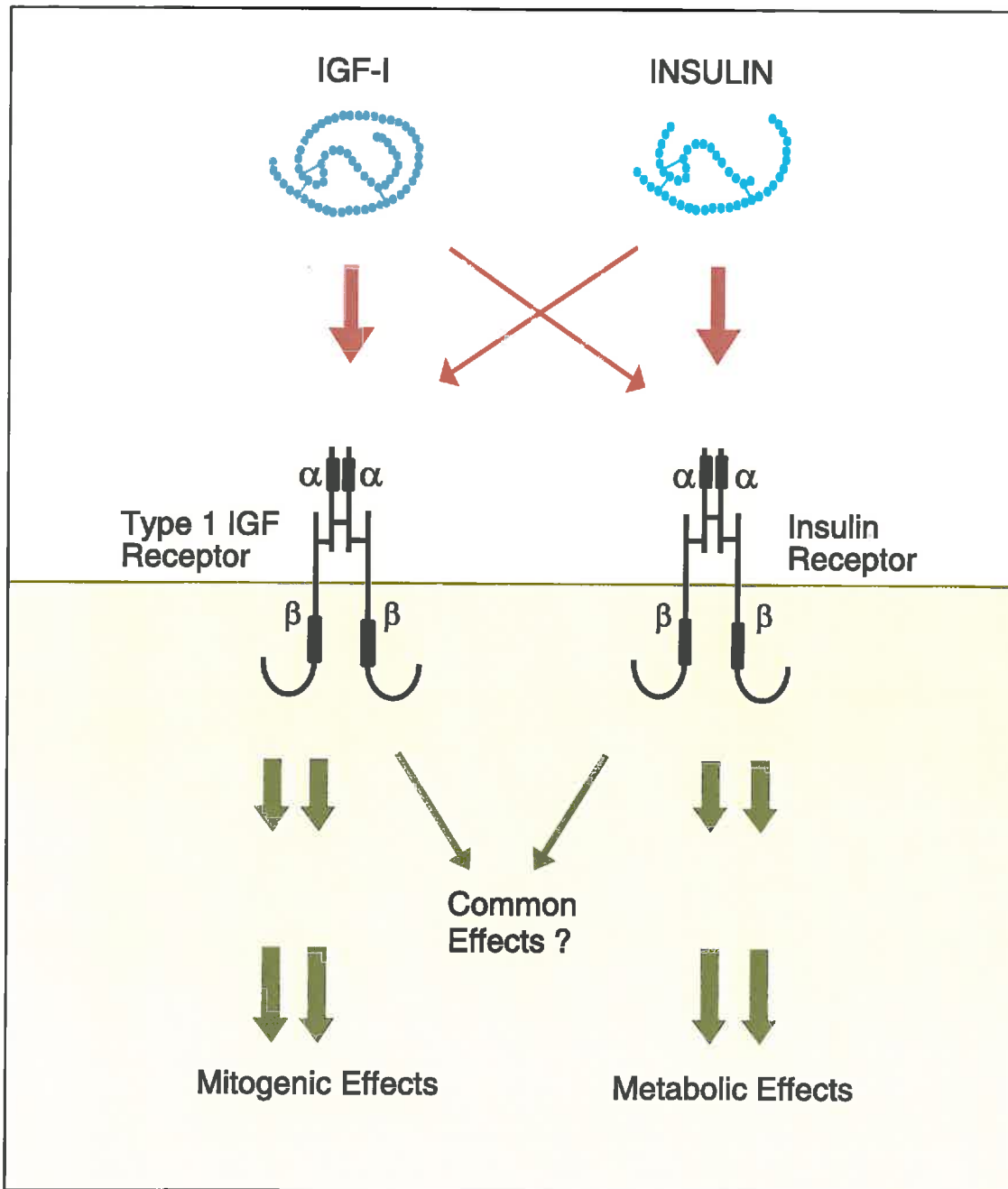
Figure 1.5 The insulin and type 1 IGF receptors may elicit multiple divergent and convergent signalling pathways. Each receptor may have separate or divergent signalling mechanisms for specific biological effects while other effects are shared through common or convergent signalling pathways. Binding of insulin to the insulin receptor has inherently metabolic effects. Conversely, IGF-I binding to the type 1 IGF receptor elicits an inherent mitogenic response.

1.4 Structure-Function Relationships

The large number of protein interactions exhibited by the IGFs provides an interesting study of the relationship between protein structure and function. IGFs bind to the type 1 and type 2 IGF receptors, the insulin receptor and the various IGFBPs with varying affinities. The unique properties of the IGFs not shared with insulin are that they associate with the type 2 IGF receptor and the IGFBPs. In addition, each ligand has weaker affinity for the cognate receptor. IGF analogues with altered binding specificities can be used as tools for investigating the action of the various receptors and IGFBPs which associate with the IGFs. As previously mentioned IGF analogues with reduced affinity for the IGFBPs have illustrated the potential actions of the IGFBPs. IGF-II analogues with selective reductions in the binding affinity for either the type 1 or type 2 IGF receptor may be used to address the role of the type 2 IGF receptor. Similarly, IGF analogues with altered binding specificity for the type 1 IGF and insulin receptors may aid in understanding which actions of IGF-I are mediated via the type 1 IGF receptor and which are due to the cross-reactivity with the insulin receptor.

1.4.1 IGF-insulin hybrids

Early work aimed at identifying determinants involved in receptor binding and IGFBP binding utilized chemically synthesized hybrid peptides in which domains from IGF-I were added to, or exchanged with, those of insulin. The lack of interaction between insulin and both the type 2 IGF receptor and IGFBPs could therefore enable identification of various domains involved in the these interactions. As IGF-I and insulin share common receptor binding regions, this approach was limited to identifying residues that confer receptor binding specificity rather than critical receptor binding regions.

King *et al*, (1982) showed that the addition of the D-domain of IGF-I to the insulin peptide reduces its ability to bind the insulin receptor and stimulate glucose oxidation in rat adipocytes. In contrast the D-domain hybrid was 2-3-fold more potent than insulin in stimulating DNA synthesis in human fibroblasts. This study suggested that the D-domain of IGF-I contributes to the mitogenic potency of this peptide but does not necessarily indicate that this domain is required for high affinity binding between IGF-I and the type 1 IGF receptor. In a similar study, the D-domain of IGF-II was

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attached to the insulin A-chain (Ogawa *et al.*, 1984). This hybrid did not exhibit increased mitogenic activity or type 1 IGF receptor binding and was unable to bind the type 2 IGF receptor (De Vroede *et al.*, 1985).

A hybrid in which the B-chain of insulin was substituted with the B-domain of IGF-I was shown to bind crude preparations of IGFBPs while exhibiting significant reductions for the IGF type 2 receptor (De Vroede *et al.*, 1985). Hybrid molecules containing the A- and D-domains of IGF-I added to the insulin B-chain were unable to bind IGFBPs and exhibited 3-5 fold higher affinity for the type 1 IGF receptor than insulin (Tseng *et al.*, 1987). The main conclusions from these early hybrid studies were that the B-domain of IGF-I is involved in the interaction with IGFBPs while the A-chain is important for type 2 IGF receptor binding. Subsequently, Cara *et al.*, (1990) generated a hybrid in which a C-region of IGF-I was connected to B22 of insulin. This hybrid polypeptide was found to have improved binding for the type 1 IGF receptor and decreased affinity for the insulin receptor suggesting the C-domain of IGF-I is important in determining receptor binding specificity.

1.4.2 IGF Fragments

Konishi *et al*, (1989) have chemically synthesised a series of overlapping fragments of bovine IGF-I and IGF-II and assessed their potency in stimulating the proliferation of rat L6 myoblasts. Two fragments of IGF-I (positions 21-45 and 31-55) and two fragments of IGF-II (positions 20-44 and 30-54) were shown to elicit a biological response. Although the homology between IGF-I and IGF-II is low in these regions, the hydrophobic profiles are similar. Furthermore the substitution of Ile^{35} in the two IGF-II fragments by Serine inactivated these fragments suggesting Ile^{35} of bovine IGF-II may be important for activity. Hence they proposed that the hydrophobic profile in these regions of IGF-I and IGF-II may be essential for the biological response. The disadvantage with this approach is that the fragments may adopt flexible conformations in solution. Moreover, these fragments were found to have low activity compared to intact IGF and consequently the region identified in contributing to activity may not be critical for receptor binding.

1.4.3 IGF analogue studies

The generation of IGF analogues through site-directed mutagenesis has identified the role of various amino acid positions. However, like the IGF-insulin hybrid studies, amino acid substitutions of certain residues outside the functional binding sites may cause conformational changes, improper folding, temperature and enzymatic destabilization. These changes may indirectly affect receptor binding affinity and biological potency and therefore complicate proper interpretation of mutational studies. In order to minimize these effects, various studies have substituted positions of IGF with the analogous amino acids in insulin. This approach is less likely to affect the conformation and folding of IGF-I than the use of random or alanine substitution.

1.4.3.1 Type 1 IGF and Insulin Receptor Binding Regions

Early models of the receptor binding surface of insulin suggested the involvement of Gly^{A1}, Gln^{A5}, Tyr^{A19}, Asn^{A21}, Val^{B12}, Tyr^{B16}, Arg^{B22}, Gly^{B23}, Phe^{B24}, Phe^{B25} and Tyr^{B26} (Pullen *et al.*, 1976). Most of these amino acid positions are preserved or conservatively substituted in IGFs. This suggests that the receptor binding region and binding mechanism are conserved with IGFs and may account for the cross-reactivity of IGF with the insulin receptor (Blundell *et al.*, 1978; 1983). Mutational studies have confirmed the hydrophobic residues involved in insulin dimerization, Phe^{B24}, Phe^{B25} and Tyr^{B26} are important for high affinity association between insulin and the insulin receptor (Tager *et al.*, 1980; Kobayashi *et al.*, 1982; Nakagawa & Tager, 1987). Truncation of amino acid residues B^{26}-B^{30} results in a subtle change in the affinity of insulin for the insulin receptor while further deletion of residues Phe^{B24} and Phe^{B25} significantly disrupts binding affinity. In contrast, substitution of Phe^{B24} with Gly and various D-amino acids are surprisingly well tolerated (Mirmira & Tager, 1989). Flexibility in this region of the insulin B-chain and exposure of the highly conserved residues Ile^{A2} and Val^{A3} has been proposed as critical for biological activity (Derewenda *et al.*, 1991; Hua *et al.*, 1991; Murray-Rust *et al.*, 1992). The crystal structure of insulin (Smith *et al.*, 1984) displaying residues important for insulin receptor binding is presented in Figure 1.6.

Maly and Luthi, (1988) have shown that when bound to the receptor, IGF-I residues Tyr^{24}, Tyr^{31}, and Tyr^{60} are protected from iodination, indicating that these residues form part of, or are close to, the receptor binding region. Indeed, IGF analogues $[Leu^{24}]$ IGF-I and $[Ser^{24}]$ IGF-I have

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dramatically reduced binding affinity for the type 1 IGF receptor without affecting type 2 IGF receptor binding or IGFBP binding (Cascieri et $al.$, 1988b). However, substitution of the conserved aromatic residues Phe[23], Tyr[24], Phe[25] of IGF-I with the corresponding residues Phe[B24], Phe[B25] and Tyr[B26] of insulin has minimal effect on type 1 IGF receptor or insulin receptor binding (Cascieri et $al.$, 1988b). Several studies have confirmed the corresponding Tyr[27] of IGF-II is involved in maintaining IGF-II affinity for the type 1 IGF receptor. IGF-II mutated at Tyr[27] to Leu or Glu reduces type 1 IGF receptor affinity by 25- and 54-fold respectively (Bürgisser et $al.$, 1991). Using human placental membranes rather than type 1 receptor overexpressing cell lines, Beukers et al, (1991) found [Leu[27]] IGF-II has a 100-fold lower affinity than IGF-II for the type 1 IGF receptor. These studies support the involvement of Tyr[24] of IGF-I in type 1 IGF receptor binding interaction and suggest this residue has a similar role to the corresponding Phe[B25] of insulin.

Replacement of Phe[26] of IGF-II with Ser results in a 5- and 20-fold decrease in type 1 IGF and insulin receptor binding respectively while [Leu[43]] IGF-II was found to have practically no affinity for these two receptor types (Sakano et $al.$, 1991). The analogous positions in IGF-I are amino acid residue Phe[23] and Val[44]. Both residues are highly conserved between IGF-I, IGF-II and insulin and have been implicated in the association between insulin and the insulin receptor (Murray-Rust et $al.$, 1992). This suggests that Phe[23] and Leu[44] of IGF-I are also important in maintaining high affinity binding of IGF-I for the type 1 IGF receptor.

Bayne et al, (1989) have further demonstrated the role of the C-domain of IGF-I in type 1 IGF receptor interaction by generating analogues in which the C-region has been replaced with a 4-residue glycine bridge. The analogue [1-27, (Gly)$_4$, 38-70] IGF-I shows 30-fold less affinity than IGF-I for the type 1 IGF receptor while maintaining affinity for the IGFBPs (Bayne et $al.$, 1989). Additionally, replacement of Tyr[31] with alanine reduces type 1 IGF receptor binding by 6-fold (Bayne et $al.$, 1990). Since the commencement of my PhD project, Zhang et $al.$, (1994) have generated a series of IGF-I analogues with N-terminal fusion peptides in which positively charged residues in the C- and D-domain have been substituted with alanine. Alanine substitutions of Arg[36] and Arg[37] reduce type 1 IGF receptor binding by 15-fold while increasing insulin receptor binding by

29-fold (Zhang *et al.*, 1994). These studies demonstrate that the G-domain of IGF-I is also important in determining receptor binding specificity and that residues Tyr^{31}, Arg^{36} and Arg^{37} are involved with high affinity type 1 IGF receptor interactions. IGF-I amino acid residues implicated through mutational studies in the interaction with the IGF type 1 receptors are illustrated in Figure 1.6.

Mutational studies have also suggested the involvement of the D-domain and C-terminal region of the B-domain α-helix in conferring receptor binding specificity. Substitution of Gln^{15}-Phe^{16} of IGF-I with the corresponding Tyr^{B16}-Leu^{B17} residues of insulin results in a 9-fold increase in insulin receptor binding affinity while having a marginal effect on type 1 IGF receptor binding affinity (Bayne *et al.*, 1988). An IGF-I analogue lacking 8 C-terminal amino acids (D-domain) has minimal effect on type 1 IGF receptor binding and increases insulin receptor binding by 2-fold (Bayne *et al.*, 1988). In contrast, the deletion of the analogous 6 amino acids in IGF-II reduces affinity for the type 1 receptor by 5-fold (Roth *et al.*, 1991). Alanine substitutions of the Lys residues at positions 65 and 68 in IGF-I reduce type 1 IGF receptor binding and increase insulin receptor binding, further demonstrating the importance of the D-domain in conferring receptor binding specificity (Zhang *et al.*, 1994).

1.4.3.2 Type 2 IGF Receptor Binding Region

IGF/insulin hybrid studies have indicated the involvement of the A-domain in type 2 IGF receptor binding (De Vroede *et al.*, 1985). Similarly, substitution of positions 42-56 of IGF-I with residues 1-15 of the insulin A chain significantly reduces affinity for the type 2 IGF receptor (Cascieri *et al.*, 1988b). An IGF-I analogue in which Phe^{49}-Arg^{50}-Ser^{51} are replaced with the corresponding Thr-Ser-Ile of insulin was shown to have >10-fold reduced affinity for the type 2 IGF receptor while maintaining normal affinity for both the IGFBPs in acid-treated human serum and the type 1 IGF receptor (Cascieri *et al.*, 1989). Alanine substitutions of Arg^{55} and Arg^{56} of IGF-I was found to increase type 2 IGF receptor binding by 7-fold and suggests the presence of these positively charged residues impairs IGF-I binding to the type 2 IGF receptor. Indeed the arginine substitution of analogous positions Ala^{54} and Leu^{55} in IGF-II dramatically affects binding to the type 2 IGF receptor (Sakano *et al.*, 1991). Similar to the observation with IGF-I (Cascieri *et al.*, 1989), changing

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Phe[48]-Arg[51]-Ser[50] of IGF-II with the corresponding amino acids of insulin also decreases IGF type 2 receptor binding. In summary, these studies suggest that positions 49-51 and 55-56 of IGF-I play an important role in type 2 IGF receptor interaction. Other undetermined structural features in the A-domain may also be involved in binding this receptor. IGF-I amino acid positions involved in the interaction with the type 2 IGF receptor are shown in Figure 1.7.

1.4.3.3 IGFBP Binding Region

Studies with insulin-IGF hybrids first illustrated the importance of the B-region of IGF in IGFBP binding. Various mutational studies indicate that the importance of the extreme N-terminal region of IGFs in this interaction. A naturally occurring variant des(1-3)IGF-I has a greatly reduced affinity for IGFBPs (Szabo *et al.*, 1988; Forbes *et al.*, 1988). The analogue [Gln[3], Ala[4]] IGF-I was found to have a 4-fold lower affinity than IGF-I for human serum IGFBPs (Bayne *et al.*, 1988). Chemically synthesized analogues of IGF-I truncated by 1 to 5 N-terminal amino acid residues have demonstrated the importance of the N-terminal region in binding bovine IGFBP-2 and IGFBPs secreted by L6 rat myoblasts (Bagley *et al.*, 1989). Furthermore IGF-I analogues with Arg and Gly substitutions at positions 3 shows a 230- and 59-fold respective loss in affinity for bovine IGFBP-2 relative to IGF-I (King *et al.*, 1992). Analogues [Arg[3]] IGF-I and [Gly[3]] IGF-I also show similar loss in affinity for IGFBPs secreted by rat L6 myoblasts (Francis *et al.*, 1992). These studies identify Glu[3] of IGF-I being crucial in the high affinity interaction with the IGFBPs.

Different IGFBPs have been shown to have differential structural requirements for high affinity IGF-I binding (Clemmons *et al.*, 1992). The analogue [Tyr[15], Leu[16]] IGF-I has 10- and 100-fold reduced affinity for IGFBP-4 and IGFBP-5 respectively while maintaining affinity for IGFBP-1, -2 and -3. In contrast, [Gln[3], Ala[4]] IGF-I was found to have 13-fold reduced affinity for IGFBP-3 with more significant loss in affinity for the other IGFBPs. The analogue, [Thr[49], Ser[50], Ile[51]] IGF-I has normal to 6-fold lower affinity for IGFBP-3 while showing greater than 20-fold reduced affinity for the other IGFBPs (Clemmons *et al.*, 1992; Baxter *et al.*, 1992). These studies suggest that the N-terminal region of IGF-I is more important than residues 49-51 for IGFBP-3 interaction (Clemmons *et al.*, 1992). IGF-I positions important for high affinity binding to the IGFBPs are illustrated in Figure 1.7.

1.4.4 Summary

A small number of mutational studies have identified the critical role of Phe^{23}, Tyr^{24}, Tyr^{31}, Arg^{36}, Arg^{37} and Val^{44} in type 1 IGF receptor association. Structural similarities between the IGFs and insulin suggest the type 1 IGF and insulin receptor binding regions are largely conserved. This conservation can enable the prediction of other residues that may form a putative hydrophobic binding core involved in the type 1 IGF receptor interaction. This receptor binding region of IGF-I may include Val^{11}, Gly^{42}, Ile^{43} Val^{44} and Tyr^{60}. Other mutational studies have demonstrated structural determinants in the C- and D-domain of IGF attenuate insulin receptor binding. However, unknown elements located on the A- and B-domains must also be important in conferring receptor binding specificity.

The substitution of IGF regions with non-homologous amino acids of insulin has shown that distinct but overlapping regions of the IGFs are involved in the interaction with the type 2 receptors and the IGFBPs. The residue Glu^{3} has been shown to be critical in high affinity association with the IGFBPs with further contribution from the regions Gln^{15}-Phe^{16} and Phe^{49}-Arg^{50}-Ser^{51}. The Phe^{49}-Arg^{50}-Ser^{51} region of IGF-I is also important in type 2 IGF receptor association while basic residues Arg^{55} and Arg^{56} impair IGF-I binding to this receptor.

1.5 Aims of Project

The IGFs provide an interesting model for examining structure-function relationships because of their low molecular weight and the large number of interactions which they exhibit. Relatively few residues have been shown to interact with the type 1 IGF and insulin receptors. A number of invariant residues shared between IGF-I and insulin may be involved with IGF-I binding to these two receptor types. The aim of this study was to identify IGF-I residues within the proposed receptor binding region that are important in defining ligand-receptor specificity. In particular, my principal goal was to generate IGF-I analogues with selectively reduced affinity for the insulin receptor and therefore enhanced specificity for the type 1 IGF receptor.

Despite a large effort aimed at understanding the intrinsic signalling differences between the type 1 IGF receptor and the insulin receptor, it is still unclear how these receptors account for the different physiological roles of IGF-I and insulin. Early observations that IGF-I can interact with the insulin receptor and that bolus injections of IGF-I elicits hypoglycaemic responses led to the view that this hormone mediates its metabolic effects through the insulin receptor. Based on this assumption it was believed that IGF-I analogues with reduced affinity for the insulin receptor might lessen the hypoglycaemic response and thus have greater therapeutic potential. Studies using various cell types generally show IGF-I can exert metabolic effects via its own receptor rather than the insulin receptor. Although IGF-I analogues with altered affinity for the insulin receptor may not prove to be therapeutically beneficial they may serve as useful tools in addressing the significance of receptor binding specificity in the insulin and IGF-I systems. Furthermore, such analogues may be used to clarify intrinsic signalling differences between the type 1 IGF and insulin receptors.

The following chapter outlines the strategy used for mutational selection and describes the production and physico-chemical characterization of the IGF-I analogues. Assessment of receptor binding properties and biological potencies for these analogues are presented in Chapter 3. As part of a broader study of the IGFBP binding surface of IGF-I, two analogues,

[Leu^8] IGF-I and [Ser^{15}] IGF-I, were assayed against the 6 IGFBPs. The aim of this study was to identify specific residues on the B-domain α-helix that are involved in IGFBP association. Results and findings from this study are discussed in Chapter 4.

Chapter 2

Production and Physicochemical Characterization of IGF-I Analogues

2.1   Introduction

As discussed in Section 1.5, the primary aim of this IGF structure-function study was to identify IGF-I residues important for defining ligand-receptor specificity and ultimately to generate IGF-I analogues with reduced affinity for the insulin receptor. In this chapter I will outline the strategies used for the selection and production of the IGF-I analogues: [Leu^8] IGF-I, [Ser^{15}] IGF-I, [Phe^{59}] IGF-I, [Leu^{62}] IGF-I and [Ser^{15}, Leu^{62}] IGF-I. Preliminary physicochemical analyses of these analogues by N-terminal sequencing, reverse phase HPLC, SDS PAGE, Mass Spectroscopy and Far-UV CD spectroscopy are also discussed. Biological Characterization of these analogues will be described in Chapter 3.

2.1.1 Rational Design

Few general principles governing protein-protein association had been established at the commencement of this study.   Early analyses based on a small number of structurally determined protein complexes demonstrated that protein interfaces share a large hydrophobic component surrounded by polar and charged residues (Janin & Chothia, 1990). The burial of non-polar surfaces during complex formation and the creation of complementary intermolecular hydrogen bonds and salt bridges provide the free energy in favor of association. Protein-protein binding specificity is commonly attributed to amino acid residues that lie at the periphery of the binding interface. If there are evolutionary pressures for maximizing the specificity of the interaction, these residues will mutate in order to introduce unfavorable electrostatic or steric effects.

Sequence comparison of insulins across many animal species has been useful in predicting amino acid residues responsible for high affinity binding of insulin with the insulin receptor.

Such predictions are based on the observation that residues with structural and functional importance undergo fewer mutations during evolution. Insulins from the hystricomorph rodents, such as the guinea pig, casiragua, porcupine and coypu, are more substituted than those of any other mammal (Baja et al. 1986). This divergence provides evolutionary information that may be used for predicting receptor binding regions on insulin or residues involved in conferring binding specificity. Divergence in primary sequence of the hystricomorph insulins has yielded a number of unusual properties. The substitution of the His at position B10 impairs self-association (Wood et al., 1975; Horuk et al., 1980) while substitution of hydrophobic hexamer surface residues B14, B17 and B20 with more hydrophilic residues stabilizes the monomeric form (Blundell et al., 1978). Elevated growth promoting effects of hystricomorph insulins suggests that they may be functionally and evolutionarily closer to the IGFs than other insulins (King & Kahn, 1981). Principles governing evolution at a molecular level would suggest that the 27 amino acid positions conserved between human insulin and hysticomorph insulins represent sites which may have structural or functional importance (Figure 2.1). Indeed, mutational analysis has confirmed the role of highly conserved insulin residues Gly^{A1} (Brandenburg et al., 1975), Ile^{A2}, Val^{A3} (Nakagawa & Tager, 1992), Leu^{B6} (Nakagawa & Tager, 1991), Val^{B12} (Brange et al., 1988), and Phe^{B24} (Tager et al. 1980) in high affinity receptor binding.

Comparison of the monomeric crystal structure of insulin with the solution structure of IGF-I demonstrates the conservation of structurally important core residues and a large hydrophobic surface region (Figure 2.2). This hydrophobic surface region of insulin consisting of Val^{A3}, Tyr^{A19}, Val^{B12}, Leu^{B15}, Phe^{B24} and Phe^{B25} represents a putative receptor binding core and is encircled by polar and charged residues that are highly conserved across all mammalian insulins (Figure 2.1). Conservation of these hydrophobic residues also in IGF-I suggests they may have a critical role in maintaining high affinity binding between IGF-I and the type 1 IGF receptor. In addition, these residues may also account for the cross-reactivity of IGF-I with the heterologous receptor. Divergent peripheral residues surrounding this putative hydrophobic receptor binding region may govern the specificity of IGF-I receptor interaction. Therefore, class specific residues that are conserved within insulins but differ in IGF-I are

potentially important for defining receptor binding specificity. Of the amino acid residues identical in human and hystricomorph insulins, 9 positions not conserved in IGF-I provide target sites for altering the specificity of IGF-I (Figure 2.1). Position Ala[8], Gln[15], and Ala[62] of IGF-I, corresponding to highly conserved insulin residues Ser[B9], Tyr[B16] and Asn[A21], were chosen for amino acid substitution due to their proximity to the putative hydrophobic receptor binding core. Previous studies have demonstrated that the C- and D-domains of IGF-I are involved in determining ligand binding specificity (Cascieri *et al.*, 1988b; Bayne *et al.*, 1989). The Met at position 59 of IGF-I lies on the A-domain and extends into a cleft between the C- and D-domains. Position Met[59] was targeted for amino acid substitution because of its location between the C- and D-domains and its closeness to receptor binding residue Tyr[24].

Conservative substitutions were chosen in order to minimize adverse effects on the structural integrity of IGF-I. The choice of amino acid substitutions was based on the size and hydrophobicity of corresponding insulin residues. Substitutions were intended to decrease binding to the insulin receptor by maximizing differences in amino acid side chain character between IGF-I and insulin. Amino acids Ala[8] and Ala[62] were substituted with Leu to increase the hydrophobic index at each position thereby enhance divergence from the corresponding polar residues Ser[B9] and Asn[A21] of insulin. Similarly a Phe substitution was chosen for Met[59] with the aim of increasing both the hydrophobic index and side chain size at this position, thus enhancing divergence from the corresponding small polar residue Asn[A18] of insulin. A substitution of Gln[15] with Ser was introduced to significantly reduce the side chain length at this position relative to the analogous bulky Tyr[B16] of insulin. The presence of Ser at position 15 of amphioxus IGF-I (Chan *et al.*, 1990) suggests the introduction of this mutation would not affect structural integrity.

IGF-I (B-domain)

Human

```
        1                        10              20                29
Human   G P E T L C G A E L V D A L Q F V C G D R G F Y F N K P T
                    L           S                        L
```

Insulin (B-chain)

```
            B1              B10                B20              B30
Human       F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A
Guinea Pig  F V S R H L C G S N L V E T L Y S V C Q D D G F F Y I P K D
Casiragua   Y V G Q R L C G S Q L V D T L Y S V C K H R G F - Y R P S E
Porcupine   F V N Q H L C G S H L V E A L Y L V C G N D G F F Y R P K A
Coypu       Y V S Q R L C G S Q L V D T L Y S V C R H R G F - Y R P N D
```

IGF-I (A-domain)

```
        42          50              60
Human   G I V D E C C F R S C D L R R L E M Y C A
                                            F   L
```

Insulin (A-chain)

```
            A1          A10              A20
Human       G I V E Q C C T S I C S L Y Q L E N Y C N
Guinea Pig  G I V D Q C C T G T C T R H Q L Q S Y C N
Casiragua   G I V D Q C C T N I C S R N Q L L T Y C N
Porcupine   G I V D Q C C T G V C S L Y Q L Q N Y C N
Coypu       G I V D Q C C T N I C S R R Q L M S Y C N D
```

Figure 2.1 Sequence homology between IGF-I B- and A-domains and insulin B- and A-chains. Open boxes indicate positions conserved between human IGF-I, human insulin and hystricomorph insulins. Residues conserved through the insulins but different in human IGF-I are highlighted with shaded boxes. Corresponding positions in IGF-I represent potential target sites for altering receptor binding specificity. Four positions were selected for substitution due to their proximity to the proposed receptor binding region. Amino acid substitutions, (indicated by arrows), were chosen to enhance differences between insulin and IGF-I at each position while maintaining a conservative change.
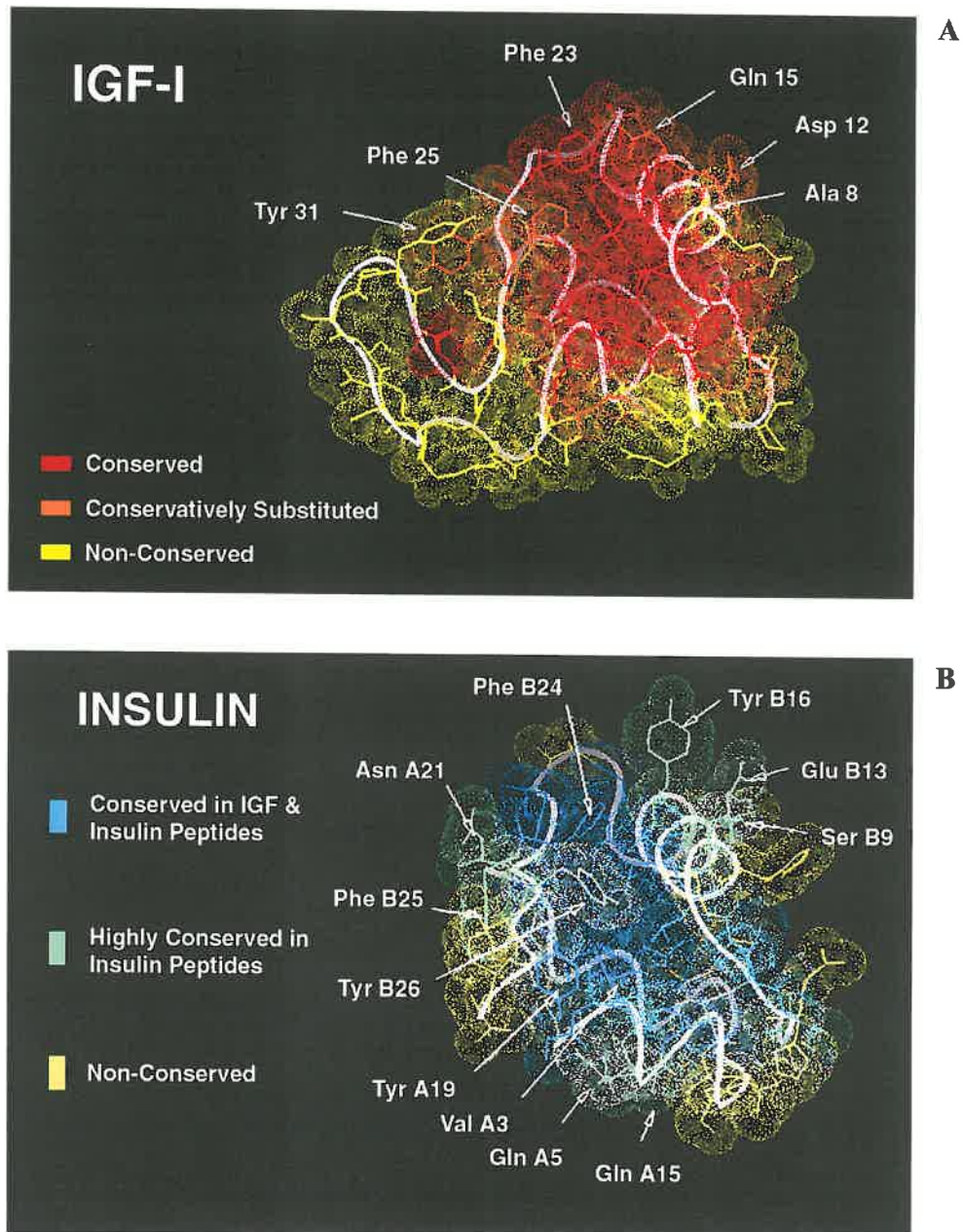
Figure 2.2 Structural homology between insulin and IGF-I. (A) The solution structure of IGF-I demonstrating homology to human insulin. (B) The monomeric crystal structure of human insulin demonstrating homology to IGF-I and other mammalian insulins.

2.2 Materials

2.2.1 General Chemicals and Reagents

Acrylamide, agarose, ampicillin, bovine serum albumin (BSA: radioimmunoassay grade), bromophenol blue, Coomassie Brilliant Blue (R250), dithioerythritol (DTE), isopropyl-β-D-galactoside (IPTG), polyethylene glycol 6000 (PEG 6000), polyoxethylene-sorbitan monolaurate (Tween 20), and sodium dodecyl sulphate (SDS) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.. 2-amino-2-hydroxymethylpropane-1,3-diol (Tris) and xylene cyanol were purchased from BDH Chemicals, Merck Pty. Ltd., Kilsyth, VIC, Australia. Bis-acrylamide was purchased from BioRad Laboratories Inc., Herates, CA, U.S.A.. Ethylenediaminetetraacetic acid (EDTA) was obtained from Boehringer Mannheim Australia, Sydney, NSW, Australia. 2-hydroxyethyl disulphide was from Aldrich, Milwaukee, WIS, U.S.A.. All other chemicals were analytical reagent grade.

2.2.2 Molecular Biology Reagents

Restriction endonucleases were purchased from either Pharmacia Biotech, Sydney, NSW, Australia or New England Biolabs Inc., MA, U.S.A.. The Altered Sites™ *in vitro* mutagenesis system was from Promega Corporation, Sydney, NSW, Australia. Calf intestinal alkaline phosphatase (CIP) was purchased from Boehringer Mannheim Australia, Sydney, NSW, Australia. GENECLEAN II kit was from Bio101 Inc., La Jolla, CA, U.S.A.. All other molecular biology materials, including dideoxynucleotide sequencing kits and synthetic oligonucleotides, were obtained from Bresatec Ltd., Adelaide, SA, Australia.

Escherichia coli strains

DH5α   *sup*E44, Δ*lac*U169, *hsd*R17, *rec*A1, *end*A1, *gyr*A96, λ-, *rel*A1, [φ80*lac*ZΔM15]

JM109   *thi*, *sup*E44, Δ(*lac-pro*AB), *end*A1, *hsd*R17 (rk-, mk+), *rec*A1, *gyr*A96,λ-, *rel*A1, [F′, *tra*D36, *pro*AB, *lac*IqZΔM15]

JM101   *sup*E, *thi*, Δ(*lac-pro*AB) [F′, *tra*D36, *pro*AB, *lac*IqZΔM15]

BMH 71-18 mut S   thi, supE, Δ(*lac-pro*AB), [mutS::Tn10], [F′, proA+B+, *lac*IqZΔM15]

2.2.3 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 addition of 1.2 % (w/v) agar. Minimal media (Min A) contains 50 mM Na_2HPO_4, 22 mM KH_2PO_4, 8 mM NaCl, 20 mM NH_4Cl, 0.4 % (w/v) glucose, and 1.5 mM thiamine. Luria-Bertain (LB) media contains 1 % (w/v) bactotryptone, 0.5 % (w/v) bactoyeast extract, 0.17 M NaCl, pH 7.0. Bactotryptone and bactoyeast extract were purchased from DIFCO Laboratories, MI, U.S.A.

2.2.4 Chromatography Materials

High performance liquid chromatography (HPLC) equipment including the radial compression Novapak C4 column was from Millipore Waters, Sydney, NSW, Australia. The reverse-phase microbore C4 column was purchased from Brownlee Laboratories, Santa Clara, California, U.S.A.. Fast Protein Liquid Chromatography (FPLC) equipment including the resins: Sephadex G-25M, S-Sepharose Fast Flow, and Sepharose Cl-6B were purphased from Pharmacia Biotech, Sydney, NSW, Australia. The C18 Matrex silica was obtained from Amicon, Danvers, MA, U.S.A.. HPLC grade acetonitrile was purchased from BDH Chemicals Ltd., Kilsyth, VIC, Australia and trifluoroacetic acid (TFA) was from Fluka Chemie, Buchs, Switzerland. Milli-Q water was obtained using Milli-Q filtration apparatus, Millipore-Waters, Sydney, NSW, Australia. Solvents were filtered through 0.22 μm GV filters purchased from Millipore-Waters.

2.2.5 Reference Peptides and Molecular Weight Markers

Recombinant human IGF-I (receptor grade), was provided by GroPep Pty. Ltd., Adelaide, SA, Australia. Human insulin was purchased from CSL-Novo Pty. Ltd., North Rocks, NSW, Australia. Low molecular weight markers were purchased from Pharmacia Biotech, North Ryde, NSW, Australia.

2.3 Methods

2.3.1 Molecular Biology Methods

2.3.1.1 Growth and Transformation of *E. coli*

JM101, JM109 and BMH 71-18 mut S were maintained on Min A medium while DH5α was maintained on LB medium. Strains were streaked out onto LB agar plates and grown overnight at 37°C. BMH 71-18 mut S was inoculated into 2 ml of Min A containing 12.5 μg/ml tetracycline while JM101, JM109 and DH5α were inoculated into 2 ml of LB. Strains were subcultured by a 1/100 dilution and grown at 37° C until the culture reached an optical density at 600 nm of between 0.3 to 0.6. Following centrifugation at 3000 g for 5 min, cell pellets were resuspended in a 2.5 ml solution containing 50 mM $CaCl_2$, 20 mM $MgCl_2$ and left at 4° C for at least 1 h. Competent cell aliquots of 200 μl were incubated with 5 μl ligation reaction or plasmid DNA for 40 min. Cells were heat shocked at 42° C for 2 min, before 100 μl of LB media containing 20 mM glucose was added. Cells were incubated at 37° C for 30 min and 200 to 500 μl of the transformation mix were plated onto LB or Min A plates containing the appropriate antibiotics. Overnight incubation at 37° C was allowed for colony formation.

2.3.1.2 Alkaline Lysis Method for Plasmid DNA Preparation

Single colonies were used to inoculate 2 ml of LB containing 100 μg/ml ampicillin and grown overnight at 37° C. Aliquots of 1.5 ml were centrifuged at 2000 g for 5 min, the supernatants were aspirated, and each pellet was resuspended in 90 μl of a solution containing 25 mM Tris pH 8.0, 10 mM EDTA, 15 % (w/v) sucrose. A 180 μl aliquot of the lysis solution (0.2 M NaOH, 1 % (w/v) SDS) was added to each sample and mixed thoroughly through gentle inversion. After a 2 min incubation at room temperature, 135 μl of 3M sodium acetate pH 4.6 was added, mixed, and centrifuged at 10000 g for 20 min at room temperature. Contaminating RNA was removed from the DNA containing supernatant by digestion with 2 μl of Dnase-free Rnase A (10 mg/ml) at 37° C for 20 min. An equal volume of Tris pH 8.0 saturated phenol:chloroform (1:1), was then added, mixed, and centrifuged at 10000g for 5

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min at room temperature. The aqueous layer (approximately 400 µl) was recovered and DNA was precipitated with 1 ml of 100% ethanol at room temperature for 15 min. DNA was pelleted by centrifugation at 10000g for 20 min at room temperature, washed with 500 µl 70 % (v/v) ethanol before resuspension in 40 µl of 0.1 mM EDTA, pH 8.0. To estimate DNA quality and purity, 1 µl samples were electrophoresed on 1 % agarose mini-gels.

2.3.1.3 Boiling Method for Plasmid DNA Preparation

Plasmid DNA used in mutagenesis and sequencing was prepared by the following method. Cell pellets obtained from 1.5 ml of overnight cultures (as described above), were resuspended in a solution containing 0.1 M NaCl, 10 mM Tris pH 8.0, 1 mM EDTA, 5 % (w/v) Triton X-100 and then lysed by incubation at 100° C for 2 min with 10 µl of lysozyme (12.5 mg/ml in 10 mM Tris pH 8.0). The mixture was centrifuged at 10000 g for 15 min at 4° C and the supernatant was recovered. An equal volume of isopropanol was added and the mixtures were incubated on ice for 5 min. Precipitated DNA was pelleted by centrifugation at 10000g for 10 min at room temperature, washed twice with 1 ml 70 % (v/v) cold ethanol, and resuspended in 20 µl of 0.1 mM EDTA pH8.0. DNA quantity and purity was assessed by agarose mini-gel electrophoresis.

2.3.1.4 GENECLEAN DNA Fragment Isolation

DNA fragments larger than 400 bp were purified using the GENECLEAN kit, according to the manufacturer's instructions. Fragments were separated by electrophoresis on a TBE (50 mM Tris pH8.0, 40 mM boric acid, 0.1 mM EDTA) agarose gel, stained with ethidium bromide, visualized and excised under long wave ultra violet (UV) light. Gel slices were solubilized by heating for 5 min at 55° C with 500 µl of NaI solution and 50 µl of TBE modifier. DNA was allowed to bind to 5 µl of glassmilk solution for 5 min at room temperature. The solid phase was pelleted by centrifugation at 10000 g for 5 min and washed 3 times with 200µl 'New Wash' solution. The glassmilk beads were resuspended in 10 µl of water and incubated at 60° C for 10 min to elute bound DNA. The elution was repeated twice and the supernatants were pooled. DNA recovery was assessed by electrophoresis on 1% agarose mini-gels.

2.3.1.5 Electroelution isolation of DNA.

DNA fragments less than 400 bp were isolated using electroelution. DNA fragments were separated by electrophoresis on a 2% TBE agarose gel, stained with ethidium bromide, visualised and excised under long wave UV light. Dialysis tubing was prepared by boiling in 1 mM EDTA for 2 min. Gel slices were placed in dialysis tubing with 400 µl of 10 mM Tris pH 7.5, 0.1mM EDTA and electrophoresed for 1h at 75 mA. The DNA was ethanol precipitated by the addition of 3 M sodium acetate, pH 5.2 (1/10th volume), cold ethanol (2.5 volumes), 20°C for 4 h, before centrifugation at 10000g. The DNA pellet was washed with 70% (v/v) cold ethanol, dried and the recovery was assessed by electrophoresis on 1% agarose mini-gels.

2.3.1.6 Sepharose CL-6B Spin Column DNA Purification

Sepharose CL-6B spin columns were used to further enhance DNA purity by removing small nucleic acids, proteins and salt prior to mutagenesis and sequencing. Mini-columns were made by piercing a 0.5 ml Eppendorf tube with a 21 gauge needle, placing a drop of acid washed glass beads in the base and then overlaying the beads with 500 µl of Sepharose Cl-6B equilibrated in 10 mM Tris pH 7.5, 0.1 mM EDTA. The spin columns were placed within 1.5 ml Eppendorf tubes which collected flow-through during centrifugation at 500 g for 3 min at room temperature. Spin-columns were primed by initial centrifugation and the flow-through discarded. DNA samples of 50 µl were loaded onto the dry Sepharose CL-6B column bed, centrifuged, and the flow-through containing the DNA collected. DNA recovery was assessed by electrophoresis on 1% agarose mini-gels.

2.3.1.7 Dephorphorylation of vector DNA and Ligation

Cleaved vector DNA was dephosphorylated by incubation in a solution containing 2 U of CIP, 50 mM Tris pH 7.5, 10 mM $MgCl_2$ for 15 min at 37° C. A further 2 U of CIP was added and reincubated as above. The CIP was removed by phenol:chloroform extraction by adding 50 µl of Tris pH 8.0 saturated phenol and 50 µl of chloroform, followed by centrifugation at 10000g for 2 min. The aqueous phase was recovered and DNA ethanol precipitated as described in section 2.3.1.5. Ligation reactions of 10 µl were set up with a 3 molar excess of insert DNA

over linearized vector in 50 mM Tris pH7.5, 10 mM MgCl$_2$, 1 mM DTT, 1mM ATP with 2U of T4 DNA ligase. Negative controls consisted of vector alone without DNA insert. Ligations were allowed to proceed overnight at 14° C. The ligation mix was then transformed into competent *E. coli* as described in section 2.3.1.1.

2.3.1.8 DNA Sequencing

DNA sequencing was used to verify correct ligation of insert DNA and specific mutations. The DNA sequencing method was based on the dideoxy-sequencing procedure of Sanger *et al.* (1977). Template dsDNA was incubated with 2 μl of 10 mg/ml DNase-free RNase A for 15 min at 37° C and denatured in 0.2 M NaOH, 0.2 mM EDTA by a further 15 min incubation at 37° C. The denatured DNA was purified on a Sepharose CL-6B spin column as described in section 2.3.1.6. Universal sequencing primer (approximately 50 ng) was combined with approximately 2 μg of denatured DNA template in an annealing buffer consisting of 40 mM Tris pH 7.5, 20 mM MgCl$_2$, 50 mM NaCl (total volume of 10 μl). Primer annealing was achieved by incubation at 65° C for 5 min, 37° C for 30 min then room temperature for a further 30 min. The annealed primer was labelled for 5 min at room temperature with 1 μl 0.1 M DTT, 2 μl labelling nucleotide mixture (1.5 μM dGTP, 1.5 μM dCTP, 1.5 μM dTTP), 5 μCi [α-^{32}P]dATP and 1 U of Sequenase®. Labelling reactions were terminated by transferring 3.5 μl of the reaction mix into 2.5 μl of each of the four dideoxy nucleotide solutions (80 μM dNTP, 50 mM NaCl, 8 μM ddNTP) and incubating at 37° C for 5 min. Sequencing reaction were prepared for electrophoresis by the addition of 4 μl of a solution containing 95 % (v/v) deionized formamide, 20 mM EDTA, 0.05 % (w/v) bromophenol blue and 0.05 % (w/v) xylene cyanol followed by incubation at 95° C for 5 min. Samples were loaded onto a 0.3 mm, 7 M Urea, TBE, 6 % polyacrylamide gel (w/v, 25:1, acrylamide: bis-acrylamide) and electrophoresed at 40 W. The gel was fixed by soaking in 10 % (v/v) acetic acid, 20 % (v/v) ethanol, transferred to Whatman 3MM paper and dried under vacuum at 70° C for 1 h. The dried gel was exposed overnight on X-ray film.

2.3.1.9 Purification and Phosphorylation of Oligomers

Oligomers, synthesized by Bresatec Ltd., were further purified and phosphorylated prior to mutagenesis. Lyophilized oligomers were resuspended and heat denatured in an equal volume of 95 % (v/v) de-ionized formamide. Approximately 20 μg in 15 μl was electrophoresed at 500 V for 1 h on a 0.5 mm, 7 M Urea, 50 mM Tris pH8.0, 0.1 mM EDTA, 40 mM boric acid, 20 % polyacrylamide gel (w/v, 25:1, acrylamide:bis-acrylamide). Oligomer bands were visualized by shadowing under UV light, excised and eluted from the gel by overnight diffusion into 200 μl water. The DNA was precipitated with 2 volumes of ethanol and 1/10th volume of 3 M sodium acetate pH 5.2 at -80° C for 1 h. The precipitated oligomers were pelleted by centrifugation at 10000 g for 5 min, washed with 70 % (v/v) ethanol and resuspended in 30 μl water. Samples were diluted to 200 pmoles in a solution containing 100 mM Tris pH 8.0, 10 mM $MgCl_2$, 5 mM DTT, 0.5 mM ATP and 5′ phosphorylated using 4.5 U of T4 polynucleoide kinase (total volume 30 μl) for 45 min at 37° C. Kinase activity was finally inactivated by heating at 65° C for 10 min.

2.3.1.10 Generation of Mutant Clones

The Altered Sites™ *in vitro* mutagenesis system was used in the generation of mutant IGF-I cDNA clones (Lewis & Thompson, 1990). This mutagenesis system is based on the use of a secondary correction primer that recovers ampicillin resistance in pSELECT-1. Procedures supplied by the manufacturer were modified to circumvent the requirement for M13 helper phage in generating ssDNA. The cloning and mutagenesis procedures (including alterations) are described below and in Figure 2.3.

IGF-I cDNA present as an Eco R1 - Hind III insert in pBluescriptSK+ was subcloned into pSelect-1 by the following method. Vectors were transformed into DH5α (section 2.3.1.1), plasmid DNA was prepared by the alkaline-lysis method (section 2.3.1.2), and digested with Xba I and Kpn I. The pSELECT-1 vector fragment and IGF-I cDNA fragment were isolated using GENECLEAN (section 2.3.1.4), ligated (section 2.3.1.7), transformed into competent DH5α (section 2.3.1.1), and plated on LB agar containing 10 μg/ml tetracycline, 50 μg/ml IPTG, 120 μg/ml BCIG. Transformant selection was based on *lac* Z gene disruption and the

functional loss of β-galactosidase activity observed in white colonies. The pSELECT/IGF-I vector was prepared by the boil method (section 2.3.1.3), denatured in 0.2 M NaOH, 0.2 mM EDTA at 37°C for 15 min, desalted on a Sepharose CL-6B spin column (section 2.3.1.6), and used as an alternative template to phage packaged ssDNA. Oligonucleotides were designed such that at least 12 codons of complementary DNA flanked the region where mutations were to be introduced. The following synthetic oligonucleotides were used to direct these changes (5′ to 3′): [Leu^8] IGF-I, GTCAACCAGTTCCAGACCGCACAGGG; [Ser^{15}] IGF-I, CCGCAAACGAAAGACAGAGCGTCAACC; [Phe^{59}] IGF-I, GGAGCGCAGTAGAATTC CAGACGACG; [Leu^{62}] IGF-I, CCGGTTTCAGCGGCAGGCAGTACATTTCC; [Ser^{15}, Leu^{62}] IGF-I, CCGCAAACGAAAGACAGAGCGTCAACC and CCGGTTTCAGCGGCAG GCAGTACATTTCC.

These oligomers, including the ampicillin repair oligo were phosphorylated (section 2.3.1.9) and annealed by the methods described by the manufacturer. Controls including no oligomers and repair oligomer only were included. Synthesis of the mutant strand was achieved by the addition of 6 µl of a solution containing 50 mM Tris pH7.5, 2.5 mM of each dNTP, 5 mM ATP, 20 mM DTT, with 2U of T4 DNA ligase and 1U of T4 DNA polymerase. Reaction mixes were incubated at 37° C for 2 h followed by a further overnight incubation at 14° C with an additional 2U of T4 DNA ligase. The total reaction was used to transform competent BMH 71-18 mut S (section 2.3.1.1). Twelve colonies from each mutagenesis reaction were selected off LB agar containing 200 µg/ml ampicillin, 15 µg/ml tetracycline, the plasmid DNA isolated (section 2.3.1.2), and retransformed into JM109 (section 2.3.1.1) using ampicillin/tetracycline selection. Plasmid DNA isolated using the boiling method (section 2.3.1.3) was used to screen for successful mutagenesis by DNA sequencing (section 2.3.1.8).

The IGF-I mutated coding regions were subcloned into the [Met^1]-pGH(1-11)-Val-Asn-IGF-I expression vector described by Francis *et al.* (1992) (Figure 2.4) by the following method. The pSELECT/IGF-I mutant vectors and the expression vector were isolated using the alkaline lysis method (section 2.3.1.2), and digested with Hpa I and Hind III. The IGF-I mutant fragments isolated by electroelution (section 2.3.1.5), and the linearized expression

vector isolated by GENECLEAN (section 2.3.1.4), were ligated and transformed into JM101. DNA sequencing (Section 2.3.1.8), was used to verified the integrity of the complete IGF-I coding sequence.

2.3.2 Expression

IGF-I mutant expression vectors were transformed into JM101 and grown in 5 ml of Min A containing 200 µg/ml ampicillin for 2.5 h at 37°C. Expression was induced with 250 µM IPTG with 1 ml samples taken prior to IPTG addition and after incubation at 37°C for 1 h. These un-induced and induced samples were centrifuged for 5 min at 10000 g and the pellets lysed with 100 µl of lysis solution containing 2 % (w/v) SDS, 10 % (v/v) β-mercaptoethanol. Expression was analyzed by SDS-polyacrylamide electrophoresis on a 20 % polyacrylamide separating gel (w/v, 40:1, acrylamide:bis-acrylamide) with a 4% stacking gel run at 20 mA for 2h. The gel was fixed and stained in a solution containing 0.1 % (w/v) Coomassie Brilliant Blue, 50 % (v/v) methanol, 10 % (v/v) acetic acid and destained in a solution containing 5 % (v/v) methanol, 10 % (v/v) acetic acid.

2.3.3 Fermentation and Inclusion Body Isolation

Fermentations of the JM101 transformants were done in 2 litre bioreactors (Applikon Bioreactor, Schiedam, Holland) as described by King *et al.* (1992). Overnight cultures were inoculated into 1 l minimal media containing 200 mM glucose, 2.3 mM $MgSO_4$, 30 mM NH_4Cl, 6.9 mM K_2SO_4, 12 mM KH_2PO_4, 18 mM Na_2HPO_4, 0.3 mM $Na_3citrate$, 30 µM $MnSO_4$, 30 µM $ZnSO_4$, 3 µM $CuSO_4$, 72 µM $FeSO_4$, 0.12 mM thiamine, pH7.0 and grown at 37° C, 55 % pO_2 until the optical density at 600 nm was approximately 8.0. The culture was induced with 250 µM IPTG, grown for a further 5 h before storage at 4° C. Fermentations of [Leu[8]] IGF-I, [Ser[15]] IGF-I, [Phe[59]] IGF-I and [Leu[62]] IGF-I were performed by Mrs. C. Senn, Bresatec Ltd., Adelaide, Australia, while [Ser[15], Leu[62]] IGF-I fermentation was performed by myself. Cells were disrupted by 4 passes through a homogeniser at 9000 psi and inclusion bodies pelleted by centifugation at 10000 g for 20 min at 4° C. Inclusion bodies were washed twice by resuspension in 100 ml of 30 mM NaCl, 10 mM KH_2PO_4 pH 7.8 and centrifuged at 4300 g and 3000g for 20 min. Isolated inclusion bodies were stored at 4° C.

2.3.4 Standard C4 Analytical HPLC / Peptide Quantitation

All chromatography buffers were prepared in Milli-Q water and filtered through 0.22 μm filters prior to use. Recovery of peptide at each purification step was monitored using a Brownlee microbore C4 column (2.1 x 100 mm) using a linear gradient of 15 to 50 % (v/v) acetonitrile over 35 min in the presence of 0.1 % TFA at a flow rate of 0.5 ml/min. Peptide elution was monitored by absorbance at 215 nm. This analytical method was also used to quantitate peptide recovery by converting peak area to protein concentration using calculated extinction coefficients (Buck *et al.*, 1989). This conversion is based on the following relationship: **μg eluted protein$_y$ = AF$_{215}$ / IF x Q$_{rel.y}$,** (where **AF$_{215}$** is the sum of the integrated peak area and flow rate; **IF** is the instrument factor and was taken as 1 and **Q$_{rel.y}$** is a correction factor allowing for contributions of amino acid substitutions to **AF$_{215}$** for protein$_y$).

The calculated Q value for IGF-I, was determined by dividing the extinction coefficient for the peptide at 215 nm (251009 mol^{-1} cm^{-1}) by the molecular weight of the protein (Mr$_{IGF-I}$ = 7648.36) and thus gave a Q$_{IGF-I}$ value of 32.88 (Buck *et al,.* 1989). Quantified IGF-I standard provided by GroPep Pty. Ltd. was run using the standard C4 analytical HPLC method described above and gave a linear response to protein concentration. Three aliquots of each IGF-I analogue were analyzed using the standard HPLC analytical method and the peak integration areas were averaged. Analogues were quantified using Q values calculated from Q$_{IGF-I}$ and the peak integration areas. The molecular weights, extinction coefficients and Q values of IGF-I and the IGF-I analogues are given in Table 2.1.

2.3.5 Purification of IGF-I Analogues

The purification procedure used for generating IGF-I analogues was based on the methods described by King *et al.* (1992), Francis *et al.* (1992) and Upton *et al.* (1992).

2.3.5.1 Refolding

Inclusion bodies were solubilized in 8 M urea, 40 mM glycine, 100 mM Tris, 0.5 mM $ZnCl_2$, and 20 mM DTT, pH 9.1 at 10% (w/v). The supernatant was desalted on FPLC apparatus using a 50×200 mm column packed with Sephadex G-25 M (Pharmacia-LKB Pty Ltd, North Ryde, Australia) and equilibrated with 8 M urea, 40 mM glycine, 100 mM Tris, 0.5 mM $ZnCl_2$, and 1.6 mM DTT, pH 9.1 at a flow rate of 10 ml/min. Refolding was achieved by diluting the eluate to a final protein concentration of 0.125 mg/ml in 2 M urea, 40 mM glycine, 100 mM Tris, 10 mM EDTA, 0.4 mM DTT, pH 8.5 in the presence of 1 mM 2-hydroxyethyl disulphide. After stirring for 16 h at 4°C, the reaction was stopped by acidification to pH 2.5 with HCl. The folded IGF-I fusion peptide was concentrated and desalted on a 26×200 mm C18 Matrex silica LC (Amicon, Danvers, MA, U.S.A.) column equilibrated with a solution containing 12% (v/v) acetonitrile in 0.1% (v/v) TFA. Peptide was eluted by applying a linear gradient from 12% to 50% acetonitrile in 0.1% (v/v) TFA over 30 min at a flow rate of 5 ml/min. A single fraction was pooled and lyophilized.

2.3.5.2 Fusion Peptide Cleavage and Isolation of the Bioactive Fraction

The [Met^1-pGH(1-11)-Val-Asn-] fusion peptide was cleaved by solubilizing the lyophilized protein in a solution containing 2 M Urea, 2 M hydroxylamine-HCl, 100 mM Tris, pH 9.0 (adjusted with LiOH) at a final protein concentration of 0.5 mg/ml. The cleavage reaction was performed at 45°C for 14 h and terminated by acidification to pH 2.5 with HCl. The reaction solution containing cleaved peptides was concentrated and desalted on a 26×200 mm C18 Matrex silica LC column as described above. A single fraction containing the cleaved IGF was loaded onto a 26×200 mm Sepharose Fast Flow S (Pharmacia-LKB Pty Ltd) column equilibrated with 1 M acetic acid. The column was then washed with 50 mM ammonium acetate, pH 4.8 to elute endotoxins, while IGF was eluted with a 0 to 2 M NaCl gradient in 50 mM ammonium acetate, pH 4.8, over 30 min at a flow rate of 5 ml/min. A single fraction was

subsequently purified by reverse-phase HPLC on a 25 × 100 mm radial compression C4 Novapak column (Waters-Millipore) column equilibrated in 20% (v/v) acetonitrile, 0.1% (v/v) TFA. Peptide was eluted by applying a linear gradient from 20% to 50% (v/v) acetonitrile in 0.1% (v/v) TFA over 100 min at a flow rate of 5 ml/min. Fractions containing biologically active IGF, determined by protein synthesis stimulation in L6 rat myoblasts (Section 3.3.1) were pooled, lyophilized and quantified as described in section 2.3.4.

2.3.6 Analysis

2.3.6.1 N-terminal Protein Sequencing

N-terminal protein sequences were determined on a gas phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.) using the Edman degradation method (Hunkapiller & Hood, 1983). Peptide sequencing was performed by Miss D. Turner, Department of Biochemistry, University of Adelaide, Australia.

2.3.6.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The following protocol for SDS PAGE is an adaptation of the method of Laemmli (1970). A 15% polyacrylamide separating gel was prepared by combining 6 ml of 40% acrylamide (w/v, 40:1, acrylamide:bis-acrylamide), 4.5 ml of MQ water, 3 ml of resolving gel buffer (1.4 M Tris-HCl pH 8.8 containing 0.5 % (w/v) SDS), 70 μl of 10% Ammonium persulphate (APS) and 7 μl TEMED. The mixture was poured into 0.75 mm spaced gel plates to within 3 cm of the top of the plates. Water was layered over the separating gel solution and the gel solution was left to polymerize for 1 h at RT. A 4% stacking gel was prepared by combining 1.5 ml of stacking gel solution (0.8 M Tris-HCl pH 6.8 containing 0.5% (w/v) SDS), 0.75 ml of 40% acrylamide (w/v, 40:1, acrylamide:bis-acrylamide), 5.25 ml of MQ water, 100 μl of 10% APS and 10 μl of TEMED. Following removal of water from the separating gel interface, the stacking gel solution poured and the comb was inserted. The gel was left to polymerize for 1 h at RT.

Fermentation and inclusion body samples were run under reducing conditions while purified IGF-I analogues were analyzed under non-reducing conditions. Load solutions were prepared by adding 10 μl of sample to 10 μl of SDS sample buffer (0.125M Tris-HCl pH 6.8 containing 4% (w/v) SDS, 10% (v/v) glyerol and 0.1% (w/v) Bromophenol Blue). For reduced samples 5% (v/v) β-mercaptoethanol was added to the SDS sample buffer before use.

The gels were electrophoresed in a running buffer (0.025M Tris-HCl pH 8.8 containing 0.2M Glycine and 0.02% (w/v) SDS) at a constant current of 20 mA for 2h. The gels were fixed and stained in a solution containing 0.1 % (w/v) Coomassie Brilliant Blue R250, 50 % (v/v) methanol, 10 % (v/v) acetic acid and destained in a solution containing 5 % (v/v) methanol, 10 % (v/v) acetic acid.

2.3.6.3 Mass Spectroscopy

Electrospray mass spectral analysis was performed on a VG Biotech Quattro mass spectrometer (VG Biotech Ltd., Altrincham, Cheshire, UK). Analysis was performed by Dr. M. Sheil, Department of Chemistry, University of Wollongong, Wollongong, NSW, Australia.

2.3.6.4 Far-UV circular dichroism (CD) spectroscopy

CD measurements between 250 and 180 nm were made at room temperature using an AVIV 60DS spectropolarimeter calibrated with 0.6 mg/ml D(+)-10-camphorsulphonic acid. Protein samples were prepared in 20mM potassium phosphate buffer pH 7.2, to a concentration of 0.1 mg/ml. Using a cell of 1 mm pathlength, spectra were recorded at 0.4nm intervals and a scan rate of 2 nm/min.

Insert

Amp^S

Recombinant dsDNA

1. Denature dsDNA with NaOH / EDTA

Recombinant template DNA (Amp^S, Tet^R)

Insert

Amp^S

2. Anneal ampicillin repair oligo and mutagenic oligo

mutagenic oligo

Amp^R oligo

Amp^S

3. Synthesize mutant strand with T4 DNA polymerase and ligate

Amp^R

Amp^S

4. Transform BMH 71-18 mut S. Grow in media + ampicillin

5. Prepare mini-prep DNA

6. Transform JM109 and select mutants on ampicillin plates

7. Screen for mutants by direct sequencing

Site-directed mutation

Amp^R

Figure 2.3 Schematic representation of the Altered Sites in vitro mutagenesis procedure. The requirement for M13 helper phage generation of ssDNA was circumvented with a NaOH/EDTA denaturing step traditionally used in DNA sequencing (Section 2.3.1.8 & 2.3.1.10).
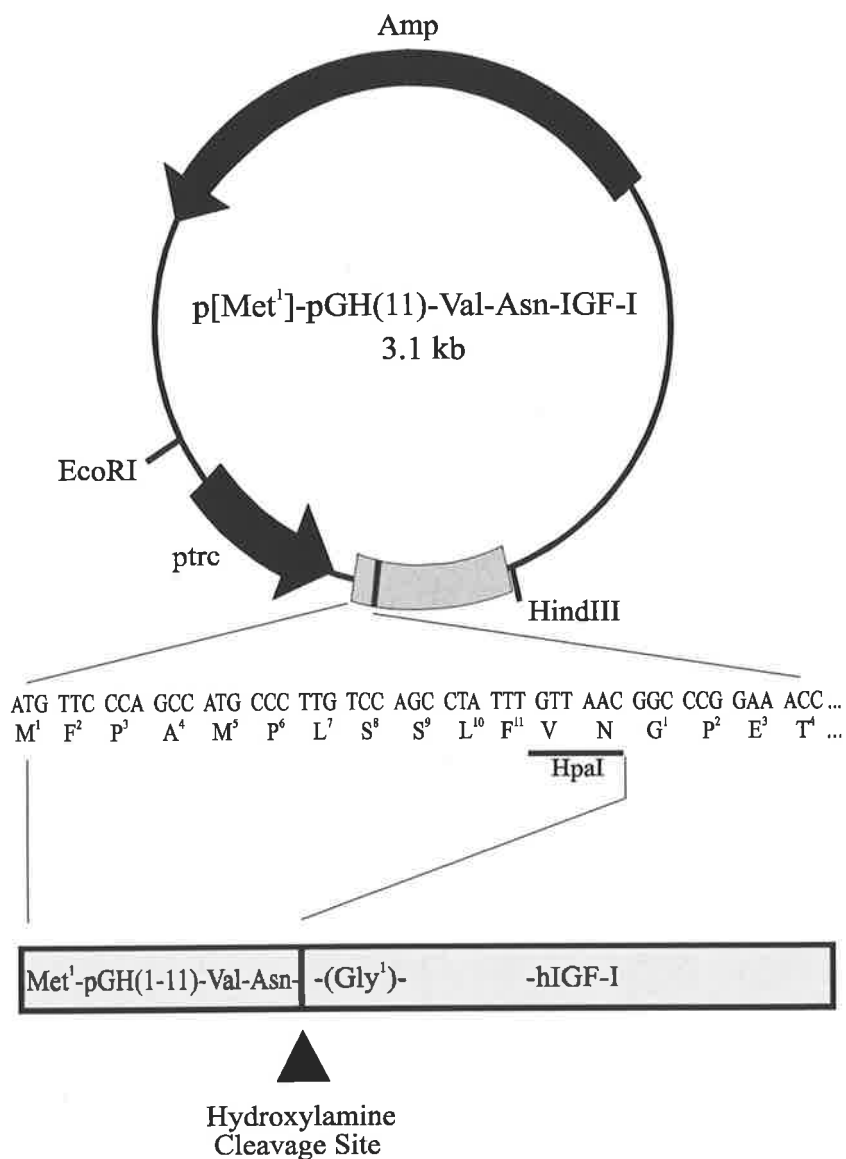
Figure 2.4 The p[Met¹]-pGH(1-11)-Val-Asn-IGF-I expression system and IGF-I fusion product. DNA and corresponding amino acid sequences in the N-terminal coding region are given in an exploded view of the vector with the hydroxylamine-labile Asn-Gly bond highlighted on the fusion peptide. The expression vector also contains a trc promoter (ptrc), an optimised ribosome binding site, a transcription termination sequence and an ampicillin resistance gene (Amp) (King *et al.,* 1992).

Table 2.1   Molecular weights, extinction coefficients, and Q values of IGF-I and the IGF-I analogues used in peptide quantitation. (Section 2.3.4) Method described by Buck *et al.* (1989). Q values are the ratio of the extinction coefficient to the molecular weight for the peptide.

Peptide	Molecular Weight (g mol^{-1})	Extinction Coefficient (mol^{-1} cm^{-1} , 215 nm)	Q Value (g^{-1} cm^{-1}, 215 nm)
IGF-I	7648.36	251009	32.88
[Leu^8] IGF-I	7692.5	251009	32.63
[Ser^{15}] IGF-I	7607.3	248163	32.62
[Phe^{59}] IGF-I	7664.4	258217	33.69
[Leu^{62}] IGF-I	7690.5	251009	32.64
[Ser^{15},Leu^{62}] IGF-I	7649.4	251009	32.81

2.4 Results

2.4.1 Expression and Purification of IGF-I Analogues

Mutant cDNA clones were generated using the Altered Sites *in vitro* mutagenesis system modified to circumvent the requirement for M13 helper phage generated ssDNA. This novel alteration to the mutagenesis procedure using dsDNA denatured in 0.2 M NaOH, 0.2 mM EDTA (Figure 2.3), enabled faster and simpler generation of template DNA. Sequencing showed successful mutation in 75 % of ampicillin resistant clones.

The IGF-I mutated coding regions were subcloned into the [Met^1]-pGH(1-11)-Val-Asn-IGF-I expression vector described by Francis *et al*. (1992) (Figure 2.4), transformed into *E. coli* JM101 cells and expression was induced with IPTG. SDS-polyacrylamide gel analysis of total cell protein in small scale fermentations revealed an IPTG inducible 9 kDa band for all transformed cultures (Figure 2.5). These predominant 9 kDa bands are consistent with the expected molecular masses for the mutant IGF fusion proteins. Fermentations yielded 1.2 g to 1.8g wet weight of inclusion bodies per litre of growth media.

Downstream processing procedures were based on the methods of King *et al*. (1992) and were consistent for all IGF-I analogues with the exception of the final large-scale reverse phase HPLC gradient. Standard C4 HPLC analysis showed similar folding and fusion peptide cleavage patterns and efficiencies for all IGF analogues. Thus, only representative results for the purification of [Leu^{62}] IGF-I are described below.

[Leu^{62}] IGF-I inclusion bodies (1.8 g wet weight), which had been solubilized and desalted under reducing conditions in 8 M urea, contained 105 mg total protein. Standard C4 HPLC analysis detected the presence of a major peak at a retention time of 28.7 min (90 % of total protein) and a minor peak at a retention time of 30.3 min (Figure 2.6A). Refolding, achieved by dilution into oxidizing conditions yielded 71mg total soluble protein and was observed by a shift in the elution time of the major peak to 20.6 min on the standard C4 HPLC analytical

gradient (Figure 2.6B). The refolded peptides were subsequently concentrated and desalted on a C18 silica matrix column and lyophilized. Cleavage of the fusion peptide was achieved using hydroxylamine and resulted in the formation of a number of protein species with the major peak eluting at 15.8 min on standard analytical HPLC (Figure 2.6C). Peptides generated from the hydroxylamine cleavage were concentrated and desalted on a C18 silica matrix column before processing on a Fast Flow S Sepharose ion exchange column. Standard C4 HPLC analysis of the Flow S Sepharose eluate indicated the removal of a peptide species eluting at a retention time of 18.0 min after hydroxylamine cleavage (Figure 2.6D). Cleavage products were resolved through two reverse phase HPLC steps with fractions from the first chromatographic step analyzed for bioactivity (as determined by protein synthesis stimulation in L6 rat myoblasts). The major peak eluting at a retention time of 15.8 min on standard HPLC analysis was identified as the primary biologically active cleavage product. The second reverse phase HPLC step yielded 6 mg of [Leu^{62}] IGF-I which eluted as a single peak on the standard HPLC analysis (Figure 2.6E).

2.4.2 Physicochemical Analysis

Final HPLC chromatography gradients were modified to allow for differences in retention times for each analogue as determined by standard HPLC analysis. Variation in the acetonitrile concentrations required for IGF-I analogue elution are given in Table 2.2. Slight increases in retention times were observed for all analogues relative to IGF-I with [Phe^{59}] IGF-I showing the largest deviation from IGF-I, equivalent to 0.7 % acetonitrile. The final analogue yields ranged from 500 μg for [Leu^{8}] IGF-I to 6 mg for [Leu^{62}] IGF-I.

Final purity of the IGF mutants was accessed by standard analytical C4 HPLC, reducing SDS-polyacylamide gel electrophoresis, N-terminal peptide sequencing and mass spectrometry. Each IGF mutant eluted as a single peak off a C4 HPLC column using a 1%/min acetonitrile gradient (Figures 2.8A to 2.12A) and migrated as a single band at the expected molecular weight of 8 kDa on reducing SDS-polyacrylamide gel electrophoresis (Figure 2.7). Peptide sequencing of all IGF-I mutants confirmed N-terminal identity with IGF-I and indicated that each preparation was greater than 98% pure. N-terminal sequencing also indicated that

correct substitutions had been introduced for the B-domain mutants [Leu^8] IGF-I and [Ser^{15}] IGF-I. Peptide identity was also confirmed by electrospray mass spectrometry (Figures 2.8B to 2.11B). This analysis indicated varying degrees of microheterogeneity in each of the analogue preparations with additional peaks detected at molecular weights of +16, +32 and +53 (Table 2.3). Far-UV CD spectra of native IGF-I and the IGF analogues are presented in Figure 2.13. Only minimal deviations in wavelength intensities and profiles were observed between the analogues and native protein.
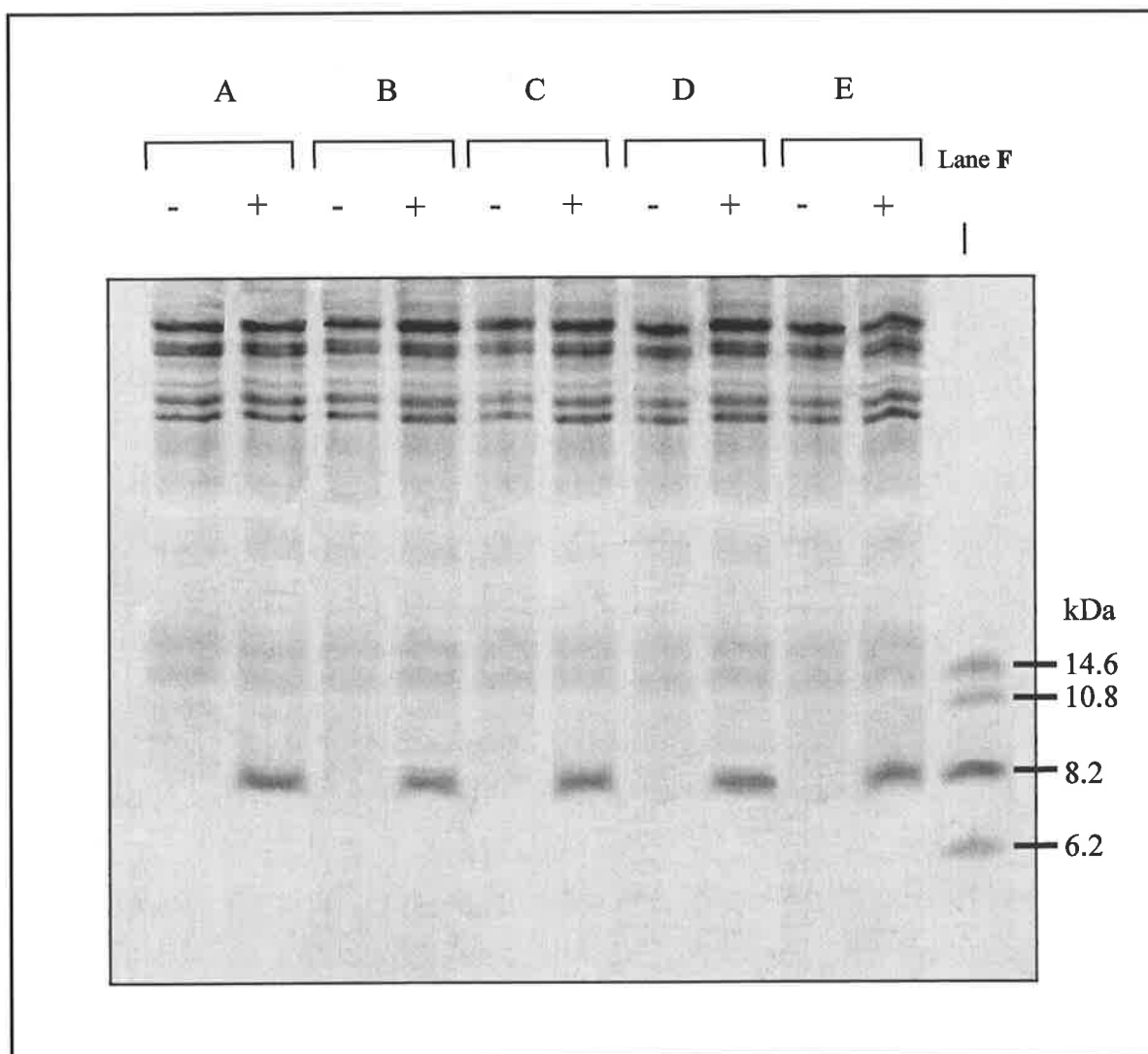
Figure 2.5 SDS-polyacrylamide gel analysis of transformed JM101 induced with IPTG (+) alongside non-induced controls (-). Lanes labelled A through to E represent total cellular protein of *E.coli* cells transformed with mutated expression vectors encoding for the five IGF-I analogues: [Leu^8] IGF-I, **A**; [Ser^{15}] IGF-I, **B**; [Phe^{59}] IGF-I, **C**; [Leu^{62}] IGF-I, **D**; and [Ser^{15}, Leu^{62}] IGF-I, **E**. Low molecular weight markers are labelled with their respective molecular masses in lane **F**.
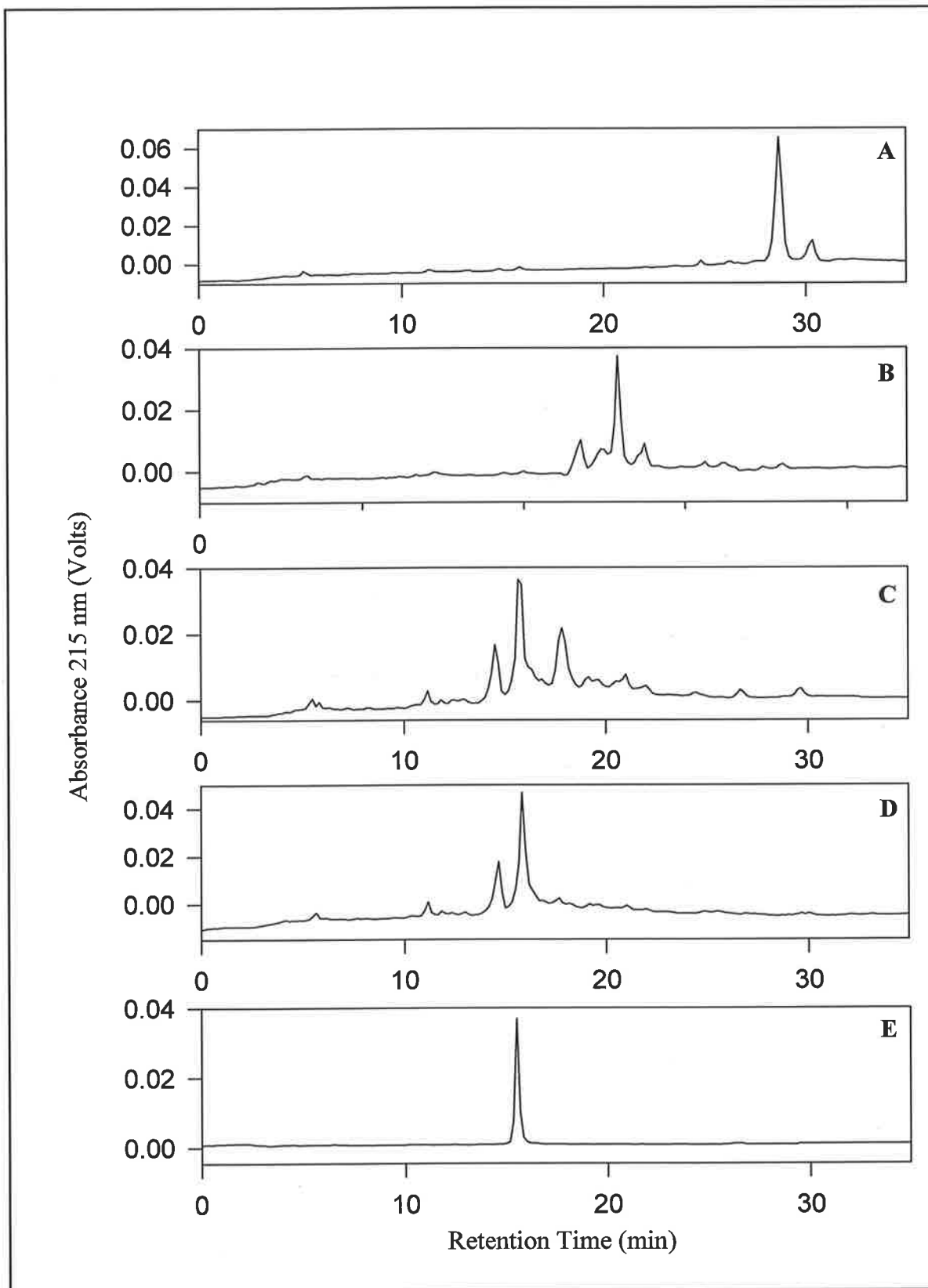
Figure 2.6   C4 HPLC chromatograms representing various stages of [Leu^{62}] IGF-I production.   (A), solubilized inclusion bodies; (B), refold material; (C), hydroxylamine cleavage products; (D), Fast Flow S-Sepharose fraction; and (E), reverse phase HPLC purified final product.

Table 2.2. Comparison of C4 reverse-phase HPLC retention times for IGF-I analogues.

Peptide	Retention Time (min)	Acetonitrile Concentration (% v/v)
IGF-I	16.6	31.6
[Leu^8] IGF-I	17.0	32.0
[Ser^{15}] IGF-I	16.8	31.8
[Phe^{59}] IGF-I	17.2	32.3
[Leu^{62}] IGF-I	17.0	32.0
[Ser^{15}, Leu^{62}] IGF-I	17.1	32.1
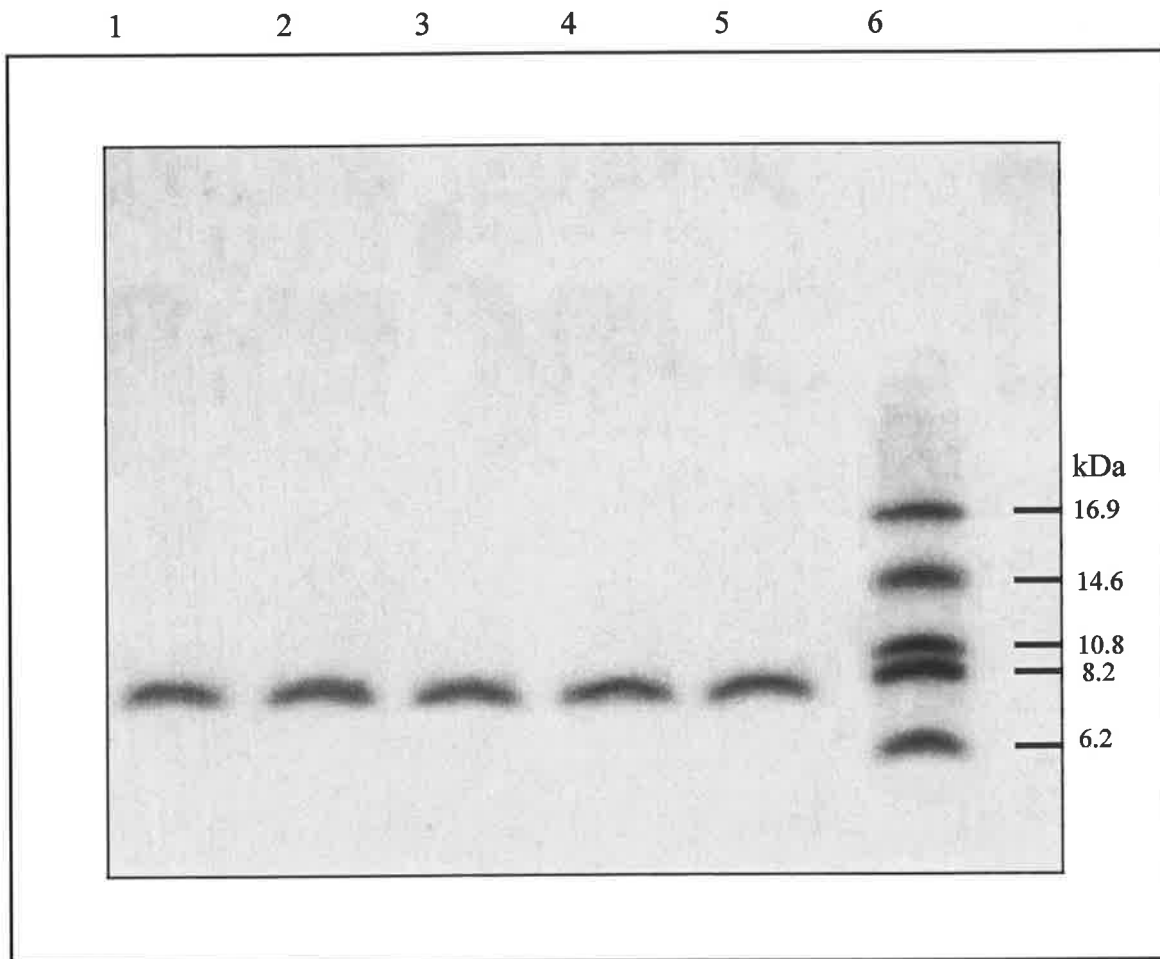
Figure 2.7 **Reducing SDS Polyacrylamide Gel Electrophoresis of purified IGF analogues**: [Leu^8] IGF-I, lane 1; [Ser^{15}] IGF-I, lane 2; [Phe^{59}] IGF-I, lane 3; [Leu^{62}] IGF-I, lane 4; and [Ser^{15}, Leu^{62}] IGF-I, lane 5. Low molecular weight markers are labeled with their respective molecular weights in lane 6.
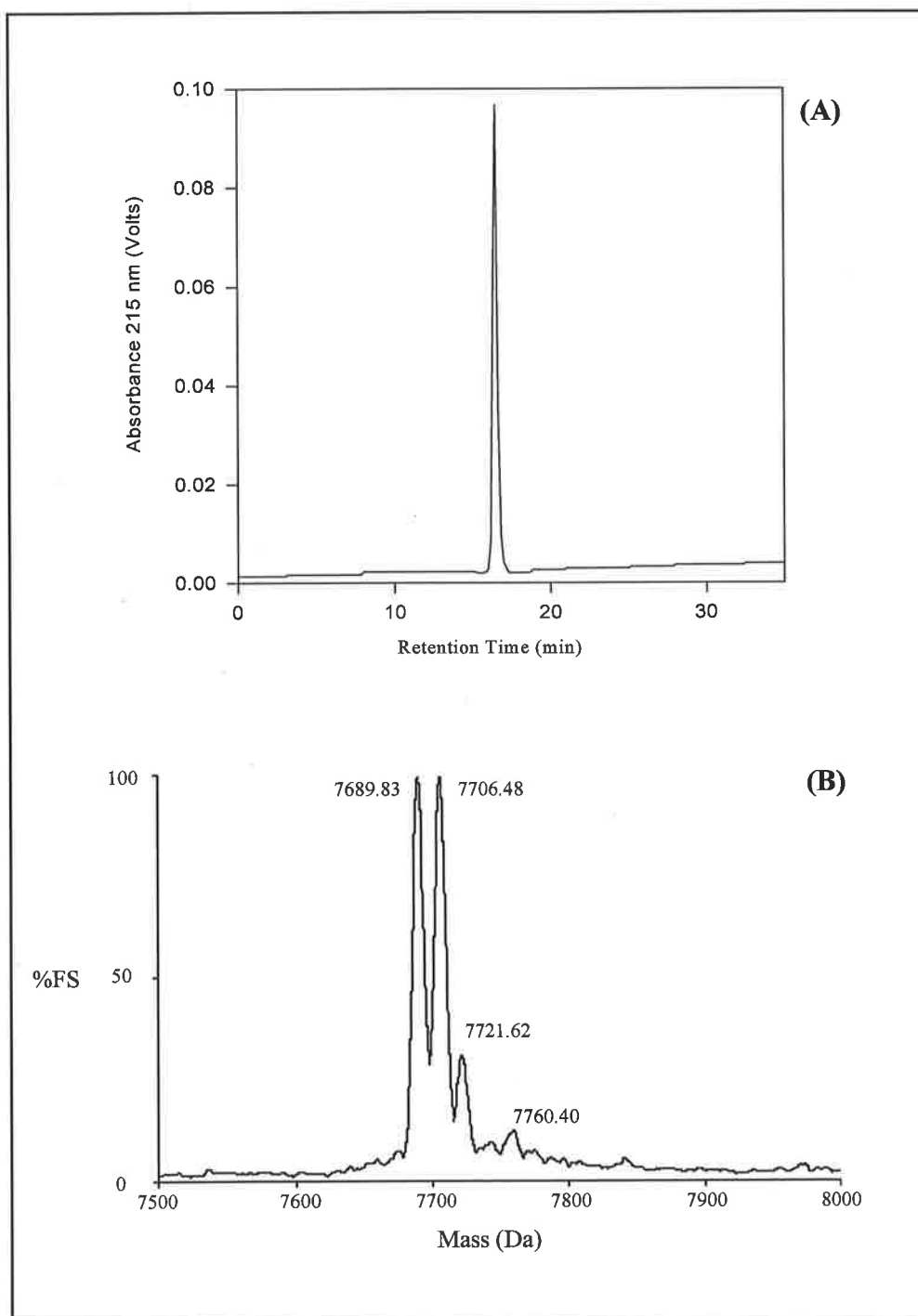
Figure 2.8 Analysis of purified [Leu^8] IGF-I by C4 HPLC and Mass Spectroscopy. (A) C4 reverse phase HPLC chromatography as described in section 2.3.4 and **(B)** electrospray mass spectroscopy. Peak molecular weights on the electrospray mass analysis are given in Daltons.
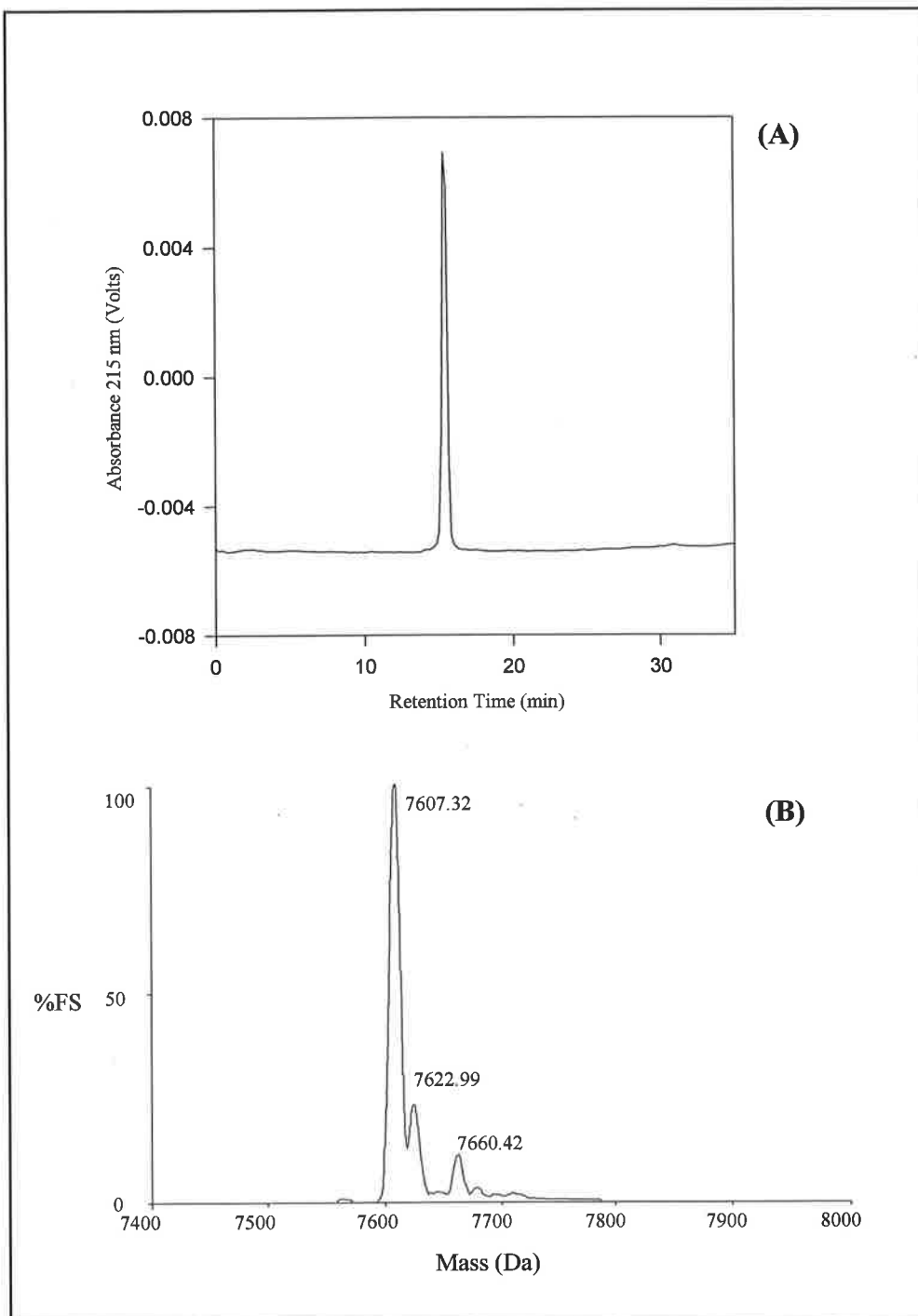
Figure 2.9   Analysis of purified [Ser^{15}] IGF-I by C4 HPLC and Mass Spectroscopy. (A)
C4 reverse phase HPLC chromatography as described in section 2.3.4 and **(B)** electrospray
mass spectroscopy. Peak molecular weights on the electrospray mass analysis are given in
Daltons.
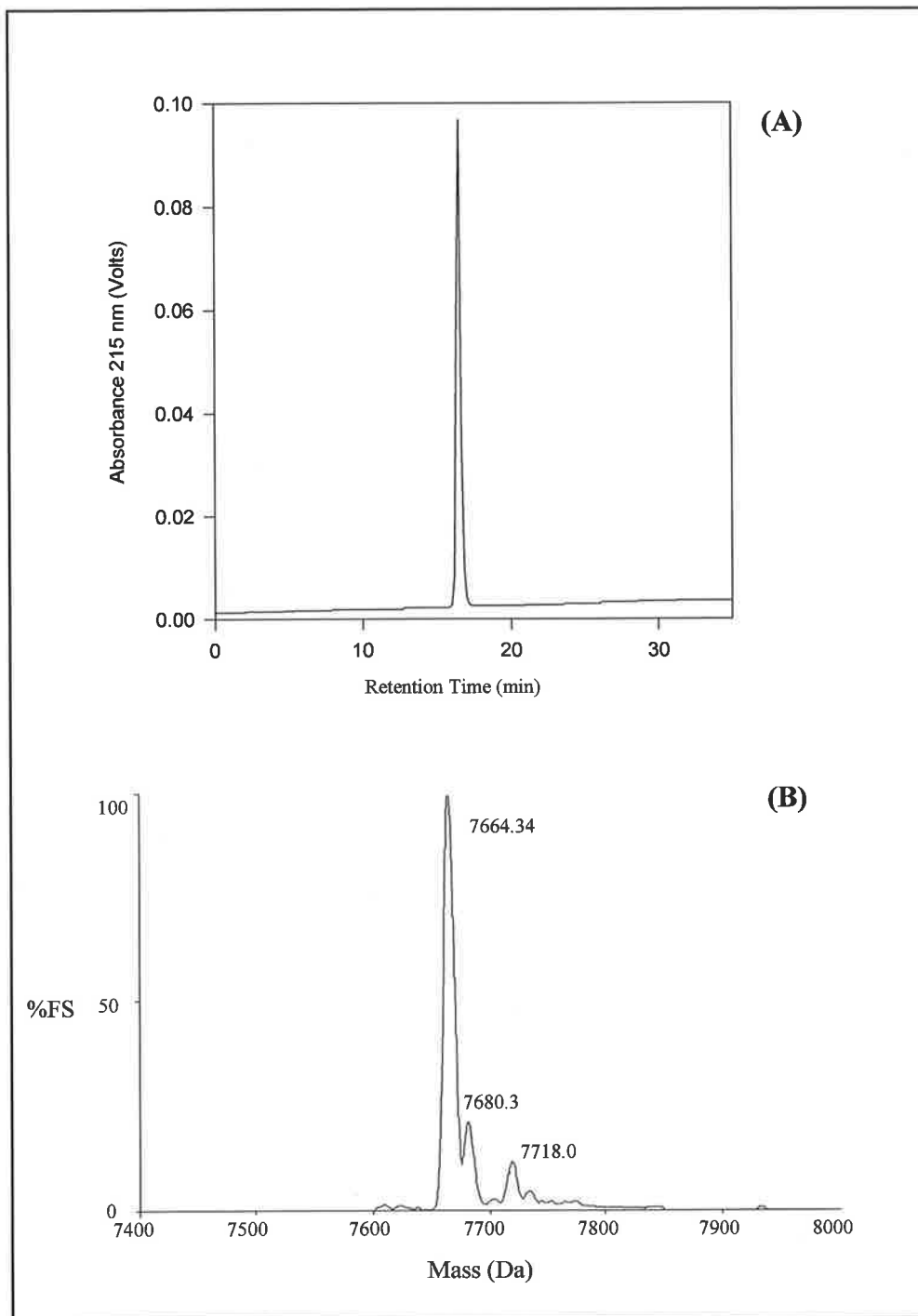
Figure 2.10 Analysis of purified [Phe[59]] IGF-I by C4 HPLC and Mass Spectroscopy. (A)
C4 reverse phase HPLC chromatography as described in section 2.3.4 and **(B)** electrospray
mass spectroscopy. Peak molecular weights on the electrospray mass analysis are given in
Daltons.
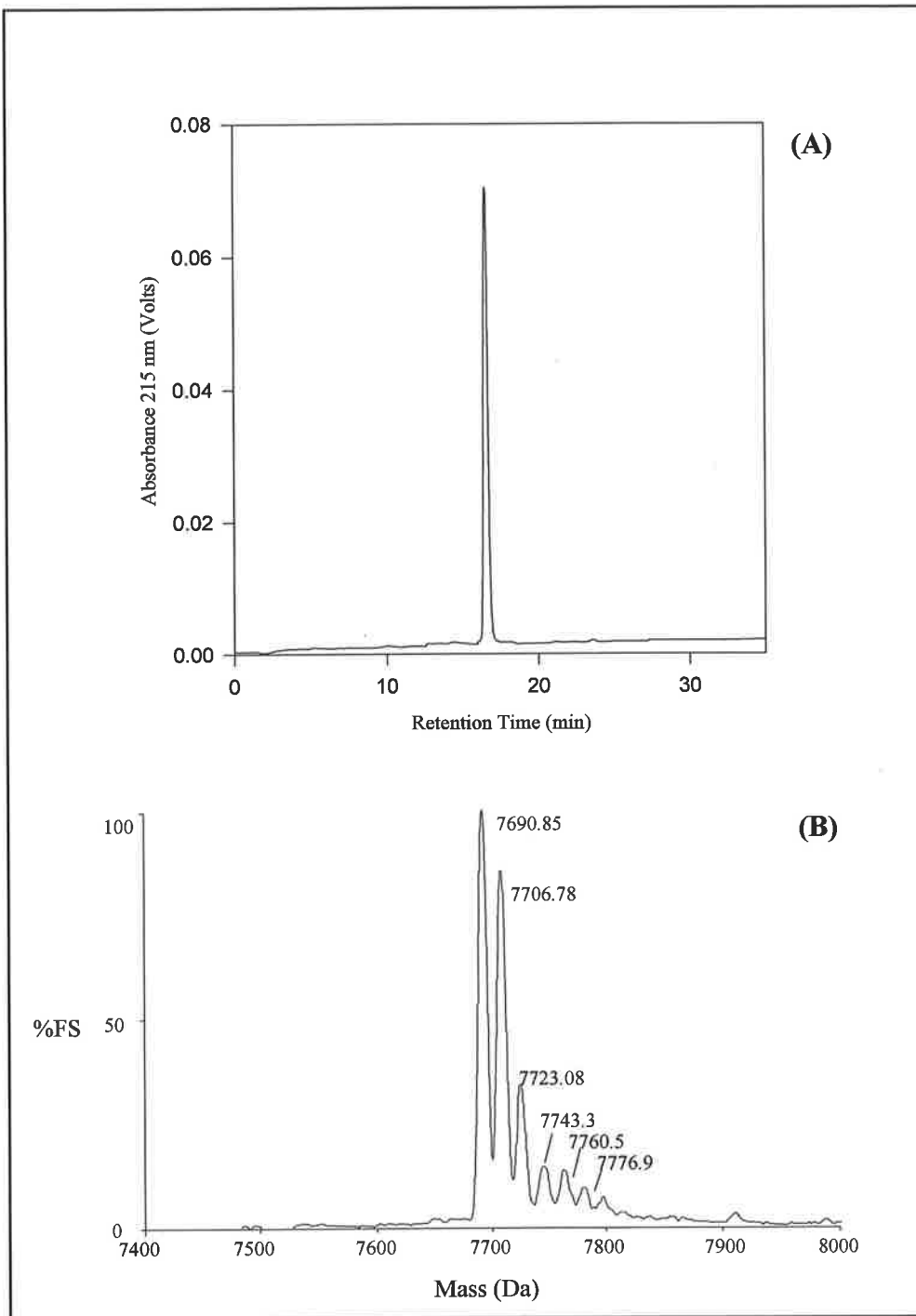
Figure 2.11 Analysis of purified [Leu[62]] IGF-I by C4 HPLC and Mass Spectroscopy. (A) C4 reverse phase HPLC chromatography as described in section 2.3.4 and **(B)** electrospray mass spectroscopy. Peak molecular weights on the electrospray mass analysis are given in Daltons.
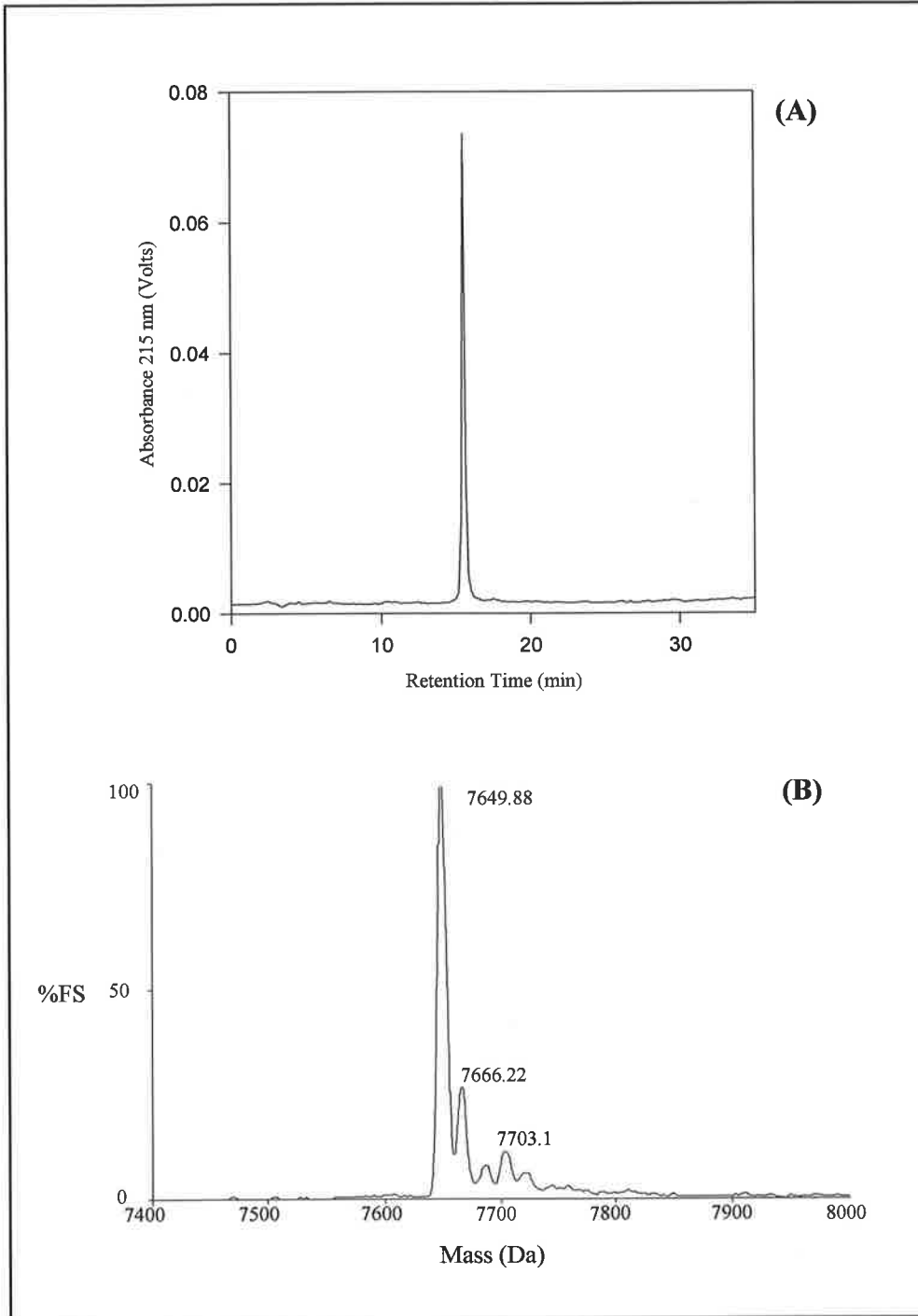
Figure 2.12 Analysis of purified [Ser^{15}, Leu^{62}] IGF-I by C4 HPLC and Mass Spectroscopy. (A) C4 reverse phase HPLC chromatography as described in section 2.3.4 and **(B)** electrospray mass spectroscopy. Peak molecular weights on the electrospray mass analysis are given in Daltons.

Table 2.3. Calculated molecular weights, experimental molecular weights, and microheterogeneity for the IGF-I analogues.

Peptide	Calculated Molecular Weight (Da)	Experimental Molecular Weight (Da)	Additional Peaks (Da)
IGF-I	7648.36	-	-
[Leu^8] IGF-I	7692.5	7691.83	+16.6 , +32.8
[Ser^{15}] IGF-I	7607.3	7607.32	+15.7 , +53.1
[Phe^{59}] IGF-I	7664.4	7664.34	+16.1 , +53.7
[Leu^{62}] IGF-I	7690.5	7690.9	+15.9 , +32.2 +52.4 , +69.6
[S^{15}, L^{62}] IGF-I	7649.4	7649.6	+16.3 , +53.8
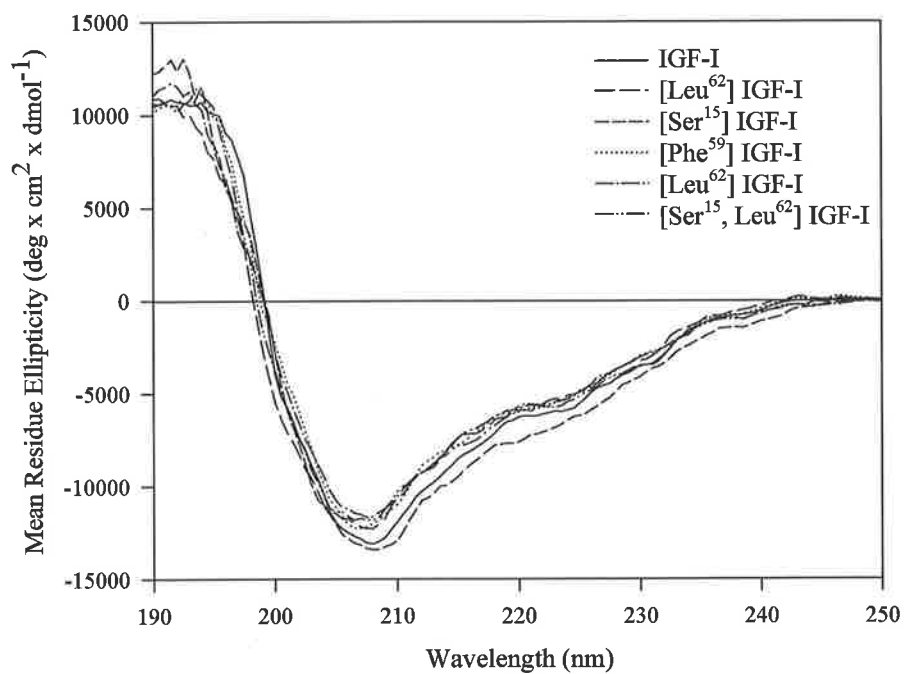
Figure 2.13 Circular Dichroism spectra of IGF-I and the IGF-I analogues. Spectra were recorded using the method described in Section 2.3.6.4.

2.5 Discussion

2.5.1 Analogue Production

This Chapter describes the production of IGF-I analogues from *E. coli* inclusion bodies based on the methods of King *et al.*, (1992) and Francis *et al.*, (1992). Recombinant production of IGFs in *E. coli* has been reported by several groups but generally high level expression requires the use of a N-terminal fusion peptide (Peters *et al.*, 1985; Forsberg *et al.*, 1989). The fusion protein analogues of IGF-I contained a 13 amino acid N-terminal fusion partner comprising the first 11 amino acids of pig growth hormone linked via a Val-Asn dipeptide. This dipeptide provided a Hpa I restriction site for the insertion of mutated coding sequences and a hydroxylamine cleavage site between the Asn and Gly[1] of the IGF-I analogues (Figure 2.4). In addition to enabling high level expression *in E. coli*, this N-terminal fusion peptide has been shown to increase the yield of the correctly folded IGF-I isomer (Milner *et al.*, 1995). Fermentation expression levels, refolding yields and recoveries were roughly consistent across all IGF-I analogues and were comparable to those observed for native IGF-I.

2.5.2 Physicochemical Characterization

Mutational analysis is a powerful approach to identifying the involvement of specific amino acid residues in protein-protein interactions. Meaningful interpretation of the biological effects of amino acid substitutions requires some assessment of structural integrity. To identify a direct functional role for a given amino acid position, the possibility of indirect structural changes must be excluded. Furthermore, the presence of different types of impurities such as host cell proteins or modified forms of the protein can affect the properties relative to the native protein. A series of physicochemical characterizations were performed on the IGF-I analogues to assess the quality, identity and integrity of the preparations, and are discussed below.

Refolding IGF-I generates 'mismatched' IGF isoforms, containing non-native disulfide bond arrangements. These isoforms have significant reductions in type 1 IGF receptor binding

affinity and biopotency relative to native folded IGF-I (Hober *et al.*, 1992; Milner *et al.*, 1995). Although peptide mapping for confirming native disulfide formation was not included in this study, C4 HPLC analysis showed identical refold profiles for all analogues and native IGF-I, with the main peak representing the native isoform. Additional evidence for correct disulfide bond formation was provided by Far-UV spectral analysis. Only minimal deviations in the wavelength intensities and profiles were observed between the analogues and native IGF-I. In contrast, significant variations in wavelength intensities occur between the spectra of mis-matched IGF-I and that of native IGF-I (Hober *et al.*, 1992). CD spectral analysis of the analogues therefore suggests that the selective substitutions had no significant effect on the secondary structure content. Small increases in the C4 HPLC retention times of the analogues relative to native IGF-I can be attributed to increased surface hydrophobicity caused by the introduction of more hydrophobic side-chains.

In addition to confirming the identity of the IGF analogues, mass spectrometry identified varying degrees of microheterogeneity. The presence of modified forms with +16 and +32 Da molecular masses for all IGF analogues was indicative of hydroxamation of Asn and Gln residues and/or methionine sulphoxide formation. Several IGF-I variants have been identified and characterized including the oxidation of Met[59], mis-incorporation of norleucine for Met[59] during biosynthesis and the proteolysis of the peptide bond between Arg[36] and Arg[37] (Forsberg *et al.*, 1990). Hydroxamates of Gln[15], Asn[26] and Gln[40] of IGF-I, formed during hydroxylamine cleavage of the fusion-peptide, have also been reported (Canova-Davis *et al.*, 1992). As might be expected, IGF-I analogues with substitutions at positions Met[59] and Gln[15] showed the smallest levels of microheterogeneity. Variation in microheterogeneity between [Leu[8]] IGF-I and [Leu[62]] IGF-I may be attributed to slight variations in incubation times and temperatures used during the hydroxylamine cleavage step. The presence of microheterogeneity in the analogue preparations is unlikely to influence their biological actions since both methoxide and hydroxamate forms have the same biological activities as unmodified IGF-I (Canova-Davis *et al.*, 1992; Forsberg *et al.*, 1990).

Chapter 3

Biological Characterisation of IGF-I Analogues.

3.1 Introduction

Insulin-like growth factor-I (IGF-I) is a 70 amino acid polypeptide that shares structural and functional similarities with insulin. The B-domain residues 1-29 and A-domain residues 42-62 of IGF-I share a high degree of sequence and structural similarity to the B- and A-chains of human insulin (Blundell *et al.* 1978; Cooke *et al.* 1991). The 12 amino acid C-domain of IGF-I, linking the B- and A-domains, and an 8 amino acid C-terminal extension termed the D-domain have no counterpart in insulin. Despite the high degree of structural and sequence similarity, IGF-I and insulin have distinct physiological roles. IGF-I is primarily regarded as a mitogenic agent involved in the regulation of cell proliferation while insulin is involved in the regulation of metabolic processes such as glucose uptake, glycogen biosynthesis and lipogenesis (Froesch *et al.* 1985).

Since commencing this study De Meyts (1994) has proposed two a site binding model for insulin and IGF-I where the ligands cross-link the two receptor α-subunits. These models account for kinetic experiments that demonstrate both the insulin and the type 1 IGF receptors bind one ligand with high affinity and a second with lower affinity. Cross-linking of the receptor α-subunits may be required for signal activation as is the case for other tyrosine kinase and cytokine receptors, however, the molecular basis of ligand association and receptor activation are still unclear.

Both IGF-I and insulin induce a biological response by first binding to cell surface receptors which activate divergent signalling pathways. Like the two ligands, the insulin and type 1 IGF receptors share a high degree of similarity. The IGF type 1 receptor binds IGF-I with high affinity and insulin with low affinity. IGF-I also cross-reacts with low affinity for the insulin receptor. These observations suggest an analogous mechanism of ligand-receptor

association for IGF-I and insulin with structural differences between the ligands and between the receptors determining binding specificity.

A number of invariant residues shared between IGF-I and insulin have been proposed as being involved with IGF-I binding to the IGF type 1 receptor and its cross-reactivity with the insulin receptor. These include Val^{11}, Arg^{21}, Gly^{22}, Phe^{23}, Tyr^{24} and Val^{44} (Blundell *et al.*, 1978: Cooke *et al.*, 1991). Before commencing this study, mutational work confirmed Tyr^{24} as a critical type 1 IGF receptor binding residue (Cascieri *et al.*, 1988b; Bayne *et al.*, 1990). Substitutional analysis also demonstrated potential involvement of Tyr^{60} of IGF-I in both insulin and type 1 IGF receptor association (Bayne *et al.*, 1990). However the buried side-chain of the Tyr at this position led the authors to suggest that substitutional effects may be the result of conformational changes. The Ala substitution of the C-domain Tyr^{31} also suggested the involvement of this residue in high affinity binding of IGF-I with the type 1 IGF receptor (Bayne *et al.*, 1990). The NMR derived solution structure for IGF-I highlighting these residues is shown in Figure 3.1.

Protein-protein binding specificity is commonly attributed to amino acid residues that lie at the periphery of the binding interface. Such residues may effect specificity through unfavourable electrostatic or steric interactions (Clackson & Wells, 1995). Previous studies using various assay systems demonstrate insulin has a 100- to 200-fold higher affinity for the insulin receptor than IGF-I. Deletion of the D-domain of IGF-I suggests that this region is not critical for type 1 IGF receptor binding but it may sterically hinder IGF-I binding to the insulin receptor (Bayne *et al.*, 1988). Similarly, replacement of the C-domain of IGF-I with a 4 residue Gly bridge resulted in a subtle increase in insulin receptor binding affinity (Bayne *et al.*, 1988). An IGF-I mutant containing both of these C- and D-domain alterations increases insulin receptor binding by 7-fold (Bayne *et al.*, 1988). These studies suggest that additional determinants of IGF-I specificity must lie within the A- and B-domains.

The divergence in sequence between hystricomorph insulins and those of other mammals has enabled prediction of insulin residues that may govern receptor binding specificity (See

Section 2.1.1). Of the potential sites identified, IGF-I residues Ala^8, Gln^{15}, and Ala^{62}, corresponding to the highly conserved insulin residues Ser^{B9}, Tyr^{B16} and Asn^{A21}, were chosen for substitution due to their positioning around the proposed receptor binding region. The Met at position 59 was also targeted for amino acid substitution because of its location between the C- and D-domains and its proximity to the critical receptor binding residue Tyr^{24}. These positions were substituted with the intention of selectively reducing insulin receptor binding affinity (See Section 2.1.1).

Both Ala^8 and Ala^{62} represent hydrophobic residues that are located at opposite ends of the proposed hydrophobic receptor binding region. Equidistant from these residues, Gln^{15} is located on the C-terminal region of the B-domain α-helix and is within 4
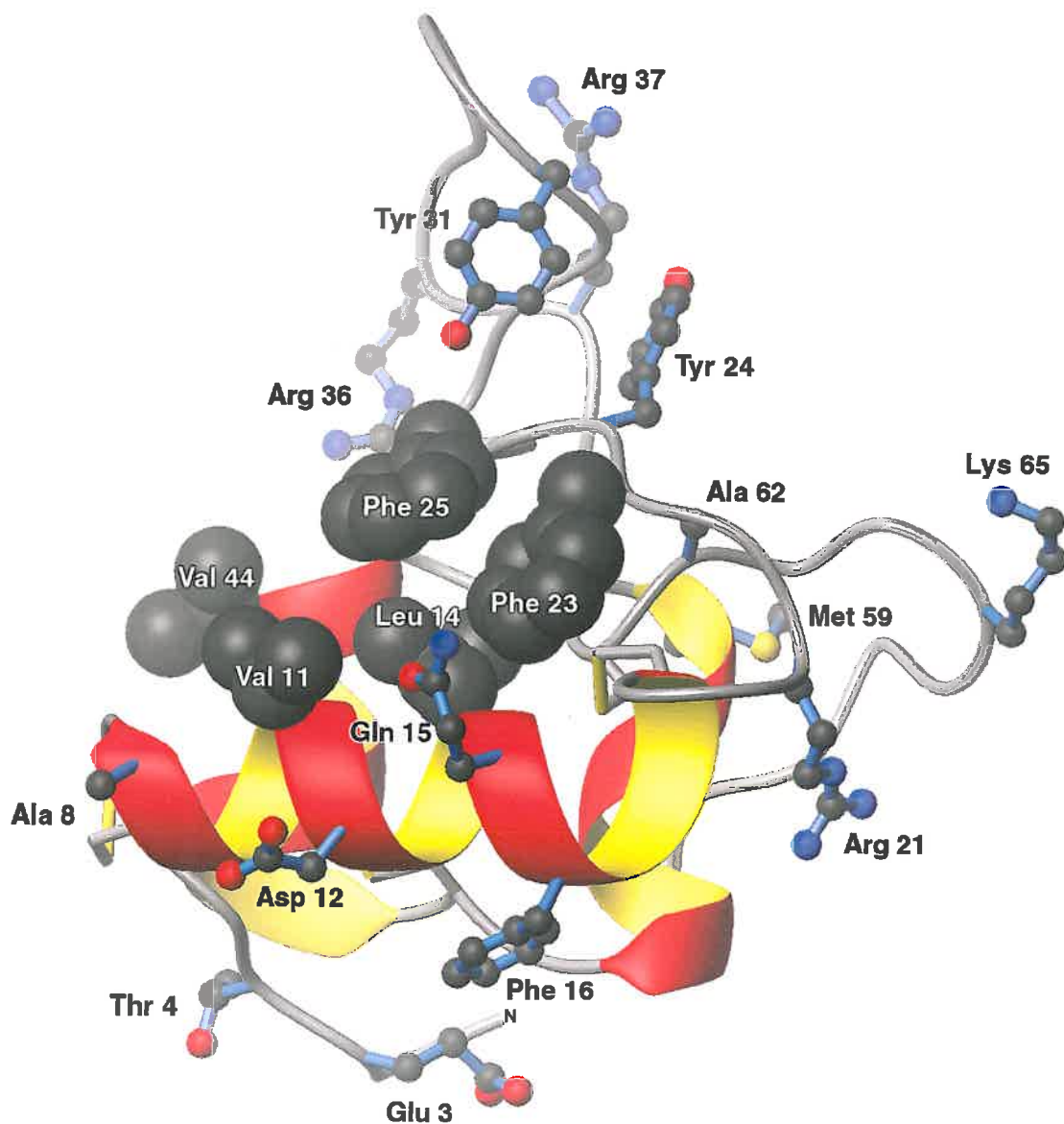
Figure 3.1 The solution structure of IGF-I displaying the proposed IGF type 1 receptor binding region. Heavy atoms of hydrophobic residues in the putative binding core are space-filled. Mutational studies have identified Tyr^{24} and Tyr^{31} are important for high affinity binding with the type 1 IGF receptor (Cascieri *et al*. 1988b; Bayne *et al*. 1990). Residues targeted for mutation are also shown along with Glu^3 of the IGFBP binding region. (Structure determined by Cooke et al., 1991. Image generated using MOLMOL).

3.2 Materials

IGF-1R/3T3 mouse fibroblasts transfected with human type 1 IGF receptor cDNA (Lammers *et al.* 1989) were a gift from Dr. A. Ullrich (Max-Planck Institut für Biochemie, Martinsried, Germany). NIH 3T3 HIR3.5 fibroblasts (Whittaker *et al.* 1987) transfected with human insulin receptor isoform-B (HIR-B) cDNA were a gift from Dr. J. Whittaker (Department of Medicine, SUNY, Stony Brook, NY, U.S.A.). HIRc-B rat 1 fibroblasts (McClain *et al.* 1987) transfected with human insulin receptor isoform-A (HIR-A) cDNA were a gift from Dr. J. Olefsky (University of California, San Diego).

Iodinated IGF-I and insulin (used in soluble receptor binding assays) were produced by Dr. Maria Soos, Department of Clinical Biochemistry, University of Cambridge, UK. $[^{125}I]$-IGF-I with specific activities of 100-150 Ci/g was prepared by S. Knowles, Cooperative Research Centre for Tissue Growth and Repair, Adelaide, SA, Australia. The IGF-I analogue $[His^4]$ IGF-I was produced by Barbara Magee, Biochemistry Department, University of Adelaide. Recombinant hIGF-I was supplied by GroPep Pty Ltd, Adelaide, Australia. Bovine serum albumin (Radioimmunoassay grade BSA) and 4-(2-hydroxylethyl)-1-piperazine-ethanesulphonic acid (HEPES) were purchased from Sigma, St Louis, MO, U.S.A. $[4,5-^3H]$ leucine was obtained from Amersham Australia Pty. Ltd., Sydney NSW, Australia. All other reagents were analytical grade.

Plasticware for cell culture and assays were from Nunc, Roskilde, Denmark. L6 rat myoblasts (ATTC CRL 1458) were purchased from American Type Tissue Culture Rockville, MD, U.S.A. Hanks' balanced salts and fetal calf serum were purchased from Flow Laboratories, North Ryde, NSW, Australia. Dulbecco's Modified Eagle's Medium (DMEM) was obtained from Gibco, Glen Waverly, NSW, Australia. Streptomycin and penicillin were obtained from Glaxo, Boronia, VIC, Australia.

3.3 Methods

3.3.1 Stimulation of protein synthesis in L6 Rat Myoblasts

Stimulation of protein synthesis in L6 rat myoblasts was measured over an 18 h period using the method described by Francis *et al.* (1986). Confluent monolayers were incubated in 24 place multiwells at 37°C for 2 h in 1 ml of DMEM containint 1μCi/ml [^{3}H] leucine together with 0.1 ml of sample. Lyophilized samples of IGF-I and IGF-I analogues were 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 18h, the monolayers were washed twice with Hanks' salts 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 above that which occurred in control buffer.

3.3.2 Cell-Based Type 1 IGF receptor binding

Peptide binding to the IGF type 1 receptor of rat L6 myoblasts was measured as described previously (Ross *et al.* 1989). ^{125}I-IGF-I was added in the presence of increasing concentrations of unlabelled peptides in a total volume of 0.5 ml HEPES buffer (0.01 mol Hepes/l, 0.12 mol NaCl/l, 5 mmol KCl/l, 1.2 mmol MgSO$_4$.7H$_2$O/l and 8 mmol glucose/l at pH 7.6) containing 0.5% (w/v) BSA to confluent cell monolayers in 24-place multiwells. Following an 18 h incubation at 4°C monolayers were washed with Hanks' salts to remove unbound ^{125}I-IGF-I. 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 that occurring in the absence of unlabelled IGF peptide.

3.3.3 L6 rat myoblast secreted IGFBPs binding

Analogue affinities for the IGFBPs secreted by L6 myoblasts were measured as described by Szabo *et al.*, (1988). Briefly, serum-free conditioned medium was collected after 24 h incubation, centrifuged at 10,000 g for 5 min and filtered through a 0.2 μm filter. A range of unlabelled IGF and IGF analogue concentrations were incubated with 50μl of conditioned

media in a total volume of 0.25ml phosphate buffer (50mM sodium phosphate, 2.5 g/l BSA, pH 6.5). After incubation at 4°C for 18 h, free ^{125}I-IGF-I was separated by the addition of 1.0 ml activated charcoal suspension (phosphate buffer containing 5 g/l activated charcoal and 0.2 g/l protamine sulphate) followed by centrifugation. The supernatant was removed for gamma counting to determine the percentage of bound ^{125}I-IGF-I. Binding was expressed as a percentage of the bound ^{125}I-IGF-I the absence of competing peptide.

3.3.4 Receptor preparations and soluble receptor binding

Materials and methods were as described by Soos & Siddle (1989) and Soos *et al.* (1990). Briefly, IGF-1R/3T3 mouse fibroblasts, NIH 3T3 HIR3.5 fibroblasts and HIRc-B fibroblasts were partially purified from 3×10^8 cells. Cells were washed with phosphate buffered saline containing protease inhibitors, centrifuged and solubilized for 1 h at 4°C in 20 ml of 50mM Hepes, pH 7.4, containing protease inhibitors and 1% (v/v) Triton X-100. Clarified supernatant was mixed for 18 h at 4°C with 1 ml of WGA-Sepharose and the resin washed with 20 ml of 50 mM Hepes, pH 7.4 (containing 0.15 M NaCl and 0.1% Triton X-100). Binding to the soluble IGF type 1 receptor and insulin receptor was determined by using ^{125}I-IGF-I and ^{125}I-insulin, respectively. Increasing concentrations of IGF-I analogues were incubated with solubilized receptors and labelled peptide (approx. 30 000 d.p.m. in a total volume of 0.25 ml) for 18 h at 4°C. Receptor bound radioligand was determined by precipitation of soluble receptors with PEG 6000. Binding was expressed as the percentage of that occurring in the absence of unlabelled IGF peptide.

3.4 Results

3.4.1 Type 1 IGF receptor binding properties

IGF type 1 receptor binding was examined using soluble IGF type 1 receptors and rat L6 myoblasts. IGF binding to the type 1 receptors of L6 myoblasts is shown in Figure 3.2. The effective dose for half maximal competition to tracer binding (ED_{50}) was used to compare relative binding affinity for the type 1 IGF receptor. In order of decreasing affinity, the derived ED_{50} values were: [His^4] IGF-I (0.37 nM); IGF-I (0.90 nM); [Ser^{15}] IGF-I (1.4 nM); [Leu^{62}] IGF-I (7.8 nM); [Phe^{59}] IGF-I (11 nM); [Ser^{15}, Leu^{62}] IGF-I (15 nM); and [Leu^8] IGF-I (21 nM). Type 1 IGF receptor binding affinity was also measured using soluble IGF type 1 receptors. Competitive binding dose-response curves are presented in Figure 3.3. In order of decreasing affinity for the soluble IGF type 1 receptor the ED_{50} values were: [His^4] IGF-I (0.025 nM), IGF-I (0.030 nM), [Ser^{15}] IGF-I (0.038 nM), [Leu^{62}] IGF-I (0.23 nM), [Phe^{59}] IGF-I (0.16 nM), [Ser^{15}, Leu^{62}] IGF-I (0.18 nM), and [Leu^8] IGF-I (0.18 nM).

Binding affinities for both assays, expressed relative to IGF-I, are presented in Table 3.1. The analogues [Ser^{15}] IGF-I and [His^4] IGF-I show subtle changes in type 1 IGF receptor affinity relative to IGF-I. Based on the soluble IGF type 1 receptor binding, [Leu^8] IGF-I, [Phe^{59}] IGF-I and [Leu^{62}] IGF-I show 6-, 5- and 8-fold respective losses in affinity. In contrast, more significant changes in type 1 IGF receptor binding affinity was observed for a number of analogues using the L6 myoblast assay. The most striking differences were observed with [Leu^8] IGF-I and [Phe^{59}] IGF-I where relative affinity for the Type 1 IGF cell surface receptors showed 24- and 12-fold losses respectively.

3.4.2 Insulin receptor binding properties

Soluble forms of the two insulin receptor isoforms (HIR-A and HIR-B) were used to determine affinity of the IGF-I analogues for the cognate receptor. Competitive binding dose response curves for HIR-A and HIR-B are given in Figures 3.4 and 3.5 respectively. In decrease order of affinity, concentrations required to give half-maximal competition with ^{125}I-insulin for HIR-A binding are: insulin (0.50 nM); [His^4] IGF-I (6.0 nM); IGF-I (40 nM);

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[Ser^{15}] IGF-I (56 nM); [Leu^{62}] IGF-I (80 nM); [Ser^{15}, Leu^{62}] IGF-I (330 nM); [Phe^{59}] IGF-I (670 nM); and [Leu^8] IGF-I (1100 nM). The distinct insulin isoforms differ due to alternate splicing of exon 11. The HIR-B isoform contains an 12 amino acids located close to the C-terminus of the α-subunit (Seino & Bell, 1989; Moller *et al.*, 1989). The ED$_{50}$ values for competitive binding to HIR-B, in order of decreasing affinity were: insulin (1.7 nM); [His^4] IGF-I (310 nM); IGF-I (1100 nM); [Ser^{15}] IGF-I (1400 nM); [Leu^{62}] IGF-I (2300 nM); [Ser^{15}, Leu^{62}] IGF-I (5500 nM); [Phe^{59}] IGF-I (>10000 nM); and [Leu^8] IGF-I (>10000 nM). Binding affinities of the analogues for both insulin receptor isoforms, were expressed relative to IGF-I, are presented in Table 3.1. HIR-A displayed a characteristic 3-fold higher affinity for insulin and substantially higher affinity for IGF-I than HIR-B. These results agree with those previously reported (Soos *et al.* 1990; Yamaguchi *et al.* 1993). The two insulin receptor isoforms showed similar relative binding affinities for the IGF analogues (Table 3.1).

Based on HIR-A binding, substitutions at positions 8 and 59 have resulted in an approximate 28- and 17-fold respective loss of insulin receptor affinity. [Leu^8] IGF-I and [Phe^{59}] IGF-I bound significantly less well to the insulin receptor compared with IGF type 1 receptor binding, indicating these analogues have altered specificity that is preferential for the type 1 IGF receptor. [Leu^{62}] IGF-I was only half as potent as IGF-I in insulin receptor affinity compared with a significant loss in IGF type 1 receptor binding potency. [His^4] IGF-I showed an approximate 7-fold increase in HIR-A binding compared to IGF-I and similar affinity for the IGF type 1 receptor. Therefore, [Leu^{62}] IGF-I and [His^4] IGF-I are more insulin-like than IGF-I-like with respect to binding specificity. [Ser^{15}] IGF-I was equipotent to IGF-I in insulin receptor binding. The reduced type 1 IGF receptor binding and insulin receptor binding of the double mutant, [Ser^{15}, Leu^{62}] IGF-I, appears to be additive in each case. The relative receptor binding specificities of these analogues are given in Figure 3.6.

3.4.3 Biological potency and IGFBP binding properties

Biological potencies of the IGF-I analogues were assessed by measuring the stimulation of protein synthesis in rat L6 myoblasts. The ED$_{50}$ values in order of potency were: [Ser^{15}] IGF-I (1.9 nM); [Phe^{59}] IGF-I (2.1 nM); [His^4] IGF-I (2.4 nM); IGF-I (3.0 nM); [Leu^{62}] IGF-I

(3.3 nM); [Ser^{15}, Leu^{62}] IGF-I (3.8 nM); and [Leu^8] IGF-I (5.1 nM). Dose response curves are shown in Figure 3.7 and potencies measured relative to IGF-I are presented in Table 3.1. Surprisingly, [Phe^{59}] IGF-I, [Leu^{62}] IGF-I, [Ser^{15}, Leu^{62}] IGF-I and [Leu^8] IGF-I display greater than expected biological potencies based on both soluble and cell based measurements of IGF type 1 receptor binding affinity. Indeed for these four analogues, the ratio of relative biological potency to relative IGF type 1 receptor binding was 18, 7.6, 13 and 14 respectively. We evaluated binding to IGFBPs to determine whether these inconsistencies could be due to modification of analogue binding to IGFBPs secreted by L6 myoblasts (Figure 3.8). In decreasing order of affinity, concentrations required to give half maximal competition with ^{125}I-IGF-I for IGFBP binding were: [Phe^{59}] IGF-I (0.79 nM); IGF-I (0.82 nM); [Ser^{15}] IGF-I (0.93 nM); [His^4] IGF-I (1.1 nM); [Leu^8] IGF-I (1.1 nM); [Leu^{62}] IGF-I (1.8 nM); and [Ser^{15}, Leu^{62}] IGF-I (2.3 nM). All analogues with the exception of [Leu^8] IGF-I reached similar maximal responses to IGF-I. Only subtle changes in the relative affinity of the IGF analogues for IGFBPs secreted from L6 myoblasts was observed (Table 3.1).

Table 3.1 Potencies of IGF-I analogues expressed relative to IGF-I. Values are expressed as a ratio of the ED_{50} for IGF-I to the ED_{50} of the IGF analogue. The %RSD (relative standard deviation) <15% across all assays.

Assay	IGF-I	[His^4]	[Leu^8]	[Ser^{15}]	[Phe^{59}]	[Leu^{62}]	[Ser^{15}Leu^{62}]
Soluble Receptor							
IGF type 1 receptor	1.0	1.2	0.17	0.79	0.19	0.13	0.17
HIR-A	1.0	6.7	0.036	0.71	0.059	0.50	0.12
HIR-B	1.0	3.5	<0.11	0.79	<0.11	0.48	0.20
L6 myoblasts							
IGF type 1 receptor	1.0	2.4	0.042	0.64	0.081	0.12	0.06
IGFBP	1.0	0.75	0.75	0.88	1.0	0.46	0.36
Protein synthesis	1.0	1.3	0.59	1.58	1.43	0.91	0.79
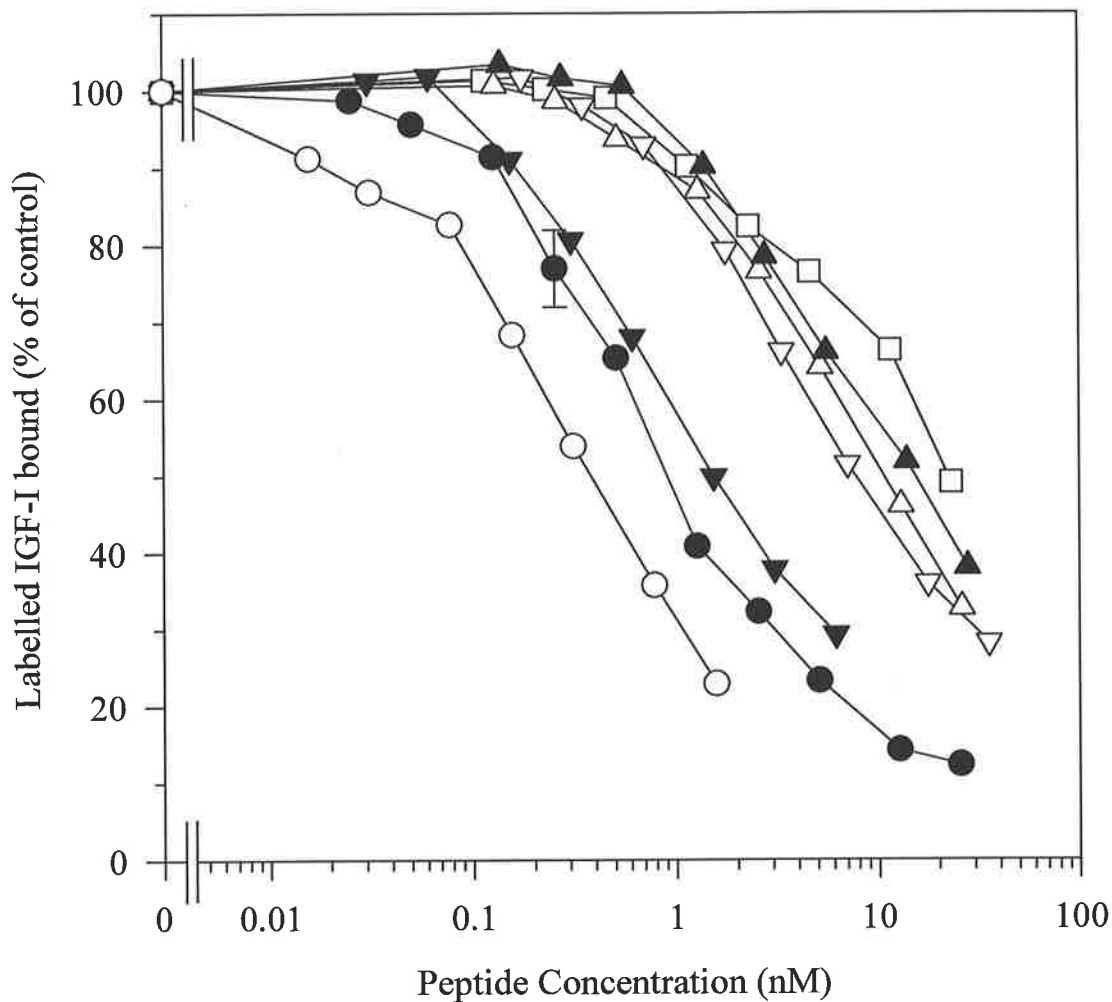
Figure 3.2. Binding of IGF analogues to type 1 IGF receptor in rat L6 myoblasts.
Competition of ^{125}I-IGF-I binding to the type 1 IGF receptor with: [His[4]] IGF-I (O), [Leu[8]]
IGF-I (□), [Ser[15]] IGF-I (▼), [Phe[59]] IGF-I (△), [Leu[62]] IGF-I (▽), [Ser[15], Leu[62]] IGF-I (▲)
and hIGF-I (●). Values are the means of triplicate determinations in three individual
experiments at each peptide concentration. The S.E.M. values are indicated by error bars
when larger than 4%.
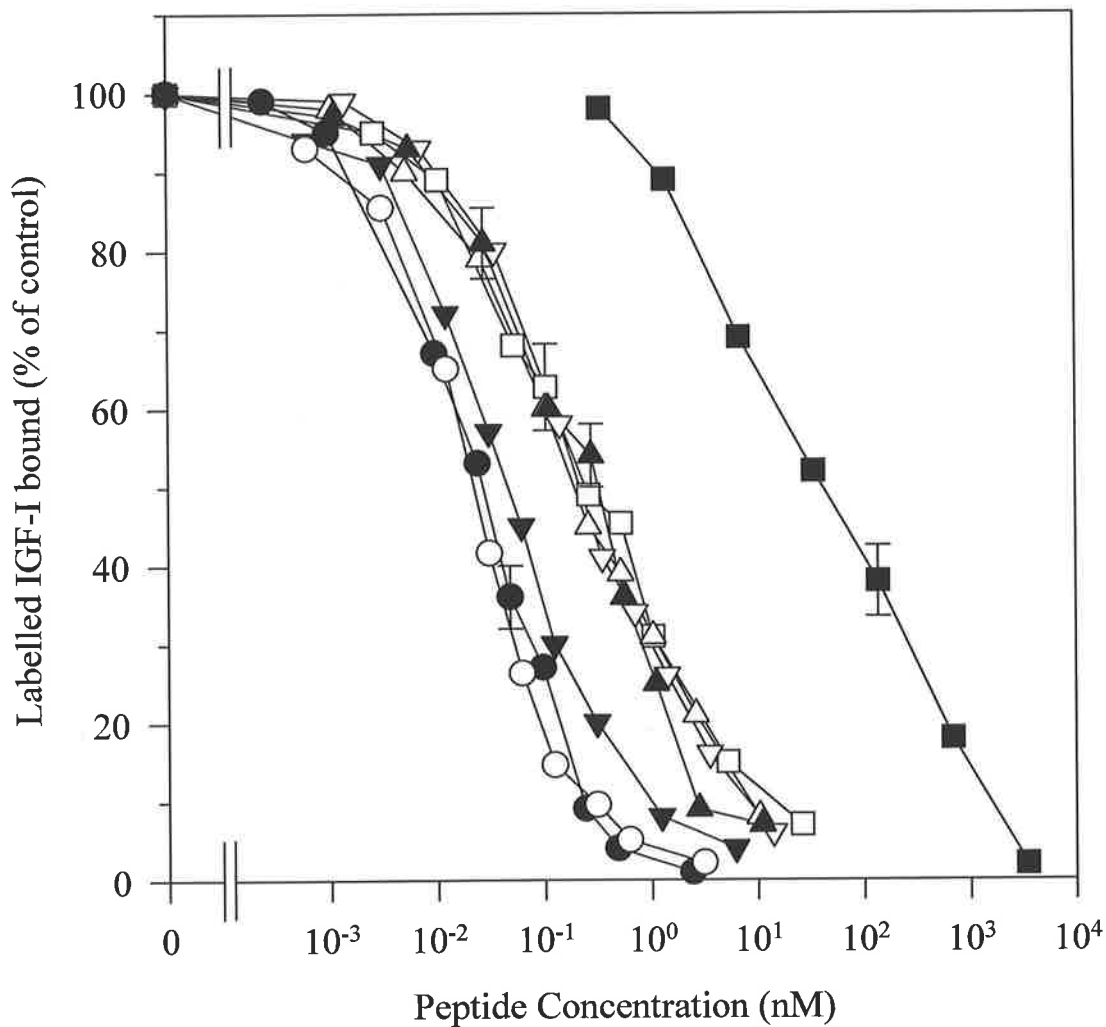
Figure 3.3. Binding of IGF analogues to the soluble Type 1 IGF receptor. Competition of ^{125}I-IGF-I binding to the type 1 IGF receptor with: [His^4] IGF-I (O), [Leu^8] IGF-I (□), [Ser^{15}] IGF-I (▼), [Phe^{59}] IGF-I (△), [Leu^{62}] IGF-I (▽), [Ser^{15}, Leu^{62}] IGF-I (▲), hIGF-I (●) and insulin (■). Values are the means of triplicate determinations in three individual experiments at each peptide concentration. The S.E.M. values are indicated by error bars when larger than 4%.

Figure 3.4 Binding of IGF analogues to the HIR-A isoform of the insulin receptor. Competition for ^{125}I-Insulin binding by: [His^4] IGF-I (O), [Leu^8] IGF-I (□), [Ser^{15}] IGF-I (▼), [Phe^{59}] IGF-I (△), [Leu^{62}] IGF-I (▽), [Ser^{15}, Leu^{62}]IGF-I (▲), hIGF-I (●) and insulin (■). Values are the means of triplicate determinations in three individual experiments at each peptide concentration. The S.E.M. values are indicated by error bars when larger than 4%.

Figure 3.5 Binding of IGF analogues to the HIR-B isoform of the insulin receptor.
Competition for ^{125}I-Insulin binding by: [His[4]] IGF-I (O), [Leu[8]] IGF-I (□), [Ser[15]] IGF-I (▼),
[Phe[59]] IGF-I (△), [Leu[62]] IGF-I (▽), [Ser[15], Leu[62]]IGF-I (▲), hIGF-I (●) and insulin (■).
Values are the means of triplicate determinations in three individual experiments at each
peptide concentration. The S.E.M. values are indicated by error bars when larger than 4%.

Figure 3.6 Receptor binding specificities of the IGF-I analogues. The ratio of relative binding potencies of IGF type 1 receptor binding to human insulin receptor binding. Values for these potencies are given in Table 3.1.
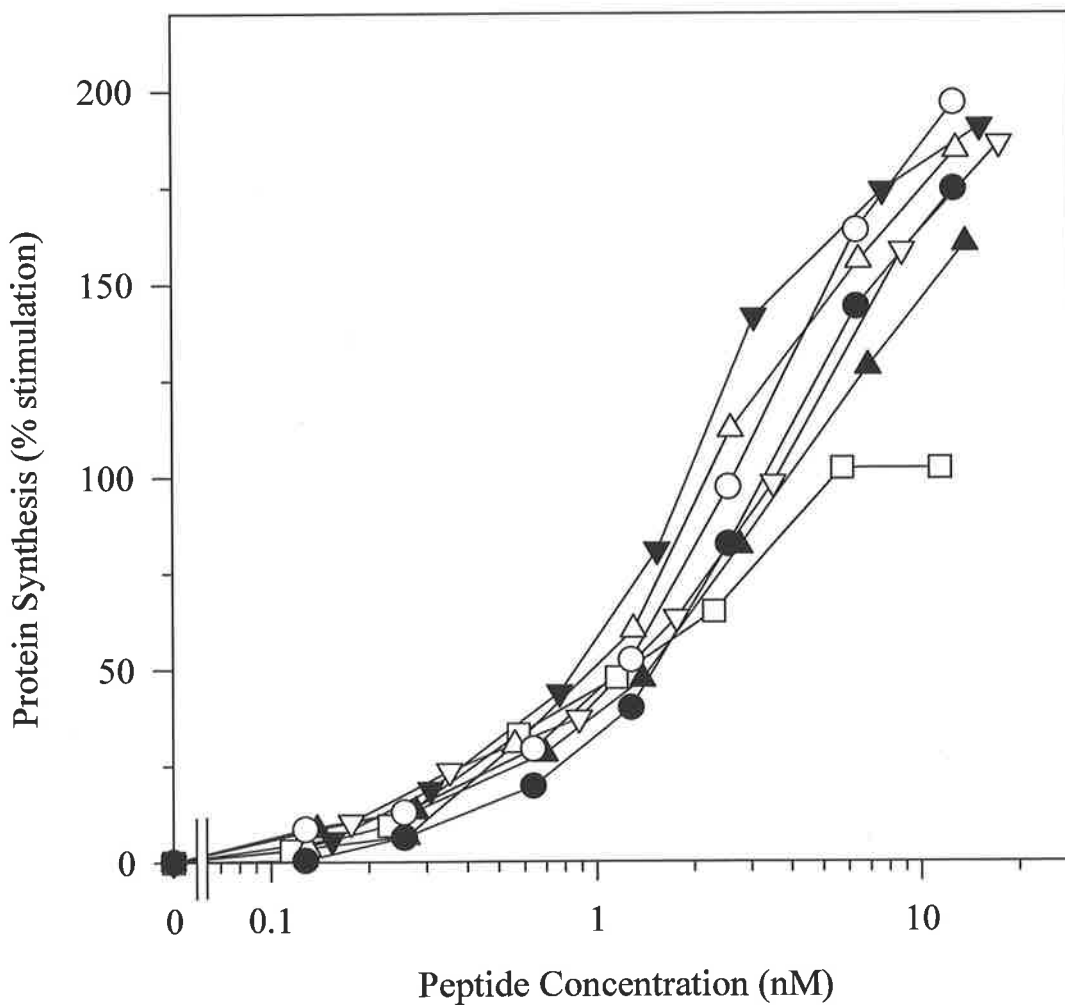
Figure 3.7 Stimulation of protein synthesis by IGF-I analogues measured in rat L6 myoblasts. Analogues include: [His^4] IGF-I (○), [Leu^8] IGF-I (□), [Ser^{15}] IGF-I (▼), [Phe^{59}] IGF-I (△), [Leu^{62}] IGF-I (▽), [Ser^{15}, Leu^{62}] IGF-I (▲) and hIGF-I (●). Values are the means of triplicate determinations in two individual experiments at each peptide concentration. The S.E.M. values are indicated by error bars when larger than 4%.
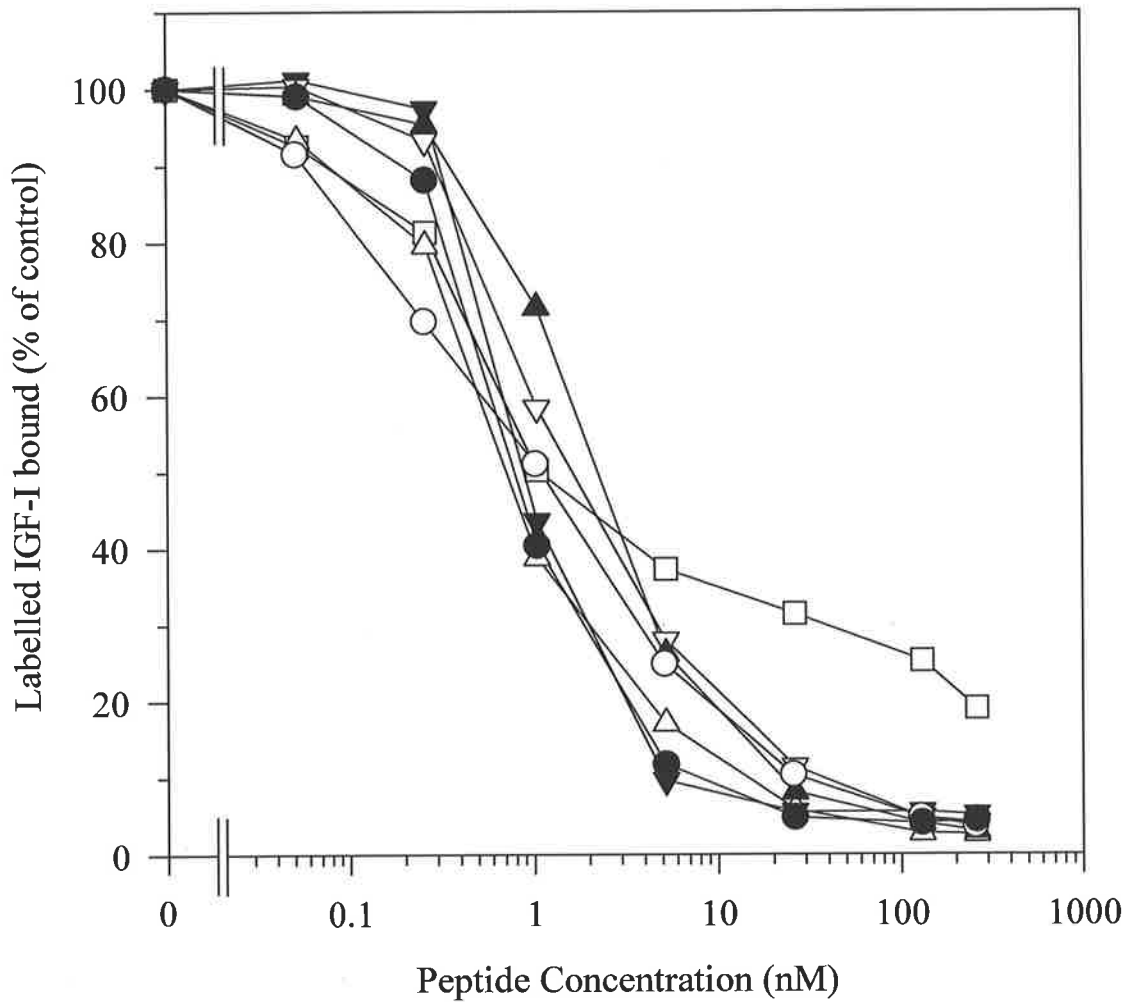
Figure 3.8 Binding of IGF analogues to IGFBPs in rat L6 myoblast conditioned media. Competition of ^{125}I-IGF-I binding to the type 1 IGF receptor with: [His^4] IGF-I (O), [Leu^8] IGF-I (□), [Ser^{15}] IGF-I (▼), [Phe^{59}] IGF-I (△), [Leu^{62}] IGF-I (▽), [Ser^{15}, Leu^{62}] IGF-I (▲) and hIGF-I (●). Values are the means of triplicate determinations in three individual experiments at each peptide concentration. The S.E.M. values are indicated by error bars when larger than 4%.

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3.5 Discussion

Blundell *et al.* (1978) have proposed that a number of invariant residues shared between IGF-I and insulin are involved with IGF-I binding to the IGF type 1 receptor and its cross-reactivity with the insulin receptor. The Tyr^{24} of IGF-I has been shown to play a similar role in high affinity IGF type 1 receptor binding (Cascieri *et al.* 1988b) as the corresponding $Phe^{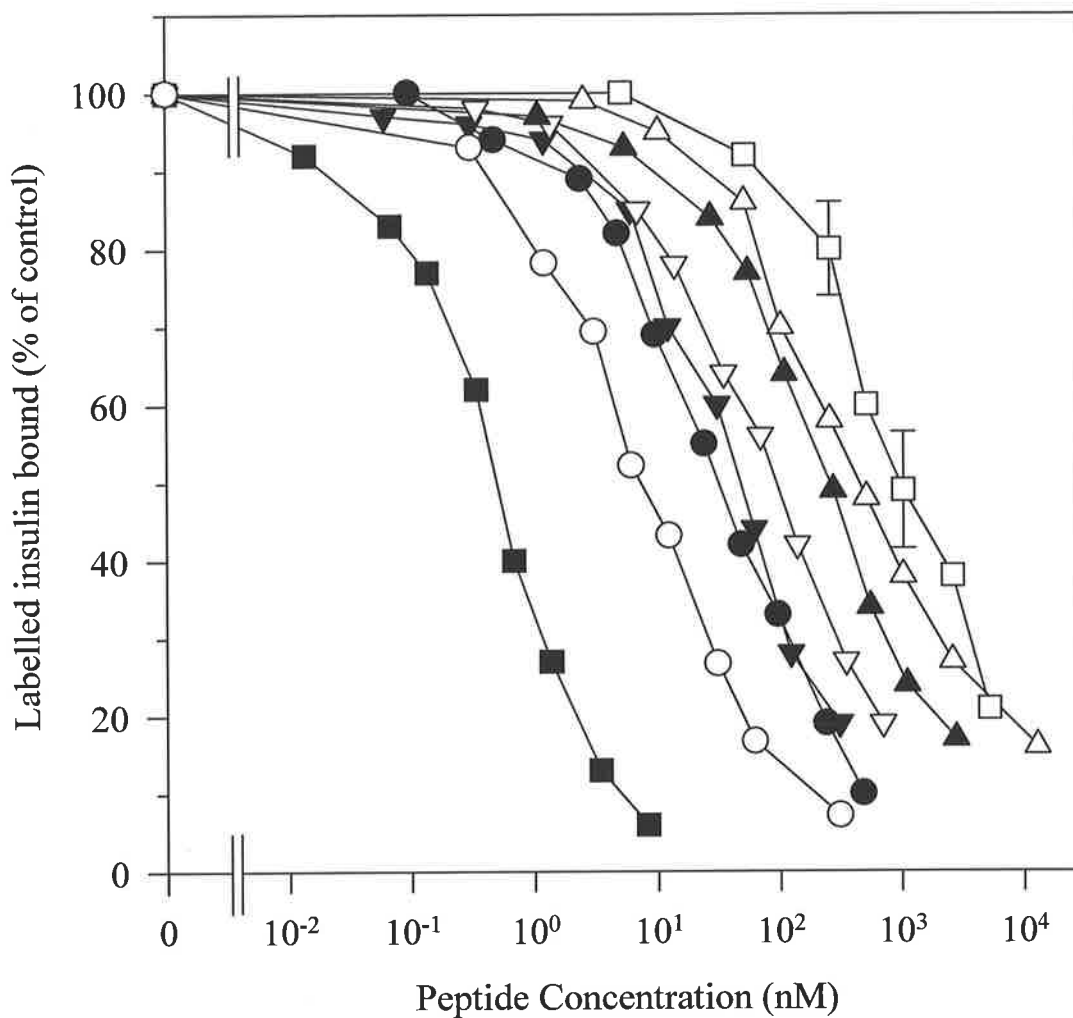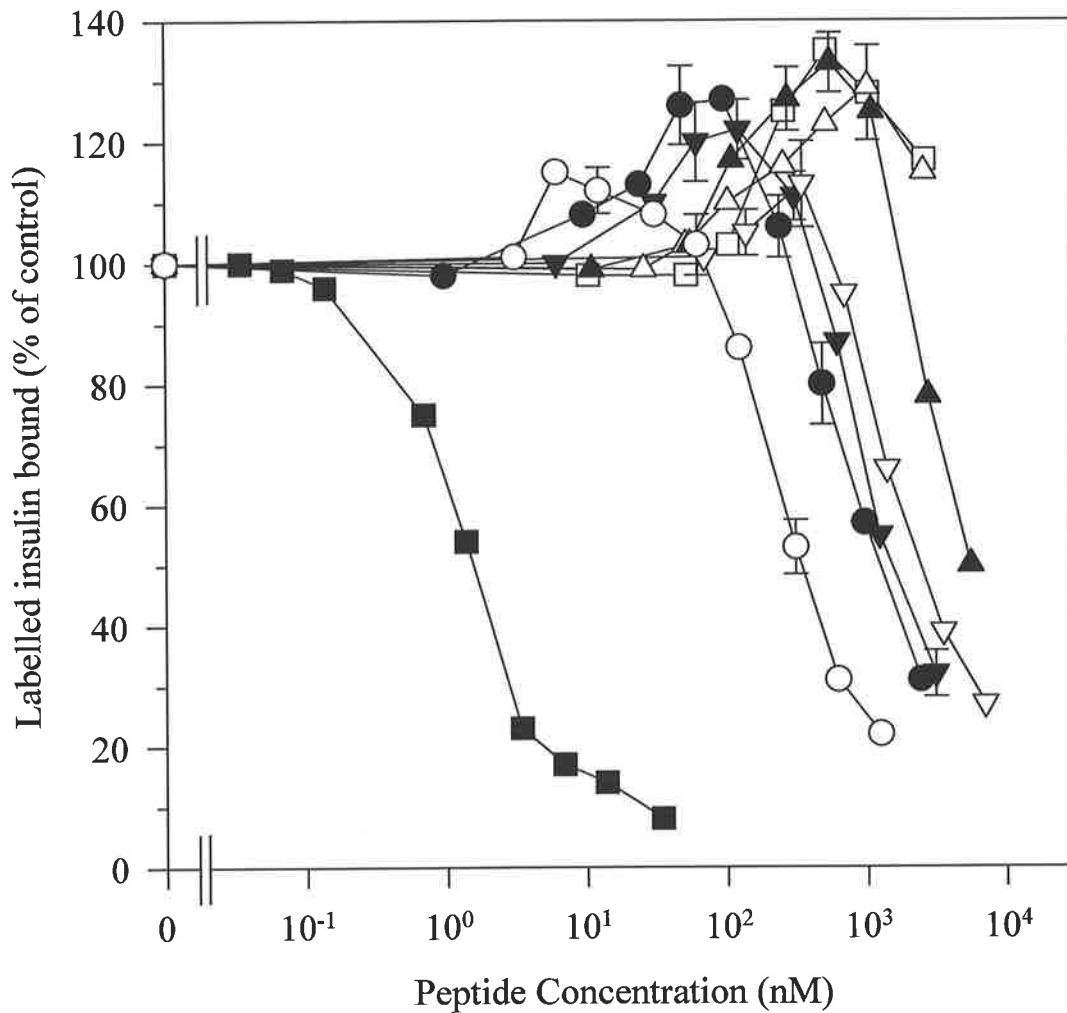B25}$ of insulin has in insulin receptor binding (Tager *et al.* 1980). Our aim in this study was to identify IGF-I residues on the A- and B-domains that are adjacent to the critical receptor binding region and may be involved in defining ligand-receptor specificity. Conservative substitutions were chosen with the intention of maintaining structural integrity while introducing a sufficient change for potential alteration of cross-reactivity with the insulin receptor. Recently published results (Jansson *et al.*, 1997) concerning the effects of alanine substitutions within the B-domain helix of IGF-I (residues Val^{11}, Asp^{12}, Gln^{15} and Phe^{16}) indicate that gross structural perturbations can result from single residue changes in this region. The loss of α helical structure seen with their mutants highlights the importance of careful selection for substitution, and suggests monitoring analogue conformation is necessary to avoid misinterpretation of binding kinetics. Far-UV CD spectral analysis of $[Leu^{8}]$ IGF-I, $[Ser^{15}]$ IGF-I, $[Phe^{59}]$ IGF-I, $[Leu^{62}]$ IGF-I and $[Ser^{15}, Leu^{62}]$ IGF-I, (presented and discussed in Chapter 2), suggests that these analogues maintain their gross native structure.

Our results demonstrate that a number of IGF-I substitutions affect binding to both forms of the soluble insulin receptor (HIR-A and HIR-B) in a similar manner. The binding affinity of IGF-I with the soluble HIR-A was approximately 3-fold higher than HIR-B. No significant change in relative binding affinity was observed with the IGF-I analogues for these two insulin receptor isoforms, suggesting none of the IGF-I positions studied contribute to differences in HIR-A and HIR-B binding characteristics.

Type 1 IGF receptor binding affinities were measured using both soluble receptors and L6 rat myoblasts. The rat L6 myoblast cell line is useful in assessing type 1 IGF receptor binding characteristics due to low insulin receptors numbers and high levels of the type 1 IGF receptor (Ballard *et al.*, 1986). Some discrepancies in relative type 1 IGF receptor binding affinities

were observed between these two assay systems. The cause of these discrepancies is unclear, however it may reflect the non-physiological conditions used in the soluble receptor assays. The presence of cell surface bound IGFBPs in the L6 myoblast assay may also have an affect. For comparative purposes, IGF type 1 receptor and insulin receptor binding characteristics of the analogues will be restricted to the results obtained from the soluble IGF type 1 and insulin receptor binding assays.

3.5.1 Position 4 of IGF-I

The analogue [His^4] IGF-I was included in this study as a useful comparison against the other analogues. Based on previous homologous substitutions in the extreme N-terminal region of IGF-I (Bayne et al., 1988), we predicted this analogue would have increased insulin receptor binding affinity. Indeed, substitution of Thr^4 with His resulted in a 4 to 7-fold increase in insulin receptor binding with no significant change in relative affinity for the type 1 IGF receptor.

3.5.2 Position 8 of IGF-I

Amino acid Ala^8 of IGF-I is the first residue of the B-domain α-helix and is contiguous with the putative hydrophobic receptor binding region (Figure 3.1). This position is commonly referred to as the N-Cap of an α-helix (Harper & Rose, 1993). The N-Cap is critical in establishing the first hydrogen bond of the α-helix and is therefore important for stabilising the helical structure. To enhance divergence from the corresponding polar residue Ser^{B9} of insulin, the hydrophobic index at this position of IGF-I was changed by substituting Ala^8 with Leu. [Leu^8] IGF-I had significantly lower relative affinity for the insulin receptor (28-fold) than the IGF type 1 receptor (6-fold).

While the Leu^8 substitution clearly affects receptor binding specificity, the loss in IGF type 1 receptor binding may not indicate a direct involvement of position 8 of IGF-I in receptor binding. This loss might reflect subtle structural alterations in the N-terminus of the α-helix. Despite no evidence of this from far-UV CD spectra, minor structural changes may be outside the sensitivity of this technique. Studies on N-cap residues and α-helix stability suggest that

this substitution may be structurally unfavourable (Harper & Rose, 1993; Chakrabartty & Baldwin, 1995). The Leu substitution of Ala^8 at the start of the α-helix could therefore inhibit solvation of the N-terminal region or the large side-chain of Leu could form additional disruptive Van der Waal's contacts with closely aligned residues. Alternatively, the introduction of a bulky Leu at this position might simply impede native receptor binding through steric hindrance. In any event, this analogue demonstrates that the N-terminal region of the B-domain α-helix is more critical for high affinity insulin receptor binding than type 1 IGF receptor binding.

3.5.3 Position 15 of IGF-I

Amino acid Gln^{15} of IGF-I is positioned at the C-terminal region of the B-domain α-helix and is closely aligned with Phe^{23} of IGF-I (Figure 3.1). The analogous Tyr^{B16} of insulin has been proposed as being involved in high affinity binding between insulin and the insulin receptor. Substitution of Gln^{15}-Phe^{16} of IGF-I with the corresponding Tyr^{B16}-Leu^{B17} of insulin increases insulin receptor binding without altering IGF type 1 receptor binding (Bayne *et al.* 1988). Using a similar strategy as described for Ala^8, changing Gln^{15} to Ser was expected to enhance divergence from the analogous residue Tyr^{B16} of insulin. The analogue [Ser^{15}] IGF-I had minimal effect on both type 1 IGF receptor and insulin receptor binding affinity. Based on the results of Bayne *et al.* (1988), this suggests that Phe^{16} rather than Gln^{15} may be more critical in defining the receptor binding specificity of IGF-I. Less conservative substitutions at this position may also better define the role of this region in determining receptor binding specificity. Subsequent mutations at this position ([Ala^{15}] IGF-I and [Glu^{15}] IGF-I), appear to reduce the association rate with the type 1 IGF receptor by approximately 2-fold (Jansson *et al.*, 1997). However, CD analysis suggested this change is due to structural disruption in the B-domain α-helix.

3.5.4 Position 59 of IGF-I

Previous studies illustrate that structural determinants in the C- and D-domains are involved in maintaining receptor binding specificity between the two receptors. Bayne *et al.* (1989) have shown an IGF-I analogue with a C-domain (Gly)$_4$ replacement has 30-fold reduced

affinity for the IGF type 1 receptor while having slightly enhanced affinity for the insulin receptor. They have also shown that a modified IGF-I lacking the D-domain has normal affinity for the IGF type 1 receptor and 2-fold enhanced affinity for the insulin receptor. Zhang et al. (1994) have subsequently demonstrated positively charged residues Arg[36], Arg[37], Lys[65] and Lys[68] of the C- and D-domain contribute to receptor binding specificity and high affinity binding to the IGF type 1 receptor. The Met[59] side chain lies within 8Å of Tyr[24] and extends into a cleft formed between the C- and D-domains. Of the amino acid positions targeted for substitution, this position is the most remote from the putative receptor binding region (Figure 3.1).

Phenylalanine substitution was chosen with the aim of lowering cross-reactivity with the insulin receptor by increasing both the hydrophobic index and side chain size at this position, thus increasing divergence from the corresponding small polar residue Asn[A18] of insulin. Previous substitutions at this position have had minimal effects on IGF type 1 receptor binding (Peters et al. 1985 Cascieri et al. 1986). [Phe[59]] IGF-I showed reduced binding to both the insulin receptor (17-fold) and the IGF type 1 receptor (5-fold). As predicted by our selection strategy, the altered specificity of [Phe[59]] IGF-I favoured IGF type 1 receptor over insulin receptor binding. This may be due to steric effects produced by reduced flexibility in the C- and D-domains although the direct involvement of this residue in defining receptor binding affinity cannot be ruled out. Like [Leu[8]] IGF-I, [Phe[59]] IGF-I represents a novel IGF-I analogue that is less "insulin-like" with respect to receptor binding (Figure 3.6).

3.5.5 Position 62 of IGF-I

To introduce more divergence from the corresponding polar residue Asn[21] of insulin, Ala[8] of IGF-I was substituted with Leu. Similar to Ala[8], Ala[62] is contiguous with the putative receptor binding surface. In contrast to [Leu[8]] IGF-I and [Ser[15]] IGF-I, [Leu[62]] IGF-I had minimal impact on insulin receptor binding (2-fold loss) but reduced affinity to the IGF type 1 receptor (8-fold loss) relative to IGF-I. Interestingly, this change in receptor binding specificity was contrary to what was predicted by our substitution rationale. Like [His[4]]

IGF-I, [Leu^{62}] IGF-I showed receptor binding that is more "insulin-like" than IGF-I (see Figure 3.6).

The loss of type 1 IGF-I receptor binding affinity with [Leu^{62}] IGF-I does not necessarily suggest a direct role for Ala^{62} in receptor binding. The solution structure of IGF-I shows that a distance of less than 4

(King *et al.*, 1992). Our data suggests that none of the positions targeted for substitution in this study are critical for binding to IGFBPs secreted by L6 myoblasts. Therefore, the lack of correlation between IGF type 1 receptor binding affinity and biological potency cannot be attributed to alteration in IGFBP binding affinity.

In the majority of IGF-I analogue studies, biological activity appears to be directly related to receptor binding affinity. In general, amino acid alterations that result in reduced receptor binding affinity also produce a comparable reduction in the *in vitro* biological potency. However, the more extensive structure-function analysis of insulin has yielded a number of mutant insulin examples where receptor binding affinity does not correlate with biological responses such as the stimulation of glucose oxidation, lipogenesis and DNA synthesis (Schwartz *et al.* 1982; Chu *et al.* 1987; Bornfeldt *et al.* 1991). These studies indicate that with insulin analogues there is no simple relationship between receptor binding and biological response. The hystricomorph insulins and insulin analogues mutated at positions A^{13} and B^{17} have been shown to exhibit higher mitogenic potency relative to receptor binding affinity (De Meyts 1994). The potency of these insulin analogues in stimulating a mitogenic response was shown to be inversely related to the ligand-receptor dissociation rate rather than the equilibrium receptor binding affinity (De Meyts 1994). More recent characterization of super-active insulin analogues has demonstrated that receptor dissociation rates below 40% of the native peptide have up to 7-fold increased mitogenic potency relative to their metabolic response (Hansen *et al.*, 1996). The high mitogenic potency of these analogues was suggested to result from sustained activation of the insulin receptor tyrosine kinase domain and sustained phosphorylation of the Shc signaling protein (Hansen *et al.*, 1996). Similar to what has been proposed for the super-active insulins, changes in dissociation rates from the IGF type 1 receptor may account for the higher than expected biological potencies shown by $[Leu^8]$ IGF-I, $[Phe^{59}]$ IGF-I and $[Leu^{62}]$ IGF-I.

Chapter 4

Involvement of the B-Domain of IGF-I in IGFBP Interactions.

4.1 Introduction

As discussed in detail in Chapter 1, IGF-I and IGF-II are anabolic, single-chain polypeptides of 70 and 67 amino acid residues respectively. Subtle evolutionary changes in the A- and B-domains of the IGFs allow them to associate with the 6 IGFBPs. The physiological effects of IGF-I and IGF-II are regulated by this interaction with the IGFBPs. In contrast, insulin does not bind the IGFBPs, but self-associates to form dimers, tetramers and hexamers in solutions.

Although several structure-function studies have implicated numerous dispersed regions of IGF-I in IGFBP association, no clear interface has been defined. Substitution of various A-chain insulin residues for non-homologous IGF-I and IGF-II sequences demonstrates a possible involvement of A-domain residues Phe^{49}, Arg^{50}, Ser^{51} of IGF-I (Clemmons *et al.*, 1992; Bach *et al.*, 1993). However, the generation of hybrid peptides has demonstrated that the B-domain is primarily involved in the association of the IGFBPs (De Vroede *et al.*, 1985; Clemmons et al., 1990). Substitution of Gln^{15}-Phe^{16} of IGF-I with the analogous Phe^{B16}-Leu^{B17} of insulin suggests the C-terminal end of the B-domain α-helix is important for high affinity association with the IGFBPs (Bayne *et al.*, 1988; Clemmons *et al.*, 1992). Chemically synthesized analogues of IGF-I truncated by 1 to 5 N-terminal amino acid residues has also demonstrated the importance of the N-terminal region of the B-domain in binding bovine IGFBP-2 and IGFBPs secreted by L6 rat myoblasts (Bagley *et al.*, 1989). IGF-I analogues with Arg and Gly substitutions identify Glu^3 of IGF-I as being crucial in the high affinity interaction with the IGFBPs (King *et al.*, 1992; Francis *et al.*, 1992). These studies also demonstrated that IGF-I fusion analogues (consisting of a $[Met^1]$-pGH(1-11)-Val-Asn N-terminal extension), such as Long $[Arg^3]$ IGF-I have significantly reduced affinities for the

IGFBPs relative to their cleaved counterparts suggesting the orientation of the B-domain fusion peptide affects IGFBP association (Laajoki *et al.*, 1997; Laajoki *et al.*, 2000).

The data presented in this Chapter was carried out as part of a broader study on the involvement of the B-domain α-helix in IGFBP association (Magee *et al.*, 1999). This study confirmed the key role of Phe^{16} in IGFBP interaction and proposed other closely aligned residues Leu^5 and Leu^{54} may form a hydrophobic IGFBP binding pocket (Figure 4.1). IGF-I residues Ala^8 and Gln^{15} are positioned at opposite ends of the B-domain α-helix, peripheral to the putative hydrophobic IGFBP binding surface but are relatively isolated from the critical residue Glu^3. In order to assess the potential involvement of these residues in IGFBP association, [Leu^8] IGF-I and [Ser^{15}] IGF-I were assayed for their affinity with each of the 6 IGFBP. The analogues [Ser^{16}] IGF-I and [Arg^3] IGF-I or Long [Arg^3] IGF-I have been included for comparative purposes.
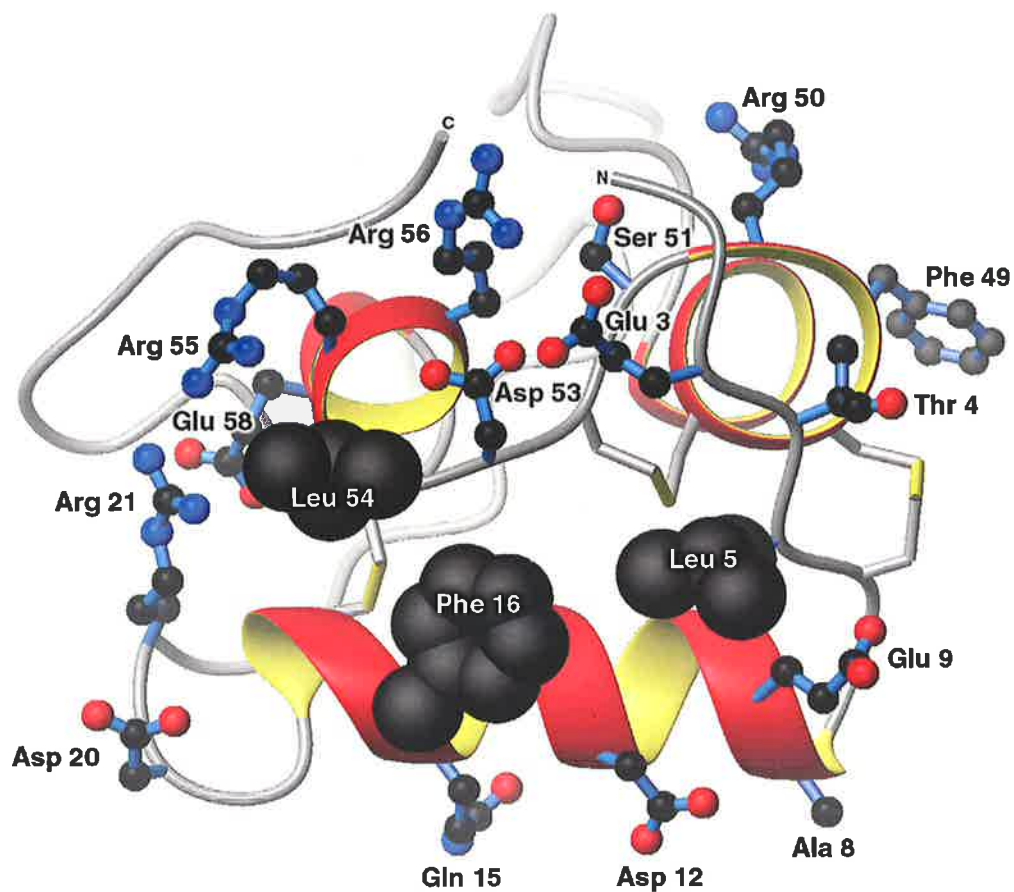
Figure 4.1 The solution structure of IGF-I displaying surface residues of the IGFBP binding surface. Heavy atoms of residues in the putative hydrophobic binding core are space-filled. Other residues implicated in IGFBP association include Glu[3] and Phe[49]-Arg[50]-Ser[51]. (Structure determined by Cooke *et al.*,1991. Image generated using MOLMOL).

4.2 Materials

All materials and buffers used are listed in the general materials sections of Chapter 2 and Chapter 3. The analogue [Ser^{16}] IGF-I was designed and constructed by Barbara Magee, Department of Biochemistry, Adelaide University, Australia. LongR^3 IGF-I and [Arg^3] IGF-I were supplied by GroPep Pty Ltd., Adelaide, Australia. Purified, lyophilized IGFBPs were kindly donated by Prof. R. C. Baxter, Kolling Institute of Medical Research, Sydney, Australia (natural human IGFBP-3 and IGFBP-6); Dr. S. Mohan, Pettis VA Medical Center, Loma Linda CA, U.S.A. (natural human IGFBP-4 and IGFBP-5); Dr. G. Forsberg, KabiGen, Stockholm, Sweden, (recombinant IGFBP-1); and Dr. M. Rechler, NIH, Bethesda, U.S.A. (rat IGFBP-2, purified from BRL-3A culture supernatant).

4.3 Methods

IGFBP affinities were assessed for the IGF-I variants using the charcoal binding assay described by Martin and Baxter (Martin and Baxter, 1986) with modifications suggested by Szabo *et al.* (Szabo *et al.*, 1988). Briefly, various concentrations of IGF-I or analogue, 10,000 cpm ^{125}I-labelled IGF-I (approximately 0.2ng) and 10ng of the appropriate IGFBP were suspended in a total of 0.25ml phosphate buffer [50mM sodium phosphate, 2.5 g/L BSA (radioimmunoassay grade), pH 6.5]. After incubation at 4°C for 18h, bound tracer was separated from free by the addition of 1.0ml activated charcoal suspension (phosphate buffer, as above, containing 5.0 g/L activated charcoal and 0.2 g/L protamine sulphate). Protamine sulphate was omitted from IGFBP-1 assays as suggested by Conover *et al.*, 1988. After centrifugation (10,000 g for 3 min.) to sediment charcoal and uncomplexed IGFs, 0.625 ml of supernatant was removed for gamma-counting to determine quantities of bound ^{125}I-labelled IGF. Results were expressed as the percentage of complexed ^{125}I-labelled IGF in the absence of competing analogue. Two competitive binding assays were conducted for each IGFBP and IGFs were tested in triplicate at each dilution.

4.4   Results

The affinities of analogues for IGFBPs 1-6 were determined by competitive binding assays. Dose-response curves for these assays are presented in Figures 4.2-4.7. Relative binding affinities, calculated from the ratio of the ED_{50} for IGF-I to the ED_{50} of the analogue, are presented in Table 4.1. The results for each of the B-domain mutations are discussed below.

The conservative replacement of Ala 8 of IGF-I with Leu produced a non-uniform loss in affinity for IGFBPs 1-5. Unfortunately, the affinity of IGFBP-6 for [Leu[8]] IGF-I was not determined due to a shortage of material. The most significant effect of this substitution was an approximate 80-fold loss in affinity for IGFBP-2. Less critical losses in affinity, of between 6- and 12-fold, were observed for IGFBP-1, -3 and -4 while IGFBP-3 affinity was reduced by 3-fold.

Replacement of Gln at position 15 with Ser resulted in very subtle effects on the binding affinity with IGFBPs. No significant change in affinity was observed with IGFBP-3 and -6 while only marginal losses were observed with IGFBP-1 and -2. The most significant effect of this analogue was an approximate 2-fold loss in binding for IGFBP-4 and IGFBP-5.

As previously presented (Magee *et al.*, 1999), substitution of Phe at position 16 of IGF-I showed significant losses in binding affinity for all 6 IGFBPs. Similar to what was observed with [Leu[8]] IGF-I, substitution at position 16 resulted in differential losses in binding affinity across the six IGFBPs studied. Association with IGFBP-2 and -3 was reduced by approximately 15-fold while a greater than 29-fold loss was observed with IGFBP-1. The binding affinity between [Ser[16]] IGF-I and IGFBP-4 was approximately 80-fold less than that of IGF-I while the relative loss in affinity for IGFBP-5 and -6 was greater than 330-fold and 90-fold respectively.

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The analogues Long [Arg^3] IGF-I and [Arg^3] IGF-I showed the lowest binding affinities for all 6 IGFBPs. Indeed no competition with iodinated IGF-I was observed for IGFBP-4, -5 and -6 across the concentration range tested. The combined effects of the N-terminal extension and charge reversal at position 3 result in greater than 150-fold losses in affinity for these IGFBPs. Subtle competition between Long [Arg^3] IGF-I and iodinated IGF-I was observed with for IGFBP-1 although the concentrations range required for half-maximal competition was not achieved (Fig. 4.2). Unfortunately, Long [Arg^3] IGF-I was not tested against IGFBP-2 and -3. However, the substitution of Glu3 with Arg was shown to reduce binding affinity for IGFBP-2 and -3 by 300-fold and 100-fold respectively.

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Table 4.1 Relative affinities of IGF analogues for IGFBPs 1-6. The values are expressed as a ratio of the ED_{50} for IGF-I to the ED_{50} of the IGF. ED_{50} values for IGF-I are given in parenthesis. The %RSD (relative standard deviation) <10% across all assays. N.D. = not determined.

Assay	IGF-I	[Leu^8]	[Ser^{15}]	[Ser^{16}]	[Arg^3]	LongR^3
IGFBP-1	1.0 (10.8 nM)	0.16	0.85	< 0.041	N.D.	<0.02
IGFBP-2	1.0 (0.16 nM)	0.013	0.69	0.050	0.0031	N.D.
IGFBP-3	1.0 (0.094 nM)	0.14	0.93	0.072	0.0097	N.D.
IGFBP-4	1.0 (1.1 nM)	0.08	0.51	0.013	N.D.	<0.0038
IGFBP-5	1.0 (0.84 nM)	0.33	0.55	< 0.003	N.D.	<0.0019
IGFBP-6	1.0 (2.7 nM)	N.D.	1.13	< 0.013	N.D.	<0.0066
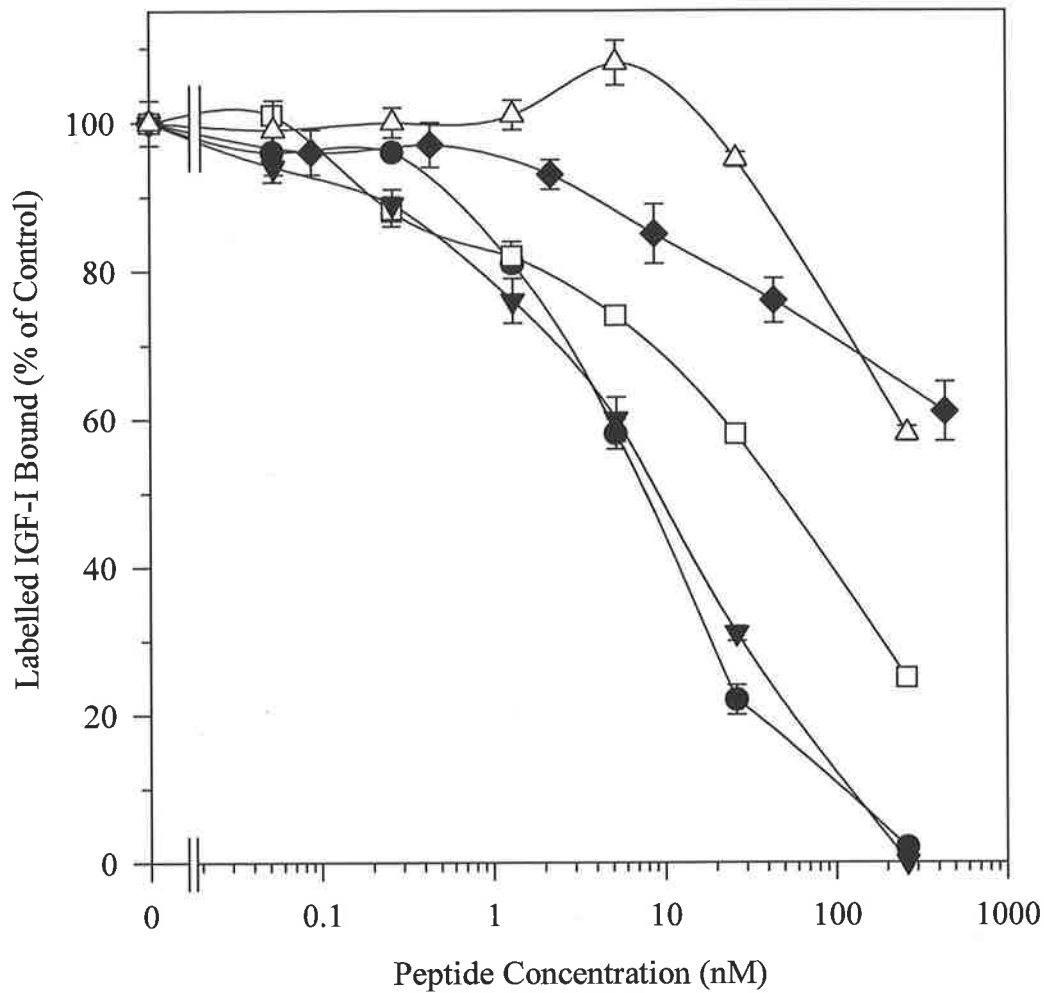
Figure 4.2 Binding of IGF-I analogues to recombinant human IGFBP-1. Competition of ^{125}I-IGF-I binding by: [Leu^8] IGF-I ($\square$), [Ser^{15}] IGF-I ($\blacktriangledown$), [Ser^{16}] IGF-I ($\triangle$), LongR^3 IGF-I ($\blacklozenge$) and IGF-I ($\bullet$). Values are the means of triplicate determinations in two individual experiments at each peptide concentration. S.E.M. values are indicated as error bars.
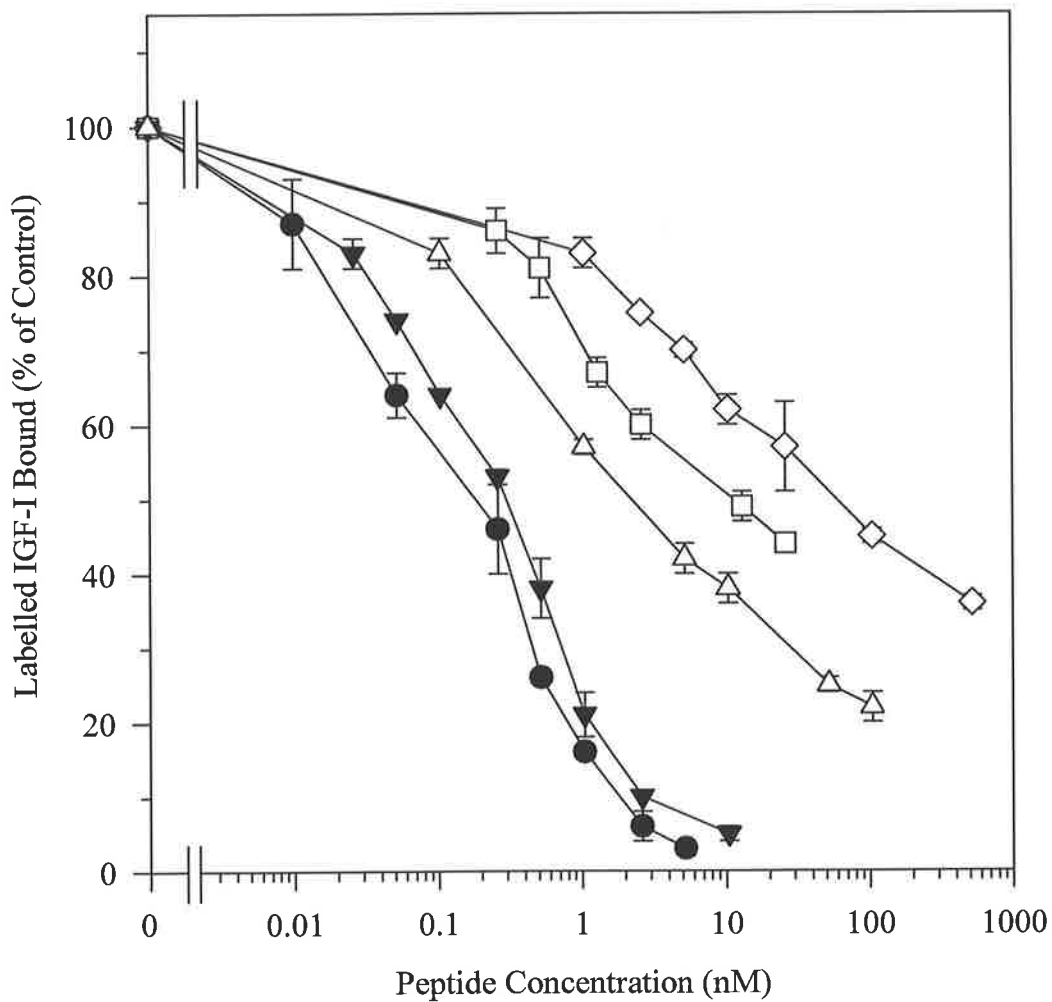
Figure 4.3 Binding of IGF-I analogues to natural rat IGFBP-2. Competition of ^{125}I-IGF-I binding by: [Leu^8] IGF-I (□), [Ser^{15}] IGF-I (▼), [Ser^{16}] IGF-I (△), [Arg^3] IGF-I (◇) and IGF-I (●). Values are the means of triplicate determinations at each peptide concentration. S.E.M. values are indicated as error bars.
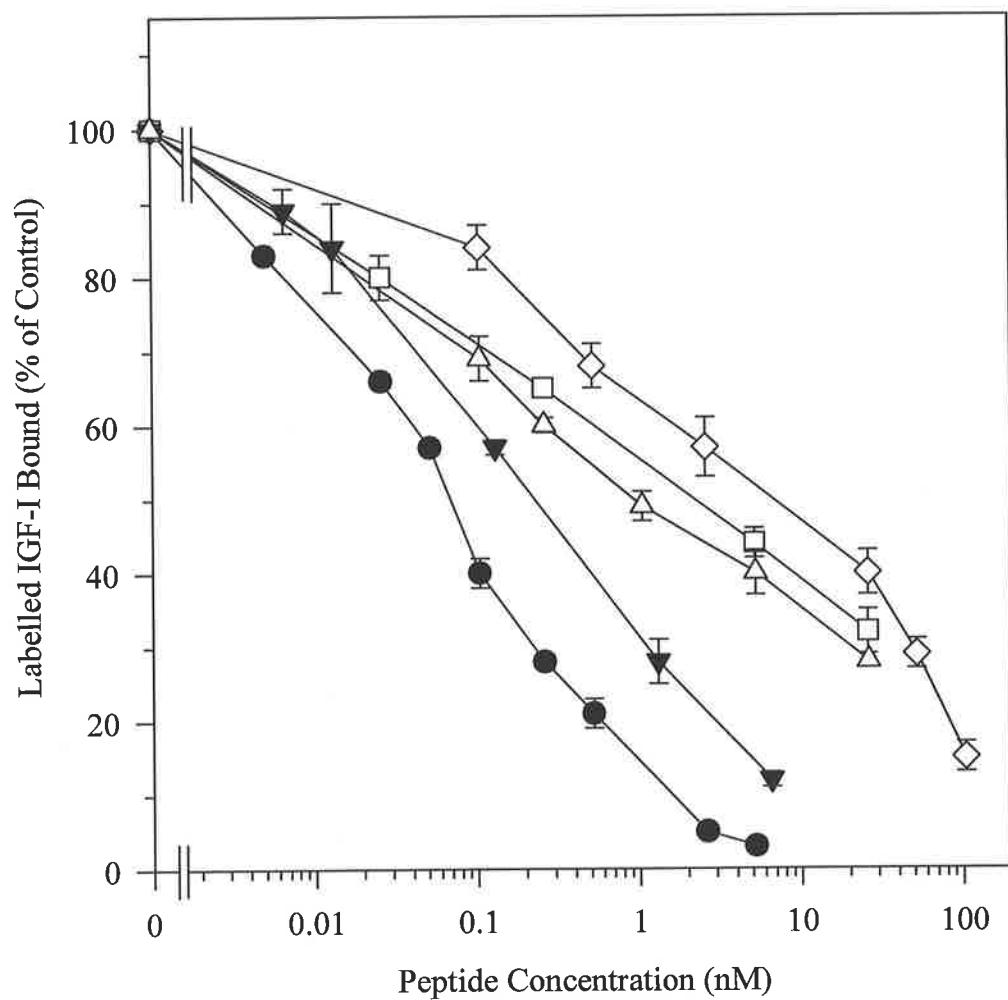
Figure 4.4 Binding of IGF-I analogues to natural human IGFBP-3. Competition of ^{125}I-IGF-I binding by: [Leu[8]] IGF-I (□), [Ser[15]] IGF-I (▼), [Ser[16]] IGF-I (△), [Arg[3]] IGF-I (◇) and IGF-I (●). Values are the means of triplicate determinations at each peptide concentration. S.E.M. values are indicated as error bars.
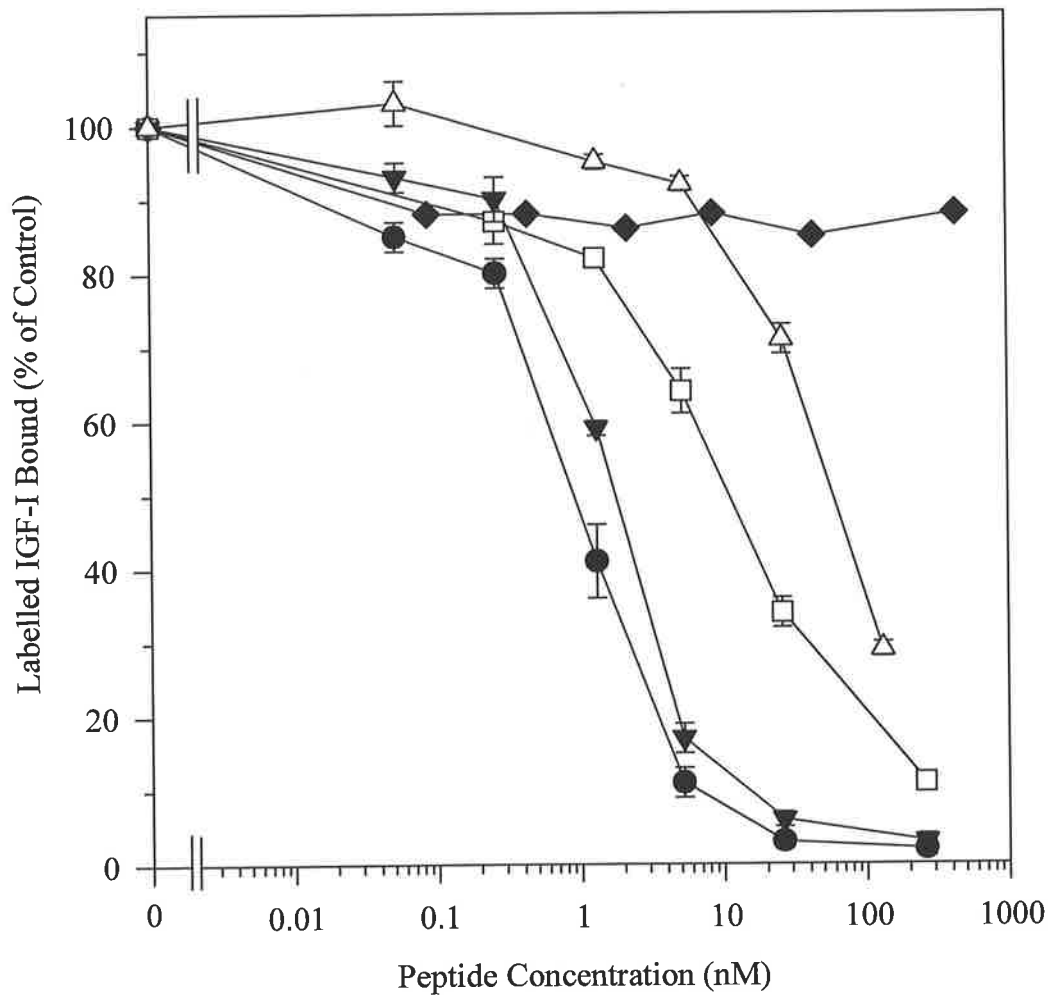
Figure 4.5 Binding of IGF-I analogues to natural human IGFBP-4. Competition of ^{125}I-IGF-I binding by: [Leu[8]] IGF-I (□), [Ser[15]] IGF-I (▼), [Ser[16]] IGF-I (△), LongR[3] IGF-I (◆) and IGF-I (●). Values are the means of triplicate determinations in two independent experiments at each peptide concentration. S.E.M. values are indicated as error bars.
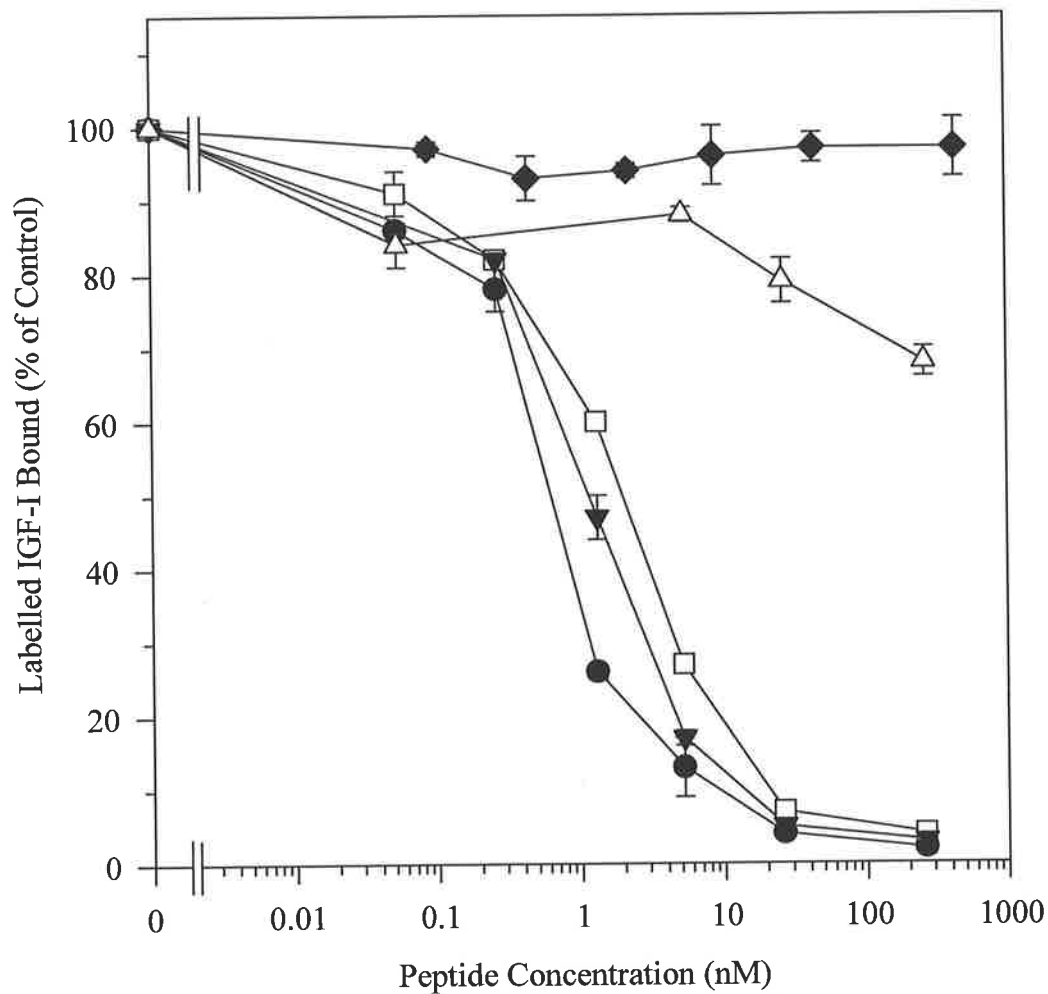
Figure 4.6 Binding of IGF-I analogues to natural human IGFBP-5. Competition of ^{125}I-IGF-I binding by: [Leu[8]] IGF-I (□), [Ser[15]] IGF-I (▼), [Ser[16]] IGF-I (△), LongR[3] IGF-I (◆) and IGF-I (●). Values are the means of triplicate determinations in two independent experiments at each peptide concentration. S.E.M. values are indicated as error bars.
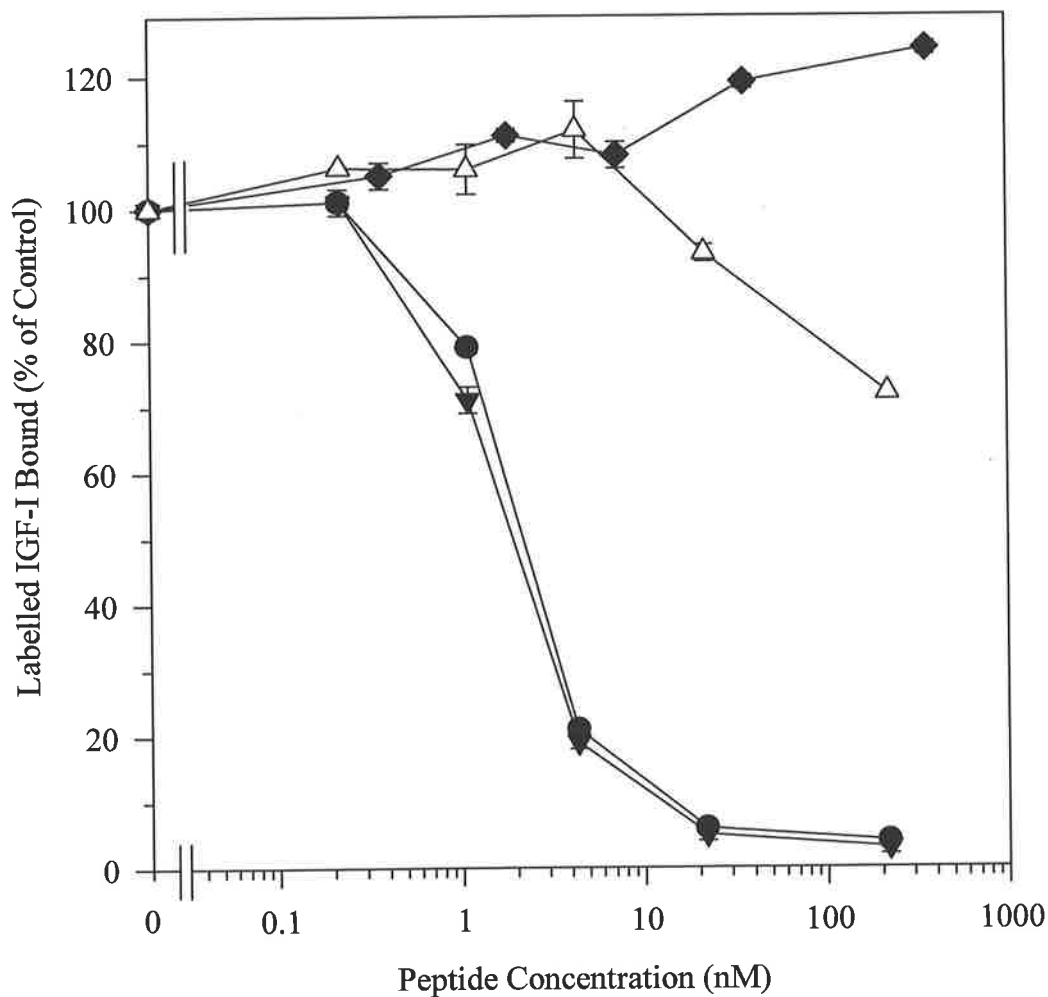
Figure 4.7 Binding of IGF-I analogues to natural human IGFBP-6. Competition of ^{125}I-IGF-I binding by: [Ser^{15}] IGF-I (▼), [Ser^{16}] IGF-I (△), LongR^3 IGF-I (◆) and IGF-I (●). Values are the means of triplicate determinations in two independent experiments at each peptide concentration. S.E.M. values are indicated as error bars.

4.5 Discussion

The extensive structure-function studies of Bayne and others (Bayne *et al.*, 1988; Clemmons *et al.*, 1992) have demonstrated the involvement of the C-terminal region of the IGF-I B-domain α helix in high affinity binding with the IGFBPs. Replacement of residues 15 and 16 using an homologous substitution approach, selectively reduces the affinity of IGF-I for IGFBP-4 and IGFBP-5 by 13- and 100-fold, respectively (Clemmons *et al.*, 1992). Although the resulting analogue, [Tyr^{15}, Leu^{16}] IGF-I, was not structurally characterized, no significant change in IGF type 1 receptor binding and IGFBP-3 binding affinities were observed, suggesting the insulin-like substitution did not affect structural integrity. Subsequently, Ala substitution of Phe^{16} has been shown to induce a significant loss of helical content that results in a 50-fold reduction in IGFBP-1 affinity and a 37-fold reduction in type 1 IGF receptor binding affinity (Jansson *et al.*, 1997). Less significant, but notable deleterious effects were also reported for Glu and Ala substitutions of Gln^{15}. In contrast, far-UV CD spectral analysis for [Ser^{15}] IGF-I (Section 2.4.2) and [Ser^{16}] IGF-I (Magee *et al.*, 1999) suggest that Ser substitutions at these positions are structurally well tolerated.

The conservative substitution of Gln^{15} with Ser was primarily chosen for modulating binding specificity with the insulin and type 1 IGF receptors (see Chapter 3). Unlike Phe^{16}, Gln^{15} and the analogous Tyr B^{16} of insulin are polar residues, peripheral to both the hydrophobic type 1 IGF receptor binding region and the putative hydrophobic IGFBP binding surface of IGF-I (Leu^{5}, Phe^{16} and Leu^{54}). Competitive binding studies show [Ser^{16}] IGF-I has significantly reduced affinity for all IGFBPs while [Ser^{15}] IGF-I has a marginal effect on IGFBP binding affinity relative to IGF-I (Table 4.1). Bayne *et al.* (1988), suggest that the shift of the aromatic ring to a more solvent exposed position in insulin and [Tyr^{15}, Leu^{16}] IGF-I may selectively inhibit association of IGFBP-4 and -5. Our results show that the substitution of Gln^{15} with Ser has no substantial effect on IGFBP association with the greatest relative loss in affinity of 50% observed with IGFBP-4 and -5. This marginal decrease in affinity for IGFBP-4 and -5, coupled with the significant decreases in affinity that these IGFBPs have for [Ser^{16}] IGF-I, suggests that the IGFBP binding characteristics of [Tyr^{15}, Leu^{16}] IGF-I are largely, if not entirely, conferred by the Leu substitution at position 16.

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Substitution of Phe[16] resulted in differential losses in binding affinity across the six IGFBPs studied. This observation is roughly consistent with that reported for [Tyr[15], Leu[16]] IGF-I in that IGFBP-4 and -5 show the greatest loss in affinity (50-fold and >330-fold, respectively). In contrast, [Ser[16]] IGF-I also has significantly lower binding affinities to IGFBP-1, -2 and -3 while the Tyr[15]-Leu[16] substitution does not substantial affect binding to these IGFBPs.

As observed with [Ser[16]] IGF-I, the six IGFBPs responded differentially to the Leu substitution of Ala[8]. IGFBP-2 was most sensitive to substitutions of Ala[8], with an 80-fold reduced affinity to [Leu[8]] IGF-I while this substitution produced a marginal loss (3-fold) in IGFBP-5 binding affinity. The binding affinities to [Leu[8]] IGF-I were surprisingly similar to those observed with [Lys[9]] IGF-I for IGFBPs 1-5 (Magee *et al.*, 1999). The similar effects of these mutants are most obvious for IGFBP-2 where both Leu[8] and Lys[9] substitutions result in a greater reduction in affinity than observed with the substitution of Phe[16]. The comparatively small changes in their association with IGFBP-5 contrasts with previous studies that predict a larger binding pocket and very stringent requirements for IGFBP-5 binding (Clemmons *et al.*, 1992).

While the Leu[8] substitution may introduce functional groups that directly affect IGFBP association, the results may reflect subtle structural alterations in the N-terminus of the α-helix. As discussed in Section 3.5.2, the Leu substitution of Ala[8] could affect the stability of the α-helix. Alternatively, the bulky Leu residue may sterically hinder IGFBP association with closely aligned residues or regions important in this interaction.

Previous studies demonstrate that L6 myoblasts predominantly secrete IGFBP-4 (Gargosky *et al.*, 1990; McCusker *et al.*, 1994). The competitive binding characteristics of [Leu[8]] IGF-I and [Ser[15]] IGF-I for IGFBPs secreted from rat L6 myoblasts has been presented in Section 3.4.3. The analogue [Leu[8]] IGF-I showed lower binding affinity for purified human IGFBP-4 than that predicted by the competitive binding assays using rat L6 myoblast conditioned media. A similar discrepancy between these two assay systems was also observed with Long [Arg[3]] IGF-I. At the highest concentration tested (450nmol/l), Long [Arg[3]] IGF-I showed no competition with labelled IGF-I for purified IGFBP-4. Analysis of this analogue using L6

myoblast conditioned media has demonstrated effective competition at significantly lower concentrations (Francis *et al.*, 1992), while ED_{50} values for IGF-I are roughly consistent across both assays. The discrepancies observed between these assays are possibly the result of differences in salt concentration or pH. Variation in the primary sequences of human (La Tour *et al.*, 1990) and rat (Shimasaki *et al.*, 1990) IGFBP-4 or varying levels of glycosylation may also influence the assay results.

Mutational studies have shown that structural determinants on the B-domain of IGF-I are critical for IGFBP association (Clemmons *et al.*, 1990). The IGF-I B-chain mutant ([Phe^{-1}, Val^1, Asn^2, Gln^3, His^4, Tyr^{15}, Leu^{16}] IGF-I), has greater than 2000-fold lower affinity to serum IGFBPs (predominantly IGFBP-3) relative to IGF-I. The essential role of Glu^3 of IGF-I in high affinity association with IGFBPs has been established through N-terminal truncation studies (Bagley *et al.*, 1989) and mutagenesis (King *et al.*, 1992). The analogue [Arg^3] IGF-I showed 300- and 100- fold lower relative affinity for IGFBP-2 and IGFBP-3 respectively. In contrast, recent biosensor analysis of [Ala^3] IGF-I suggests Ala substitution at this positions has no effect on the binding affinity for immobilized IGFBP-3 (Dubaquié & Lowman, 1999). Furthermore, Ala substitution of Phe^{16}, known to destabilize the B-domain α-helix (Jansson *et al.*, 1997), was also shown to have a marginal effect on IGFBP-3 association as measured by biosensor analysis (Dubaquié & Lowman, 1999). This also contrasts with our observations for [Ser^{16}] IGF-I where a 14-fold relative loss in IGFBP-3 affinity was observed. It is unclear whether these inconsistencies are due to the nature of the amino acid substitutions or methodological differences. Interestingly, despite IGFBP-3 having the highest affinity for IGF-I of all 6 IGFBPs, the extensive alanine scanning studies of Dubaquié & Lowman. (1999), do not identify critical residues involved in this interaction. This led the authors to propose that the IGF-I main chain backbone is probably involved in the interaction with IGFBP-3. Our soluble IGFBP assays demonstrate a greater than 100-fold difference in IGF-I binding affinity between IGFBP-3 and IGFBP-1. In contrast, a 10-fold difference was observed for biosensor analysis of IGF-I affinity between immobilized IGFBP-3 and IGFBP-1 (Dubaquié & Lowman, 1999). These observations suggest significant differences in the behavior of immobilized IGFBP-3 and the soluble form.

Chapter 5

Discussion

At the commencement of this Ph.D. research project in 1992, there was general confusion regarding the specific differences between the IGF-I and insulin signaling pathways. A lot of debate has centered on whether the metabolic responses of IGF-I are mediated through the type 1 IGF receptor or due to the cross-reactivity with the insulin receptor. Similarly, it was unclear whether the mitogenic responses stimulated by insulin were mediated through the insulin receptor or the type 1 IGF receptor. Furthermore, the lack of evidence for distinct differences between the insulin and type 1 IGF receptor signaling systems suggested that these two receptors may be mutually redundant at some level. This led to the proposition that the physiological differences between insulin and IGF-I are largely due to differential receptor expression across various tissue types. The primary aim of this study was to identify IGF-I residues within the proposed receptor binding region that are important in defining ligand-receptor specificity. In particular, my principal goal was to generate IGF-I analogues with selectively reduced affinity for the insulin receptor and therefore enhanced specificity for the type 1 IGF receptor. Such analogues may provide tools for addressing intrinsic signalling differences and similarities between the insulin and IGF type 1 receptors. Similarly, generation of IGF analogues with selective reductions in IGFBP binding affinity has been useful in addressing the physiological role of these proteins in IGF actions. The B-domain IGF-I analogues generated in this study were also included in a larger investigation of the IGFBP binding region of IGF-I.

Since commencing this project, numerous studies have focused on analyzing the contribution of individual amino acids at a protein-protein interface and have led to increased understanding of the principles governing protein-protein interactions (Lo Conte *et al.*, 1999; Bogan & Thorn, 1998). These studies suggest that molecular interactions are characterized by shape and electrostatic complementarity, with the energy involved in protein-protein association being directly related to the burial of surface residues. Long range electrostatic interactions and Brownian dynamics are critical factors affecting the initial process of protein-protein association. These electrostatic interactions increase the time interval that two proteins remain spatially close thus allowing sufficient opportunity

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for rotation into an optimal orientation for binding. A typical interface consists of a small number of centrally located hydrophobic amino acid residues that contribute most of the free energy of binding. In addition to conferring binding specificity, peripheral residues surrounding this central binding core are also important for excluding solvent from key binding residues. Protein unfolding simulations have shown that solvation of hydrophobic residues is critical during the unfolding process (Karplus & Sali, 1995). A similar mechanism possibly operates during the dissociation of a protein-protein complex. It has been proposed that such peripheral residues establish a non-aqueous environment that surrounds essential binding residues thus increasing the interaction affinity by slowing the dissociation rate (Bogan & Thorn, 1998).

In relation to the IGF-I system, mutational work has identified the critical role of Tyr^{24} in type 1 IGF receptor binding (Cascieri *et al.* 1988). A number of residues shared between insulin and IGF-I including Val^{11}, Leu^{14}, Phe^{23}, Ile^{43} and Val^{44} are spatially close to Tyr^{24} and form a small hydrophobic surface (Fig. 3.1). This region may represent the receptor-binding core of IGF-I. In accordance with the general characteristics governing protein-protein interactions, peripheral residues surrounding this region are most likely to contribute to conferring receptor-binding specificity. Previous studies (Cascieri *et al.*, 1988b; Bayne *et al.*, 1989), which had demonstrated the importance of C- and D-domains in conferring receptor-binding affinity, suggest however that additional determinants of IGF-I specificity must lie within the A- and B-domains. This study was largely successful in using evolutionary information contained within sequence homology of IGF-I and insulin for identifying A- and B-domain residues important for conferring receptor-binding specificity. Amino acid substitutions of His^4, Ala^8, Met^{59} and Ala^{62} clearly resulted in modulation of receptor binding specificity. The analogues $[Phe^{59}]$ IGF-I and $[Leu^8]$ IGF-I showed 28-fold and 17-fold respective losses in insulin receptor binding affinity relative to IGF-I, albeit with an associated ~5-fold loss in type 1 IGF receptor binding. In contrast, the Leu substitution of Ala^{62} resulted in a marginal decrease in insulin receptor binding while reducing affinity to the type 1 IGF receptor by 8-fold relative to IGF-I. Substitution of Thr^4 with His (the corresponding amino acid of insulin) demonstrated that this amino acid position may be more important for insulin receptor binding than type 1 IGF receptor binding.

The studies presented in the thesis have identified a number of A- and B-domain positions in IGF-I that are important for maintaining high receptor binding affinity and receptor binding specificity. Moreover, the substitutions made here indicate that the receptor specificity of IGF-I evolved with a number of sequence changes within the B- and A-domains that collectively contribute to the observed receptor binding properties of IGF-I. IGF-I analogues with altered receptor binding specificity may prove useful in addressing the nature of the biological responses elicited through the IGF type 1 receptor and insulin receptor. Our study supports the conclusion that the co-evolution of the IGF-I and insulin receptor/ligand systems has resulted in subtle structural differences in the A- and B-regions of each ligand important for defining receptor binding specificity.

The Ala substitution of positively changed residues within the C- and D-domains demonstrate that residues Arg^{36}, Arg^{37}, Lys^{65} and Lys^{68} contribute to the receptor binding specificity of IGF-I (Zhang *et al.*, 1994). Substitution of Arg residues in the C-domain result in a 15-fold relative loss in type 1 IGF receptor binding affinity while increasing insulin receptor binding by 29-fold. Similarly, D-domain Ala substitutions of the two Lys residues reduces type 1 IGF receptor binding by 10-fold but enhance insulin receptor binding by 6-fold. This study suggests positively charged residues in the C- and D-domains inhibit IGF-I association with the insulin receptor while contributing to the binding affinity for the type 1 IGF receptor. The presence of these positively charged residues may increase the association rate of IGF-I for the type 1 IGF receptor in a similar process to that described for the association between barnase and barstar (Schreiber & Fersht, 1995). Long range electrostatic forces between the positively charged C- and D-domains of IGF-I and complementary elements on the receptor may result in the rapid formation of a weakly specific complex. As described for the barnase-barstar interaction, this might be followed by a more precise high affinity docking between IGF-I and the IGF type 1 receptor involving key binding residues such as Tyr^{24} on the hydrophobic patch (Fig. 3.1). Further evidence for the involvement of the C- and D-domain regions in receptor association is provided by earlier IGF-I peptide analogue studies. Two synthetic IGF-I peptide fragments including amino acid residues 29-37 and 60-69 have been shown to inhibit phosphorylation of the type 1 IGF receptor and growth of several cell lines (Pietrzkowski *et al.*, 1992). These two peptides may antagonize IGF-I by blocking important negatively charged binding

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elements on the type 1 IGF receptor. The presence of Tyr residues in each peptide, corresponding to Tyr^{31} and Tyr^{60} of IGF-I, may also contribute to the antagonism observed for these peptides.

Hodgson *et al.*, (1995) have reported two IGF-I analogues with enhanced specificity for the type 1 IGF receptor without altering receptor binding affinity or biological potency. The analogues [Phe^{60}] IGF-I and [Ile^{11}] IGF-I were shown to selectively decrease insulin receptor binding relative to IGF-I by 2.6- and 3.8-fold respectively. In contrast to the positions targeted for substitution in this study, Tyr^{60} and Leu^{11} are highly conserved across all insulin and IGF peptides. More recent studies show that the disulphide-swapped isomer of IGF-I has a ~200-fold lower affinity for the insulin receptor than native IGF-I while reducing type 1 IGF receptor binding affinity by 30-fold (Gill *et al.*, 1999). This result suggests that the internal rearrangement of IGF-I caused by mis-matched disulfide bonding is more disruptive to insulin receptor binding regions than to those involved in type 1 IGF receptor binding. Modeling of this IGF isomer led the authors to suggest that displacement of the C-region may hinder receptor interaction with Val^{44} which they propose to be more important for insulin receptor binding than type 1 IGF receptor binding. Like Tyr^{60} and Leu^{11}, Val^{44} is highly conserved across different insulin species. These studies suggest that highly conserved residues in addition to divergent residues peripheral to the receptor binding region contribute to the binding specificity of IGF-I.

Before commencing this study the prevailing view of IGF-I receptor binding and subsequent signaling was that biological activity is directly related to receptor binding affinity. This was supported by numerous IGF-I analogue studies where reduced receptor binding affinity was shown to produce a comparable reduction in the *in vitro* biological potency. The only exceptions to this general rule were IGF-I analogues with additional losses in binding affinity for the IGFBPs. It has been established that the high biological potencies of these analogues is the result of increased availability of free IGF-I for cell surface receptors (Clemmons *et al.*, 1990; King *et al.*, 1992; Francis *et al.*, 1992). To the best of our knowledge, [Leu^{8}] IGF-I, [Phe^{59}] IGF-I, [Leu^{62}] IGF-I and [Ser^{15}, Leu^{62}] IGF-I represent the first examples of IGF-I analogues where the observed biological potencies do not correlate with receptor binding affinity and IGFBP binding properties. The more extensive studies of insulin analogues suggests that there is no simple relationship between equilibrium

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binding and the biological response in the analogous insulin system. Insulin analogues with enhanced mitogenic potency relative to binding affinity have a slower dissociation rate from the insulin receptor. Indeed, the mitogenic response of these insulin analogues is related to the residence time of the ligand-receptor interaction rather than the equilibrium receptor binding affinity (De Meyts, 1994). This enhancement of mitogenic potency has been suggested to result from sustained activation of the insulin receptor tyrosine kinase domain and sustained phosphorylation of the Shc signaling protein (Hansen *et al.*, 1996).

It is plausible that slower dissociation rates from the IGF type 1 receptor may account for the higher than expected mitogenic potencies of [Leu^8] IGF-I, [Phe^{59}] IGF-I and [Leu^{62}] IGF-I. Although the kinetic binding properties of these analogues were not assessed in this study, subsequent biosensor analysis of the receptor binding kinetics for the double mutant [Ser^{15}, Leu^{62}] IGF-I has been assessed (personal communication with Dr. Briony Forbes, Department of Biochemistry, Adelaide University). As predicted, the dissociation rate of [Ser^{15}, Leu^{62}] IGF-I was significantly reduced relative to native IGF-I while its association rate was only marginally reduced. These analogues may therefore prolong the half-life of the ligand-receptor complex in a parallel manner to that described for super-active insulins by sustaining receptor activation. These findings suggest that, like the insulin receptor, residence time rather than equilibrium binding affinity may also be an important factor in determining the magnitude of the mitogenic response from the type 1 IGF receptor. At the molecular level, substitutions peripheral to the binding core may introduce additional Van de Waals' interactions not present in the native ligand-receptor interface. These additional interactions may prolong the half-life of the ligand-receptor complex by strengthening the non-aqueous barrier surrounding essential binding residues. Jansson *et al.* (1997) have subsequently reported two IGF-I fusion proteins with type 1 IGF receptor and IGFBP binding affinities that do not correlate with mitogenic activity. Surprisingly, the IGF-Z fusion protein was shown to have higher than expected biological potency and only marginally longer receptor residence times. The authors suggest that a significant reduction in association rate may account for the enhanced potency of this IGF-I analogue.

It has become increasingly clear that timing factors for the insulin and IGF-I systems are important determinants for signaling specificity. Theoretical examination of the time-dependent signaling specificity of the insulin receptor suggests that if signaling is transmitted through a single effector, the ligand association kinetics must be slower than the dissociation rate. To account for no known effector molecules with slow kinetic interactions Shymko *et al.*, (1999) proposed a signaling model with two effector molecules. In this model, where two effector molecules must bind coincidentally with the ligand for signaling to occur, at least one of the effectors must have slow binding kinetics relative to insulin. Alternatively, the slow kinetic component could be a signaling event located further down the signaling chain rather than being an effector molecule (Shymko *et al.*, 1999). The mitogenic signaling pathway of the insulin receptor should therefore include an activation step with slow kinetics relative to the residence time of insulin. In contrast, the metabolic pathway should involve components with rapid kinetic interactions with the receptor. Blakesley *et al.* (1996) have proposed that the transient nature of the phosphorylated insulin receptor may preferentially activate metabolic pathways while any effector molecules that induce or stabilize phosphorylation of the receptor may activate mitogenic signaling pathways. These authors also suggest that v-Src may play an important role in defining intrinsic signaling differences between the insulin and IGF type 1 receptors. While the type 1 IGF receptor is phosphorylated by v-Src, the insulin receptor is largely unaffected (Xu *et al.*, 1995).

It is possible that much of the observed difference between IGF and insulin receptor function can be attributed to the unique interaction of IGFs with the IGFBPs. Interaction of the IGFs with the type 1 IGF receptor requires dissociation of the high affinity IGF-IGFBP complex. These complexes may prevent IGF from cross-reacting with the relatively low affinity insulin receptor. Slow release and presentation of IGFs to the type 1 IGF receptor can be achieved by proteolytic cleavage, dephosphorylation or association of IGFBPs with the plasma membrane, thus enabling additional control upstream of receptor binding and signaling events.

Mutational studies have established the important role of Glu^3 (King *et al.*, 1992; Francis *et al.*, 1992) and Gln^{15}-Phe^{16} (Clemmons *et al.*, 1992) in the high affinity binding between IGF-I and the IGFBPs. As part of a larger study on the involvement of the B-domain α-helix in IGFBP

association, the analogues [Leu^8] IGF-I and [Ser^{15}] IGF-I were assayed against IGFBPs 1-6. This study demonstrated the essential role of Phe^{16} in IGFBP interaction and proposed other closely aligned residues Leu^5 and Leu^{54} may form a hydrophobic IGFBP binding pocket (Fig. 4.1) (Magee *et al.*, 1999). Substitution of Gln^{15} with Ser was shown to have marginal effect on binding affinities for each of the 6 IGFBPs. This observation coupled with the poor IGFBP binding characteristics of the analogue [Ser^{16}] IGF-I (Magee *et al.*, 1999) suggest that the IGFBP binding characteristics of [Tyr^{15}, Leu^{16}] IGF-I (Clemmons *et al.*, 1992) are entirely conferred through the substitution of Phe^{16}. The Leu substitution of Ala^8 resulted in differential reductions in binding affinities across the 6 IGFBPs tested, with surprising similarities to those observed for [Lys^9] IGF-I (Magee *et al.*, 1999). These changes to the N-terminal region of the B-domain α-helix were shown to have greatest impact on IGFBP-2 binding affinity while only subtle reductions in IGFBP-5 binding were observed. These results are in contrast with previous studies (Clemmons *et al.*, 1992) that suggest IGFBP-5 has a larger binding pocket with very stringent requirements for association with IGF-I.

It is becoming increasing clear that the insulin and type 1 IGF receptors are both capable of mediating mitogenic and metabolic responses. This is supported by recent gene knockout studies in mice (Lamothe et al., 1998) that suggest the insulin and type 1 IGF receptors are not fully redundant however, quantitative differences are still lacking. IGF-I treatment of patients with severe insulin resistance is a practical example of this redundancy (Clemmons *et al.*, 2000). To date, no IGF-I analogues with modified receptor binding specificity have been used to gain further insight into the intrinsic differences between the IGF and insulin systems. However, the extensive amount of work directed towards mapping the interactive regions of IGF-I by groups described within this thesis has greatly improved our understanding of the molecular basis of IGF-I actions. We eagerly await structural determination of both IGF-I and insulin receptor complexes to trigger a new era in IGF structure-function studies.

REFERENCES

Abbott, A. M., Bueno, R., Pedrini, M. T., Murray, J. M. & Smith, R. J. (1992). Insulin-like growth factor I receptor gene structure. *Journal of Biological Chemistry* **267**, 10759-10763.

Adamo, M., Roberts, C. T. Jr. & LeRoith, D. (1992). How distinct are the insulin and insulin-like growth factor I signalling systems? *Biofactors* **3**, 151-157.

Adams, T. E., Epa, V. C., Garrett, T. P. J. & Ward, C. W. (2000). Structure and function of the type 1 insulin-like growth factor receptor. *Cellular and Molecular Life Sciences* **57**, 1050–1093.

Adashi, E. Y., Resnick, C. E. & Rosenfeld, R. G. (1990). Insulin-like growth factor I (IGF-I) and IGF-II hormonal action in cultured rat granulosa cells: mediation via type I but not type II IGF receptors. *Endocrinology* **126**, 216-222.

Aitken, A., Jones, D., Soneji, Y. & Howell, S. (1995). 14-3-3 proteins: biological function and domain structure. *Biochemistry Society Transactions* **23**, 605-611.

Albiston, A. L. & Herrington, A. C. (1990). Cloning and characterization of the growth hormone-dependent insulin-like growth factor-binding protein (IGFBP-3) in the rat. *Biochemical and Biophysical Research Communications* **166**, 892-897.

Ando, A., Momomura, K., Tobe, K., Yamamoto-Honda, R., Sakura, H., Tamori, Y., Kaburagi, Y., Koshio, O., Akanuma, Y., Yazaki, Y., Kasuga, M. & Kadowaki, T. (1992). Enhanced insulin induced mitogenesis and mitogen-activated protein kinase activities in mutant insulin receptors with substitution of two COOH-terminal tyrosine autophosphorylation sites by phenylalanine. *Journal of Biological Chemistry* **267**, 12788-12796.

Andress, D. L. (1998). Insulin-like growth factor binding protein-5 (IGFBP-5) stimulates phosphorylation of the IGFBP-5 receptor. *American Journal of Physiology – Endocrinology and Metabolism* **274**, E744-E750.

Andress, D. L. (1995). Heparin modulates the binding of insulin-like growth factor (IGF) binding protein-5 to a membrane protein in oesteoblastic cells. *Journal of Biological Chemistry* **270**, 28289-28296.

Andress, D. L., Loop, S. M., Zapf, F. & Kiefer, M. C. (1993). Carboxy-truncated insulin-like growth factor binding protein-5 stimulated mitogenesis in osteoblast-like cells. *Biochemical and Biophysical Research Communications* **195**, 25-30.

Andress, D. L. & Birnbaum, R. S. (1991). A novel human insulin-like growth factor binding protein secreted by osteoblast-like cells. *Biochemical and Biophysical Research Communications* **176**, 213-218.

Bach, L. A., Hsieh, S., Sanko, K., Fujiwara, H., Perdue, J. F. & Rechler, M. M. (1993). Binding of mutants of human insulin-like growth factor II to insulin-like growth factor binding proteins 1-6. *Journal of Biological Chemistry* **268**, 9246-9254.

Bagley, C. J., May, B. L., Szabo, L., McNamara, P. J., Ross, M., Francis, G. L., Ballard, F. J. & Wallace, J. C. (1989). A key functional role for the insulin-like growth factor 1 N-terminal pentapeptide. *Biochemical Journal* **259**, 665-671.

Baja, M., Blundell, T. L., Hoeuk, T. L., Pitts, J. E., Wood, S. P., Gowan, L. K., Schwabe, C., Wollmer, A., Gliemann, J. & Gammeltoft, S. (1986). Coypu insulin. Primary structure, conformation and biological properties of a hystricomorph rodent insulin. Biochemical Journal **238**, 345-351.

Bala, R. M., Lopatka, J., McCoy, E. & McArthur, R. G. (1981). Serum immunoreative somatomedin levels in normal adults, pregnant woman at term, children at various ages, and children with constitutionally delayed growth *Journal of Clinical Endocrinology and Metabolism* **52**, 508-512.

Ballard, F. J., Tomas, F. M., Read, L. C., Knowles, S. E., Owens, P. C., Lemmey, A. B., Martin, A. A., Wells, J. R. E., Wallace, J. C. & Francis, G. L. (1991). Effects of IGF-I and IGF analogues on growth during catabolic states in rats. In *Modern Concepts of Insulin-like Growth Factors*, pp. 617-627. ED. E. M. Martin. New York (U.S.A.): Elsevier.

Ballard, F. J., Francis, G. L., Ross, M., Bagley, C. J., May, B. & Wallace, J. C. (1987). Natural and synthetic forms of insulin-like growth factor-I (IGF-I) and the potent derivative, destripeptide IGF-I: Biological activities and receptor binding. *Biochemical and Biophysical Research Communications* **149**, 398-404.

Ballard, F. J., Read, L. C., Francis, G. L., Bagley, C. J. & Wallace, J. C. (1986). Binding properties and biological potencies of insulin-like growth factors in L6 myoblasts. *Biochemical Journal* **233**, 223-230.

Bar, R. S., Boes, M., Dake, B. L., Sandra, A., Bayne, M., Cascieri, M. & Booth, B. A. (1990). Tissue localization of perfused endothelial cell IGF binding protein is markedly altered by association with IGF-I. *Endocrinology* **127**, 3243-3245.

Bautista, C., Baylink, D. J. & Mohan, S. (1991). Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: a potential candidate for fixing IGF-II in human bone. *Biochemical and Biophysical Research Communications* **176**, 756-763.

Baxter, R. C. (2000). Insulin-like growth factor (IGF)-binding proteins: interactions with IGFs and intrinsic bioactivities. *American Journal of Physiology – Endocrinology and Metabolism* **278**, E967-E976.

Baxter, R. C. & Skriver, L. (1993). Altered ligand specificity of proteolysed insulin-like growth factor binding protein-3. *Biochemical and Biophysical Research Communications* **196**, 1267-1273.

Baxter, R. C., Bayne, M. L. & Cascieri, M. A. (1992). Structural determinants for binary and tertiary complex formation between insulin-like growth factor-I (IGF-I) and IGF binding protein-3. *Journal of Biological Chemistry* **267**, 60-65.

Baxter, R. C. & Martin, J. L. (1989). Binding proteins for the insulin-like growth factors: structure, regulation and function. *Progress in Growth Factor Research* **1**, 49-68.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Miller, R. E. & Cascieri, M. A. (1990). The roles of tyrosines 24, 31and 60 in the high affinity binding of insulin-like growth factor I to the type 1 insulin-like growth factor receptor. *Journal of Biological Chemistry* **265**, 15648-15652.

Bayne, M. L., Applebaum, J., Underwood, D., Chicchi, G. G., Green, B. G., Hayes, N. S., & Cascieri, M. A. (1989). The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type 1 IGF receptor. *Journal of Biological Chemistry* **264**, 11004-11008.

Bayne, M. L., Applebaum, J., Chicchi, G. G., Hayes, N. S., Green, B. G. & Cascieri, M. A. (1988). Structural analogues of human insulin-like growth factor I with reduced affinity for serum binding proteins and the type 2 insulin-like growth factor receptor. *Journal of Biological Chemistry* **263**, 6233-6239.

Behringer, R. R., Lewin, T. M., Quaife, C. J., Palmiter, R. D. & Brinster, R. L. (1990). Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. *Endocrinology* **127**, 1033-1040.

Bell, G. I., Gerhard, D. S., Fong, N. M., Sanchez-Pescador, R. & Rall, L. B. (1985). Isolation of the human insulin-like growth factor genes: insulin-like growth factor -II and insulin genes are contiguous. *Proceedings of the National Academy of Sciences U.S.A.* **82**, 6450-6454.

Bell, G. I., Stempien, M. M., Fong, N. M. & Rall, L. B. (1986). Sequences of liver cDNAs encoding two different mouse insulin-like growth factor-I precursors. *Nucleic Acids Research* **14**, 7873-7882.

Bell, G. I., Stempien, M. M., Fong, N. M. & Seino, S. (1990). Sequence of cDNA encoding guinea pig IGF-I. *Nucleic Acids Research* **18**, 4275.

Beukers, M. W., Youngman, O., Zhang, H., Ling, N. & Rosenfeld, R. G. (1991). (Leu^{27}) insulin-like growth factor II is highly selective for the type 2 IGF receptor in binding crosslinking and thymidine incorporation experiments. *Endocrinology* **128**, 1201-1203.

Binkert, C., Landwehr, J., Mary, J. L., Schwander, J. & Heinrich, G. (1989). Cloning, sequence analysis and expression of a cDNA encoding a novel insulin-like growth factor binding protein (IGFBP-2). *EMBO Journal* **8**, 2497-2502.

Blakesley, V. A., Scrimgeour, A., Esposito, D. & Le Roith, D. (1996). Signaling via the insulin-like growth factor-I receptor: Does it differ from insulin receptor signaling? *Cytokine & Growth Factor Reviews* **7**, 153-159.

Blum, W. F., Ranke, M. B. & Bierich, J. R. (1986). Isolation and partial characterization of six somatomedin-like peptides from human plasma Cohn fraction IV. *Acta Endocrinologica* **111**, 271-284.

Blundell, T. L., Bedarkar, S., Rinderknecht, E. & Humbel, R. E. (1978). Insulin-like growth factor: a model for tertiary structure accounting for immunoreactivity and receptor binding. *Proceedings of the National Academy of Sciences, U.S.A.* **75**, 180-184.

Blundell, T. L., Bedarkar, S. & Humbel, R E. (1983). Tertiary structures, receptor binding, and antigenicity of insulin-like growth factors. In *Polypeptide growth factors and the regulation of cell growth and differentiation. Federation Proceedings* **42**, 2592-2597.

Bogan, A. A. & Thorn, K. S. (1998). Anatomy of hot spots in protein interfaces. *Journal of Molecular Biology* **280**, 1-9.

Bornfeldt, K. E., Gidlöf, R. A., Wasteson, A., Lake, M., Skottner, A. & Arnqvist, H. J. (1991). Binding and biological effects of insulin, insulin analogues and insulin-like growth factors in rat aortic smooth muscle cells. Comparison of maximal growth promoting activities. *Diabetologia* **34**, 307-313.

Bortz, J. D., Rotwein, P., DeVol, D., Bechtel, P. J., Hansen, V. A. & Hammerman, M. R. (1988). Focal expression of insulin-like growth factor I in rat kidney collecting duct. *Journal of Cellular Biology* **107**, 811-819.

Brandenburg, D., Biela, M., Herbertz, L. & Zahn, H. (1975). Partial synthesis and properties of des-A1-glycine-insulin. *Hoppe Seylers Z Physiol Chem* **356**, 961-979.

Brange, J., Ribel, U., Hansen, J. F., Dodson, G., Hansen, M. T., Havelund, S., Melberg, S. G., Norris, F., Norris, K. & Snel, L. (1988). Monomeric insulins obtained by protein engineering and their medical implications. *Nature* **333**, 679-682.

Braulke, T., Causin, C., Waheed, A., Junghans, U., Hasilik, A., Maly, P., Humbel, R. E. & von Figura, K. (1988). Mannose-6-phosphate/insulin-like growth factor II receptor: distinct binding sites for mannose 6-phosphate and insulin-like growth factor II. *Biochemical and Biophysical Research Communications* **150**, 1287-1293.

Brewer, M. T., Stetler, G. L., Squires, C. H., Thompson, R. C., Busby, W. H. & Clemmons, D. R. (1988). Cloning, characterization and expression of a human insulin-like growth factor binding protein. *Biochemical and Biophysical Research Communications* **152**, 1289-1297.

Brissenden, J. E., Ullrich, A & Francke, U. (1984). Human chromosomal mapping of the gene for insulin-like growth factors I and II and epidermal growth factor. *Nature* **310**, 781-784.

Brown, H., Sanger, F., & Kitai, R. (1955). The structure of sheep and pig insulins. *Biochemical Journal* **60**, 556-565.

Brown, A. L., Chiariotti, L., Orloski, C. C., Mehlman, T., Burgess, W. H., Ackerman, E. J., Bruni, C. B. & Rechler, M. M. (1989). Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors. *Journal of Biological Chemistry* **264**, 5148.

Buck, M. A., Olah, T. A., Weitzmann, C.J & Cooperman, B. S. (1989). Protein estimation by the product of integrated peak area and flow rate. *Analytical Biochemistry* **182**, 295-299.

Bürgisser, D. M., Roth, D. V., Giger, R., Lüthi, C., Weigl, S., Zarn, J. & Humbel, R. E. (1991). Mutants of human insulin-like growth factor II with altered affinities for the type 1 and type 2 insulin-like growth factor receptor. *Journal of Biological Chemistry* **266**, 1029-1033.

Butler, A. A., Yakar, S., Gewolb, I. H., Karas, M., Okubo, Y. & LeRoith, D. (1998). Insulin-like growth factor-I receptor signal transduction: at the interface between physiology and cell biology. *Comparative Biochemistry and Physiology Part B* **121**, 19-26.

Canalis, E., Centrella, M. & McCarthy, T. L. (1991). Regulation of insulin-like growth factor-II production in bone cell cultures. *Endocrinology* **129**, 2457-2462.

Canova-Davis, E., Eng, M., Mukku, V., Reifsnyder, D. H., Olson, C. V. & Ling, V. T. (1992). Chemical heterogeneity as a result of hydroxylamine cleavage of a fusion protein of human insulin-like growth factor I. *Biochemical Journal* **285**, 207-213.

Cara, J. F., Mirmira, R. G., Nakagawa, S. H. & Tager, H. W. (1990). An insulin-like growth factor I / insulin hybrid exhibiting high potency for interaction with the type I insulin-like growth factor and insulin receptors of placental plasma membranes. *Journal of Biological Chemistry* **265**, 17820-17825.

Carlsson-Skwirut, C., Jornvall, H., Holmgren, C., Andersson, C., Berman, T., Lundquist, G., Sjogren, B. & Sara, V. (1986). Isolation and characterization of variant IGF-I and IGF-II from adult human brain. *FEBS Letters* **201**, 46-50.

Cascieri, M. A., Chicchi, G. G., Applebaum, J., Green, B. G., Hayes, N. S. & Bayne, M. L. (1989). Structural analogs of human insulin-like growth factor (IGF) I with altered affinity for type 2 IGF receptors. *Journal of Biological Chemistry* **264**, 2199-2202.

Cascieri, M. A., Saperstein, R., Hayes, N. S., Green, B. G., Chicchi, G. G., Applebaum, J. & Bayne M. L. (1988a). Serum half-life and biological activity of mutants of human insulin-like growth factor I which do not bind to serum binding proteins. *Endocrinology* **123**, 373-381.

Cascieri, M. A., Chicchi, G. G., Applebaum, J., Hayes, N. S., Green, B. G. & Bayne, M. L. (1988b). Mutants of human insulin-like growth factor I with reduced affinity for the type 1 insulin-like growth factor receptor. *Biochemistry* **27**, 3228-3233.

Cascieri, M. A., Chicchi, G. G., Hayes, N. S.& Strader, C. D. (1986) [Thr-59]-insulin-like growth factor I stimulates 2-deoxyglucose transport in BC3H1 myocytes through the insulin-like growth factor receptor, not the insulin receptor. *Biochimica Biophysica Acta* **886**, 491-499.

Causin, C., Waheed, A., Braulke, T., Junghans, U., Maly, P. & Humbel, R. E. (1988). Mannose-6-phosphate/insulin-like growth factor-II binding proteins in human serum and urine. *Biochemical Journal* **252**, 795-799.

Chaiken, R. L., Moses, A. C., Usher, P. & Flier, J. S. (1986). Insulin stimulation of aminoisobutyric acid transport in human skin fibroblasts is mediated through both insulin and type 1 insulin-like growth factor receptors. *Journal of Clinical Endocrinology and Metabolism* **63**, 1181-1184.

Chakrabartty, A. & Baldwin, R. L. (1995) Stability of alpha-helices. *Advances in Protein Chemistry* **46**, 141-176.

Chan, S. J., Cao, Q. P. & Steiner, D. F. (1990) Evolution of the insulin superfamily: cloning of a hybrid insulin/insulin-like growth factor cDNA from amphioxus. *Proceedings of the National Academy of Science U.S.A.* **87**, 9319-9323.

Chochinov, R. H., Mariz, I. K., Hajek, A. S. & Daughaday, W. H. (1977). Characterization of a protein in mid-term human amniotic fluid which reacts in the somatomedin C radio-receptor assay. *Journal of Clinical Endocrinology and Metabolism* **44**, 902-908.

Chou, C. K., Dull, T. J., Russell, D. S., Gherzi, R., Lebwohl, D., Ullrich, A. & Rosen, O. R. (1987). Human insulin receptors mutated at the ATP-binding site lack protein kinase activity and fail to mediate postreceptor effects of insulin. *Journal of Biological Chemistry* **262**, 1842-1847.

Chou, P. Y. & Fasman, G. D. (1974). Prediction of protein confirmation. *Biochemistry* **13**, 222-245.

Chu, Y. C., Wang, R. Y., Burke, G. T., Chanley, J. D. & Katsoyannis, P. G. (1987). Possible involvement of the A20-A21 peptide bond in the expression of the biological activity of insulin. 1. [21-Desasparagine,20-cysteinamide-A]insulin and [21-desasparagine,20-cysteine isopropylamide-A]insulin. *Biochemistry* **26**, 6966-6971.

Clark, J. L. & Steiner D. F. (1969). Insulin biosynthesis in the rat: demonstration of two proinsulins. *Proceedings of the National Academy of Science U.S.A.* **62**, 278-285.

Clackson, T. & Wells, J. A. (1995). A hot spot of binding energy in a hormone-receptor interface. *Science* **267**, 383-386.

Claustres, M., Chatelain, P. & Sultan, C. (1987). Insulin-like growth factor-I stimulates human erythroid colony formation *in vitro*. *Journal of Clinical Endocrinology and Metabolism* **65**, 78-82.

Clemmons, D. R., Moses, A. C., McKay, M. J., Sommer, A., Rosen, D. M. & Ruckle, J. (2000). The combination of insulin-like growth factor I and insulin-like growth factor-binding protein-3 reduces insulin requirements in insulin-dependent type 1 diabetes: evidence for in vivo biological activity. *Journal of Clinical Endocrinology and Metabolism* **85**, 1518-1524.

Clemmons, D. R., Dehoff, M. L., Busby, W. H., Bayne, M. L. & Cascieri, M. A. (1992). Competition for binding to insulin-like growth factor (IGF) binding protein-2, 3, 4, and 5 by IGFs and IGF analogs. *Endocrinology* **131**, 890-895.

Clemmons, D. R., Cascieri, M. A., Camacho-Hubner, C., McCusker, R. H. & Bayne, M. L. (1990). Discrete alterations of the insulin-like growth factor I molecule which alter its affinity for insulin-like growth factor-binding proteins result in changes in bioactivity. *Journal of Biological Chemistry* **265**, 12210-12216.

Clemmons, D. R., Klibanski, A., Underwood, L. E., McArthur, J. W., Ridgway, E. C., Beitnis, I. Z. & Van Wyk, J. J. (1981). Reduction of plasma immunoreactive somatomedin C during fasting in humans. *Journal of Clinical Endocrinology and Metabolism* **53**, 1247-1250.

Clemmons, D. R., Underwood, L. E., Ridgway, E. C., Kliman, B., Kjellberg, R. N. & Van Wyk, J. J. (1980). Estradiol treatment of acromegaly. Reduction of immunoreactive somatomedin C and improvement of metabolic status. *American Journal of Medicine* **69**, 571-575.

Cooke, R. M., Harvey, T. S. & Campbell, I. D. (1991). Solution structure of human insulin-like growth factor 1: a nuclear magnetic resonance and restrained molecular dynamics study. Biochemistry **30**, 5484-5491.

Congote, L. F. & Esch, F. (1987). The major erythrotropin of bovine Cohn fraction V has the N-terminal sequence of insulin-like growth factor II with isoleucine at position 35. *Biochemical and Biophysical Research Communications* **149**, 1026-1032.

Conover, C. A (1992). Potentiation of insulin-like growth factor (IGF) action by IGF-binding protein-3: studies of underlying mechanism. *Endocrinology* **130**, 3191-3199.

Conover, C. A., Ronk, M., Lombana, F. & Powell, D. R. (1990). Structural and biological characterization of bovine insulin-like growth factor-binding protein-3. *Endocrinology* **127**, 2795-2803.

Conover, C. A., Liu, F., Powell, D., Rosenfeld, R. G. & Hintz, R. L. (1988). Insulin-like growth factor binding proteins from cultured human fibroblasts. Characterization and hormonal regulation. *Journal of Clinical Investigation* **83**, 852-850.

Conover, C. A., Misra, P., Hintz, R. L. & Rosenfeld, R. G. (1986). Effect of an anti-insulin-like growth factor I receptor antibody on insulin-like growth factor II stimulation of DNA synthesis in human fibroblasts. *Biochemical and Biophysical Research Communications* **139**, 501-508.

Daughaday, W. H. & Reeder, C. (1966). Synchonous activation of DNA synthesis in hypophysectomized rat cartilage by growth hormone. *Journal of Laboratory and Clinical Medicine* **68**, 357-368.

Daughaday, W. H., Hall, K., Raben, M.S., Salmon, W. D. Jr., Van den Brande, J. L. & Van Wky, J. J. (1972). Somatomedin: Proposed designation for sulphation factor. *Nature* **235**, 107.

Daughaday, W. H. & Trivedi, B. (1987). Absence of serum growth hormone binding protein in patients with growth hormone receptor deficiency (Laron dwarfism). *Proceedings of the National Academy of Sciences U.S.A.* **84**, 4636-4640.

Daughaday, W. H. & Rotwein, P. (1989). Insulin-like growth factors. Peptide, messenger nucleic acid and gene structures, serum and tissue concentrations. *Endocrine Reviews* **10**, 68-91.

De Chiara, T. M., Efstratiadis, A. & Robertson, E. J. (1990). A growth deficiency phenotype in heterzygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* **345**, 78-80.

De Chiara, T. M., Robertson, E. J. & Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell* **64**, 849-859.

De Meyts, P. (1994). The structural basis of insulin and insulin-like growth factor-I receptor binding and negative co-operativity, and its relevance to mitogenic versus metabolic signalling. *Diabetologia* **37**, S135-S148.

Derewenda, U. & Dodson, G. G. (1993) The structure and sequence of insulin. in *Molecular Structures in Biology* (Diamond, R., Koetzle, T. F., Pout, K. & Richardson, J. S., Eds.) pp 260-277, Oxford University Press, Oxford U.K.

Derewenda, U., Derewenda, Z., Dodson, E. J., Dodson, G. G., Bing, X. & Markussen, J. (1991). X-ray analysis of the single chain B29-A1 peptide-linked insulin molecule. *Journal of Molecular Biology* **220**, 425-433.

DeVroede, M. A., Rechler, M. M., Nissley, S. P., Joshi, S., Burke, G. T. & Katsoyannis, P. G. (1985). Hybrid molecules containing the B-domain of insulin-like growth factor I are recognized by carrier proteins of the growth factor. *Proceedings of the National Academy of Sciences U.S.A.* **82**, 3010-3014.

Dodson, E. J., Dodson, G. G., Hodgkin, D. C. & Reynolds, C. D. (1979). Structural relationships in the two-zinc insulin hexamer. *Canadian Journal of Biochemistry* **57**, 467-479.

Dubaquie, Y. & Lowman, H. B. (1999). Total alanine-scanning mutagenesis of insulin-like growth factor I (IGF-I) identifies differential binding epitopes for IGFBP-1 and IGFBP-3. *Biochemistry* **38**, 6386-6396.

Dulak, N.C. & Temin, H. M. (1973). A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts. *Journal of Cellular Physiology* **81**, 153-160.

Dull, T. J., Gray, A., Hayflick, J. S. & Ullrich, A. (1984). Insulin-like growth factor II precursor gene organization in relation to insulin gene family. *Nature* **310**, 777-781.

Ebina, Y., Araki, E., Taira, M., Shimada, F., Mori, M., Craik, C. S., Siddle, K., Pierce, S. B., Roth, R. A. & Rutter, W. J. (1987). Replacement of lysine residue 1030 in the putative ATP-binding region of the insulin receptor abolishes insulin- and antibody-stimulated glucose uptake and receptor kinase activity. *Proceedings of the National Academy of Sciences U.S.A.* **84**, 704-708.

Emler, C. A. & Schalch, S. S. (1987). Nutritionally induced changes in hepatic insulin-like growth factor-I (IGF-I) gene expression in rats. *Endocrinology* **120**, 832-834.

Firth, S. M., Ganeshprasad, U. & Baxter, R. C. (1998). Structural determinants of ligand and cell-surface binding of insulin-like growth factor binding protein-3. *Journal of Biological Chemistry* **273**, 2631-2638.

Flier, J. S., Usher, P. & Moses, A. C. (1986). Monoclonal antibody to the type 1 insulin-like growth factor receptor blocks IGF-I receptor-mediated DNA synthesis: clarification of the mitogenic mechanisms of IGF-I and insulin in human skin fibroblasts. *Proceedings of the National Academy of Sciences U.S.A.* **83**, 664-668.

Florini, J. R. & Magri, K. A. (1989). Effects of growth factors on myogenic differentiation. *American Journal of Physiology* **256**, C701-C711.

Forbes, B., Ballard, F. J. & Wallace, J. C. (1990). An insulin-like growth factor-binding protein purified from medium conditioned by a human lung fibroblast cell line (He[39]L) has a novel N-terminal sequence. *Journal of Endocrinology* **124**, 2289-2296.

Forbes, B., Szabo, L., Baxter, R. C., Ballard, F. J. & Wallace, J. C. (1988). Classification of the insulin-like growth factor binding proteins into three distinct binding categories according to their binding specificities. *Biochemical and Biophysical Research Communications* **157**, 196-202.

Forsberg, G., Palm, G., Ekebacke, A., Josephson, S. & Hartmanis, M. (1990). Separation and characterization of modified variants of recombinant human insulin-like growth factor I derived from a fusion protein secreted from *Escherichia coli*. *Biochemical Journal* **271**, 357-363.

Forsberg, G., Baastrup, B., Brobjer, M., Lake, M., Jornvall, H. & Hartmanis, M. (1989). Comparison of two cleavage methods for preparation of a truncated form of recombinant human insulin-like growth factor I from a secreted fusion protein. *BioFactors* **2**, 105-112.

Francis, G. L., Ross, M., Ballard, F. J., Milner, S. J., Senn, C., McNeil, K. A., Wallace, J. C., King, R. & Wells, J. R. E. (1992). Novel recombinant fusion protein analogues of insulin-like growth factor (IGF)-I indicate the relative importance of IGF-binding protein and receptor binding for enhanced biological potency. *Journal of Molecular Endocrinology* **8**, 213-223.

Francis, G. L., Read, L. C., Ballard, F. J., Bagley, C. J., Upton, F. M., Gravestock, P. M. & Wallace, J. C. (1986). Purification and partial sequence analysis of insulin-like growth factor-I from bovine colostrum. *Biochemical Journal* **233**, 207-213.

Francis, G. L., Upton, F. M., Ballard, F. J., McNeil, K. A. & Wallace, J. C. (1988). Insulin-like growth factors 1 and 2 in bovine colostrum: Sequences and biological activities compared with those of a potent truncated form. *Biochemical Journal* **251**, 95-103.

Francis, G. L., McNeil, K. A., Wallace, J. C., Ballard, F. J. & Owens, P. C. (1989a). Sheep insulin-like growth factors I and II: sequences, activities and assays. *Endocrinology* **124**, 1173-1183.

Francis, G. L., Owens, P. C., McNeil, K. A., Wallace, J. C. & Ballard, F. J. (1989b). Purification, amino acid sequences and assay cross-reactivities of porcine insulin-like growth factor - I and -II. *Journal of Endocrinology* **122**, 681-687.

Froesch, E. R., Schmid, C., Schwander, J. and Zapf, J. (1985). Actions of insulin-like growth factors. *Annual Review of Physiology* **47**, 443-467.

Froesch, E. R., Burgi, H., Ramsier, E. B., Bally, P. & Labhart, A. (1963). Antibody-suppressible and non-suppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity. *Journal of Clinical Investigation* **42**, 1816-1834.

Froesch, E. R., Zapf, J., Audhya, T. K., Benporath, E., Segen, B. J. & Gibson, K. D. (1976). Nonsuppressible insulin like activity and thyroid hormones: major pituitary-dependent sulphation factors for cartilage. *Proceedings of the National Academy of Science U.S.A.* **73**, 2904-2908.

Frost, J. P. & Linda, T. (1991). Insulin-like growth factor-binding protein-1 is phosphorylated by cultured human endometrial stromal cells and multiple protein kinases in vitro. *Journal of Biological Chemistry* **266**, 18082-18088.

Furlanetto, R. W., Dey, B. R., Lopaczynski, W. & Nissley, S. P. (1997). 14-3-3 proteins interact with the insulin-like growth factor receptor but not the insulin receptor. *Biochemical Journal* **327**, 765-771.

Furlanetto, R. W., DiCarlo, J. N. & Wisehart, C. (1987). The type II insulin-like growth factor receptor does not mediate deoxyribonucleic acid synthesis in human fibroblasts. *Journal of Clinical Endocrinology and Metabolism* **64**, 1142-1149.

Gargosky, S. E., Walton, P. E., Wallace, J. C. & Ballard, F. J. (1990). Insulin-like growth factor-I (IGF-I) and IGF-binding proteins both decline in the rat during late pregnancy. *Journal of Endocrinology* **127**, 383-390

Garrett, T. P. J., McKern, N. M., Lou, M. Z., Frenkel, M. J., Bentley, J. D., Lovrecz, G. O. & Ward, C. W. (1998). Crystal structure of the first three domains of the type-1 insulin-like growth factor receptor. *Nature* **394,** 395-399.

Gelato, M. C., Kiess, W., Lee, L., Malozowski, S., Rechler, M. M. & Nissley, P. (1988). The insulin-like growth factor II/mannose-6-phosphate receptor is present in monkey serum. *Journal of Clinical Endocrinology and Metabolism* **67**, 669-675.

Gill, R., Verma, C., Wallach, B., Urso, B., Pitts, J., Wollmer, A., De Meyts, P. & Wood, S. (1999). Modelling of the disulphide-swapped isomer of human insulin-like growth factor-1: implications for receptor binding. *Protein Engineering* **12,** 297-303.

Gillespie, C., Read, L. C., Bagley, C. J. & Ballard, F. J. (1990). Enhanced potency of truncated insulin-like growth factor-I (des(1-3)IGF-I) relative to IGF-I in lit/lit mice. *Journal of Endocrinology* **127**, 401-405.

Gowan, L. K., Hampton, B., Hill, D. J., Schlueter, R. J. & Perdue, J. F. (1987). Purification and characterization of a unique high molecular weight form of insulin-like growth factor II. *Endocrinology* **121**, 449-458.

Guler, H. P., Zapf, J., Schmid, C. & Froesch, E. R. (1989). Insulin-like growth factors I and II in healthy man. Estimations of half-lives and production rates. *Acta Endocrinologica* **121**, 753-758.

Guler, H. P., Zapf, J. & Froesch, E. R. (1987). Short term metabolic effects of recombinant human insulin-like growth factor I in healthy adults. *New England Journal of Medicine* **317**, 137-140.

Gustafson, T. A. & Rutter, W. J. (1990). The cysteine-rich domains of the insulin-like growth factor I receptors are primary determinants of hormone binding specificity: evidence from receptor chimeras. *Journal of Biological Chemistry* **265**, 18663-18667.

Haig, D. & Graham, C. (1991). Genomic imprinting and the strange case of the insulin-like growth factor II receptor. *Cell* **64**, 1045-1046

Hall, K. & Tally, M. (1989). The somatomedin-insulin-like growth factors. *Journal of International Medicine* **225**, 47-54.

Hall, K., Engberg, G., Hellem, E., Lundin, G., Ottosson-Seeberger, A., Sara, V., Trygstad, O. & Ofverholm, U. (1984). Somatomedin levels in pregnancy: longitudinal study in healthy subjects and patients with growth hormone deficiency. *Journal of Clinical Endocrinology and Metabolism* **59**, 587-594.

Hampton, B., Burgess, W. H., Marshak, D. R., Cullen, K. J. & Perdue, J. F. (1989). Purification and characterization of an insulin-like growth factor II variant from human plasma. *Journal of Biological Chemistry* **264**, 19155-19160.

Hansen, B. F., Danielsen, G. M., Drejer, K., Sørensen, A. R., Wiberg, F. C., Klein, H.L. & Lundemose, A.G. (1996). Sustained signalling from the insulin receptor after stimulation with insulin analogues exhibiting increased mitogenic potency. *Biochemical Journal* **315**, 271-279.

Harper, E. T. & Rose, G. D. (1993). Helix stop signals in proteins and peptides: the capping box. *Biochemistry* **32**, 7605-7609.

Hartmann, H., Meyer-Alber, A. & Braulke, T. (1992). Metabolic actions of insulin-like growth factor II in cultured adult rat hepatocytes are not mediated through the insulin-like growth factor II receptor. *Diabetologia* **35**, 216-223.

Hasselbacher, G. K. & Humbel, R. (1982). Evidence for two species of insulin-like growth factor II (IGF-II and "big"IGF-II) in human spinal fluid. *Endocrinology* **110**, 1822-1824.

Hernandez, E. R., Roberts, C. T. Jr., Le Roith, D. & Adashi, E. Y. (1989). Rat ovarian insulin-like growth factor I (IGF-I) gene expression is granulosa cell specific: 5′ untranslated mRNA variant representation and hormonal regulation. *Endocrinology* **125**, 572-574.

Hernandez, E. R., Roberts, C. T. Jr., Hurwitz, A., Le Roith, D. & Adashi, E. Y. (1990). Rat ovarian insulin-like growth factor II gene expression is theca-interstitial cell-exclusive: hormonal regulation and receptor distribution. *Endocrinology* **127**, 3249-3251.

Hintz, R. & Liu, F. (1977). Demonstration of specific plasma protein binding sites for somatotrophin. *Journal of Clinical Endocrinology and Metabolism* **45**, 988-995.

Hobba, G. D., Lothgren, A., Holmberg, E., Forbes, B. E., Francis, G. L. & Wallace, J. C. (1998). Alanine screening mutagenesis establishes tyrosine 60 of bovine insulin-like growth factor binding protein-2 as a determinant of insulin-like growth factor binding. *Journal of Biological Chemistry* **273**, 19691-19698.

Hober, S., Forsberg, G., Palm, G., Hartmanis, M. & Nilsson, B. (1992). Disulfide exchange folding of Insulin-like Growth Factor I. *Biochemistry* **31**, 1749-1756.

Hodgson, D. R., May, F. E. B. & Westley, B. R. (1995). Mutations at positions 11 and 60 of insulin-like growth factor 1 reveal differences between its interactions with the type 1 insulin-like-growth-factor receptor and the insulin receptor. *European Journal of Biochemistry* **233**, 299-309.

Holly, J. M. P., & Wass, J. A. H. (1989). Insulin-like growth factors: autocrine, paracrine or endocrine? New perspectives of the somatomedin hypothesis in light of recent developments. *Journal of Endocrinology* **122**, 611-618.

Honeggar, A. & Humbel, R. E. (1986). Insulin-like growth factor I and II in fetal and adult bovine serum. *Journal of Biological Chemistry* **261**, 569-575.

Horuk, R., Goodwin, P., O'Connor, K., Neville, R. W. J. & Lazarus N. R. (1979). Evolutionary change in the insulin receptors of hystricomorph rodents. *Nature* **279**, 439-440.

Horuk, R., Blundell, T. L., Lazarus, N. R., Neville, R. W. J., Stone, D. & Wollmer, A. (1980). A monomeric insulin from the porcupine (*Hystrix cristata*) an Old World insulin. *Nature* **286**, 822-824.

Hua, Q. X., Shoelson, S. E., Kochoyan, M. & Weiss, M. A. (1991). Receptor binding redefined by a structural switch in a mutant human insulin. *Nature* **354**, 238-241.

Huhtala, M. -L., Koistinen, R., Palomaki, P., Partanen, P., Bohn, H. & Seppala, M. (1986). Biologically active domain in somatomedin-binding protein. *Biochemical and Biophysical Research Communications* **141**, 263-270.

Hunkapiller, M.W.& Hood, L.E. (1983). Protein sequence analysis: automated micro-sequencing. *Science* **219**, 650-659.

Hwa, V., Oh, Y. & Rosenfeld, R. G. (1999). The insulin-like growth factor binding protein (IGFBP) superfamily. *Endocrine Reviews* **20**, 761-787.

Hynes, M. A., Van Wyk, J. J., Brookes, P. J., D'Ercole, A. J., Jansen, M. & Lund, P. K. (1987). Growth hormone dependence of somatomedin-C/insulin-like growth factor-II messenger ribonucleic acids. *Molecular Endocrinology* **1**, 233-242.

Jacobs, S., Kull, F. C., Earp, H. S., Svoboda, M. E., Van Wyk, J. J. & Cuatrecasas, P. (1983). Somatomedin-C stimulates the phosphorylation of the β subunit of its own receptor. *Journal of Biological Chemistry* **258**, 9581-9884.

Janin, J. & Chothia, C. (1990). The structure of protein-protein recognition sites. *Journal of Biological Chemistry* **265**, 16027-16030.

Jansen, M., Van Schaik, F. M. A., Ricker, A. T., Bullock, B., Woods, D. E., Gabbay, K. H., Nussbaum, A. L., Sussenbach, J. S. & Van der Brande, J. L. (1983). Sequence of cDNA encoding insulin-like growth factor I precursor. *Nature* **306**, 609-611.

Jansson, M., Andersson, G., Uhlén, M., Nilsson, B. & Kördel, J. (1998). The insulin-like growth factor (IGF) binding protein 1 binding epitope on IGF-I probed by heteronuclear NMR spectroscopy and mutational analysis. *Journal of Biological Chemistry* **273**, 24701-24707.

Jansson, M., Uhlen, M. & Nilsson, B. (1997). Structural changes in insulin-like growth factor (IGF) I mutant proteins affecting binding kinetics rates to IGF binding protein 1 and IGF-I receptor. *Biochemistry* **36**, 4108-4117.

Jones, J. I., Gockerman, A., Busby, W. J., Wright, G. & Clemmons, D. R. (1993). Insulin-like growth factor binding protein 1 stimulates cell migration and binds to the alpha 5 beta 1 intergrin by means of its Arg-Gly-Asp sequence. *Proceedings of the National Academy of Science U.S.A.* **90**, 10553-10557.

Jones, J. I., D'Ercole, A. J., Camacho-Hubner, C. & Clemmons, D. R. (1991). Phosphorylation of insulin-like growth factor (IGF)-binding protein 1 in cell culture and in vitro: effects on affinity for IGF-I. *Proceedings of the National Academy of Sciences U.S.A.* **88**, 7481-7485.

Jones, S. & Thornton, J. M. (1996). Principles of protein-protein interactions. *Proceedings of the National Academy of Sciences U.S.A.* **93**, 13-20.

Kadowaki, T., Koyasu, S., Nishida, E., Tobe, K., Izumi, T., Takaku, F., Sakai, H., Yahara, I. & Kasuga, M. (1987). Tyrosine phosphorylation of common and specific sets of cellular proteins rapidly induced by insulin, insulin-like growth factor I, and epidermal growth factor in an intact cell. *Journal of Biological Chemistry* **262**, 7342-7350.

Kalus, W., Zweckstetter, M., Renner, C., Sanchez, Y., Georgescu, J., Grol, M., Demuth, D., Schumacher, R., Dony, C., Lang, K. & Holak, T. A. (1998). Structure of the IGF-binding domain of the insulin-like growth factor binding protein-5 (IGFBP-5): implications for IGF and IGF-I receptor interactions. *EMBO Journal* **17**, 6558-6572.

Karey, K. P., Marquardt, H. & Sirbasku, D. A. (1989). Human platelet-derived mitogens. Identification of insulin-like growth factors I and II by purification and Na α amino acid sequence analysis. *Blood* **74**, 1084-1092.

Karplus, M. & Sali, A. (1995). Theoretical studies of protein folding and unfolding. *Current Opinion in Structural Biology* **5**, 58-73.

Kasuga, M., Van Obberghen, E., Nissley, S. P. & Rechler, M. M. (1981). Demonstration of two subtypes of insulin-like growth factor receptors by affinity crosslinking. *Journal of Biological Chemistry* **256**, 5305-5308.

Keiss, W., Greenstein, L. A., White, R. M., Lee, L., Rechler, M. M. & Nissley, S. P. (1987). Type II insulin-like growth factor receptor is present in rat serum. *Proceedings of the National Academy of Sciences U.S.A.* **84**, 7720-7724.

Kellerer, M., Lammers, R., Ermel, B., Tippmer, S., Vogt, B., Obermaier-Kusser, B., Ullrich, A. & Häring, H-U. (1992). Distinct α-subunit structures of human insulin receptor A and B variants determine differences in tyrosine kinase activities. *Biochemistry* **31**, 4588-4596.

Kiefer, M. C., Masiarz, F. R., Bauer, D. M. & Zapf, J. (1991a). Identification and molecular cloning of two new 30 kDa insulin-like growth factor binding-proteins isolated from adult human serum. *Journal of Biological Chemistry* **266**, 9043-9049.

Kiefer, M. C., Ioh, R. S., Bauer, D. M. & Zapf, J. (1991b). Molecular cloning of a new human insulin-like growth factor binding protein. *Biochemical and Biophysical Research Communications* **176**, 219-225.

Kim, H-S, Nagalla, S. R., Oh, Y., Wilson, E., Roberts, C. T. & Rosenfeld, R. G. (1997). Identification of a family of low-affinity insulin-like growth factor binding proteins (IGFBPs): Characterization of connective tissue growth factor as a member of the IGFBP superfamily. *Proceedings of the National Academy of Science U.S.A.* **94**, 12981-12986.

King, G. L., Kahn, C. R. & Samuels, B. (1982). Synthesis and characterization of molecular hybrids of insulin and insulin-like growth factor I. *Journal of Biological Chemistry* **257**, 10869-10873.

King, G. L. & Kahn, C. R. (1981). Non-parallel evolution of metabolic and growth-promoting functions of insulin. *Nature* **292**, 644-646.

King, R., Wells, J. R. E., Krieg, P., Snoswell, M., Brazier, J., Bagley, C. J., Wallace, J. C., Ballard, F. J., Ross, M. & Francis, G. L. (1992). Production and characterization of recombinant insulin-like growth factor-I (IGF-I) and potent analogues of IGF-I, with Gly or Arg substitutioned for Glu^3, following their expression in *Escherichia coli* as fusion proteins. *Journal of Molecular Endocrinology* **8**, 29-41.

Kjeldsen, T., Andersen, A. S., Wiberg, F. C., Rasmussen, J. S., Schaffer, L., Balschmidt, P., Moller, K. B. & Moller, N. P. H. (1991). The ligand specificities of the insulin receptor and the insulin-like growth factor I receptor reside in different regions of a common binding site. *Proceedings of the National Academy of Sciences U.S.A.* **88**, 4404-4408.

Klapper, D. G., Svoboda, M. E. & Van Wyk, J. J. (1983). Sequence analysis of somatomedin-C: confirmation of identity with insulin-like growth factor I. *Endocrinology* **112**, 2215-2217.

Kobayashi, M., Ohgaku, S., Iwasaki, M., Maegawa, H., Shigeta, Y. & Inouye, K. (1982). Characterization of [Leu^{B-24}]- and [Leu^{B-25}]-insulin analogues: receptor binding and biological activity. *Biochemical Journal* **206**, 597-603.

Koistinen, R., Kalkinen, N., Huhtala, M. L., Seppälä, M., Bohn, H. & Rutanen, E. M. (1986). Placental protein 12 is a decidual protein that binds somatomedin and has an identical N-terminal amino acid sequence with somatomedin binding protein from human amniotic fluid. *Endocrinology* **118**, 1375-1378.

Konishi, Y., Kotts, C. E., Bullock, L. D., Tou, J. S. & Johnson, D. A. (1989). Fragments of bovine insulin-like growth factors I and II stimulate proliferation of rat L6 myoblast cells. *Biochemistry* **28**, 8872-8877.

Koontz, J. W. (1984). The role of the insulin receptor in mediating the insulin-stimulating growth response in Reuber H-35 cells. *Molecular and Cellular Biochemistry* **58**, 139-146.

Laajoki, L. G., Francis, G. L., Wallace, J. C., Francis, G. L., Carver, J. A. & Keniry, M. A. (2000). Solution structure and backbone dynamics of long-[Arg(3)]insulin-like growth factor-I. *Journal of Biological Chemistry* **275**, 10009-10015.

Laajoki, L. G., Breton, E. L., Shooter, G. K., Wallace, J. C., Carver, J. A. & Keniry, M. A. (1997). Secondary structure determination of ^{15}N-labelled human long-[Arg-3]-insulin-like growth factor I by multi-dimensional NMR spectroscopy. *FEBS Letters* **420** 97-102.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lammers, R., Gray, A., Schlessinger, J. & Ullrich, A. (1989). Differential signalling potential of insulin- and IGF-receptor cytoplasmic domains. *EMBO Journal* **8**, 1369-1375.

Lamothe, B., Baudry, A., Desbois, P., Lamotte, L., Bucchini, D., De Meyts, P. & Joshi, R. L. (1998). Genetic engineering in mice: impact on insulin signalling and action. *Biochemical Journal* **335**, 193-204.

La Tour, D., Mohan, S., Linkhart, T. A., Baylink, D. J. & Strong, D. D. (1990). Inhibitory insulin-like growth factor binding protein: cloning, complete sequence and physiological regulation. *Molecular Endocrinology* **4**, 1806-1814.

Laviola, L., Giogino, F., Chow, J. C., Baquero, J. A., Hansen, H., Ooi, J. J. Z., Riedel, H. & Smith, R. J. (1997). The adapter protein Grb10 associates preferentially with the insulin receptor as compared with the IGF-I receptor in mouse fibroblasts. *Journal of Clinical Investigation* **99**, 830-837.

Lee, J., Pilch, P. F., Shoelson, S. E. & Scarlata, S. F. (1997). Conformational changes of the insulin receptor upon insulin binding and activation as monitored by fluorescence spectroscopy. *Biochemistry* **36**, 2701-2708.

Lee, Y. L., Hintz, R. L., James, P. M., Lee, P. D. K., Shively, J. E. & Powell, D. R. (1988). Insulin-like growth factor binding protein complementary deoxyribonucleic acid from human HEP G2 hepatoma cells: Predicted protein sequence suggests an IGF binding domain different from those of the IGF-I and IGF-II receptors. *Molecular Endocrinology* **2**, 404-411.

Lemmey, A. B., Martin, A. A., Read, L. C. Tomas, F. M., Owens, P. C. & Ballard, F. J. (1991). IGF-I and especially the truncated analog des(1-3)IGF-I enhance growth in rats following gut resection. *American Journal of Physiology* **260**, E 213-219.

Levinovitz, A. & Norstedt, G. (1989). Developmental and steroid hormonal regulation of insulin-like growth factor II expression. *Molecular Endocrinology* **3**, 797-804.

Lewis, M. K.& Thompson, D. V. (1990). Efficient site directed in vitro mutagenesis using ampicillin selection. *Nucleic Acids Research* **18**, 3439-3443.

Li, W., Fawcett, J., Widmer, H. R., Fielder, P. J., Rabkin, R. & Keller, G-A. (1997). Nuclear transport of insulin-like growth factor-I and insulin-like growth factor binding protein-3 in opossum kidney cells. *Endocrinology* **138**, 1763-1766.

Lin, T., Wang, D., Nagpal, M. L., Chang, W. & Calkins, J. H. (1992). Down regulation of leydig cell IGF-I gene expression by interleukin-1. *Endocrinology* **130**, 1217-1224.

Lobel, P., Dahms, N. M., Breitmeyer, J., Chirgwin, S. M. & Kornfeld, S. (1987). Cloning of the bovine 215-kDa cation-independent mannose-6-phosphate receptor. *Proceedings of the National Academy of Sciences U.S.A.* **84**, 2233-2237.

Lo Conte, L., Chothia, C. & Janin, J. (1999). The atomic structure of protein-protein recognition sites. *Journal of Molecular Biology* **285**, 2177-2198.

Luo, R. Z. T., Beniac, D. R., Fernandes, A., Yip, C. C. & Ottensmeyer, F. P. (1999). Quaternary structure of the insulin-insulin receptor complex. *Science* **285**, 1077-1080.

Magee, B. A., Shooter, G. K., Wallace, J. C. & Francis, G. L. (1999). Insulin-like growth factor I and its binding proteins: a study of the binding interface using B-domain analogues. *Biochemistry* **38**, 15863-15870.

Maly, P. & Lüthi, C. (1988). The binding sites of insulin-like growth factor I (IGF-I) to type 1 IGF receptor and a monoclonal antibody. *Journal of Biological Chemistry* **263**, 7068-7072.

Marquardt, H., Todara, G. J., Henderson, L. E. & Oroszlan, S. (1981). Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures. *Journal of Biological Chemistry* **256**, 6859-6865.

Martin, J. L., Willetts, K. E. & Baxter, R. C. (1990). Purification and properties of a novel insulin-like growth factor-II binding protein from transformed human fibroblasts. *Journal of Biological Chemistry* **265**, 4124-4130.

Martin, J. L. & Baxter, R. C. (1986). Insulin-like growth factor-binding protein from human plasma. Purification and characterization. *Journal of Biological Chemistry* **261**, 8754-8760.

Massague, J. & Czech, M. P. (1982). The subunit structure of two distinct receptors for insulin-like growth factors I and II and their relationships to insulin receptor. *Journal of Biological Chemistry* **257**, 5038-5045.

Massague, J., Blinderman, L. A. & Czech, M. P. (1982). The high affinity insulin receptor mediates growth stimulation in rat hepatoma cells. *Journal of Biological Chemistry* **257**, 13958-13963.

Mathews, L. S., Norstedt, G. & Palmiter, R. D. (1986). Regulation of insulin-like growth factor I gene expression by growth hormone. *Proceedings of the National Academy of Sciences U.S.A.* **83**, 9343-9347.

Mathews, L. S., Hammer, R. E., Behringer, R. R., D'Ercole, A. J., Bell, G. I., Brinster, R. L. & Palmiter, R. D. (1988). Growth enhancement of transgenic mice expressing human insulin-like growth factor I. *Endocrinology* **123**, 2827-2833.

McClain, D. A., Maegawa, H., Levy, J., Dull, T. J., Lee, J., Ullrich, A. & Olefsky, J. M. (1988). Properties of a human insulin receptor with a C-terminal truncation: insulin binding, autophosphorylation and endocytosis. *Journal of Biological Chemistry* **263**, 8904-8911.

McClain, D. A., Maegawa, H., Lee, J., Dull, T. J., Ullrich, A. & Olefsky, J. M. (1987). A mutant insulin receptor with defective tyrosine kinase displays no biological activity and does not undergo endocytosis. *Journal of Biological Chemistry* **262**, 14663-14671.

McCusker, R. H. & Clemmons, D. R. (1994). Effects of cytokines on insulin-like growth factor-binding protein secretion by muscle cells in vitro. *Endocrinology* **134**, 2095-2102.

Merimee, T. J., Zapf, J. & Froesch, E. R. (1982). Insulin-like growth factors in fed and fasted states. *Journal of Clinical Endocrinology and Metabolism* **55**, 999-1002.

Mesiano, S., Young, I. R., Hey, A. W., Browne, C. A. & Thorburn, G. D. (1989). Hypophysectomy of the fetal lamb leads to a fall in the plasma concentration of insulin-like growth factor I (IGF-I) but not IGF-II. *Endocrinology* **124**, 1485-1491.

Miller, S. B., Martin, D. R., Kissane, J. & Hammerman, M. R. (1992). Insulin-like growth factor I accelerates recovery from ischemic acute tubular necrosis in the rat. *Proceedings of the National Academy of the Sciences U.S.A.* **89**, 11876-11880.

Milner, S. J., Francis, G. L., Wallace, J. C., Magee, B. A. & Ballard, F. J. (1995). Mutations in the B-domain of insulin-like growth factor-I influence the oxidative folding yield products with modified biological properties. *Biochemical Journal* **308**, 865-871.

Mirmira, R. G., Nakagawa, S. H. & Tager, H. S. (1991). Importance of the character and configuration of residues B24, B25, and B26 in insulin-receptor interactions. *Journal of Biological Chemistry* **266**, 1428-1436.

Mirmira, R. G. & Tager, H. S. (1989). Role of the phenylalanine B24 side chain in directing insulin interaction with its receptor. *Journal of Biological Chemistry* **264**, 6349-6354.

Mochizuki, H., Hakeda, Y., Wakatsuki, N., Usui, N., Akashi, S., Sato, T., Tanaka, K. & Kumegawa, M. (1992). Insulin-like growth factor I supports formation and activation of osteoclasts. *Endocrinology* **131**, 1075-1080.

Mohan, S., Nakao, Y., Honda, Y., Landale, E., Leser, U., Dony, C., Lang, K. & Baylink, D. J. (1995). Studies on the mechanisms by which insulin-like growth factor (IGF) binding protein-4 (IGFBP-4) and IGFBP-5 modulate IGF actions in bone cells. *Journal of Biological Chemistry* **270**, 20424-20431.

Mohan, S., Bautista, C. M., Wergedal, J. & Baylink, D. J. (1989). Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell conditioned media: A potential local regulator of IGF action. *Proceedings of the National Academy of Sciences U.S.A.* **86**, 8338-8342.

Moller, D. E., Yokota, A., Caro, J. F. & Flier, J. S. (1989). Tissue-specific expression of two alternatively spliced insulin receptor mRNA in man. *Molecular Endocrinology* **3**, 1263-1269.

Morgan, D. O., Edman, J. C., Standring, D. N., Fried, V. A., Smith, M. C., Roth, R. A. & Rutter, W. J. (1987). Insulin-like growth factor II receptor as a multifunctional binding protein. *Nature* **329**, 301-307.

Morrione, A., Valentinis, B., Resnicoff, M., Xu, S. Q. & Baserga, R. (1997). The role of mGrb10-α in insulin-like growth factor I-mediated growth. *Journal of Biological Chemistry* **272**, 26382-26387.

Moses, A. C., Nissley, S. P., Passamani, J., White, R. M. & Rechler, M. M. (1979). Further characterization of growth hormone-dependent somatomedin-binding proteins in rat serum and demonstration of somatomedin-binding proteins produced by rat liver cells in culture. *Endocrinology* **104**, 536-546.

Moses, A. C., Freinkel, A. J., Knowles, B. B. & Aden, D. P. (1983). Demonstration that a human hepatoma cell line produces a specific insulin-like growth factor carrier protein. *Journal of Clinical Endocrinology and Metabolism* **56**, 1003-1008.

Mosthaf, L., Grako, K., Dull, T. J., Coussens, L., Ullrich, A. and McClain, D. A. (1990). Functionally distinct insulin receptors generated by tissue-specific alternative splicing. *EMBO Journal* **9**, 2409-2413.

Mottola, C., MacDonald, R. G., Brackett, J. L., Mole, J. E., Anderson, J. K. & Czech, M. P. (1986). Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells. Journal of Biological Chemistry **261**, 11180-11188.

Mottola, C. & Czech, M. P. (1984). The type II IGF receptor does not mediate increased DNA synthesis in H-35 hepatoma cells. *Journal of Biological Chemistry* **259**, 12705-12713.

Moxham, C. P. & Jacobs, S. (1992). Insulin-like growth factor receptors. In *The Insulin-like Growth Factors: Structure and Biological Function*, ed. P. N. Schofield, pp. 80-109. Oxford, Oxford University Press.

Moxham, C. P., Duronio, V. & Jacobs, S. (1989). Insulin-like growth factor I receptor β-subunit heterogeneity: Evidence for hybrid tetramers composed of insulin-like growth factor I and insulin receptor heterodimers. *Journal of Biological Chemistry* **264**, 13238-13244.

Mueller, R. V., Spencer, E. M., Sommer, A., Maack, A., Suh, D. & Hunt, T. K. (1991). The role of IGF-I and IGFBP-3 in wound healing. *in Modern Concepts of Insulin-like Growth Factors* pp. 185-192. Ed. E. M. Spencer. Elsevior: New York (U.S.A.).

Murphy, L. J., Seneviratne, C., Ballejo, G., Croze, F. & Kennedy, T. G. (1990). Identification and characterization of a rat decidual insulin-like growth factor-binding protein complementary DNA. *Molecular Endocrinology* **4**, 329-336.

Murphy, L. J., Bell, G. I. & Friesen, H. G. (1987). Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. *Endocrinology* **120,** 1279-1282.

Murray-Rust, J., McLeod, A. N., Blundell T. L. & Wood, S. P. (1992). Structure and evolution of insulins: implications for receptor binding. *Bioessays* **14**, 325-331.

Myers, M. G., Backer, J. M., Siddle, K. & White, M. F. (1991). The insulin receptor functions normally in Chinese hamster ovary cells after truncation of the C terminus. *Journal of Biological Chemistry* **266**, 10616-10623.

Nagarajan, L. & Anderson, N. B. (1982). Insulin promotes the growth of F9 embryonal carcinoma cells apparently by acting through its own receptor. *Biochemical and Biophysical Research Communications* **106**, 974-980.

Nakagawa, S. H. & Tager, H. S. (1992). Importance of aliphatic side-chain structure at positions 2 and 3 of the insulin A chain in insulin-receptor interactions. *Biochemistry* **31**, 3204-3214.

Nakagawa, S. H. & Tager, H. S. (1991). Implications of invariant residue LeuB6 in insulin-receptor interactions. *Journal of Biological Chemistry* **266**, 11502-11509.

Nakagawa, S. H. & Tager, H. S. (1987). Role of the COOH-terminal B-chain domain in insulin-receptor interactions. *Journal of Biological Chemistry* **262**, 12054-12058.

Nicholson, H., Becktel, W. J. & Matthews, B. W. (1988) Enhanced protein thermostability from designed mutations that interact with alpha-helix dipoles. *Nature* **336**, 651-656.

Nicol, D. S. H. W. & Smith, L. F. (1960). The amino acid sequence of human insulin. *Nature* **181**, 483.

Nishimoto, I., Murayama, Y., Katada, T., Ui, M. & Ogata, E. (1989). Possible direct linkage of insulin-like growth factor-II receptor with guanine nucleotide binding proteins. *Journal of Biological Chemistry* **264**, 14029-14038.

Ogasawara, M., Karey, K. P., Marquardt, H. & Sirbasku, D. A. (1989). Identification and purification of truncated insulin-like growth factor I from porcine uterus. Evidence for high biological potency. *Biochemistry* **28**, 2710-2721.

Oh, Y., Muller, H. L., Lee, D., Fielder, J. P. and Rosenfeld, R. G. (1993). Characterization of the affinities of insulin-like growth factor (IGF)-binding proteins 1-4 for IGF-I, IGF-II, IGF-I/insulin hybrid, and IGF-I analogs. *Endocrinology* **131**, 890-895.

Oka, Y., Rozek, L. M. & Czech, M. P. (1985). Direct demonstration of rapid insulin-like growth factor II receptor internalization and recycling in rat adipocytes: insulin stimulates ^{125}I insulin-like growth factor II degradation by modulating the IGF-II receptor recycling process. *Journal of Biological Chemistry* **260**, 9435-9442.

Olefsky, J. M. (1990). The insulin receptor: a multifunctional protein. *Diabetes* **39**, 1009-1016.

Oliver, J. E., Aitman, T. J., Powell, J. F., Wilson, C. A. & Clayton, R. N. (1989). Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. *Endocrinology* **124**, 2671-2679.

Ooi, G. T., Orlowski, C. C., Brown, A. L., Becker, E. R., Unterman, T. G. & Rechler, M. M. (1990). Different tissue distribution and hormonal regulation of messenger RNAs encoding rat insulin-like growth factor-binding proteins-1 and -2. *Molecular Endocrinology* **4**, 321-328.

Orlowski, C. C., Brown, A. L., Ooi, G. T., Yang, Y. W., Tseng, L. Y. & Rechler, M. M. (1990). Tissue, developmental and metabolic regulation of messenger ribonucleic acid encoding a rat insulin-like growth factor-binding protein. *Endocrinology* **126**, 644-652.

Pahlman, S., Meyerson, G., Lindgren, E., Schalling, M. & Johansson, I. (1991). Insulin-like growth factor I shifts from promoting cell division to potentiating maturation during neuronal differentiation. *Proceedings of the National Academy of the Sciences U.S.A.* **88**, 9994-9998.

Peters, M. A., Lau, E. P., Snitman, D. L., Van Wyk, J. J. & Merryweather, J. P. (1985). Expression of a biologically active analogue of somatomedin-C/insulin-like growth factor I. *Gene* **35**, 83-89.

Pietrzkowski, Z., Wernicke, D., Porcu, P., Jameson, B. A. & Baserga, R. (1992). Inhibition of cellular proliferation by peptide analogues of insulin-like growth factor 1. *Cancer Research* **52**, 6447-6451.

Pullen, R. A., Lindsay, D. G., Wood, S. P., Tickle, I. J., Blundell, T. L., Wollmer, A., Krail, G., Brandenburg, D., Zahn, H., Gliemann, J & Gammeltoft, S. (1976). Receptor-binding region of insulin. *Nature* 259, 369-373.

Qin, X., Strong, D. D., Baylink, D. J. & Mohan, S. (1998). Structure-function analysis of the human insulin-like growth factor binding protein-4. *Journal of Biological Chemistry* **273**, 23509-23516.

Rinderknecht, E. & Humbel, R. E. (1978a). The amino acid sequence of human insulin-like growth factor I and its structural homology with insulin. *Journal of Biological Chemistry* **253**, 2769-2776.

Rinderknecht, E. & Humbel, R. E. (1978b). Primary structure of insulin-like growth factor II. *FEBS Letters* **89**, 2365-2369.

Roberts, C. T., Lasky, S. R. Jr., Lowe, W. L. J. R., Seaman, W. T. & LeRoith, D. (1987). Molecular cloning of insulin-like growth factor I complementary deoxyribonucleic acids: differential riboneucleic acid processing and regulation by growth hormone in extrahepatic tissue. *Molecular Endocrinology* **1**, 243-248.

Rogers, S. A. & Hammerman, M. R. (1988). Insulin-like growth factor II stimulates production of inositol triphosphate in proximal tubular basolateral membranes from canine kidney. *Proceedings of the National Academy of Sciences U.S.A.* **85**, 4037-4041.

Ross, M., Francis, G. L., Szabo, L., Wallace, J. C. & Ballard, F. J. (1989). Insulin-like growth factor (IGF)-binding proteins inhibit the biological activities of IGF-I and IGF-II but not des-(1-3)-IGF-I. *Biochemical Journal* 258, 267-272.

Roth, B. V., Bürgisser, D. M., Lüthi, C. & Humbel, R. E. (1991). Mutants of human insulin-like growth factor II: expression and characterization of analogs with a substitution of TYR27 and/or a deletion of residues 62-67. *Biochemistry Biophysics Research Communications* **181**, 907-914.

Roth, R. A. (1988). Structure of the receptor for insulin-like growth factor II: the puzzle amplified. *Science* **239**, 1296-1271.

Roth, R. A., Steele-Perkins, G., Hari, J., Stover, C., Pierce, S., Turner, J., Edman, J. C. & Rutter, W. J. (1988). Insulin and insulin-like growth factor receptors and responses. In *Cold Spring Harbor Symposia on Quantitative Biology*, Volume LIII. Cold Spring Harbor Laboratory (U.S.A.).

Rotwein, P. (1991). Structure, evolution, expression and regulation of insulin-like growth factors I and II. *Growth Factors* **5**, 3-18.

Rotwein, P. (1986). Two insulin-like growth factor I messenger RNAs are expressed in human liver. *Proceedings of the National Academy of the Sciences U.S.A.* **83**, 77-81.

Rotwein, P., Pollock, K. M., Didier, D. K. & Krivi, G. G. (1986). Organization and sequence of the human insulin-like growth factor I gene. *Journal of Biological Chemistry* **261**, 4828-4832.

Rotwein, P., Folz, R. J. & Gordon, J. I. (1987). Biosynthesis of human insulin-like growth factor I (IGF-I). *Journal of Biological Chemistry* **262**, 11807-11812.

Ruderman, N. B., Kapeller, R., White, M. F. & Cantley, L. C. (1990). Activation of phosphatidylinositol-3-kinase by insulin. *Proceedings of the National Academy of Sciences U.S.A.* **87**, 1411-1415.

Ryle, A. P., Sanger, F., Smith, L. F. & Kitai, R. (1955). The disulphide bonds of insulin. *Biochemical Journal* **50**, 541-545.

Sakano, K., Enjoh, T., Numata, F., Fujiwara, H., Marumoto, Y., Higashihashi, N., Sato, Y., Perdue, J. F. & Yamaguchi, Y. F. (1991). The design, expression and characterization of human insulin-like growth factor II (IGF-II) mutants specific for either the IGF-II/Cation-independant mannose 6-phosphate receptor or IGF-I receptor. *Journal of Biological Chemistry* **266**, 20626-20635.

Salmon, W. D. Jr. & Daughaday, W. H. (1957). A hormonally controlled serum factor which stimulated sulfate incorporation by cartilage *in vitro*. *Journal of Laboratory and Clinical Medicine* **49**, 825-836.

Salmon, W. D. Jr. & Duvall, M. R. (1970). In vitro stimulation of leucine incorporation into muscle and cartilage protein by a serum fraction with sulphation factor activity: differentiation of effects from those of growth hormone and insulin. *Endocrinology* **87**, 1168-1180.

Sara, V. R., Carlsson-Skwirut, C., Andersson, C., Hall, K., Sjogren, A., Holmgren, A. & Jornvall, H. (1986). Characterization of somatomedins from human fetal brain: identification of a variant form of insulin-like growth factor I. *Proceedings of the National Academy of the Sciences U.S.A.* **83**, 4904-4907.

Schedlich, L. J., Young, T. F., Firth, S. M. & Baxter, R. C. (1998). Insulin-like growth factor binding protein (IGFBP)-3 and IGFBP-5 share common nuclear transport pathway in T49D human breast carcinoma cells. *Journal of Biological Chemistry* **273**, 18347-18352.

Schoenle, E. J., Zapf, J., Hauri, C., Steiner, T. & Froesch, E. R. (1985). Comparison of *in vivo* effects of insulin-like growth factors I and II and of growth hormone in hypophysectomized rats. *Acta Endocrinologica* **108**, 167-174.

Schreiber, G. & Fersht, A. R. (1995). Energetics of protein-protein interactions: analysis of the barnase-barstar interface by single mutations and double mutant cycles. *Journal of Molecular Biology* **248**, 478-486.

Schumacher, R., Mosthaf, L., Schlessinger, J., Brandenburg, D. & Ullrich, A. (1991). Insulin and insulin-like growth factor I binding specificity is determined by distinct regions of their cognate receptors. *Journal of Biological Chemistry* **266**, 19288-19295.

Schwartz, G., Burke, G. T. & Katsoyannis, P. G. (1982). The importance of the B10 amino acid residue to the biological activity of insulin. *Journal of Protein Chemistry* **1**, 177-189.

Seino, S. & Bell, G. I. (1989). Alternative splicing of human insulin receptor messenger RNA. *Biochemical and Biophysical Research Communications* **159**, 312-316.

Shemer, J., Adamo, M., Wilson, G. L., Heffez, D., Zick, Y. & Le Roith, D. (1987). Insulin and insulin-like growth factor-I stimulate a common endogenous phosphoprotein substrate (pp185) in intact neuroblastoma cells. *Journal of Biological Chemistry* **262**, 15476-15482.

Shimasaki, S., Uchiyama, F., Shimonaka, M. & Ling, N. (1990). Molecular cloning of the cDNA encoding a novel insulin-like growth factor binding protein from rat and human. *Molecular Endocrinology* **4**, 1451-1458.

Shimasaki, S., Shimonaka, M., Zhang, H-P. & Ling, N. (1991a). Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP-5 in rat and human. *Journal of Biological Chemistry* **266**, 10646-10653.

Shimasaki, S., Gao, L., Shimonaka, M. & Ling, N. (1991b). Isolation and molecular cloning of insulin-like growth factor-binding protein-6. *Molecular Endocrinology* **5**, 938-948.

Shimizu, M., Webster, C., Morgan, D. O., Blau, H. M. & Roth, R. A. (1986). Insulin and insulin-like growth factor receptors and responses in cultured human muscle cells. *American Journal of Physiology* **251**, E611-E615.

Shimonaka, M., Schroader, R., Shimasaki, M. & Ling, N. (1989). Identification of a novel binding protein for insulin-like growth factors in adult rat serum. *Biochemical and Biophysical Research Communications* **165**, 189-195.

Shymko, R. M., Dumont, E., De Meyts, P. & Dumont, J. E. (1999). Timing-dependence of insulin-receptor mitogenic versus metabolic signalling: a plausible model based on coincidence of hormone and effector binding. *Biochemical Journal* **339**, 675-683.

Siddle, K. (1992). The insulin receptor and type 1 IGF receptor: comparison of structure and function. *Progress in Growth Factor Research* **4**, 301-320.

Smith, G. D., Swenson, D. C., Dodson, E. J., Dodson, G. G. & Reynolds, C. D. (1984). Structural stability in the 4-zinc human insulin hexamer. *Proceedings of the National Academy of Science U.S.A.* **22**, 7093-7097.

Smith, L. F. (1966). Species variation in the amino acid sequence of insulin. *American Journal of Medicine* **40**, 662-666.

Smith, L. F. (1972). Amino acid sequence of insulins. *Diabetes* **21**, *Supplement 2*, 457-460.

Soos, M. A., Field, C. E. & Siddle, K. (1992). Purified hybrid insulin/insulin-like growth factor-I receptors bind insulin-like growth factor-I, but not insulin, with high affinity. *Biochemical Journal* **290**, 419-426.

Soos, M. A., Whittaker, J., Lammers, R., Ullrich, A. & Siddle, K. (1990). Receptors for insulin and insulin-like growth factor-I can form hybrid dimers. Characterisation of hybrid receptors in transfected cells. *Biochemical Journal* **270**, 383-390.

Soos, M. A. & Siddle, K. (1989). Immunological relationships between receptors for insulin and insulin-like growth factor I: Evidence for structural heterogeneity of insulin-like growth factor I receptors involving hybrids with insulin receptors. *Biochemical Journal* **263**, 553-563.

Steele-Perkins, G., Turner, J., Edman, J. C., Hari, J., Pierce, S. B., Stover, C., Rutter, W. J. & Roth, R. A. (1988). Expression and characterization of a functional human insulin-like growth factor I receptor. *Journal of Biological Chemistry* **263**, 11486-11492.

Stempien, M. M., Fong, N. M., Rall, L. B. & Bell G. I. (1986). Sequence of a placental cDNA encoding the mouse insulin-like growth factor II precoursor. *DNA* **5**, 357-360.

Stracke, M. L., Engel, J. D., Wilson, L. W., Rechler, M. M., Liotta, L. A. & Schiffmann, E. (1989). The type I insulin-like growth factor receptor is a motility receptor in human melanoma cells. *Journal of Biological Chemistry* **264**, 21544-21549.

Suh, D. Y., Hunt, T. K. & Spencer, E. M. (1992). Insulin-like growth factor I reverses the impairment of wound healing induced by corticosteroids in rats. *Endocrinology* **131**, 2399-2403.

Sussenbach, J. S. (1989). The gene structure of the insulin-like growth factor family. *Progress in Growth Factor Research* **1**, 22-40.

Szabo, L., Mottershead, D. G., Ballard, F. J. & Wallace, J. C. (1988). The bovine insulin-like growth factor binding protein purified from conditioned medium requires the N-terminal tripeptide in IGF-I for binding. *Biochemical and Biophysical Research Communications* **151**, 207-214.

Tager, H., Thomas, N., Assoian, R., Rubenstein, A., Saekow, M., Olefsky, J. & Kaiser, E. T. (1980). Semisynthesis and biological activity of porcine [Leu^{B24}] insulin and [Leu^{B25}] insulin. *Proceedings of the National Academy of Sciences U.S.A.* **77**, 3181-3186.

Takata, Y., Webster, N. J. G. & Olefsky, J. M. (1991). Mutation of the two carboxyl-terminal tyrosines results in an insulin receptor with normal metabolic signalling but enhanced mitogenic signaling properties. *Journal of Biologiacl Chemistry* **266**, 9135-9139.

Tamura, K., Kobayashi, M., Ishii, Y., Tamura, T., Hashimoto, K., Nakamura, K., Niwa, M. & Zapf, J. (1989). Primary structure of rat insulin-like growth factor-I and its biological activities. *Journal of Biological Chemistry* **264**, 5616-5621.

Thies, R. S., Ullrich, A. & McClain, D. A. (1989). Augmented mitogenesis and impaired metabolic signaling mediated by a truncated insulin receptor. *Journal of Biological Chemistry* **264**, 12820-12825.

Thissen, J. P., Underwood, L. E., Maiter, D. M., Maes, M., Clemmons, D. R. & Ketelslegers, J. M. (1991). Failure of IGF-I infusion to promote growth in protein-restricted rats despite normalization of serum IGF-I concentrations. *Endocrinology* **128**, 885-890.

Tomas, F. M., Knowles, S. E., Owens, P. C., Chandler, C. S., Francis, G. L., Read, L. C. & Ballard, F. J. (1992). Insulin-like growth factor I and especially IGF-I variants are anabolic in dexamethasone-treated rats. *Biochemical Journal* **282**, 91-97.

Tomas, F. M., Knowles, S. E., Owens, P. C., Read, L. C., Chandler, C. S., Gargosky, S. E. & Ballard, F. J. (1991). Effects of full length and truncated insulin-like growth factor-I on nitrogen balance and muscle protein metabolism in nitrogen-restricted rats. *Journal of Endocrinology* **128**, 97-105.

Tornqvist, H. E., Pierce, M. W., Frackelton, A. R., Nemenoff, R. A. & Avruch, J. (1987). Identification of insulin receptor tyrosine residues autophosphorylated *in vitro*. *Journal of Biological Chemistry* **262**, 10212-10219.

Tricoli, J. V., Rall, L. B., Scott, J., Bell, G. I. & Shows, T. B. (1984). Localization of insulin-like growth factor genes to chromosomes 11 and 12. *Nature* **10**, 784-786.

Tseng, L. Y. -H., Swartz, G. P., Sheikh, M., Chen, Z. Z., Joshi, S., Wang, J. -F., Nissley, S. P., Burke, G. T., Katsoyannis, P. G. & Rechler, M. M. (1987). Hybrid molecules containing the A-domain of insulin-like growth factor I and the B-chain of insulin have increased mitogenic activity relative to insulin. *Biochemical and Biophysical Research Communications* **149**, 672-679.

Tulloch, P. A., Lawrence, L. J., McKern, N. M., Robinson, C. P., Bentley, J. D., Cosgrove, L., Siddle, K. & Ward, C. W. (1999). Single-molecule imaging of human insulin receptor ectodomain and its Fab complexes. *Journal of Structural Biology* **125**, 11-18.

Ullrich, A. & Schlessinger, J. (1990). Signal transduction by receptors with tyrosine kinase activity. *Cell* **61**, 203-212.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y. C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M. & Ramachandran, J. (1985). Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes. *Nature* **313**, 756-761.

Ullrich, A., Gray, A., Tam, A. W., Yang-Feng, T., Tsubokawa, M., Collins, C., Henzsel, W., Le Bon, T., Kathuria, S., Chen, E., Jacobs, S., Francke, U., Ramachandran, J. & Fujita-Yamaguchi, Y. (1986). Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. *EMBO Journal* **5**, 2503-2512.

Upton, F. Z., Francis, G. L., Ross, M., Wallace, J. C. & Ballard, F. J. (1992). Production and characterization of recombinant chicken insulin-like growth factor-I from Escherichia coli. *Journal of Molecular Endocrinology* **9**, 83-92.

Upton, F. Z., Szabo, L., Wallace, J. C. & Ballard, F. J. (1990). Characterization and cloning of a bovine insulin-like growth factor-binding protein. *Journal of Molecular Endocrinology* **5**, 77-84.

Van Wyk, J. J., Graves, D. C., Casella, S. J. & Jacobs, S. (1985). Evidence from monoclonal antibody studies that insulin stimulates deoxyribonucleic acid synthesis through the type I somatomedin receptor. *Journal of Clinical Endocrinology and Metabolism* **61**, 639-645.

Voutilainen, R. & Miller, W. L. (1987). Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450ssc, in human steroidogenic tissues. *Proceedings of the National Academy of Sciences U.S.A.* **84**, 1590-1594.

Wang, J., Dai, H., Yousaf, N., Moussaif, M., Deng, Y., Boufelliga, A., Swamy, O. R., Leone, M. E. & Riedel, H. (1999). Grb10, a positive, stimulatory signaling adapter in platelet-derived growth factor BB-, insulin-like growth factor I- and insulin-mediated mitogenesis. *Molecular and Cellular Biology* **19**, 6217-6228.

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Wang, J. -F., Hampton, B., Mehlman, T., Burgess, W. H. & Rechler, M. M. (1988). Isolation of a biologically active fragment from the carboxy terminus of the fetal rat binding protein for insulin-like growth factors. *Biochemical and Biophysical Research Communications* **157**, 718-726.

Ward, C. W., Garrett, T. P. J., McKern, N. M., Lou, M., Cosgrove, L. J., Sparrow, L. G., Frenkel, M. J., Hoyne, P. A., Elleman, T. C., Adams, T. E., Lovrecz, G. O., Lawrence, L. J. & Tulloch, P. A (2001). The three dimensional structure of the type I insulin-like growth factor receptor. *Journal of Clinical Pathology* **54**, 125-132.

White, M. F., Shoelson, S. E., Keutmann, H. & Kahn, C. R. (1988). A cascade of tyrosine autophosphorylation in the β-subunit activities of the insulin receptor. *Journal of Biological Chemistry* **263**, 2969-2980.

Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F. & Hofmann, C. A. (1987). High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells. *Proceedings of the National Academy of Sciences U.S.A.* **84**, 5237-5241.

Weiland, M., Bahr, F., Hohne, M., Schurmann, A., Ziehm, D. & Joost, H. G. (1991). The signaling potential of the receptors for insulin and insulin-like growth factor I (IGF-I) in 3T3-L1 adipocytes: comparison of glucose transport activity, induction of oncogene c-fos, glucose transporter mRNA, and synthesis. *Journal of Cellular Physiology* **149**, 428-435.

Wilden, P. A., Kahn, C. R., Siddle, K. & White, M. F. (1992). Insulin receptor kinase domain autophosphorylation regulates receptor enzymatic function. *Journal of Biological Chemistry* **267**, 16660-16668.

Wood, S. P., Blundell, T. L., Wollmer, A., Lazarus, N. R. & Neville, R. W. (1975). The relation of conformation and association of insulin to receptor binding; x-ray and circular-dichroism studies on bovine and hystricomorph insulins. *European Journal of Biochemistry* **55**, 531-542.

Wood, W. I., Cachianes, G., Henzel, W. J., Winslow, G. A., Spencer, S. A., Hellmiss, R., Martin, J. L. & Baxter, R. C. (1988). Cloning and expression of the growth hormone-dependent insulin-like growth factor-binding protein. *Molecular Endocrinology* **2**, 1176-1185.

Xu, B., Bird, V. G. & Miller, W. T. (1995). Substrate specificity of the insulin and insulin-like growth factor I receptor tyrosine kinase catalytic domains. *Journal of Biological Chemistry* **270**, 29825-29830.

Yamaguchi, Y., Flier, J. S., Benecke, H., Ransil, B. J. & Moller, D. E. (1993). Ligand-binding properties of the two isoforms of the human insulin receptor. *Endocrinology* **132**, 1132-1138.

Yee, D., Lebovic, G. S., Marcus, R. R. & Rosen, N. (1989). Identification of an alternate type 1 insulin-like growth factor receptor β-subunit mRNA transcript. *Journal of Biological Chemistry* **264**, 21439-21441.

Young, S. C. J., Miles, M. V. & Clemmons, D. R. (1992). Determination of the pharmokinetic profiles of insulin-like growth factor binding proteins -1 and -2 in rats. *Endocrinology* **131**, 1867-1873.

Zapf, J., Kiefer, M., Merryweather, J., Musiarz, F., Bauer, D., Born, W., Fischer, J. A. & Froesch, E. R. (1990). Isolation from adult serum of four insulin-like growth factor (IGF) binding proteins and molecular cloning of one of them that is increased by IGF-I administration and in extrapancreatic tumor hypoglyceamia. *Journal of Biological Chemistry* **265**, 14892-14898.

Zapf, J., Kiefer, M., Merryweather, J., Masiarz, F., Bauer, D., Born, W., Fischer, J. A. & Froesch, E. R. (1990). Isolation from adult human serum of four insulin-like growth factor (IGF) binding proteins and molecular cloning of one of them that is increased by IGF-I administration and in extrapancreatic tumor hypoglycemia. *Journal of Biological Chemistry* **265**, 14892-14898.

Zapf, J., Hauri, C., Waldvogel, M. & Froesch, E. R. (1986). Acute metabolic effects and half-lives of intravenously administered insulin-like growth factor I and II in normal and hypophysectomized rats. *Journal of Clinical Investigation* **77**, 1768-1775.

Zapf, J., Walter, H. & Froesch, E. R. (1981). Radioimmunological determination of insulin-like growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglyceamia. *Journal of Clinical Investigation* **68**, 1321-1330.

Zapf, J., Waldvogel, M. & Froesch, E. R. (1975). Binding of nonsuppressible insulin-like activity to human serum: evidence for a carrier protein. *Archives of Biochemistry and Biophysics* **168**, 638-645.

Zha, J., Harada, H., Yang, E., Jockel, J. & Korsmeyer, S. J. (1996). Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-628.

Zhang, W., Gustafson, T. A., Rutter, W. J. & Johnson, J. D. (1994). Positively charged side chains in the insulin-like growth factor I C- and D-regions determine receptor binding specificity. *Journal of Biological Chemistry* **269**, 10609-10613.

Zumstein, P. P., Lüthi, C. & Humbel, R. E. (1985). Amino acid sequence of the variant proform of insulin-like growth factor II. *Proceedings of the National Academy of the Sciences U.S.A.* **82**, 3169-3172.