INVESTIGATION INTO THE MOLECULAR SPECIFICITY OF THE IGFs: A CHIMERIC APPROACH

By

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A Thesis submitted to the University of Adelaide, South Australia in fulfilment of the requirements for the degree of Doctor of Philosophy

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ABSTRACT

Insulin-like growth factors (IGF)-I and -II induce a variety of cellular outcomes including cell proliferation, differentiation, cell migration and protection from apoptosis. IGF-I and IGF-II share a high degree of structural similarity reflecting their high level of sequence similarity. Despite this both IGF-I and IGF-II exhibit different binding affinities to almost all proteins with which they both interact. To date few studies have investigated the structural basis whereby IGF-I and IGF-II bind with different affinities to the type-1 IGF receptor (IGF-1R), insulin receptor (IR) isoforms, type-2 IGF receptor (IGF-2R) and IGF binding proteins (IGFBPs). The major objective of this thesis is to investigate the molecular basis for the unique receptor and binding protein binding properties of IGF-I and IGF-II.

To address the question of what parts of IGF-I and IGF-II confer their unique receptor and binding protein binding affinities, chimeras of IGF-I and IGF-II were engineered where their C and D domains were exchanged either singly or together. These are the first ever whole domain chimeras of IGF-I and IGF-II. Recently the alternatively spliced isoform of the insulin receptor lacking 12 amino acids encoded by exon 11 (IR-A) has been shown to be overexpressed in foetal and cancer tissues and bind IGF-II but not IGF-I with high affinity. Binding analysis of the engineered chimeras to the IR-A showed that the C and D domains of IGF-II allow high affinity binding whereas the same domains of IGF-I preclude high affinity binding to the IR-A. The C and D domains also regulate the differential interaction of IGF-I and IGF-II with the insulin receptor isoform containing the amino acids encoded by exon 11 (IR-B). The C domain of IGF-II, in the background of IGF-II or IGF-I, also allows potent induction of phosphorylation of various tyrosine residues in the intracellular β
subunits of the IR-A and IR-B, whereas the same domain of IGF-I in the background of IGF-II did not. In addition this same domain also is the molecular basis for the differential ability of IGF-II and IGF-I to phosphorylate IRS-1 and activate PKB/Akt. Furthermore, the differential ability of IGF-II and IGF-I to induce cell survival and promote cell migration via the IR-A is due to their C domains.

The IGF-1R binds IGF-I with a 3-5 fold higher affinity than IGF-II. Here in this thesis the differential affinity of IGF-I and IGF-II for the IGF-1R is shown to be in a large part due to their C and D domains.

The IGF-2R is a receptor that does not contain any intrinsic signaling activity and in the context of the IGF system appears to regulate the concentration of IGF-II. IGF-2R binds IGF-II with high affinity but binds IGF-I with very low affinity. Reported here is the novel identification of the IGF-II C domain as an important contributor for wildtype IGF-2R binding. IGFBPs act to both potentiate and inhibit the cellular actions of the IGFs. Almost all IGFBPs bind IGF-I and IGF-II with different affinities, particularly IGFBP-6 which has a 10-60 fold higher affinity for IGF-II compared to IGF-I. Binding analysis of IGF chimeras to IGFBP-1, -2, -3 revealed that the C and D domains of the IGFs do not regulate any IGFBP affinity difference, however the IGF-II C domain may play a small role in binding to IGFBP-6.

Recently a patient exhibiting mental retardation, short stature and gonadal dysfunction was shown to be homozygous for a mutation in his IGF-I gene resulting in a protein with a methionine instead of a valine at position 44. Here in this thesis the biochemical characterization of this mutant is reported. This mutation causes almost a 90-fold reduction in IGF-1R binding affinity, while completely abrogating binding to either IR isoform. Interestingly binding to IGFBP-2, -3 and -6 is unaffected. In addition, NMR analysis showed only small perturbations in the structure localized around the site of the mutation.
In conclusion this thesis has contributed to the understanding of ligand receptor interactions of the insulin/IGF system.
STATEMENT OF ORIGINALITY

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

Adam Denley
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I am eternally indebted to my supervisors Professor John Wallace, Dr. Grant Booker and Dr. Leah Cosgrove, whose encouragement, guidance and unwavering support have allowed me to complete what at times seemed a truly monumental task. I am unbelievably thankful to Dr. Briony Forbes who has spent much of her valuable time answering my questions and discussing my work. I am also extremely appreciated of both the financial and academic support I have received from Dr. Colin Ward. I am also enormously grateful for the assistance, advice and opportunity provided to me by Dr. Charles Roberts Jr.

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This thesis is dedicated to my small but amazing family. I am forever grateful to my parents Paul and Victoria and sister Amanda who have provided me with more love and support than any one person could ever ask for. I would not be here today if it wasn’t for my mum’s cooking!! To my grandparents Nana Bet and Grandpa Jim, and Nana Irene and the late Dr. Arthur Noel Turner I thank them for their tireless encouragement and endless words of wisdom.
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Chapter 1:


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** corresponding author

Adam Denley, Leah J. Cosgrove, Grant W. Booker, Briony E. Forbes and John C. Wallace. Molecular Interactions of the IGF system.

_Manuscript in preparation for Cytokine and Growth Factor Reviews_

Chapters 2 – 4 & 6:

Adam Denley, Eric R. Bonython, Leah J. Cosgrove, Briony E. Forbes, Grant W. Booker, Colin W. Ward and John C. Wallace (2004). Structural determinants for high affinity binding of IGF-II to IR-A, the exon 11 minus isoform of the insulin receptor.

_Molecular Endocrinology 18_(10): 2502-2512

Chapter 5:

Adam Denley, Gemma V. Brierley, Grant W. Booker, Leah J. Cosgrove, John C. Wallace, Briony E. Forbes and Charles T. Roberts Jr. (2004). Differential activation of the insulin receptor isoforms by the insulin-like growth factors is determined by its C domain

_Manuscript in preparation._
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*Manuscript submitted to Protein Science.*

Chapter 8:


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*Molecular Endocrinology in press.*

And conference proceedings:


# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ALS</td>
<td>acid labile subunit</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDC</td>
<td>N-ethyl-N’-(dimethylaminopropyl) carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetra-acetate</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>Erk1/2</td>
<td>extracellular signal-related kinases 1 and 2</td>
</tr>
<tr>
<td>Eu</td>
<td>Europium</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FKHR</td>
<td>forkhead transcription factors</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast performance liquid chromatography</td>
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<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GHR</td>
<td>growth hormone receptor</td>
</tr>
<tr>
<td>Grb 2</td>
<td>growth factor receptor bound 2</td>
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<tr>
<td>GSK-3</td>
<td>glycogen synthase kinase-3</td>
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<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid</td>
</tr>
<tr>
<td>HBS</td>
<td>hepes buffered saline</td>
</tr>
<tr>
<td>rpHPLC</td>
<td>reverse phase high performance liquid chromatography</td>
</tr>
<tr>
<td>Hsp90</td>
<td>heat shock protein 90</td>
</tr>
<tr>
<td>HSQC</td>
<td>heteronuclear single quantum coherence</td>
</tr>
<tr>
<td>IB</td>
<td>inclusion body</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>concentration of inhibitor which reduces binding by 50 %</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>IGFBP</td>
<td>insulin-like growth factor binding protein</td>
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<tr>
<td>IGF-1</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IGF-2</td>
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<td>IL-2</td>
<td>interleukin 2</td>
</tr>
<tr>
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<td>interleukin 8</td>
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<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
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<td>IR</td>
<td>insulin receptor</td>
</tr>
<tr>
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<td>insulin receptor isoform B</td>
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<tr>
<td>JAK</td>
<td>janus kinase</td>
</tr>
<tr>
<td>JNK</td>
<td>Jun amino-terminal kinase</td>
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<tr>
<td>KD</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>KA</td>
<td>association constant</td>
</tr>
<tr>
<td>LB</td>
<td>Luria broth</td>
</tr>
<tr>
<td>MBP</td>
<td>maltose binding protein</td>
</tr>
<tr>
<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
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<tr>
<td>NOE</td>
<td>nuclear overhauser effect</td>
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<tr>
<td>ODx</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PDK-1</td>
<td>3-phosphoinositide-dependent protein kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>PI3K</td>
<td>phosphatidyl-inositol 3’ phosphate kinase</td>
</tr>
<tr>
<td>PKB</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>PtdIns</td>
<td>phosphatidylinositol</td>
</tr>
<tr>
<td>PTEN</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RU</td>
<td>response units</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>S.E.M.</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SH2</td>
<td>src homology 2</td>
</tr>
<tr>
<td>Shc</td>
<td>src homology/collagen proteins</td>
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<td>SHP-2</td>
<td>src homology-2 domain-containing phosphatase 2</td>
</tr>
<tr>
<td>Sos</td>
<td>son of sevenless</td>
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<tr>
<td>STAT5b</td>
<td>signal transducer and activator of transcription 5b</td>
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<tr>
<td>TEMED</td>
<td>N,N,N,N’N’-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>transforming growth factor β 1</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase plasminogen activating receptor</td>
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1

Introduction
1.1 INTRODUCTION

The IGF system is critical for the normal development and postnatal growth across all species from *C. elegans* to humans. The biological effects of IGF-I and IGF-II are mediated and regulated by a number of transmembrane receptors and soluble binding proteins. IGF-I and IGF-II exhibit high structural similarity reflecting their high degree of sequence similarity. Despite this, both IGF-I and IGF-II have different binding affinities for all proteins with which they both interact. The structural determinants of IGF-I and IGF-II that account for these affinity differences are unknown and hence is the focus of this study.

Throughout the course of evolution gene duplication and mutation have given rise to groups of families of proteins that have similar sequence and consequently similar structure. In parallel with this structural evolution each protein can adopt a more specific function, due to the mutation in its own sequence and/or also by co-operative mutations in an interacting protein and this results in retention of the duplicate protein (Fryxell, 1996). The molecular evolution of proteins can change the ability of one protein to interact with another resulting in the formation of unique productive protein-protein complexes or inhibiting unwanted or unproductive protein interactions. In addition, protein evolution can optimise specific protein-protein interactions by increasing affinity and/or specificity. This process of molecular evolution, gene duplication or mutation followed by retention, has given rise to many growth factor systems that are made up of multiple ligands and multiple receptors. The presence of many structurally related growth factors and structurally related receptors suggests that each one must have a unique function or a unique tissue or developmental expression profile to be retained throughout evolution. The fibroblast growth factor family, for example, is made up of 19 structurally related polypeptides.
(FGF1-19) and 4 structurally related receptor tyrosine kinases (FGFR1-4). Each FGF binds each FGFR with a unique affinity and this FGF-FGFR specificity is indispensable for regulating FGF responses (Plotnikov et al., 2000; Yeh et al., 2003). Distinct change to FGFR ligand binding affinity and specificity gives rise to the severe craniosynostosis syndrome, Apert syndrome (Ibrahimi et al., 2001), indicating the importance of maintaining specific sets of ligand/receptor interactions.

Most receptors have several ligands e.g. the epidermal growth factor receptor (EGFR) has 7 ligands (Harris et al., 2003), while the Erb3 and 4 have 4 ligands (Falls, 2003). Thus, ligand/receptor binding specificity may be a general mechanism for regulating the biological response of many polypeptide growth factor systems. The insulin/insulin-like growth factor (IGF) system is also an example of a complex network whereby several ligands interact with the same receptor e.g. insulin receptor (IR) binds 3 ligands. In this way the receptor binding specificity of insulin/IGF ligands influence a multitude of biological responses. In this thesis the receptor binding specificity and consequent biological response of the insulin/insulin-like growth factor family is investigated.

1.1.1 Introduction to the IGF axis

The insulin/insulin-like growth factor system is an ancient signalling system with homologous genes conserved throughout evolution from yeast to humans (Barbieri et al., 2003). The intricate network of peptide hormones, cell surface receptors and circulating binding proteins are shown in Figure 1.1. The peptide hormones, insulin, insulin–like growth factor (IGF)- I and IGF-II, bind to and activate the intrinsic tyrosine kinase activity of the insulin/IGF family of cell surface receptors; namely the insulin-like growth factor I receptor (IGF-1R), insulin receptor exon 11- (IR-A) and insulin receptor exon 11+ (IR-B). These activated receptors initiate
signalling cascades that ultimately result in regulation of a number of biological responses. Functional hybrid receptors can form between the IGF-1R and either the IR-A or IR-B. Hybrids between IR-A and IR-B are predicted to form but their presence has not been shown experimentally. The interaction of IGF ligands (IGF-I and IGF-II) with the IGF family receptors can be regulated either positively or negatively by a class of soluble high affinity binding proteins, insulin-like growth factor binding protein 1-6 (IGFBP 1-6). In addition another level of regulation of IGF-II action is that of binding to the insulin-like growth factor 2 receptor (IGF-2R). This receptor has no intrinsic signalling transduction capability and in the context of the IGF system primarily acts to internalise and degrade IGF-II. The interaction of IGF-I and IGF-II with all components of the IGF system will be discussed in this introduction.
Figure 1.1. Schematic of the IGF system. Summary of the insulin/IGF system. This figure depicts most components of the insulin/IGF system and their interactions. All six binding proteins bind IGF-I and IGF-II in the circulation. However, IGFBP-3 is the most abundant binding protein and binds the majority of circulating IGF-I and IGF-II in a 150 kDa complex with ALS. This ternary complex protects the IGFs from proteases in circulation but also prevents the IGFs traversing the vascular membrane into the extracellular space (Jones and Clemmons, 1995). Association of binding proteins to the extracellular matrix (ECM) reduces their affinity for the IGFs and allows then the binding of the IGFs to an insulin/IGF family receptor. Binding of an IGF to either the insulin receptor isoform or IGF-1R elicits a number of biological responses (Adams et al., 2000; Denley et al., 2003). The exact role of hybrid receptors in cell biology is not well understood. The IGF-2R acts to internalize and degrade IGF-II but has no signalling capabilities (Scott and Firth, 2004). An IGFBP-3 receptor has been postulated but its existence is yet to be confirmed (Firth and Baxter, 2002). (Figure adapted from F.E. Carrick, 2001 Ph.D thesis).
1.2 IGF expression and transcriptional regulation

IGF-I and IGF-II were isolated from the Cohn fraction of human serum almost 30 years ago (Rinderknecht and Humbel, 1976). IGF-I stimulates pleiotropic actions including cell proliferation, differentiation, migration or protection from apoptosis by either autocrine, paracrine or endocrine mechanisms. IGF-I mRNA has been detected in many tissues both in the developing human foetus (Han et al., 1988) and adult (Daughaday and Rotwein, 1989). While relatively little is known about the full range of physiological stimuli that regulate IGF-I production, nutritional status and GH levels are two factors that strongly influence IGF-I levels (Thissen et al., 1994). Growth hormone secreted from the pituitary stimulates IGF-I production in the liver (Mathews et al., 1986) which constitutes the major source of endocrine circulating IGF-I (Sjogren et al., 1999; Yakar et al., 1999). IGF-I production at extrahepatic sites in the mouse, including heart, lung, testis, uterus is largely independent of GH (Mathews et al., 1986). IGF-I production in these extrahepatic sites provides a pool of growth factor that can act locally in an autocrine or paracrine manner (Lupu et al., 2001). Mice with a targeted disruption of the IGF-I gene are born at 60% birth weight compared to wildtype litter mates and continue to show growth retardation postnatally so that at their linear growth plateau the mutant mice have an average bodyweight that is only 30% that of wildtype animals (Liu et al., 1993). Liver specific disruption of the IGF-I gene in mice reduced serum IGF-I levels by 75 % but did not affect birth weight, suggesting that IGF-I from extrahepatic sites is sufficient to support normal development (Yakar et al., 1999).

IGF-II production is not regulated by GH and the physiological factors that regulate IGF-II production are largely unknown. At a cellular level, IGF-II expression has recently been shown to be regulated by nutrients in skeletal myogenesis (Erbay et al., 2003) and by FGF-6 in myofibres (Armand et al., 2004). In the human foetus
IGF-II is expressed in many tissues, generally at a higher level than IGF-I (Han et al., 1988) and expression is maintained at a constant level in most tissues throughout adulthood (Daughaday and Rotwein, 1989). The IGF-II gene inherited maternally is silenced and expression occurs exclusively from the paternal allele. Targeted disruption of the paternal IGF-II allele results in mutant mice with only 60% the bodyweight of their wildtype littermates (DeChiara et al., 1990).

While mouse knockout studies have helped define the biological roles of the two IGF ligands, IGF-II primarily acts as a foetal development growth factor and IGF-I is critical for both pre and postnatal growth, the observations from such studies are not easily transposed into human biology. In both humans and mice, IGF-I is expressed both pre- and postnatally. IGF-II in humans and mice is highly expressed in the developing foetus however postnatally, humans exhibit high level expression while in mice postnatal IGF-II expression is restricted to the choroid plexus and leptomeninges (Stylianopoulou et al., 1988).

Two growth restricted humans have been identified as having almost no circulating functional IGF-I due to either deletion of a large portion of the IGF-I gene (Woods et al., 1997) or a missense mutation (Walenkamp et al., 2004). These subjects have revealed IGF-I to be important in foetal longitudinal growth as well as brain and hearing development. In adulthood IGF-I is important in bone mineralisation and gonadal function (Walenkamp et al., 2004). Several polymorphisms within the IGF-I gene and promoter regions have also been shown to be associated with varying levels of growth restriction in humans (Johnston et al., 2003; Obrepalska-Steplowska et al., 2003). In addition these human mutations indirectly reveal that IGF-II cannot totally compensate for the lack of functional IGF-I. Two variants of IGF-II have been identified however what effect the presence of these variants has on normal human
development and growth is unknown (Jansen et al., 1985; Zumstein et al., 1985; Schofield and Tate, 1987).

1.2.1 IGF Structure and Function

IGF-I and IGF-II are 70 and 67 amino acid single chain polypeptides respectively, that are classified into four “domains” (in order N to C terminus): B, C, A and D. The B and A domains of IGF-I and IGF-II have a 50% sequence similarity to the B and A chains of insulin (Rinderknecht and Humbel, 1978) (Figure 1.2). The C domain in IGF-I and IGF-II is analogous to the C peptide of proinsulin, however the IGF C domain is not proteolytically removed as in the mature insulin molecule. Both IGF-I and IGF-II contain a C-terminal extension, called the D domain that has no counterpart in insulin. The prepro forms of the IGFs are composed of an additional N-terminal signal sequence and either one of two C-terminal E domains that arise from alternative splicing of the IGF mRNA (Daughaday and Rotwein, 1989). Both the N and C terminal extensions are cleaved post-translationally (de Pagter-Holthuizen et al., 1986).

For many years, before the first successful structural studies, Blundell and colleagues lead the way in modelling the tertiary structure of the IGFs, based on the known crystal structure of insulin (Blundell et al., 1978; Gunning et al., 1982; Blundell et al., 1983). Since these pioneering models which proved to be largely correct, several groups have reported the three dimensional structure of IGF-I by both NMR (Cooke et al., 1991; Sato et al., 1993; Schaffer et al., 2003) (Figure 1.3) and X-ray crystallography methods (Vajdos et al., 2001; Zeslawski et al., 2001; Brzozowski et al., 2002). Several structures of IGF-I mutants have also provided insights into the function of wildtype IGF-I (Laajoki et al., 1997; Laajoki et al., 1998; Laajoki et al., 2000). The structures reveal the major secondary structural elements of human IGF-I are three alpha helices: Helix 1 is comprised of Gly7-Cys 18 in the B domain, helix 2
All sequenced IGF-I and IGF-II peptides from different species. Dashed lines represent identical sequences compared to the human sequence and # was used to frame shift peptides for optimal alignment between sequences. Complete amino acid sequences are given for human insulin, proinsulin and other related molecules (Adapted and updated from a leaflet produced by GroPeP Ltd., Thebarton, SA, Australia.)
involving amino acids Ile43-Cys47 and helix 3 which is Leu54-Leu57 are both located in the A domain. The three dimensional fold of IGF-I is held together by three disulphide bonds: Cys6-Cys48, Cys18-Cys61, Cys47-Cys52 for IGF-I. The overall structure of IGF-I, within the B and A domains, is similar to that seen in the crystal structure of insulin (Bentley et al., 1976; Baker et al., 1988) and the NMR structure of proinsulin (Weiss et al., 1990).

![Figure 1.3 Comparison of the three-dimensional structures of insulin, IGF-I and IGF-II.](image)

The structure of insulin (Bentley et al., 1976), IGF-I (Sato et al., 1992) and IGF-II (Torres et al., 1995) are aligned along the B domain helix shown in blue. The N and C terminus are denoted as N and C respectively. The first and second A domain helix are shown in magenta and orange respectively. The three disulphide bonds are shown in gold.

The C and D domains of the IGFs, are largely unstructured as shown by a high root mean squared deviation of backbone atoms in NMR experiments and the inability to visualize C and D domains atoms in X-ray crystallography studies. In addition, intense resonances seen in the $^{1}H-^{15}N$ HSQC spectrum of IGF-I also suggest that both the C and D domains are inherently flexible in solution (Schaffer et al., 2003). Recently two crystal structures of IGF-I, solved in the presence of detergents to reduce aggregation and molecular flexibility have provided insights into the possible physiological conformation of the C domain. Notably both structures revealed a type-II
β turn, made up of residues Gly30-Ser33 of the C domain, which is proposed to orientate Tyr31 for receptor binding (Vajdos et al., 2001; Brzozowski et al., 2002). Other small turns have been observed including residues 18-21 which form a type II' β-turn causing the B-helix to be extended and residues 24-27 which form a type VIII β-turn allowing the C domain to protrude out from the core of the molecule (Vajdos et al., 2001). It should be noted that the precise physiological orientation of the C domain may not be able to be determined from crystal structures where crystal packing forces and strong lattice contacts can force flexible regions, like the C domain, into distinct but possibly non-native conformations.

The structure of IGF-II has been less extensively studied with only two NMR structures reported (Terasawa et al., 1994; Torres et al., 1995) and no crystal structures elucidated to date. The NMR studies reveal that IGF-II has a high degree of structural similarity with IGF-I and insulin (Figure 1.3). IGF-II contains three disulphide bonds; Cys9-Cys47, Cys21-Cys60, Cys46-Cys51 that constrain the molecule. IGF-II has three alpha helices one in the B domain, Glu12-Cys21 and two in the A domain Glu44-Arg49 and Leu53-Tyr59 all of which are analogous to those in IGF-I and insulin. Given the high degree of conformational flexibility of the IGF-II C and D domains in solution, the structure in these regions is not conclusively defined (Terasawa et al., 1994; Torres et al., 1995). Backbone amides in the C domain of IGF-II exhibited strong DQF-COSY cross peaks suggesting that in this region there is a high degree of flexibility (Torres et al., 1995).

Despite the structural homology between IGF-I and IGF-II each molecule exhibits distinct functional properties. For example, the extracellular matrix protein vitronectin, binds IGF-II but not IGF-I (Upton et al., 1999). The binding of IGF-I to vitronectin is indirect and requires IGFBP-5 (Kricker et al., 2003). The molecular basis for this differential interaction with vitronectin is unclear. In addition IGF-II has been
shown to bind glypican 3, an extracellular matrix proteoglycan and postulated to contribute to the Simpson-Golabi-Behmel overgrowth syndrome (Pilia et al., 1996). Whether IGF-I also interacts with glypican 3 is unknown, however it is unlikely as Simpson-Golabi-Behmel overgrowth syndrome is similar to Beckwith-Wiederman Syndrome which is caused by deregulated IGF-II expression not IGF-I expression.

IGF-I and IGF-II have different affinities for all components of the insulin/IGF system with which they both interact. Table 1.1 shows the relative affinities of IGF-I and IGF-II for the IGF-1R, two IR isoforms, hybrid receptors, IGF2R and IGFBPs.

<table>
<thead>
<tr>
<th>Protein</th>
<th>IGF-I affinity (% of IGF-II affinity)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-1R</td>
<td>400</td>
<td>(Hodgson et al., 1996)</td>
</tr>
<tr>
<td>IGF-2R</td>
<td>&lt; 0.1</td>
<td>(Tong et al., 1988)</td>
</tr>
<tr>
<td>IR-A</td>
<td>≈10</td>
<td>(Frasca et al., 1999)</td>
</tr>
<tr>
<td>IR-B</td>
<td>N.D.</td>
<td>(Frasca et al., 1999)</td>
</tr>
<tr>
<td>Hybrid-A (IGF-1R/IR-A)</td>
<td>200</td>
<td>(Pandini et al., 2002)</td>
</tr>
<tr>
<td>Hybrid-B (IGF-1R/IR-B)</td>
<td>600</td>
<td>(Pandini et al., 2002)</td>
</tr>
<tr>
<td>IGFBP-1</td>
<td>64</td>
<td>(Bach et al., 1993)</td>
</tr>
<tr>
<td>IGFBP-2</td>
<td>30</td>
<td>(Bach et al., 1993)</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>40</td>
<td>(Bach et al., 1993)</td>
</tr>
<tr>
<td>IGFBP-4</td>
<td>53</td>
<td>(Bach et al., 1993)</td>
</tr>
<tr>
<td>IGFBP-5</td>
<td>14</td>
<td>(Bach et al., 1993)</td>
</tr>
<tr>
<td>IGFBP-6</td>
<td>1.5</td>
<td>(Bach et al., 1993)</td>
</tr>
</tbody>
</table>

Table 1.1. Relative affinities of IGF-I and IGF-II for various insulin/IGF receptors and IGFBPs. Shown is the affinity of IGF-I for all proteins listed as a percentage of the IGF-II affinity for that same protein. The values presented here are representative of those affinities reported in the literature. The affinity of IGF-I and IGF-II for all binding proteins varies substantially depending on the methods used to detect binding, source, purity and post-translational modification state of the IGFBP (Coverley and Baxter, 1991). Bach and colleagues provide one of the few reports comparing the binding of IGF-I and IGF-II to all six IGFBPs. N.D. not accurately determined i.e. no EC\textsubscript{50} given, however the affinity of IGF-II for the IR-B is higher than IGF-I for the IR-B.
The biological functions of IGF-I and IGF-II mediated by the IGF-1R and the IR will be discussed below.

1.3 Insulin-like growth factor I receptor (IGF-1R) – Structure and Function

The insulin-like growth factor I receptor (IGF-1R) is a transmembrane tyrosine kinase receptor that is expressed across a variety of foetal and adult tissues (Werner et al., 1989; Bondy et al., 1990). IGF-1R signalling is essential for embryonic development and along with GH signalling, is responsible for almost all pre and postnatal longitudinal growth (Liu et al., 2002). Mice with a targeted disruption of the IGF-1R gene have a birth weight of only 45% of normal and dies within minutes of birth due to respiratory failure (Liu et al., 1993). IGF-1R mutations, partial deletions or hemizygosity at the IGF-1R locus in humans, causing a reduced receptor number on cells or reduced sensitivity to IGF-I, lead to intrauterine growth retardation that persists into adulthood (Roback et al., 1991; Tamura et al., 1993; Peoples et al., 1995; Siebler et al., 1995; Abuzzahab et al., 2003; Okubo et al., 2003).

The IGF-1R is synthesized as a precursor that is glycosylated on the extracellular regions, dimerized and cleaved to yield separate α and β chains (reviewed in (Adams et al., 2000)). The mature processed IGF-1R is a homodimer consisting of two α and two β subunits held together by disulphide bonds (Bhaumick et al., 1981; Chernausek et al., 1981). Ligand binding to the extracellular region causes a conformational change resulting in tyrosine phosphorylation of the intracellular β subunits which then causes an increase in the intrinsic kinase activity of the receptor (Rubin et al., 1983).

While the complete structures IGF type 1 receptor (IGF-IR) and the related insulin receptor (IR) have not been determined, the structure of the first three domains (L1-cys-rich-L2) of the IGF-IR have been solved (Garrett et al., 1998). The L domains
resemble other leucine-rich repeat proteins and consist of a single-stranded, right-hand parallel β helix (Ward and Garrett, 2001), while the cys-rich region is composed of eight disulphide-bonded modules and resembles the cys-rich repeats in laminin. These three domains surround a cavity large enough to accommodate ligand (Garrett et al., 1998). Indeed alanine scanning mutagenesis of the IGF-1R confirmed the prediction of the structural studies in showing that both the L1 and cys-rich domains are critical for IGF-I binding and that the L1 domain is important for IGF-II binding (Whittaker et al., 2001; Sorensen et al., 2004). Within the insert domain, located at the C terminus of the α subunit, lies residues 692-702 also shown by alanine scanning mutagenesis to be required for wildtype binding of both IGF-I and IGF-II to the IGF-1R (Whittaker et al., 2001; Sorensen et al., 2004). The complete domain organization of the IGF-1R is similar to that of the insulin receptor (Figure 1.10).

Tissue specific mouse knockout models and a vast body of in vitro experimental data suggest that the IGF-1R can promote a variety of cellular responses such as cell proliferation, differentiation, migration and protection from apoptosis (reviewed in (Khandwala et al., 2000)). Over-expression of the IGF-1R can cause a cell to display transformed characteristics which will be discussed below.

1.3.1 IGF-1R signalling

The IGF-1R can mediate a number of biological responses of the IGFs in various tissues. In doing so the IGF-1R activates a variety of intracellular signalling pathways (reviewed in (Gray et al., 2003; O'Connor, 2003)). Due to the similar structures of the IGF-1R and the IR both initiate overlapping signalling pathways and many reviews have been the subject of whether IGF-1R and IR signalling are actually unique (Dupont and LeRoith, 2001; Nakae et al., 2001; Siddle et al., 2001; Kim and Accili, 2002). Although signalling will be discussed here in the context of the IGF-1R,
all pathways and molecular events detailed are also pertinent to IR signalling. Upon ligand binding to the IGF-1R extracellular α subunits a conformational change is proposed to be transmitted via the transmembrane domains causing the intracellular β subunit to adopt an orientation that allows transphosphorylation. Phosphorylation of tyrosine residues in the β subunits provide docking sites for adaptor proteins such as IRS-1 (insulin receptor substrate 1) and Shc (Src homology/ collagen). These proteins can then be phosphorylated by the IGF-1R kinase and these phosphorylation sites then provide binding sites for SH2 domain containing signalling molecules like Grb2 (Growth factor receptor binding protein 2). The guanine nucleotide exchange factor Sos is constitutively associated with Grb2 and it promotes the exchange of Ras-bound GDP to GTP thereby activating Ras. The succession of protein-protein interactions then constitute the mitogen-activated protein kinase (MAPK) pathway that ultimately can regulate transcription as a biological outcome (Chang and Karin, 2001).

Once IRS-1 is phosphorylated, p85, which is the regulatory subunit of PI3K (phosphoinositide 3-kinase), can bind via its two SH2 domains. The p85 subunit of PI3K binds to the catalytic subunit of PI3K, of which p110 is one isoform, which in turn induces phosphorylation of phosphatidylinositol lipids such as phosphotidyl inositol (4,5) bisphosphate (PI(4,5)P2) and phosphotidyl inositol (4) phosphate (PI(4)P1). The subsequent molecular interactions comprise the PI3K pathway which is critical for protection from apoptosis, induction of protein synthesis via mTOR and its down stream targets and cell cycle regulation (Cantrell, 2001). The JNK pathway is involved in apoptosis protection and regulation of gene transcription (Chang and Karin, 2001). These pathways are summarised in Figure 1.4.
Figure 1.4. Signalling pathways activated by the IGF-1R (or IR). Recruitment of IRS-1 and Shc to an activated IGF-1R/IR provides a mechanism for activation of the MAPK and PI3K pathway. A succession of molecular interactions results in either a change in transcription, cell proliferation, protein synthesis, protection from apoptosis or rearrangement of the cytoskeleton and promotion of migration.
1.3.2 Role of the IGF-1R in cancer

It has been postulated that the IGF-1R is an important factor in the pathogenesis and progression of certain cancers due to the potent survival signals generated by the IGF-1R and the implicit requirement of a cell to evade apoptosis if it is to become cancerous (Hanahan and Weinberg, 2000). Experiments in vitro and in vivo have revealed that over-expression of the IGF-1R allows ligand-dependent growth in serum-free media, ligand-dependent colony formation in soft agar and tumour formation in nude mice (Kaleko et al., 1990; Pietrzkowski et al., 1992). The presence of the IGF-1R is obligatory for the transforming potential of many oncogenes e.g. SV40 T antigen (Sell et al., 1993), EGFR (Coppola et al., 1994) and Ha-Ras (Sell et al., 1994), but not all oncogenes e.g. v-src and the GTPase-deficient mutant of G_{a13} (Liu et al., 1997; Valentinis et al., 1997). The progression of several malignancies, e.g. colon (Hakam et al., 1999), melanoma (Kanter-Lewensohn et al., 1998), sarcoma (Sekyi-Otu et al., 1995; Xie et al., 1999) and pancreatic carcinoma (Bergmann et al., 1995), to a more aggressive, metastatic phenotype parallels an increase in IGF-1R expression. However, the mere presence of the IGF-1R rather than the expression level has been postulated to be essential for cancer progression in the case of prostate cancer (Tennant et al., 1996; Damon et al., 2001; Pollak et al., 2004). Moreover in late stage breast cancer the IGF-1R exhibits elevated autophosphorylation and kinase activities (Resnik et al., 1998). Mice heterozygous for an IGF-1R knockout show a reduction in the tissue level expression of the IGF-1R, however the cancer incidence in these mice has not been reported (Holzenberger et al., 2003). Crossing these mice with another strain prone to develop certain cancers may shed light on the in vivo role of the IGF-1R in cancer pathogenesis.

Due to the integral nature of the IGF-1R in neoplastic formation and growth, strategies to inhibit several aspects of IGF-1R biology have been investigated as
techniques for treating cancer; 1) reducing receptor expression using antisense oligonucleotides, antisense expression vectors (Resnicoff et al., 1995; Burfeind et al., 1996; Lee et al., 1996; Pass et al., 1996; Nakamura et al., 2000; Macaulay et al., 2001), small interfering RNAs (Bohula et al., 2003) or triple helix forming homopurine oligonucleotides (Rininsland et al., 1997), 2) inhibiting ligand binding or down regulating receptor levels using antibodies (Zia et al., 1996; Burtrum et al., 2003; Ye et al., 2003; Lu et al., 2004), dominant-negative receptors (D'Ambrosio et al., 1996; Reiss et al., 1998; Adachi et al., 2002; Lee et al., 2003; Min et al., 2003) or processing enzyme inhibitors (Khatib et al., 2001) and 3) inhibiting kinase activity using tyrosine kinase inhibitors (Blum et al., 2000; Blum et al., 2003; Garcia-Echeverria et al., 2004; Mitsiades et al., 2004). Each strategy has at least been shown to inhibit cancer cell growth in vitro while most have been shown to be effective in reducing tumour growth in vivo in mouse models (except (Blum et al., 2000; Blum et al., 2003; Bohula et al., 2003)).

1.3.3 IGF residues involved in IGF-1R binding

While the structure of IGF-I, IGF-II and the first three domains of the IGF-1R have been solved, to date a structural complex of IGF-I or IGF-II with any fragment of the IGF-1R does not exist. For this reason the residues on IGF-I and IGF-II that interact with the receptor (and those of the receptor that interact with the IGFs) have been probed using other biochemical and protein engineering techniques such as mutagenesis, deletion or chimera formation between IGF-I and insulin. These findings will be discussed below highlighting the IGF residues important in IGF-1R binding, considering each IGF domain separately. Each section has a sequence alignment of the appropriate domain of IGF-I and IGF-II in the text showing the position of the mutation and the resulting change in IGF-1R binding affinity relative to IGF-I.
CHAPTER 1 – Introduction

B Domain

Substitution of tyrosine 24 in the IGF-I B domain by either serine or leucine resulted in either a 16-fold or 32-fold loss in IGF-1R binding affinity respectively (Cascieri et al., 1988; Bayne et al., 1990). Substitution of phenylalanine 23 by glycine decreased IGF-1R binding by 48-fold (Hodgson et al., 1996). Despite these findings not all residues in the B domain have been shown to be critical for IGF-1R binding. An example is the substitution of the first 16 amino acids with the first 17 of insulin, thereby introducing 10 sequence differences, had almost no effect on IGF-1R binding (Bayne et al., 1988). Contrary to the findings of Bayne and colleague mutations of alanine 8 to leucine resulted in a 6-fold decrease in IGF-1R binding (Shooter et al., 1996). This highlights the potential of the introduced amino acid to determine the change in IGF-1R binding and reveals the inherent problem in interpreting mutagenesis data.

Substitution of valine 11 by threonine, but not isoleucine, resulted in a 3-fold decrease in IGF-1R binding affinity relative to wildtype IGF-I (Hodgson et al., 1995). Mutation of various residues within the B domain α-helix resulted in decreased IGF-1R affinities as determined by surface plasmon resonance analysis (Jansson et al., 1997). Far-UV circular dichroism spectra indicated however that the mutations had caused reduced α-helical content despite the targeted residues being the most solvent exposed in the B domain α-helix. Consequently the reduction in IGF-1R binding affinity of 2.5-fold when valine 11 was mutated to alanine, 4-fold when apartate 12 was mutated to alanine, 2-fold when glutamine 15 was mutated to alanine or 37-fold when phenylalanine 16 was mutated to alanine does not accurately reflect the effect of removing the individual side chains past the β-carbon but is more likely due to a local structural change (Jansson et al., 1997). In a separate study by Jansson et al. mutation of arginine 21 to alanine caused a 3-fold decrease in IGF-1R binding, as determined by
surface plasmon resonance studies, but had wildtype affinity for IGFBP-I (Jansson et al., 1998).

The IGF-II B domain has not been studied as extensively as the IGF-I B domain. However, Sakano et al. highlighted the importance of phenylalanine 26 in IGF-II in the IGF-1R interaction by mutating this residue to serine and observing a 5-fold decrease in binding affinity to the human placental IGF-1R (Sakano et al., 1991). More striking was the substitution of tyrosine 27 in IGF-II with leucine, which caused a 132-fold decrease in human placental IGF-1R binding affinity relative to recombinant human IGF-II (Sakano et al., 1991). The critical nature of tyrosine 27 in IGF-II was confirmed when it was substituted for glutamate and the resulting IGF-1R binding was decreased by 200-fold (Roth et al., 1991). Sequential deletions of the N-terminus of IGF-II revealed threonine 7 and leucine 8 are critical for IGF-1R binding (Hashimoto et al., 1995). A naturally occurring variant of human IGF-II with a substitution of serine 29 for arginine and an insertion of leucine-proline-glycine at position 30 caused a 3-fold reduction in IGF-1R binding (Hampton et al., 1989).

Figure 1.5 Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-1R binding.

C Domain

Chimeras of insulin and IGF-I have been used extensively in an attempt to map the regions of IGF-I important in binding the IGF-1R. Cara et al. attached the IGF-I C domain to the C-terminus of the insulin B chain and showed a 67-fold increase in IGF-1R binding affinity relative to native human insulin (Cara et al., 1990).
Substitution of the positively charged residues in the IGF-I C domain, arginine 36 and 37 with alanine caused a 15-fold decrease in IGF-1R affinity (Zhang et al., 1994). However, in a separate study where the same mutant was prepared only a 5-fold decrease in IGF-1R was observed (Jansson et al., 1998). Zhang et al. produced the mutant IGF-I as a fusion with an N-terminal flag tag which may have altered the binding properties of the mutant. In addition, Zhang et al. used a radiolabel competition binding assay to determine the effect of the mutation in binding to human placental IGF-1R while Jansson et al. utilized surface plasmon resonance technology (BIAcore) to determine the IGF-I mutant binding to recombinantly produced IGF-1R. Either the technique used to measure binding or the source of IGF-1R could affect the results. Replacement of the equivalent arginines in the C domain of IGF-II with glutamines reduced the IGF-1R binding affinity by only 2-fold (Edwards and Bawden, 1993). Tyrosine 31 of IGF-I is also important in IGF-1R binding as mutation to alanine causes a 6-fold affinity decrease (Bayne et al., 1990). Replacement of the entire C domain, with a tetra-glycine bridge ([1-27,Gly,38-70] IGF-I) designed to preserve the tertiary structure, caused a 30-fold decrease in IGF-1R affinity (Bayne et al., 1989). Complete deletion of the IGF-I C domain resulted in a two chain IGF with no detectable affinity for the IGF-1R (Gill et al., 1996). Removal of the C domain caused non-native reorientation of the A domain helices accounting for the loss in IGF-1R binding affinity (De Wolf et al., 1996).

![Sequence alignment of the C domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-1R binding.](image-url)

Figure 1.6 Sequence alignment of the C domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-1R binding.
A Domain

Exchanging residues 42-56 in IGF-I with the first 15 residues of the insulin A chain (including a substitution of threonine 41 for a isoleucine in the C domain) which effectively introduces 8 sequence differences, did not affect IGF-1R affinity for IGF-I (Cascieri et al., 1989). In addition mutation of phenylalanine 49, arginine 50 and serine 51 to that of insulin did not affect IGF-1R binding affinity (Cascieri et al., 1989). A separate mutant where arginine 55 and 56 were mutated to tyrosine and glutamine, respectively, also did not result in a reduction in IGF-1R binding (Cascieri et al., 1989). Furthermore mutation of arginine 56 to alanine only lead to a 2-fold decrease in IGF-1R binding compared to wildtype IGF-I (Jansson et al., 1998). The importance of the residues at the end of the second α-helix in maintaining high affinity IGF-1R binding was revealed when methionine 59 was mutated to phenylalanine causing a 5-fold decrease in IGF-1R binding affinity relative to wildtype IGF-I (Shooter et al., 1996) and mutation of tyrosine 60 to leucine yielded a protein with a 20-fold decrease in IGF-1R binding affinity relative to wildtype IGF-I (Bayne et al., 1990).

Mutation of valine 43 in IGF-II to leucine decreased IGF-1R affinity by 16-fold relative to wildtype IGF-II (Sakano et al., 1991). Mutation of residues alanine 54 and leucine 55 of IGF-II to the equivalent residues in IGF-I, arginine 55 and 56, did not change the IGF-1R binding affinity (Forbes et al., 2001).

![Figure 1.7 Sequence alignment of the A domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-1R binding.](image)
Deletion of the IGF-I D domain had a negligible effect on IGF-1R binding (Bayne et al., 1989), however this finding is in contrast to another study where the charged residues in the IGF-I D domain, lysine 65 and 68 were replaced with alanine causing a 10-fold decrease in IGF-1R affinity (Zhang et al., 1994). The contribution of the D domain to IGF-1R binding affinity appears different in the context of either IGF-I or IGF-II as deletion of the D domain of IGF-II did result in a 5-fold decrease in IGF-1R binding affinity (Roth et al., 1991).

![Sequence alignment of the D domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-1R binding.](image1)

![Summary of Mutagenesis studies. NMR structure of IGF-I (Sato et al., 1993) is depicted in surface mode. The colour are as in the text and refer to changes in IGF-1R affinity as a result of mutation. Figure constructed using Insight II.](image2)

**Figure 1.9 Summary of Mutagenesis studies.** NMR structure of IGF-I (Sato et al., 1993) is depicted in surface mode. The colour are as in the text and refer to changes in IGF-1R affinity as a result of mutation. Figure constructed using Insight II.

### 1.3.4 Summary of IGF residues involved in IGF-1R binding

Taken together these data indicate that while certain residues and the secondary structure in the B and A domains are involved in IGF-1R, the C domain and certain residues in the D domain are indispensable for high affinity IGF-1R binding. IGF-II
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has been largely unstudied with respect to the residues that are important for binding the IGF-1R.

1.3.5 Mechanism of IGF binding to the IGF-1R

The exact mechanism of IGF-I or IGF-II binding to the IGF-1R is poorly understood. Few models have been put forward as possible mechanisms for IGF binding to the IGF-1R due to the lack of both structural and kinetic data of the IGF/IGF-1R interaction. In addition, Scatchard plots of IGF-I binding to the IGF-1R have been reported as both linear and curvilinear, stimulating confusion as to whether the receptor has only one or in fact two different affinities for an IGF ligand. Despite this, two seminal reviews were published by Pierre De Meyts during 1994, in which he proposed a possible ligand-binding mechanism of the IGF-1R (De Meyts, 1994a; De Meyts et al., 1994b). His “cross-linking” model for IGF binding to the IGF-1R is analogous to that which he had originally proposed for insulin binding to the IR and therefore will be discussed in the IR section 1.4.9.

Sequence analysis and limited structural information suggests that both IR and IGF-IR are very similar (Ullrich et al., 1986). It is therefore not surprising that all three growth factors (insulin, IGF-I and IGF-II) can interact with both receptors to initiate signalling cascades. The following section will discuss the structure and function of the insulin receptor.

1.4 Insulin receptor

The insulin receptor (IR) is a transmembrane glycoprotein that mediates the pleiotropic actions of insulin. The many biological roles that the IR plays have been investigated using tissue specific mouse knockout studies. Such studies have shown the IR to be important in neovascularization (Kondo et al., 2003), adipogenesis
(Entingh et al., 2003), pancreatic insulin secretion in response to glucose (Kulkarni et al., 1999), glucose disposal in muscle and adipose (Lauro et al., 1998) and regulation of hepatic glucose synthesis (Michael et al., 2000). Dysfunctional insulin receptors and/or insulin receptor mediated signalling has been implicated in a wide variety of diseases ranging from type 2 diabetes to cancer, underlying its importance in human pathology (Sesti, 2000; Sesti et al., 2001; De Meyts and Whittaker, 2002). The literature on the role of the insulin receptor in type 2 diabetes is extensive and several comprehensive reviews have been published recently (Virakamaki et al., 1999; Kahn and Flier, 2000; Sesti, 2000; Sesti et al., 2001; Zick, 2001; Mauvais-Jarvis et al., 2002).

1.4.1 Insulin receptor (IR) – structure and function

The insulin receptor is a transmembrane glycoprotein with two extracellular α subunits and two membrane spanning β subunits that form a β-α-α-β arrangement (Czech, 1982). The domain organisation of the receptor is shown in Figure 1.10 (see reviews (Adams et al., 2000; Siddle et al., 2001; De Meyts and Whittaker, 2002)). The major ligand binding determinants are located within the α subunits (Schaefer et al., 1990; Brandt et al., 2001) and the intrinsic tyrosine kinase domain is located in the cytoplasmic portions of the β subunits (Kasuga et al., 1982). Using chimeric insulin/IGF-I receptors the cysteine rich domain has been identified as the major IGF-I ligand binding site while both the amino- and carboxy-terminal regions of the α subunit constitute the insulin binding site (Andersen et al., 1990; Kjeldsen et al., 1991; Schumacher et al., 1991). Cross-linking experiments have shown insulin binding to residues in the amino terminus of the α subunit (Wedekind et al., 1989) as well as in the L2 domain (Fabry et al., 1992) and to residues 704-718 at the carboxy-terminus of the α subunit (Kurose et al., 1994) (see Figure 1.10). Reconstitution of full
holoreceptor affinity for insulin has been reported by fusing the first three domains of
the α subunit with the first fibronectin type III domain, which are residues encoded by
exon 10, and 16 amino acids from the C-terminus of the α subunit (Brandt et al.,
2001). This suggests that the α subunits contain all the required information for high
affinity insulin binding (Brandt et al., 2001). Alanine scanning mutagenesis more
recently characterised the energetic contribution of many amino acids within these
regions to the insulin binding event. Specifically amino terminal residues in four
distinct regions 1-(Asp12, Ile13, Arg14 and Asn-15), 2-(Gln-34, Leu-36, Met-38, Phe-
29 and Glu-44), 3-(Phe-64 and Tyr-67) and 4-(Phe-89, Asn-90 and Tyr-91) and
C-terminal residues Thr-704, Phe 705, Glu-706 and His 710 contribute most to free
energy of insulin binding (see Figure 1.10) (Williams et al., 1995; Mynarcik et al.,
1996).

Figure 1.10 Structure of the insulin receptor and exon 11 encoded sequence.
A) Domain organisation of the IR: L1 and L2; large domains 1 and 2 (Leucine-rich
repeats); CR, cysteine rich domain; Fn0, Fn1, Fn2, fibronectin type III domains; Ins,
insert domain; Ex11, exon 11 encoded peptide; TM, transmembrane domain; JM,
Juxtamembrane domain; TK, tyrosine-kinase domain; CT, carboxy-terminal tail. Solid
arrows represent the major receptor sites involved in insulin binding (see text for
details). Y, indicates potential phosphorylation sites at Y960 in the juxtamembrane
domain, Y1158, Y1162, Y1163 in the tyrosine-kinase domain and Y1316 and Y1322
in the carboxy terminal tail (Hubbard, 1997).
B) Sequence alignment of IR-A, IR-B and IGF1R. The exon 11 peptide in IR-B has 12
amino acids at position 717-728 (numbering as in (Ebina et al., 1985)). The IR-A lacks
this region (numbering as in (Ullrich et al., 1985)). The IGF-1R is “IR-A like”, lacking
an exon 11 encoded analogous peptide (numbering as in (Ullrich et al., 1986)). Solid
line indicates site of furin cleavage of the proreceptors, which upon digestion yield the
separate α and β subunits.
Insulin receptors are capable of binding two molecules of insulin (Yip and Jack, 1992). However, curvilinear Scatchard plots indicate that the receptor binds each molecule of insulin with different affinities. A model for insulin binding to the IR has been proposed where a molecule of insulin cross links separate epitopes on each α subunit constituting high affinity binding and a second molecule of insulin can bind to only one α subunit with lower affinity (negative cooperativity) (De Meyts, 1994a; Schaffer, 1994)(reviewed by De Meyts and Whittaker (De Meyts and Whittaker, 2002) and a more detailed discussion of IR binding models is in section 1.4.9). Reduction of class I disulphide bonds of the receptor, yielding αβ monomers, results in a 10 fold loss of affinity for insulin binding suggesting that dimerisation of αβ monomers confers intact IR binding affinity (Boni-Schnetzler et al., 1987). Receptor deletion constructs have shown that the first two fibronectin type III domains provide disulphide bonds for α subunit dimerisation while the IR characteristic of negative cooperativity is conferred by the second fibronectin domain (Molina et al., 2000; Surinya et al., 2002).

Upon ligand binding the receptor undergoes a conformational change allowing the cytoplasmic β subunits to transphosphorylate tyrosine residues 1158, 1162 and 1163 (Frattali et al., 1992). Phosphorylation of these tyrosine residues releases the autoinhibitory mechanism of the catalytic loop revealing the kinase active site and allowing unobstructed access of ATP and protein substrates leading to activation of the receptor’s intrinsic kinase activity (Hubbard, 1997). Tyrosine 960 in the juxtamembrane domain is phosphorylated by the kinase domain and then provides a docking site for adaptor proteins such as Insulin Receptor Substrates 1-4 (IRS 1-4) and Shc. Docking of these and other adaptor molecules to the receptor then allows their phosphorylation by the IR kinase. Tyrosine phosphorylation of the IRS proteins provides docking sites for other downstream signalling components that contain Src
homology 2 (SH2) domains such as p85, the regulatory subunit of PI3K, which ultimately results in the mediation of a large number of insulin effects including glycogen synthesis (Shepherd et al., 1995), mitogenesis and inhibition of apoptosis. Phosphorylation of IRS and Shc also recruits other proteins, for example Grb2, via its Src homology 2 (SH2) domains, which in turn activates the guanine-nucleotide-exchange factor Sos (son of sevenless). This activates the mitogen-activated protein kinase (MAPK) cascade critical for the biological response of insulin (Figure 1.4 shows signalling through the IGF-1R but all signalling pathways depicted are also activated by the IR). Despite similarities in IR and IGF-1R signalling, there are many differences, reviewed in (Blakesley et al., 1996; Dupont and LeRoith, 2001; Nakae et al., 2001; Kim and Accili, 2002). For a detailed review of insulin signalling, see (Virkamaki et al., 1999; Siddle et al., 2001)).

Many knockout studies have revealed the importance of the insulin receptor in normal growth and fuel homeostasis. Mice homozygous for ablation of the IR are born with only a 10% reduction in birth weight compared to their wildtype littermates, but die within several days from diabetic ketoacidosis (Louvi et al., 1997). This phenotype suggests that although the IR is not critical for embryonic growth it is vital for normal metabolic processes. Mice with tissue specific knockouts of the IR have been instrumental in more specifically elucidating the role of the IR in several normal physiological and pathophysiological processes (for extensive reviews see (Mauvais-Jarvis et al., 2002; Kitamura et al., 2003)). Briefly, tissue specific knockouts in mice have shown the IR to be involved in neovascularization (Kondo et al., 2003), adipogenesis (Entingh et al., 2003), pancreatic insulin secretion in response to glucose (Kulkarni et al., 1999), glucose disposal in muscle and adipose (Lauro et al., 1998) and regulation of hepatic glucose production (Michael et al., 2000; Fisher and Kahn, 2003).
1.4.3 Insulin receptor isoforms

In the initial reports of the primary sequence of the insulin receptor two sequences were identified which differed by 12 amino acids (Ebina et al., 1985; Ullrich et al., 1985). Further characterization of the intron/exon organization of the insulin receptor gene revealed that the 12 amino acid difference was the result of alternative splicing of exon 11, a 36 base-pair exon (Seino et al., 1989). In the mature receptor the amino acids derived from the 36 base pairs are found at the extreme C-terminus of the α subunits (Figure 1.10).

Alternative splicing of exon 11 is regulated both developmentally and in a tissue specific manner (Moller et al., 1989; Seino and Bell, 1989; Mosthaf et al., 1990; Frasca et al., 1999). Elements within intron 10 and exon 11 act to regulate the splicing process (Kosaki et al., 1998). Intron 10 and exon 11 both contain sequences that can enhance inclusion or exclusion of exon 11. The precise mechanism and splicing factor(s) involved are to date not known. However the RNA processing factor CUG-binding protein has been shown to bind to an 110 nucleotide sequence within intron 10 of the IR transcript. Its overexpression decreases the inclusion of exon 11 in NIH 3T3 fibroblasts (Savkur et al., 2001). Two other putative IR mRNA splicing proteins, CELF-6 and Muscleblind 1, have been show to promote exon 11 exclusion or inclusion respectively (Ho et al., 2004; Ladd et al., 2004).

The seemingly small sequence difference between the isoforms results in the receptors having quite different properties. Inclusion of the exon 11 (IR-B or IR exon 11 +) encoded peptide results in a receptor that has decreased affinity for IGF-I and IGF-II and only a small decrease in affinity for insulin (Mosthaf et al., 1990; McClain, 1991; Yamaguchi et al., 1991; Yamaguchi et al., 1993; Kotzke et al., 1995). The IR-B has greater autophosphorylation and kinase activities (Kellerer et al., 1992; Kosaki et al., 1995) as well as altered internalisation kinetics (Vogt et al., 1991). Despite a
relatively small effect on insulin binding affinity alanine scanning of the proposed insulin binding site on both IR isoforms has revealed differences in the energetic contribution of receptor side chains, suggesting different modes of insulin binding (Whittaker et al., 2002). This suggests that there is considerable accommodation for structural differences induced by the extra 12 amino acids to allow almost equal binding affinities for insulin. As there is a greater effect on IGF binding it would now be interesting to compare the IR isoform alanine mutants for their IGF binding properties to further delineate the binding site and specific interactions with IGF-I or IGF-II.

In some cases insulin activation of the two IR isoforms results in a similar outcome. For example, insulin binding to both IR-A or IR-B stimulates glucose uptake and thymidine incorporation, with IR-A responding similarly to (Yamaguchi et al., 1991), or better than IR-B (McClain, 1991). In addition, insulin signalling can induce a downregulation of IR-A in some (McClain, 1991) but not other (Yamaguchi et al., 1991, 1993) transfected cells overexpressing the receptor. In contrast, there appear to be isoform-specific insulin-induced signalling differences. Insulin stimulates both insulin production and β-glucokinase transcription in pancreatic β cells transfected with either IR-A or IR-B. However, insulin is more efficient at stimulating insulin expression through the class I PI3-K pathway in IR-A expressing islets whereas signalling though the IR-B preferentially activates an alternate PI3-K class II pathway ultimately leading to increased β-glucokinase transcription (Leibiger et al., 2001), although this has not been demonstrated by other groups. It has been suggested that an increase in the relative expression of IR-B by pancreatic β cells may decrease insulin-stimulated insulin gene expression. Interestingly, the differential ability of IR-A and IR-B to activate insulin and glucokinase transcription is dependent on their isoform-specific cell localisation (Uhles et al., 2003).
Similar to insulin, IGF-II binding to IR-A and IR-B can result in different biological outcomes. For instance, in 32D cells transfected to express IRS-1, IR-A is more effective than IR-B, when activated by IGF-II, in protecting cells from apoptosis induced by IL-3 withdrawal (Sciacca et al., 2003). In 32D cells not transfected to express IRS-1, IR-B cells were more effective in inducing differentiation in response to IGF-II activation than the IR-A, as measured by mRNA levels of the differentiation marker MPO (Sciacca et al., 2003). The IR-A but not the IR-B induces nuclear translocation of IRS-1, the biological consequence of this difference is currently unknown (Wu et al., 2003), however IGF-I induced nuclear translocation of IRS-1 in mouse embryonic fibroblasts causes an increase in ribosomal RNA synthesis (Tu et al., 2002; Sun et al., 2003). These different responses to insulin and IGF-II may have interesting implications for the role of the IR-A in disease as discussed below.

1.4.4 Insulin receptor isoform tissue distribution

Normal Physiology

The relative expression of the insulin receptor isoforms varies tissue specifically, developmentally and pathophysiologically supporting the proposal that the two isoforms have unique functions. In normal human physiology the IR-A is expressed predominantly in foetal tissues such as the kidney, muscle, liver and fibroblasts (Frasca et al., 1999), and exclusively in the adult spleen, peripheral blood cells and adult brain (Seino and Bell, 1989; Mosthaf et al., 1990). The IR-B, however, is expressed primarily in the liver and adipose tissue (Moller et al., 1989; Mosthaf et al., 1990). Differentiation leads to the expression of IR-B in preference to IR-A (Frasca et al., 1999) as demonstrated by treating HepG2 cells with dexamethasone (Kosaki and Webster, 1993; Kosaki et al., 1995; Frasca et al., 1999). Therefore, this
would suggest that the IR-A isoform could be regarded as the foetal form whereas IR-B exists in adult tissues and differentiated cell types. Hormonal and metabolic factors regulate the alternative splicing. For example, insulin and high glucose concentrations induce the expression of IR-B in some insulin responsive cell lines and tissues (Kosaki and Webster, 1993; Norgren et al., 1994; Sell et al., 1994; Hribal et al., 2003).

**The Insulin receptor in Disease**

The biophysical and biochemical properties of the isoforms are being investigated as possible causes for various disease states. The Italian groups of Belfiore, Sesti and Lauro have recently contributed greatly to the understanding of the two IR isoforms in disease and in particular in cancer and type 2 diabetes. Interestingly, increased prevalence of the lower kinase activity of IR-A is postulated to be the cause of insulin resistance in myotonic dystrophy (Savkur et al., 2001; Savkur et al., 2004). In addition, expression of IR isoforms has been investigated in tissues in hypertension (Lou et al., 1996), obesity (Anderson et al., 1993), fasting (Vidal et al., 1995), aging (Vidal et al., 1995; Wiersma et al., 1997) and leprechaunism (van der Vorm and Maassen, 1994).

**1.4.5 The role of the insulin receptor isoforms in cancer**

Early in 1999 Frasca et al. (Frasca et al., 1999) and Sciacca et al. (Sciacca et al., 1999) from the same research group, were the first to demonstrate differential expression of the IR isoforms in cancer tissue versus normal tissue. Preferential expression of the IR-A isoform occurs in many cancers including those of the lung, colon (Frasca et al., 1999), breast (Frasca et al., 1999; Sciacca et al., 1999), ovaries (Kalli et al., 2002), thyroid (Vella et al., 2002) and smooth and striated muscle
The IR-B is also downregulated in hepatoblastomas (von Horn et al., 2001). Interestingly, in thyroid cancer not only is IR-A preferentially expressed but there is an increase in overall level of IR-A as the cancer progresses (Vella et al., 2002). Expression of the IR-A isoform in cancer correlates well with previous observations that IR-A is expressed in dedifferentiated cells (Kosaki and Webster, 1993; Frasca et al., 1999).

These observations have provided an interesting complexity to the role of IGFs in cancer. There is substantial evidence that activation of the IGF-1R plays a major role in the development and progression of certain cancers (reviewed by LeRoith and Roberts (LeRoith and Roberts, 2003)). Binding of IGFs to the IGF-1R results in cancer cell proliferation and survival. However, Morrione et al. demonstrated that stimulation by IGF-II and insulin but not IGF-I resulted in proliferation and survival of an R- fibroblast cell line, devoid of IGF-1R, transfected to express the IR (Morrione et al., 1997). Importantly, activation of IR-A in breast cancer cell lines by IGF-II also leads to cell proliferation (Sciacca et al., 1999). Also, the observation that IGF-II can protect cancer cells from apoptosis by activation of IR-A in vitro (Sciacca et al., 2002) suggests a critical role for this isoform in cancer. The IR-A has a similar affinity to the IGF-1R for IGF-II suggesting that in a cell expressing both receptors IGF-II may activate either or both receptors possibly leading to an enhanced effect on cell growth and survival (Sciacca et al., 1999).

The fact that IGF-II is over expressed by many cancers such as sporadic adrenocortical tumours (Gicquel et al., 1994), colorectal cancer (Renehan et al., 2000) and breast cancer (Quinn et al., 1996) where the IR-A is the predominant isoform adds further to the argument that IR-A is involved in cancer. Over expression of IGF-II often results from LOI (loss of imprinting) of the IGF-II gene. This relaxation of the imprinting of the maternal gene has been identified as a possible risk marker for
colorectal cancer (Cui et al., 2003) and changes in the imprinting of the IGF-II locus may predispose development of Wilms tumours in embryonic kidneys (Okamoto et al., 1997). Over expression of IGF-II in vitro results in a more malignant phenotype in the breast cancer cell line MCF-7 (Cullen et al., 1992). In addition, the type 2 IGF receptor (IGF-2R) mediates IGF-II internalisation and degradation and is frequently mutated in cancers leading to loss of IGF-II binding and therefore resulting in increased circulating IGF-II levels (Byrd et al., 1999; Devi et al., 1999). Loss of IGF-2R expression promotes IGF-II activation of IRS-1, a substrate of the IR (Osipo et al., 2001) whereas a colorectal cancer cell line transfected to over express the IGF-2R showed decreased growth and enhanced apoptosis relative to non-transfected cells (Souza et al., 1999). This suggests that IGF-II in conjunction with the IR-A may be providing an autocrine/paracrine growth stimulatory loop in certain tumours and may promote cancer progression. Supporting this hypothesis is the observation that increased expression of both IGF-II and IR-A occurs as thyroid cancer progresses (Vella et al., 2002). Furthermore, experimental evidence supports this hypothesis. IGF-II has been shown to be more effective than insulin at stimulating cell migration and invasion in an IGF-1R deficient leiomyosarcoma cell line SKUT-1, that express 95% IR-A (Sciacca et al., 2002). Interestingly in the same study a blocking antibody against IGF-II decreased the ability of cells to migrate suggesting that autocrine production of the IGF-II mediates cancer cell motility. Most of the studies investigating the role of IR isoforms and IGF-II in cancer have used cell lines deficient in IGF-1R and overexpressing the IR-A or IR-B. Further analysis of responses in cells expressing receptor levels reflecting cancer cells in vivo will be necessary to confirm the function of the different isoforms.

Recently it has been shown that insulin and IGF-II may induce differential gene regulation through the IR-A (Pandini et al., 2003). Several genes involved in
protection from apoptosis were identified as upregulated by IGF-II to a larger extent than insulin when signalling through the IR-A e.g. acidic nuclear phosphoprotein 32 (ANP32) and TDAG51 (Pandini et al.). Novel findings by the group also included the upregulation of angiogenic genes, Mrp/prl and proliferin, by insulin but not IGF-II. These differences in expression were small but correlated well with real time PCR analyses. The significance of these findings remains to be determined but they indicate that potentially there is differential signalling when insulin or IGF-II stimulate the IR-A.

The factors leading to the switch to IR-A in cancer are not understood but presumably result from the cumulative mutations arising in cancer. Interestingly, hyperinsulinemia has been linked to increased risk of developing colon cancer (Giovannucci, 2001) and patients with colorectal cancer and non insulin dependent diabetes mellitus type 2 diabetes have a poorer prognosis (Meyerhardt et al., 2003). Hyperinsulinemia has also been linked in some studies to the preferential expression of IR-A (Huang et al., 1994) in type 2 diabetes as will be discussed below. Both insulin and IGF-II can promote cell survival through IR-A activation in vitro (Sciacca et al., 2003). This suggests a possible role for the IR-A in regulating the genesis and progression of cancer in response to both insulin and IGF-II.

1.4.6 The role of insulin receptor isoforms in type 2 diabetes

Non-insulin-dependent diabetes mellitus (type 2 diabetes) is a disorder that is characterised by the following metabolic dysfunctions: peripheral insulin resistance, increased hepatic glucose production and ultimately impaired insulin secretion. Many research groups have been investigating the etiology of these traits that together give rise to type 2 diabetes. The establishment of tissue specific knockouts of the insulin receptor suggest that type 2 diabetes most likely arises from insulin resistance in all
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insulin responsive tissues (liver, adipose, skeletal muscle and insulin secreting pancreatic β cells) (Kitamura et al., 2003). Hyperphosphorylation of serine and threonine residues on IRS proteins plays a central role in insulin resistance (Paz et al., 1997; Zick, 2001). Despite concentrated efforts by many researchers, the role of the insulin receptor is not clearly understood and an association of one of the insulin receptor isoforms with the pathogenesis of type 2 diabetes has not yet been convincingly demonstrated.

Many of the studies into the expression ratios of the IR isoforms in type 2 diabetes have reported conflicting results. There are many possible explanations for these conflicting observations including the use of a variety of detection methods or contamination of tissue samples. Reasons for the discrepancies are also reviewed in Sesti et al. (Sesti, 2000), (Sesti et al., 2001). Some researchers report an increase in the expression of the IR-B in skeletal muscle or adipocytes of type 2 diabetes patients relative to tissues from non-type 2 diabetes subjects using detection at both the protein and RNA levels (Kellerer et al., 1993; Norgren et al., 1993; Sesti et al., 1995). However, several groups report no difference (Benecke et al., 1992; Anderson et al., 1993) and others have reported an increase in the relative abundance of the IR-A in type 2 diabetes (Norgren et al., 1994). In vivo evidence in hyperinsulinemic monkeys has shown higher amounts of IR-A mRNA in muscle compared to nonhyperinsulinemic monkeys (Huang et al., 1994). The progression from prediabetic diabetes mellitus (hyperinsulinemia) to late diabetes mellitus (hypoinsulinemia) may parallel an increase in the relative abundance of the IR-B in insulin-responsive tissues suggesting that the transcription from the insulin gene may be down regulated due to the isoform switch (Huang et al., 1994).

A model could be proposed to accommodate these observations. It is plausible to suggest that the hyperinsulinemia of prediabetes mellitus is promoted by increased
transcription of the insulin gene following signalling through IR-A and that hyperinsulinemia is caused by the increase in relative abundance of the IR-A. Leibiger et al. (Leibiger et al., 2001) have demonstrated that signalling through IR-A by insulin leads to an increase in insulin production. Subsequently in progression to later stage diabetes mellitus the greater affinity for IR-A leads to activation of this receptor, its downregulation and subsequent signalling through the low affinity IR-B (McClain, 1991). How these observations come together to result in type 2 diabetes is yet to be determined. The lower affinity of IR-B might further exacerbate the effects of insulin resistance. A recent study has shown that chronic hyperglycaemia leads to a decrease in IR expression and also promotes the incorporation of the exon 11 encoded peptide (IR-B) in human pancreatic islets (Hribal et al., 2003). The combination of lowered IR levels and preferential IR-B expression could explain the reduction in the total amount of insulin produced. However, it is possible that alternative models exist for the involvement of the different IR isoforms in type 2 diabetes as the disease arises from a large number of different mutations rather than a single identifiable genetic mutation. It is therefore possible that more than one model exists against different type 2 diabetes backgrounds.

In summary, it has so far been impossible to conclusively identify which isoform is associated with type 2 diabetes due to discrepancies in the literature. These discrepancies may in fact reflect the complex nature of progression of type 2 diabetes. There may be switching of the isoform at different stages of the disease in different tissues. Also, some discussion exists in the literature on the differences in affinities and kinase activities between the two isoforms. The significance of these properties is unclear. Further analysis of models of type 2 diabetes is essential to allow definitive conclusions to be drawn.
1.4.7 Residues of IGF that are involved in IR binding

The absolute binding affinities of insulin, IGF-II and IGF-I for the IR-A and IR-B vary substantially between studies and few studies have examined the binding of insulin, IGF-II and IGF-I to the IR isoforms in the one experiment. As a result the ratio of binding affinities of insulin: IGF-II : IGF-I for the IR-A and IR-B is unknown. For binding to the IR-B, both IGF-II and IGF-I have lower affinities than they have for the IR-A and the exact relative ratio of IR-B binding affinities of the three ligands is not known.

The finding that IGF-II binds with a higher affinity than IGF-I to the IR was relatively recent (Frasca et al., 1999) and most of the studies investigating IGF binding to the IR were performed with IGF-I analogues rather than IGF-II analogues. In addition, many studies did not exclusively use the IR-A or IR-B but instead many used membrane preparations that could contain varying levels of each isoform.

The insulin molecule has been mutated extensively, including the total alanine scanning mutagenesis of both B and A chains (Kristensen et al., 1997) to reveal a great deal about the role of insulin amino acids in IR binding. Despite the structural homology of the IGF-II and insulin B and A domains and that both insulin and IGF-II bind IR with high affinity, few mutations have been made in IGF-II that are equivalent to those in insulin shown to disrupt IR binding.

The sequence alignment in each subsequent section highlights the position and effect of published mutations on IR binding. Shooter et al. 1996 is the only study to have analysed the role of IGF amino acids in binding specifically to either the IR-A or the IR-B (Shooter et al., 1996). Most early studies used placental membranes as sources of insulin receptor which maybe a combination of both the IR-A and IR-B (Moller et al., 1989). Hence the results detailed in each sequence alignment is probably a combination of the effect of IGF mutations on binding to both the IR-A and IR-B.
B Domain

Substitution of the first 16 IGF-I amino acids with the first 17 of insulin only resulted in a 4-fold increase in affinity for placental insulin receptors relative to human IGF-I (Bayne et al., 1988).

Mutation of IGF-I at threonine 4 to histidine resulted in a 7-fold and 4-fold increase in affinity for the IR-A and IR-B respectively, while substitution of alanine 8 with leucine yielded a protein with a 28-fold decrease in affinity for the IR-A and a greater than 10-fold decrease in IR-B binding affinity (Shooter et al., 1996). Substitution of glutamine 15 with serine did not change the binding affinity to either the IR-A or IR-B isoforms (Shooter et al., 1996). Valine 11 in IGF-I when substituted with either isoleucine or threonine was 3.8 or 8.8 fold poorer in insulin receptor affinity relative to wildtype human IGF-I (Hodgson et al., 1995). IGF-I with a mutation at phenylalanine 23 to glycine resulted in a greater than 12-fold decrease in insulin receptor affinity (Hodgson et al., 1996), while an IGF mutant of the adjacent tyrosine 24 for leucine or serine was 10- or 2-fold less potent for placental insulin receptors (Cascieri et al., 1988).

IGF-II residues, phenylalanine 26 and tyrosine 27, appear to be more critical for IR binding, than the equivalent IGF-I residues. Mutation of phenylalanine 26 to serine and tyrosine 27 to leucine caused a 20-fold and an 80-fold decrease in IR binding respectively (Sakano et al., 1991). N-terminal deletions of IGF-II indicated that threonine 7 is crucial for IR binding as des (1-6)-IGF-II had only a 2 fold reduction in IR binding however des-(1-7)-IGF-II had only 10 % the IR affinity of wildtype IGF-II (Hashimoto et al., 1995). The positive role of this threonine in IGF-II in IR binding is in contrast to the negative role it appears to play in IGF-I binding to the IR (Shooter et al., 1996). Mutation of leucine 8 in IGF-II to glycine reduces IR binding by 250-fold (Hashimoto et al., 1995).
To Insulin B chain

\[
\begin{align*}
\text{IGF-I} & \quad E\text{TLCGAELVDALQFVGCDRGFYFNKPT}^{29} \\
\text{IGF-II} & \quad AYPSETLCGGELVDTQFVCGDRGFYFSRPA^{32}
\end{align*}
\]

Figure 1.11 Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated affect IR binding. Binding studies reported rarely discerned the IR isoform used and hence the results are most likely a combination of binding to both the IR-A and IR-B.

**C Domain**

Tyrosine is a bulky aromatic side chain amino acid that in IGF-I at position 31 is “prominently displayed”, protruding directly out from the main chain, in the C domain due to a type II \( \beta \)-turn (Vajdos et al., 2001). While tyrosine 31 is important in maintaining high affinity binding to the IGF-1R it appears to hinder IR binding as mutation to alanine resulted in a small but significant 2-fold increase in human placental insulin receptor binding (Bayne et al., 1990). Removal of the positively charged side chains of arginine 36 and 37 in the C domain increased the IR affinity by 29-fold compared to wildtype IGF-I (Zhang et al., 1994). In contrast this same mutant exhibited a decrease in IGF-1R binding affinity, revealing the opposing role of these arginine residues in IR and IGF-1R binding. The mutation of arginine 37 and 38 in IGF-II, both to glutamine, reduced the affinity for the IR by 10-fold (Edwards and Bawden, 1993).

Removal of the entire IGF-I C domain and replacement with a 4-glycine bridge caused a 2-fold increase in IR binding affinity (Bayne et al., 1989) and an addition of the IGF-I C domain to the C-terminus of the insulin B chain causes a 3.5-fold decrease
in IR affinity compared to wildtype (Cara et al., 1990). Another insulin/IGF hybrid, however in this case a single chain peptide, comprised of insulin with the IGF-I C domain had an IR affinity not appreciably different from that of native human insulin (Kristensen et al., 1995).

A Domain

Exchanging residues 42-56 in IGF-I with the first 15 residues of the insulin A chain (including a substitution of threonine 41 for a isoleucine in the C domain) yielded a 7-fold increase in IR binding (Cascieri et al., 1989). Replacement of phenylalanine 49, arginine 50 and serine 51 in IGF-I with the equivalent residues in insulin, threonine, serine and isoleucine, caused a 2-fold increase in IR binding affinity relative to human IGF-I (Cascieri et al., 1989). The residues phenylalanine, arginine and serine are conserved in IGF-II and the analogous experiment where these residues were replaced with those of insulin did not change IR binding affinity relative to recombinant human IGF-II (Sakano et al., 1991). These results indicate that A domain residues conserved in IGF-I and IGF-II may differentially interact with the IR. Substitution of both alanine 54 and leucine 55 in IGF-II for arginine, which is found at the equivalent position in IGF-I, did not change IR binding affinity relative to IGF-II (Sakano et al., 1991).
Mutation of valine 43 in IGF-II to leucine caused a large 220-fold reduction in IR binding affinity (Sakano et al., 1991). The equivalent valine in insulin when mutated causes similar large decreases in IR binding affinity (Kobayashi et al., 1986; Nanjo et al., 1987) and recently cross-linking studies have shown that valine 3 in the insulin A chain contacts the IR (Xu et al., 2004). Mutation of tyrosine 60 to phenylalanine while preserving the aromatic nature of the side chain does however result in a 2.6 fold decrease in IR binding affinity (Hodgson et al., 1995). Replacement of tyrosine 60 with leucine yielded a protein with almost no detectable affinity for the IR (Bayne et al., 1990). Tyrosine 60 (or tyrosine 59 in IGF-II) stabilises helices I and II and forms part of the protein core (Cooke et al., 1991; Sato et al., 1992; Sato et al., 1993; Terasawa et al., 1994). Therefore mutation of tyrosine 60 may not directly change an interface interaction with the IR but may in fact alter the overall conformation of IGF-I. The NMR analysis of long [Leu60]-IGF-I does suggest perturbations in the overall tertiary structure (Laajoki et al., 1998).

![Figure 1.13 Sequence alignment of the A domains of IGF-I and IGF-II highlighting residues that when mutated affect IR binding. Binding studies reported rarely discerned the IR isoform used and hence the results are most likely a combination of binding to both the IR-A and IR-B.](image)

**D Domain**

In a study of the positive charges in the IGF-I D domain, mutation of lysine 65 and 68 caused a 6-fold increase in IR binding potency relative to IGF-I (Zhang et al.,
1994). Removal of the IGF-I D domain resulted in a 2-fold increase in IR binding affinity (Bayne et al., 1989). While an IGF-II analog with a deletion of its D domain has been made, its binding to the IR has not been examined (Roth et al., 1991).

![Sequence alignment of the D domains of IGF-I and IGF-II highlighting residues that when mutated affect IR binding. Binding studies reported rarely discerned the IR isoform used and hence the results are most likely a combination of binding to both the IR-A and IR-B.](image)

**Figure 1.14** Sequence alignment of the D domains of IGF-I and IGF-II highlighting residues that when mutated affect IR binding. Binding studies reported rarely discerned the IR isoform used and hence the results are most likely a combination of binding to both the IR-A and IR-B.

![Summary of Mutagenesis studies. NMR structure of IGF-I (Sato et al., 1993) is depicted in surface mode. The colours are as in the text and refer to changes in IR affinity as a result of mutation. Figure constructed using Insight II.](image)

**Figure 1.15** Summary of Mutagenesis studies. NMR structure of IGF-I (Sato et al., 1993) is depicted in surface mode. The colours are as in the text and refer to changes in IR affinity as a result of mutation. Figure constructed using Insight II.

### 1.4.8 Summary of IGF residues involved in IR binding

Residues within the B domain of IGF-I and IGF-II are critical for high affinity binding to the IR. Residues within the IGF-I C and D domain appear to hinder binding to the
IR, whereas the role of the IGF-II C domain in IR binding has not been studied extensively. The determinants for the difference in IR binding affinity of IGF-II and IGF-I are presently unknown.

1.4.9 Mechanism of insulin binding to the IR

As mentioned in section 1.3.5 the mechanism of IGF binding to the IGF-1R is not understood in great detail. Likewise, due to the lack of structural information the mechanism of insulin (or IGF) binding to the IR is also poorly understood. Several models of insulin binding to the IR have been proposed by Yip (Yip, 1992), Fabry et al. (Fabry et al., 1992), Lee et al. (Lee et al., 1993), Soos et al. (Soos et al., 1993), De Meyts (De Meyts, 1994a) and Schaffer (Schaffer, 1994). The binding of IGF-I and presumably IGF-II as well to the IGF-1R has been hypothesised to occur in a similar mechanism to that proposed for insulin binding to the IR (De Meyts, 1994a). In addition, no model for IGF binding to the IR has been put forward, however it would be predicted on the basis of the current models that it is equivalent to the mechanism of insulin binding to the IR. While either of the mentioned models may in fact turn out to be correct, Schaffer was the first to propose a “cross linking” model that takes into account all biochemical and biophysical data on insulin binding to the IR (Schaffer, 1994). The binding model proposed by De Meyts (De Meyts, 1994a) is also extremely ingenious and indeed more refined, so it will also be discussed along with that proposed by Schaffer (Schaffer, 1994) (Figure 1.16 and 1.17).

Mutations in insulin at either positions A1, A21, B12, B24 or B25 caused large reductions in binding affinity and clustered together in a contiguous patch on the insulin three-dimensional structure. This has been termed the “classical binding site” and in Schaffer’s model termed “binding site 1”. A second receptor binding site on insulin was identified involving A13 and B17 as mutation at these sites caused very
slow receptor binding kinetics and the mutants exhibited lower \textit{in-vitro} potencies in a lipogenesis assay than would be expected from their receptor binding affinities (Schaffer, 1994). This site is termed "binding site 2" in Schaffer's model. An analogous hypothesis is drawn for the IGF molecule where the IGF binding site 1 constitutes the equivalent amino acids to those in insulin and binding site 2 lies in the IGF C domain.

Each insulin receptor \(\alpha\)-subunit has been postulated to contain two distinct ligand binding sites termed receptor binding sites 1 and 2. The exact regions of the IR that correspond to site 1 and 2 are unknown although as already discussed in section 1.4.1 cross linking, mutagenesis and chimeric receptor studies have revealed the L1, L2 domains and 16 amino acids in the insert domain as important ligand binding determinants.

Binding of insulin's binding site 1 to site 1 on the receptor constitutes part B in the model and this is the initial interaction of ligand with receptor (Figure 1.16). The second stage involves cross linking of site 2 on insulin with site 2 on the receptor, depicted in part C. The binding energy in part C is provided by both site 1 and site 2 and is therefore representative of the high affinity binding mode observed in Scatchard analyses. This conformation then allows reorientation of the intracellular \(\beta\)-subunits for productive transphosphorylation and activation of signalling pathways (Figure 1.16). The unoccupied second site 1 on the receptor can provide a binding site for a second insulin molecule but without a second site to provide more binding energy this corresponds to the low affinity mode seen in Scatchard analyses.
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Figure 1.16 Mechanism of insulin binding to the IR proposed by Schaffer (Schaffer, 1994). This figure has been adapted from that in (Schaffer, 1994).

Figure 1.17 Mechanism of insulin binding to IR proposed by De Meyts (De Meyts, 1994a). This figure has been adapted from that in (De Meyts, 1994a).

The model proposed by De Meyts suggests that the IR has an “internal symmetry” whereby binding sites 1 and 2 face each other at each end of the α-subunits (De Meyts, 1994a) (Figure 1.17). In addition, De Meyts proposed that the cysteine rich domains of the α-subunits act as hinges allowing opposite binding sites to be cross-linked (De Meyts, 1994a). This mechanism of binding is in contrast to that proposed by Schaffer (Figure 1.16), however this more refined binding mechanism can more easily explain the properties of negative cooperitivity (e.g. unlabelled insulin
accelerating the dissociation of prebound labelled insulin and the loss of this accelerated dissociation at high concentrations of insulin (De Meyts, 1994a).

The models proposed by Fabry (Fabry et al., 1992) and Yip (Yip, 1992) suggest one insulin molecule contacts two sites within the same α-subunit, contrary to the cross linking models of both Schaffer and De Meyts detailed above.

Many of the recent models predicting activation of cytokine and growth factor receptors has been based on the early crystal structures of GH complexed with the GHR (de Vos et al., 1992). While these models seem plausible and are in line with all current biochemical and biophysical data they should be treated with some caution as highlighted in the following example. Prior to the recent success in determining the structures of EGFR, in complex with TGF-α (Garrett et al., 2002) or EGF (Ogiso et al., 2002) and unactivated EGFR (Ferguson et al., 2003), ErbB2 (Cho et al., 2003) and ErbB3 (Cho and Leahy, 2002) the exact mechanism of receptor activation was unknown. In fact the common belief was that EGFR activation occurred via ligand-mediated dimerisation events (Lemmon et al., 1997; Tzahar et al., 1997). However, the wealth of recent structural information revealed that dimerisation was mediated exclusively by receptor-receptor contacts (Burgess et al., 2003). Hence the previous EGFR binding models were incorrect. Whether the IR and IGF-1R extracellular domains have a similar orientation to that of the EGF receptor family or not is currently unknown (Garrett et al., 2002; Burgess et al., 2003; Garrett et al., 2003). However, due to the lessons learnt with the now incorrect models for EGF binding, all IR and IGF-1R binding models must be treated with some caution.

1.5 Hybrid IR/IGF-1R

Many of the studies investigating the functional role of the two IR isoforms have used IGF-1R deficient cell lines expressing either isoform. The responses of those
cells to insulin, IGF-II and IGF-I may be different to either normal or diseased cells expressing both IGF-1R and either (or both) IR isoform(s), particularly because the IGF-1R and IR can form hybrid receptors. Due to the high degree of sequence homology between the IR and the IGF-1R they can heterodimerise to form hybrids where one αβ monomer is the IR and one is the IGF-1R. Initially, it was proposed that IGF-1R/IR hybrids resulted in a receptor with a high affinity for IGF-I but not insulin (Soos et al., 1993). However, identification of the alternative splicing of the IR has lead to the analysis of hybrids of the IGF1R with either isoform of the IR (Pandini et al., 2002). Hybrids containing the IR-A (Hybrid-A) are high affinity receptors for IGF-I and IGF-II (with similar affinity to IGF-1R binding) and they do bind insulin, albeit at ~20 fold lower affinity than IR-A. Also Hybrid-A receptors have a higher affinity for IGF-I and IGF-II than the hybrids containing the IR-B (Hybrid-B)(Pandini et al., 2002). Significantly, however, Hybrid-B bind insulin poorly (Pandini et al., 2002). In all cells expressing both the IGF-1R and either IR isoform hybrids are predicted to form randomly and follow a 1:1:2 ratio for expression of IR homodimers: IGF-1R homodimers: IR/IGF-1R heterodimers. Furthermore if one receptor type is expressed at a substantially higher level the other less abundant receptor type is likely to be totally involved in hybrid formation.

Levels of hybrid IR/IGF-1R are elevated in thyroid (Belfiore et al., 1999) and breast cancer (Pandini et al., 1999). In addition there is an increased formation of hybrid receptors during the differentiation of colon carcinoma cells HT29-D4 (Garrouste et al., 1997). The recent finding that hybrids of IGF-1R and either the IR-A or IR-B have different binding and signalling properties suggests that hybrid receptor formation is another level of control where perturbations in receptor expression can regulate biological outcomes.
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Hybrid levels are increased in the muscle and adipose tissue of type 2 diabetes patients compared to non-type 2 diabetes control subjects (Federici et al., 1996; Federici et al., 1997). This lead to the prediction that the lower affinity of insulin for the hybrids may contribute to a decrease in insulin sensitivity in these tissues. In both of these the studies the type of IR isoform forming one half of the hybrid was not examined. Despite the relatively high affinity of insulin for the Hybrid-A compared to the Hybrid-B, both hybrids bind insulin with lower affinity than the classical IR homodimers. This suggests that hybrid formation with either isoform could promote insulin resistance. Classical IR-A and IR-B have been shown to localize to different areas of the plasma membrane indicating that possibly one receptor maybe more closely linked to activating a particular signalling pathway than the other if intracellular signalling components are compartmentalized (Leibiger et al., 2001; Uhles et al., 2003). Whether differential localization of hybrids containing either isoform occurs is not known. Hybrid receptors from IR-A and IR-B αβ monomers have not been identified due to a lack of isoform specific antibodies, although they are expected to form. The significance of such HybridAB receptors is yet to be determined.

Not one study of insulin, IGF-I or IGF-II analogs for binding to hybrid receptors of IGF-1R and either the IR-A or IR-B has been published. The relative difficulty in purifying hybrid receptors has prevented more extensive biochemical analyses.

1.6 The insulin-like growth factor type 2 receptor (IGF-2R)

As detailed in sections 1.2 and 1.4 the cellular effects of IGF-I and IGF-II are mediated by the insulin and IGF-I receptors, which belong to the family of transmembrane tyrosine kinase receptors (Hubbard and Till, 2000). The availability of
IGF-II for binding to these receptors is modulated in part by the type-2 IGF receptor which is structurally distinct from the IGF-1R and the IR.

The insulin like growth factor type II receptor (IGF-2R) is a 300 kDa protein that is comprised of 15 extracellular repeating domains, a 23 amino acids transmembrane domain and a 163 amino acid intracellular region (Morgan et al., 1987; Oshima et al., 1988) reviewed in (Kornfeld, 1992). The structure of domain 11 has been solved recently and consists of two-crossed β-sheets forming a flattened β barrel (Brown et al., 2002). The IGF-2R binds a wide variety of ligands including IGF-II, mannose-6-phosphate (man-6-P) containing proteins e.g. TGF-β, and recently the urokinase-type plasminogen activator receptor (uPAR) (Kreiling et al., 2003). The binding sites for man-6-P containing proteins and for IGF-II are located on separate domains of the IGF-2R: IGF-II binds primarily to domain 11 with domain 13 providing an additional binding site (Devi et al., 1998), whereas man-6-P containing proteins bind to domains 1-3 and 7-9 (Tong et al., 1989; Westlund et al., 1991). The residues on the IGF-2R that interact with IGF-II are not known. However, IGF-II has been cross linked to dom 11 and mutation of Ile 1572, within dom 11, abolished IGF-II binding (Garmroudi and MacDonald, 1994).

The IGF-2R binds IGF-II with high affinity but binds IGF-I with very low affinity and does not bind insulin (Lee et al., 1986; Ewton et al., 1987; Tong et al., 1988). Its primary function has been suggested to be sorting of enzymes in lysosomal compartments as only 10 % of the total cellular IGF-2R is found at the cell’s surface, the rest in located in lysosomes and endosomes. Mice with a homozygous knockout of the IGF-2R gene exhibit a lethal over-growth phenotype resulting from elevated serum IGF-II levels (Lau et al., 1994; Wang et al., 1994; Ludwig et al., 1996) suggesting that the IGF-2R is important in regulating the concentration of IGF-II in the circulation. Indeed the IGF-2R has been shown to mediate degradation of IGF-II (Oka et al.,
1985). The lethal phenotype of an IGF-2R knockout can be rescued by deletion of the IGF-1R or IGF-II genes (Ludwig et al., 1996). The IGF-2R sequesters IGF-II from potential receptor activating interactions (Ellis et al., 1996). Mutation or loss of expression, resulting in loss of heterozygosity (LOH), of the IGF-2R gene occurs in several cancers and correlates with a poor prognosis (De Souza et al., 1995; Oka et al., 2002; Jamieson et al., 2003). Down-regulation of the IGF-2R in cancer cells promotes increased sensitivity to IGF-II as shown by IRS-1 activation (Osipo et al., 2001). In rhabdomyosarcoma cells the IGF-2R was shown to mediate IGF-II induced motility, independent of the IGF-1R, which is contrary to other reports suggesting that the receptor acts only to internalise IGF-II (Minniti et al., 1992).

The IGF-2R has IGF-independent functions that are critical in mediating cell proliferation (Scott and Firth, 2004). For example, activation of the growth inhibitory cytokine TGF-β from its inactive latent form requires a complex of TGF-β, uPAR and the IGF-2R (Godar et al., 1999). Loss of IGF-2R expression or function in tumours may reduce the level of TGF-β activation and therefore the tumour may circumvent TGF-β induced growth inhibition. The IGF-2R has also been shown to regulate fibrinolysis, cell adhesion and migration by regulating the activity of plasminogen (Leksa et al., 2002).

1.6.1 IGF residues involved in IGF-2R binding

**B Domain**

Sequential deletions of the N-terminus of IGF-II have shown that leucine 8 is important in IGF-2R binding, as des[1-7] IGF-II has wildtype IGF-2R affinity however des[1-8] IGF-II has only 1 % wildtype IGF-II affinity for the IGF-2R (Hashimoto et al., 1995). Removal of the aromatic side chain of phenylalanine 26 in IGF-II and replacing it with serine reduced IGF-2R binding by 2.5-fold (Sakano et al., 1991).
However removal of the aromatic ring of neighbouring tyrosine 27 by substitution with leucine did not affect IGF-2R binding affinity (Sakano et al., 1991). In a separate report tyrosine 27, in IGF-II, when mutated to leucine or glutamate produced a 3.4 and 9.2 fold (Burgisser et al., 1991) or a 1.6 and 7.6 fold (Roth et al., 1991) decrease in affinity for the IGF-2R. The reasons for these differences in the effect of mutating tyrosine 27 in IGF-II on IGF-2R binding are unclear. Mutation of the corresponding tyrosine in IGF-I, tyrosine 24, to alanine did not change IGF-2R binding compared to wildtype IGF-I (Bayne et al., 1990). Exchanging the first 16 residues of IGF-I with the first 17 of insulin caused a 100-fold decrease in IGF-2R binding affinity (Bayne et al., 1988). A naturally occurring variant of human IGF-II with a substitution of serine 29 for arginine and an insertion of leucine-proline-glycine at position 30 bound the IGF-2R with equivalent affinity to wildtype human IGF-II (Yandell et al., 1999).

![Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated effect IGF-2R binding.](image)

**C Domain**

The role of the IGF-II C domain in IGF-2R binding has not been extensively studied. Mutation of arginine 37 and 38 to glutamine did not affect IGF-2R binding (Edwards and Bawden, 1993). Residues within the C domain of IGF-I do not appear to form part of the IGF-2R binding surface as [1-27,Gly^4,38-70] IGF-I, C domain
deletion, did not show any change in IGF-2R binding (Bayne et al., 1989). Alanine mutation of tyrosine 31 in IGF-I did not change IGF-2R binding (Bayne et al., 1990).

**A Domain**

Mutation of phenylalanine 48, arginine 49 and serine 50 in the A domain of IGF-II to the sequence in the equivalent region of insulin i.e. threonine, serine and isoleucine, resulted in a 115-fold decrease in IGF-2R binding affinity relative to wildtype IGF-II (Sakano et al., 1991). Similarly substitution of the residues between 42 and 56 of IGF-I with those in the equivalent positions in insulin (including a substitution of threonine 41 for a isoleucine in the C domain) resulted in a greater than 20-fold decrease in IGF-2R binding relative to wildtype IGF-I (Cascieri et al., 1989). Mutation in IGF-II of alanine 54 and leucine 55 to the equivalent in IGF-I, arginine 55 and 56, decreased IGF-2R binding by 6.6-fold (Forbes et al., 2001).

![Figure 1.19 Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated effect IGF-2R binding.](image)

**D Domain**

Deletion of the IGF-II D domain causes a 2-fold decrease in IGF-2R binding affinity (Roth et al., 1991), however deletion of the IGF-I D domain did not change its IGF-2R relative to wildtype IGF-II (Bayne et al., 1989).
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Deletion

IGF-I 63 PLPAKSA 70
IGF-II 62 -TPAKSE 67

≥ 6 fold decrease
≥ 2 fold ≤ 6 fold decrease
≤ 2 fold decrease

Figure 1.20 Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated affect IGF-2R binding.

Figure 1.21 Summary of Mutagenesis studies. NMR structure of IGF-II (Torres et al., 1995) is depicted in surface mode. The colours are as in the text and refer to changes in IGF-2R affinity as a result of mutation. Figure constructed using Insight II.

1.6.2 Summary of IGF residues involved in IGF-2R binding

Residues with the B and A domains appear to be critical for IGF-2R binding while those in the C and D domains are not. The exact role of the C domain of IGF-II in IGF-2R binding is unknown.

1.6.3 Mechanism of IGF-II binding to the IGF-2R

The exact mechanism of IGF-II binding to the IGF-2R is unknown, despite the structure of the major IGF-II binding domain (domain 11) of the IGF-2R being solved (Brown et al., 2002). Domain 13 of the IGF-2R also provides an “affinity enhancing”
domain for IGF-II binding, but both the exact structure of this domain and its relative orientation to domain 11 are unknown. The IGF-2R binding site on IGF-II is very under studied as detailed in section 1.6.1. For all these reasons a model of the mechanism of IGF-II binding to the IGF-2R to date has not been proposed.

1.7 Insulin-like growth factor binding proteins (IGFBPs)-structure and function

While the level of IGF-II is modulated by IGF-2R the bioavailability of both IGF-I and IGF-II is regulated by a family of high affinity IGFBPs (reviewed in (Jones and Clemmons, 1995; Rajaram et al., 1997; Hwa et al., 1999; Baxter, 2000; Clemmons, 2001; Firth and Baxter, 2002)). The IGFs are bound in the circulation to any one of six IGFBPs and these binding proteins act as carriers to prolong the half-life of the IGFs in the serum (Rajaram et al., 1997; Baxter, 2000). In the circulation of adult mammals the majority of IGF-I and IGF-II are in a ternary complex with IGFBP-3 and the acid-labile subunit (Baxter et al., 1992; Hashimoto et al., 1997). While IGF-I or IGF-II are bound to an IGFBP they are prevented from making a functional interaction with either the IGF-1R or IR. Thus, in most situations the binding of IGF-I or IGF-II to an IGFBP is inhibitory to the biological actions of the IGFs. However, in some experimental situations IGFBPs can potentiate the IGF action by co-localising IGFs to the cell membrane or sites in the extracellular matrix where the IGFs can be released for interaction with an IGF-1R or IR (Conover, 1991; Jones et al., 1993). Recently the IGFBPs have been shown to exhibit functions that are independent of binding the IGFs (Mohan and Baylink, 2002).

All six IGFBPs share a similar domain organization and disulphide bond pattern (Firth and Baxter, 2002). The IGFBPs consist of three domains which are the amino-terminal domain, central domain and carboxy-terminal domain, all of approximately similar size (Drop et al., 1992; Hwa et al., 1999). Recombinantly
produced fragments of several IGFBPs have shown that both the N terminal (Andress et al., 1993; Hashimoto et al., 1997; Qin et al., 1998; Vorwerk et al., 1998; Carrick et al., 2001; Vorwerk et al., 2002) and C terminal domains (Carrick et al., 2001; Headey et al., 2004) of the IGFBPs contain the IGF binding determinants. The central linker domain does not appear to play a role in IGF binding as deletion or site directed mutagenesis in this region does not alter IGF binding (Jones et al., 1993; Conover et al., 1995; Imai et al., 1997; Kalus et al., 1998; Qin et al., 1998; Rees et al., 1998).

1.7.1 IGF residues involved in IGFBP binding

Investigations into residues in the IGFs that interact with the IGFBPs have been the basis of many studies (reviewed in (Baxter, 2000)). A crystal structure of IGF-I in complex with a portion of the N-terminal domain of IGFBP-5 has since been a major advance in our understanding of the IGF-IGFBP interaction (Zeslawski et al., 2001). In addition the complete alanine scanning of IGF-I displayed on phage allowed for probing the role of almost all amino acid side chains of IGF-I in IGFBP-1 and IGFBP-3 binding (IGF-I with alanine mutants at valine 11, arginine 36 and proline 39 were not successfully expressed on phage) (Dubaqie and Lowman, 1999). Recently, the binding site of the IGFBP-6 C-terminal domain on IGF-II was defined by NMR (Headey et al., 2004).

B domain

The B domain of IGF-I was first shown to contain determinants for IGFBP binding when chimeras between insulin and IGF-I were engineered. Insulin does not bind any IGFBPs however when the B chain of insulin was substituted with the B domain of IGF-I, IGFBP binding ability was conferred (De Vroede et al., 1985).
Conversely, replacing the B domain of IGF-I with the equivalent region of insulin decreased the affinity for serum IGFBPs by 1000-fold relative to wildtype IGF-I (Bayne et al., 1988).

A naturally occurring variant of IGF-I, des-(1-3) IGF-I, highlighted the importance of the N-terminal residues for BP binding (Szabo et al., 1988). In a separate study the biological activity of IGF-I and IGF-II but not des-(1-3) IGF-I was inhibited by IGFBPs, further supporting the role of the first three residues in IGFBP recognition (Ross et al., 1989). Confirmation of the N-terminal residue(s) responsible for IGFBP recognition occurred by production of a recombinant IGF mutant with a charge reversal at position 3 from a negatively charged glutamate to a positively charged arginine (Wallace et al., 1989). This mutation resulted in a >200 fold decrease in the ability to compete $^{125}$I-IGF-I for binding to bovine IGFBP-2 (King et al., 1992). The equivalent mutation in IGF-II, glutamate 6 to arginine, reduced binding to serum IGFBPs by 125-fold relative to IGF-II confirming the importance of a negatively charged residue at this position (Francis et al., 1993). Threonine 4, which is adjacent to the critical glutamate in IGF-I, when mutated to histidine resulted in a 2-fold decrease in binding to all six IGFBPs (Magee et al., 1999). A charge reversal of the positive glutamate at position 9 in IGF-I to a negative lysine caused decreases in IGFBP-2 and −6 binding affinity of 140- and 30-fold respectively (Magee et al., 1999).

Residues valine 11, aspartate 12, glutamine 15 and phenylalanine 16, which are all located in the IGF-I B domain helix, when mutated to alanine yield decreases in IGFBP-1 binding of 3- to 50-fold relative to wildtype IGF-I (Jansson et al., 1997). All mutants however, have reduced alpha helical content as determined by circular dichroism and therefore a change in IGFBP-1 binding due to local unfolding of the helix rather than loss of side chain contacts cannot be ruled out. Other studies have also shown that mutation at glutamine 15 and phenylalanine 16 preclude high affinity
binding to serum IGFBPs (Bayne et al., 1988) or IGFBP-1 (Clemmons et al., 1990). Mutation at phenylalanine 16 to serine, which did not alter the tertiary structure as determined by CD analysis, reduced binding to IGFBPs by 14- to >220-fold depending on the IGFBP (Magee et al., 1999). Mutation of another phenylalanine, this time at position 26 in IGF-II, to leucine reduced IGFBP-1 and IGFBP-6 binding by 80-fold compared to wildtype IGF-II (Bach et al., 1993). Tyrosine 27 of IGF-II has been shown to be critical for high affinity binding to the IGF-1R and IR (Sakano et al., 1991) but its mutation to leucine only reduced binding to one of the six IGFBPs, IGFBP-6, by 2-fold (Bach et al., 1993).

Alanine scanning mutagenesis of the entire IGF-I molecule identified further residues glycine 7, leucine 10, valine 17 and phenylalanine 25 as important for the IGFBP interaction (Dubaquie and Lowman, 1999). In the studies by both Magee et al. and Dubaquie et al., some of the mutant IGFs had different decreases in IGFBP affinity compared to wildtype IGF-I depending on the IGFBP investigated (Dubaquie and Lowman, 1999; Magee et al., 1999). This was observed in other studies (Clemmons et al., 1992; Bach et al., 1993) and suggests that the binding epitopes on the IGFs are different for several IGFBPs. Taking advantage of the different IGFBP binding epitopes on the IGFs, Dubaquie and colleagues engineered an IGF mutant, E3A/F49AIGF-I, that exhibited an 80,000-fold decrease in IGFBP-1 affinity but only a 15-fold decrease in IGFBP-3 binding affinity compared to wildtype IGF-I (Dubaquie et al., 2001).

Of the mutated residues discussed above, glutamate 3, aspartate 12 and phenylalanine 16 have been shown to make contacts with mini-IGFBP-5 within 4Å (Zeslawski et al., 2001). Other B domain residues shown to make contact with mini-IGFBP-5 were threonine 4, leucine 5 and glutamate 9 (Zeslawski et al., 2001). While mini-IGFBP-5 is only a small portion of the N-terminal domain of IGFBP-5 it does
bind IGF-I and IGF-II with $K_D$ values of 37 nM and 6 nM respectively, suggesting that it contains most of the IGF binding determinants (Kalus et al., 1998). NMR studies showed the following IGF-II B domain residues underwent chemical shifts in the presence of IGFBP-6 C-terminal domain: threonine 7, glycine 10, glycine 11, glutamate 12, leucine 13, valine 14, aspartate 15, threonine 16, glutamine 18, cystine 21 and phenylalanine 28 (Headey et al., 2004).

Figure 1.22 Sequence alignment of the B domains of IGF-I and IGF-II highlighting residues that when mutated effect IGFBP binding. As stated in the text each IGFBP most likely has subtle differences in the IGF residues that they interact with. The alignment is a summary of all the current literature of all IGFBPs.

C domain

Conflicting results have been obtained regarding the role of the IGF-I and IGF-II C domain in IGFBP binding. Deletion of the entire IGF-I C domain and replacement with four glycines did not alter binding to serum purified IGFBPs (Bayne et al., 1989). The IGF residues involved in IGFBP binding were probed by chemical iodination where the accessibility of IGF tyrosine residues to labelling was compared in the presence or absence of IGFBP-2 (Moss et al., 1991). Tyrosine 31 in the IGF-I C domain was exposed for iodination while in the presence of IGFBP-2, suggesting it is not involved in the interaction with the IGFBP (Moss et al., 1991). In contrast to both these reports an NMR study examining the backbone chemical shifts of $^{15}$N and $^{13}$C labelled IGF-I in the presence of IGFBP-1, found several C domain residues
underwent chemical shift perturbation in the presence of IGFBP-1. The labelled backbone atoms of C domain residues glycine 30 and 32, arginine 36 and 37 and glutamine 40 all exhibited chemical shift differences in the presence of IGFBP-1 (Jansson et al., 1998). However, the chemical shift changes of arginine 36 and 37 were small comparative to similar NMR experiments with other IGFBPs (B.E. Forbes personal communication). Mutation of arginine 36 and 37 to alanine however, did decrease IGFBP-1 binding by 2-fold as determined by surface plasmon resonance experiments (Jansson et al., 1998). The other residues in the C domain exhibiting chemical shift changes in the study by Jansson and colleagues have not been mutated.

![Figure 1.23 Sequence alignment of the C domains of IGF-I and IGF-II highlighting residues that when mutated effect IGFBP binding.](image)

As stated in the text each IGFBP most likely has subtle differences in the IGF residues that they interact with. The alignment is a summary of all the current literature of all IGFBPs.

**A domain**

Several studies have taken advantage of the lack of IGFBP binding of insulin to probe the residues in the IGF A domain that are involved in the IGFBP interaction. Specifically phenylalanine 49, arginine 50 and serine 51 in IGF-I (Cascieri et al., 1989; Clemmons et al., 1992; Oh et al., 1993) and phenylalanine 48, arginine 49 and serine 50 (Bach et al., 1993) in IGF-II, when substituted with the equivalent residues of insulin, resulted in 6- to 500-fold reductions in IGFBP binding depending on which IGFBP was tested. Unexpectedly exchanging residues 42-56 of IGF-I with those
corresponding in insulin (including a substitution of threonine 41 for a isoleucine in the
C domain) did not affect binding to human serum purified IGFBPs despite containing
the mutations at phenylalanine 49, arginine 50 and serine 51 discussed above (Cascieri
et al., 1989).

Alanine substitutions of isoleucine 43, valine 44 and leucine 54 in IGF-I,
reduced binding to IGFBP-1 up to 4-fold but had no effect on IGFBP-3 binding
(Dubaquie and Lowman, 1999). As already mentioned, chemical iodination and NMR
studies highlighted residues in the IGF-I B domain critical for IGFBP binding and the
same studies showed several A domain residues are important also. Iodination of
tyrosine 60 of IGF-I and tyrosine 59 of IGF-II was reduced in the presence of IGFBP-2
(Moss et al., 1991). However mutation of tyrosine 60 in IGF-I did not alter binding to
purified serum IGFBPs (Bayne et al., 1990). Mutation of alanine 62 at the end of the A
domain to leucine reduced binding to purified serum binding proteins by 2-fold
(Shooter et al., 1996).

In the NMR study by Jansson et al. the only A domain residue found to have its
backbone chemical shifts perturbed by IGFBP-1 was glycine 42 (Jansson et al., 1998).
While arginine 50 did not appear to be involved in IGFBP-1 binding as determined by
NMR, mutation to alanine did decrease IGFBP-1 binding affinity (Jansson et al.,
1998).

The crystal structure of mini-IGFBP-5 in complex with IGF-I showed the IGF-I
A domain residues, cysteine 52, aspartate 53, leucine 54, leucine 57 and glutamate 58,
make contacts with mini-IGFBP-5 within 4Å (Zeslawski et al., 2001). Chemical shifts
were observed by NMR for the following IGF-II residues in the presence of the
IGFBP-6 C-terminal domain: isoleucine 42, glutamate 44, cysteine 46, cysteine 47,
phenylalanine 48, arginine 49 and cysteine 51 (Headey et al., 2004).
CHAPTER 1 - Introduction

C-terminal C domain residue

To Insulin A chain

IGF-I

\[ \text{IGIVEQCCTSICSLYQ} \]

IGF-II

\[ \text{RGIVEQCCFRSCDLALLETYCA} \]

Figure 1.24 Sequence alignment of the A domains of IGF-I and IGF-II highlighting residues that when mutated effect IGFBP binding. As stated in the text each IGFBP most likely has subtle differences in the IGF residues that they interact with. The alignment is a summary of all the current literature of all IGFBPs.

D domain

The D domain and residues contained within it have not been extensively investigated by mutagenesis for their role in IGFBP binding. Removal of the IGF-I D domain caused a 4-fold increase in serum binding protein binding affinity (Bayne et al., 1989), however when the IGF-II D domain was deleted the IGFBP-3 binding affinity was not affected (Roth et al., 1991). Backbone NMR chemical shifts of residues proline 62, lysine 65, proline 66, lysine 68, serine 69 and alanine 70 were found to be affected by IGFBP-1 binding (Jansson et al., 1998). In the same study several positive charged residues in the B and C domains but not in the D domain were mutated to alanine (Jansson et al., 1998).

Figure 1.25 Sequence alignment of the D domains of IGF-I and IGF-II highlighting residues that when mutated effect IGFBP binding. As stated in the text each IGFBP most likely has subtle differences in the IGF residues that they interact with. The alignment is a summary of all the current literature of all IGFBPs.
Figure 1.26 Summary of Mutagenesis studies. NMR structure of IGF-I (Sato et al., 1993) is depicted in surface mode. The colours are as in the text and refer to changes in IGFBP affinity as a result of mutation. Figure constructed using Insight II. Neither the residues that showed chemical shifts (Jansson et al., 1998) nor those that make contact with mini-BP-5 are shown (Zeslawski et al., 2001).

1.7.2 Summary of IGF residues involved in IGFBP binding

The determinants for high affinity binding of both IGF-I and IGF-II to any of the six IGFBPs appear to be in the B and A domains. The role of the IGF-II C domain in IGFBP binding is unclear. The reason for IGF-II having a 10- to 60-fold higher affinity for IGFBP-6 than IGF-I is unknown and may be due to elements within its C domain.

1.7.3 Mechanism of IGF-I and IGF-II binding to IGFBPs

Recombinant and naturally occurring fragments of IGFBPs have provided useful tools in determining the regions of the IGFBPs that bind IGF-I and IGF-II (as discussed in section 1.7). These studies reveal an IGF binding site in both the N and C terminal domains and also show that both the N and C terminal domains are required for wildtype affinity (reviewed in (Hwa et al., 1999)). BIAcore analyses of the kinetics of IGF-I and IGF-II binding to IGFBP-2 fragments have revealed that IGF binding to the N terminal domain occurs as a fast association phase and the C terminal domain provides stabilization of the complex (Carrick et al., 2001). This mechanism may hold true for all IGF-IGFBP interactions as similar results were obtained for
IGFBP-3 (Payet et al., 2003). NMR relaxation experiments have shown the C terminal domain of IGFBP-6 undergoes a conformational change upon binding to IGF-II, but how this contributes to the binding mechanism is unclear (Yao et al., 2004).

1.8 Comparison of the receptor and binding protein binding sites on the IGFs

A summary of the IGF-1R, IR and IGFBP binding sites on the IGF-I and the IGF-2R binding site on IGF-II is shown in Figure 1.27. From a comparison of the different protein interaction sites on IGF-I, and IGF-II in the case of the IGF-2R, it is striking that almost all regions of the IGF protein appears to be involved in at least one interaction. This is even more remarkable given that the complete binding sites of the IGF-1R, IR and IGF-2R on the IGFs are unknown and therefore once completely determined may in fact show that almost all surface-exposed amino acids are involved in an interaction. It appears that evolution has refined all the surfaces on the IGFs for protein-protein interaction. Protein-protein interactions create strong selection pressures so that most of the amino acids at interfaces are more conserved than amino acids not involved in an interaction (Teichmann, 2002). The fact that the entire IGF molecule is involved in some type of interaction may be the reason behind the slow evolution of both the IGF-I and IGF-II molecules (Nagamatsu et al., 1991).

Strikingly, the residues in IGF-I that are critical for IGF-1R appear to hinder IR binding. The opposing role of these IGF amino acids may indicate the differences in the ligand binding pockets of the IGF-1R and the IR or may reflect contrasting receptor binding mechanisms.

The IGFBP and IGF-2R binding sites are similar and yet largely distinct from those of the IR and IGF-1R. However, detailed comparisons of the IGF-1R and IGFBP binding site on IGF-II, taking into account IGF:IGFBP NMR and crystal structure studies, shows they partially overlap (Headey et al., 2004). This provides a molecular
Figure 1.27 Comparison of the IGF binding sites of the IGF-1R, IR, IGFBP and IGF-2R. Binding sites for the IGF-1R, IR and IGFBP are shown on IGF-I (Cooke et al., 1991) while the IGF-2R binding site is shown on IGF-II (Torres et al., 1995). N= N-terminus, C= C terminus. For residue labels see IGF-1R – Figure 1.9, IR – Figure 1.15, IGF-2R – Figure 1.21, IGFBP – Figure 1.26.
mechanism whereby IGFBP binding inhibits IGF-I and IGF-II binding to the IGF-1R (and most likely the IR).

Clearly from the summary in Figure 1.27 both the IR and IGF-2R binding sites on the IGFs are very poorly understood. Most studies have probed the role of certain amino acids of IGF-I in binding the IR rather than those of IGF-II. The IGF-2R binding site has not been studied in great detail, generally due to the lack of signalling capability by the IGF-2R and its perceived lack of importance in the IGF system previously. These two areas are major gaps in our understanding of the ligand/receptor interactions of the insulin/IGF system.

Shown in Figure 1.28 are the mutagenesis data summarised in Figure 1.27. However, each residue is now coloured with respect to the IGF domain in which it resides. From this diagram it is obvious that the C and D domains are involved (either positively or negatively) in IGF-1R and IR binding. However, these same domains are either dispensible or not studied with respect to IGFBP and IGF-2R binding. The B and A domains appear to be crucial for binding to all receptor and binding proteins.

1.9 AIMS AND EXPERIMENTAL STRATEGY

IGF-I and IGF-II bind to a variety of transmembrane receptors and soluble high affinity binding proteins in a complex system that regulates almost all pre- and post-natal growth. Despite the structural similarity of IGF-I and IGF-II they exhibit different binding affinities to almost all proteins with which they both interact. The molecular basis for this affinity difference for any interacting protein is unknown and hence is the focus of these studies.

Recently, the IR-A has become of great interest as a possible mediator of the growth promoting effects of IGF-II in development and cancer. The reason for the high affinity of IGF-II but the low affinity of IGF-I for the IR-A is currently unknown. To
Figure 1.28 Comparison of the relative involvement of IGF domains to the binding sites of the IGF-1R, IR, IGFBP and IGF-2R. Binding sites for the IGF-1R, IR and IGFBP are shown on IGF-1 while the IGF-2R binding site is shown on IGF-II. Molecules in the same orientation as Figure 1.27.
date investigation into the region(s) on IGF-II that allow high affinity binding to the IR-A has not been conducted. In addition the region(s) of IGF-I that preclude high affinity binding to the IR-A is also unknown.

The highest divergence of sequence between IGF-I and IGF-II is within their flexible C and D domains. As discussed in the introduction, the C and D domains have been implicated by mutagenesis and other biochemical studies to be involved in IGF-1R and IR binding while being dispensable for IGFBP binding. The role of the C and D domains in IGF-2R binding has been largely ignored.

Therefore, the first major goal of this thesis was to investigate the role of the C and D domains of IGF-I and IGF-II in determining their respective receptor and binding protein binding specificities. To determine the receptor and binding protein binding specificity of the IGFs, a chimeric approach was taken. Specifically, the C and D domains were exchanged between IGF-I and IGF-II either singly or together, creating for the first time, whole domain IGF chimeras. Production of these proteins allowed investigation into the following:

- Define differences in the role of the C and D domains of IGF-I and IGF-II in interactions with the IGF-1R, IR isoforms, IGF-2R and IGFBPs.
- Probe the signalling pathways activated by IGF-I and IGF-II through the IR isoforms.

Exchanging regions of structurally related but functionally distinct molecules, creating chimeras, allows determination as to whether the swapped regions confer specific properties. Creation of chimeric proteins has been used to delineate a diverse range of biochemical properties in a wide range of proteins. Recent examples include: the region of cdc42 that is responsible for specific binding to the activated cdc42
associated tyrosine kinase (Gu et al., 2004), region of IGFBP-5 responsible for its nuclear localization (Xu et al., 2004), the domain for localization and nuclear translocation of protein kinase C-\(\phi\) and \(\varepsilon\) (Wang et al., 2004) and the selectivity of membrane-bound fatty acid desaturases in *Caenorhabditis elegans* (Sasata et al., 2004).

Furthermore, construction of chimeras between two structurally similar but functionally unique growth factors has shed light on the ErbB binding specificity of EGF and TGF-\(\alpha\) (Kramer et al., 1994; van de Poll et al., 1995; Lenferink et al., 1997; Schmidt and Wels, 2002; Stortelers et al., 2002; Wingens et al., 2003), the heregulin receptor binding specificity of EGF and heregulin (Barbacci et al., 1995), and the FGFR binding specificity of FGF-7 (Sher et al., 1999; Sher et al., 2000). These previous reports confirm that the production of chimeric proteins is a valid method of probing binding interactions. In this thesis, chimeras of IGF-I and IGF-II are shown to be a powerful tool in dissecting the IR and IGF-1R binding specificity of the IGFs.

The second major goal of this thesis, in keeping with the broad aim to probe the structure and function of IGF ligands, was to investigate the biochemical characteristics of the first ever detected, naturally occurring mutant of IGF-I. Production of this mutant IGF-I, which had a valine to methionine substitution at position 44 allowed:

- Investigation into the role of the valine 44 in IGF-1R, IR isoform and IGFBP binding.
- Analysis of the contribution of the substituted methionine to any disruption of tertiary structure which would inadvertently reveal the role of valine 44 to normal IGF-I maintenance of tertiary structure.
- Correlation of biochemical properties of the mutant with the growth and developmental abnormalities of the affected subject.
Construction of vectors for IGF chimera expression
CHAPTER 2 – Construction of vectors for IGF chimera expression

2.1 INTRODUCTION

The reason for the differential binding affinities of IGF-II and IGF-I for almost all proteins with which they both interact is poorly understood. While extensive mutagenesis and deletion studies have revealed certain residues important in the free energy of binding of IGF-I to the IGF-1R (reviewed in (Ward et al., 2003)) the regions on IGF-II that allow it to bind with higher affinity than IGF-I to the IR isoforms, IGF-2R and a number of IGFBPs e.g. IGFBP-2 and -6 are not well defined. The proposal of this thesis is that the C and D domains of IGF-I and IGF-II may be involved in discriminating specificity of IGF-I and IGF-II binding to insulin/IGF family receptors and IGFBPs. To investigate this proposal, IGF chimeras between IGF-I and IGF-II were produced, where the C and D domains are swapped either singularly or in tandem.

To date no whole domain chimeras of IGF-I and IGF-II have been produced, however, a number of chimeras of insulin and IGF-I have been engineered and their properties studied (De Vroede et al., 1985; Joshi et al., 1985; Joshi et al., 1985; De Vroede et al., 1986; Tseng et al., 1987; Cara et al., 1990; Joshi et al., 1990; Schaffer et al., 1993; Kristensen et al., 1995; Wang et al., 2000). Different regions of both insulin and IGF-I have been exchanged: the A domain of IGF-I combined with the B domain of insulin (Tseng et al., 1987), residues 22-41 of IGF-I (9 residues of IGF-I B domain and entire C domain) connected to des-octapeptide-(B23-B30)-insulin (Cara et al., 1990; Schaffer et al., 1993) and the C domain of IGF-I inserted in insulin forming a single chain hybrid (Kristensen et al., 1995; Wang et al., 2000). These proteins have been valuable in investigating IGF-1R and IR binding determinants revealing that the C domain of IGF-I can confer high affinity binding to insulin to the IGF-1R (Cara et al., 1990). In addition such studies have challenged existing dogma relating to elements that are required for insulin receptor binding (Kristensen et al., 1995). While
these proteins have shed new light on the specificity of IGF-I/insulin binding to the IGF-1R and IR they do not provide any means to investigate the basis of receptor binding specificity of IGF-I and IGF-II.

High affinity binding of insulin to the insulin receptors has been suggested to involve movement of the C-terminus of the B chain to reveal hydrophobic residues in the A chain (Hua et al., 1991; Gill et al., 1996; Geddes et al., 2001). Cross-linking A1 Gly to B29 Lys in insulin reduces its affinity for the insulin receptor supporting the notion that flexibility is required for binding. Mature insulin has no C or D domain. Hence in this study we chose to investigate whether the flexible C and D domains of IGF-I and IGF-II influence their insulin receptor isoform binding specificity.

There are 26 residue differences between IGF-I and IGF-II and with the greatest concentration in the C and D domains (Figure 2.1). Hence to answer the question whether the C and D domains are responsible for the differential receptor binding affinities of IGF-II and IGF-I IGF chimeras where the C and D domains are exchanged either singularly or together were required to be produced.

Here in this chapter the construction of the coding sequences for wildtype human IGF-I, IGF-II and all IGF chimeras is detailed. The nomenclature of the IGF chimeras discussed throughout the rest of this thesis is as follows: IGF-I CII, IGF-I with the C domain from IGF-II; IGF-I DII, IGF-I with the D domain from IGF-II; IGF-I CIIDII, IGF-I with the C and D domains from IGF-II; IGF-II CI, IGF-II with the C domain from IGF-I; IGF-II DI, IGF-II with the D domain from IGF-I; IGF-II CIDI, IGF-II with the C and D domains from IGF-I (Figure 2.1).
CHAPTER 2 – Construction of vectors for IGF chimera expression

Figure 2.1. A, Sequence alignment of human IGF-I, IGF-II and insulin. Alignment completed using Clustal W (www.ebi.ac.uk/clustalw/), with the numbering of amino acids indicated above for IGF-I and below for insulin. B, Diagrammatic representation of the domain exchanged chimeras. Amino acid numbers and molecular weights are given. Each linear representation is divided into the domain structure, B, C, A, D with all IGF-I domains in blue and all IGF-II domains in red.
2.2 GENERAL MATERIALS

The solutions listed below in Table 2.2.1 were prepared by Mrs. Ros Hammond (Central Services Unit, School of Molecular and Biomedical Science, The University of Adelaide). Each solution was prepared using water purified by the Milli-Q Ultra Pure Water System (Millipore Pty Ltd, North Ryde, Australia) and sterilized by autoclaving.

2.2.1 Bacterial media and buffers

Luria-Bertani Medium (LB)
0.5% bacto® yeast extract, 1% (w/v) bactotryptone®, 0.17M NaCl pH 7.0

Luria-Bertani Medium (LB)
LB media + 1.5% (w/v) bacto-agar

Transformation Buffer 1
30 mM KAcetate, 100 mM RbCl, 10 mM CaCl₂(2H₂O), 50 mM MnCl₂(4H₂O), 15%
Glycerol – pH to 5.8 and sterilized through 0.22μM filter

Transformation Buffer 2
10 mM 3-N-morpholinopropanesulfonic acid (MOPS), 10 mM RbCl, 75 mM
CaCl₂(2H₂O), 15% Glycerol – pH to 6.5 and sterilized through 0.22μM filter.

2.2.2 Bacterial Strains

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<th>Name</th>
<th>Genotype</th>
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<td>supE44 ΔlacU169 hsdR17 recA1 endA1 gyrA96 λΔ relA1 [φ80lacZΔM15]</td>
</tr>
<tr>
<td>E. coli JM101</td>
<td>supE thi-1Δ(lac-proAB) F' [traD36 pro AB+ lacIΔ lacZ ΔM15]</td>
</tr>
</tbody>
</table>
CHAPTER 2 – Construction of vectors for IGF chimera expression

2.3 MATERIALS FOR MOLECULAR BIOLOGY

Bacto® yeast extract and bactotryptone® were purchased from DIFCO laboratories (MI, U.S.A.). Ampicillin, ammonium persulphate, N,N,N′,N′-tetramethyl ethylene-diamine (TEMED) and ethidium bromide were obtained from Sigma Chemical Co. (MO, U.S.A.). Hpa I and Hind III were purchased from Geneworks (Adelaide, Australia) while Hind I was obtained from New England Biolabs (MA, U.S.A.). DyNAzyme™ EXT was purchased from Finnzymes (OY, Finland). T4 DNA ligase was from Boehringer Mannheim Australia (NSW, Australia). Acrylamide 40% (w/v) was purchased from Biorad Pty. Ltd. (NSW, Australia). Durapore® 0.22μM filter was purchased from Millipore Corp. (MA, U.S.A.). SPP1/Eco RI DNA molecule weight markers and pUC19 DNA restricted with Hpa II were obtained from Geneworks (Adelaide, South Australia). Shrimp alkaline Phosphatase and 10x reaction buffer were purchased from USB corporation (OH, U.S.A.), 10x One Pho All Buffer from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.3.1 Molecular biology solutions

PCR reaction buffer

50 mM Tris-HCl pH 9.0, 15 mM (NH₄)₂SO₄, 0.1% Triton X-100, 0.005% BSA, 0.25 mM dNTPs, 1.5 mM MgCl₂

Tris Borate EDTA (TBE)

0.09 M Tris, 0.09 boric acid, 2.5 mM EDTA

10x DNA loading dye

50% (v/v) glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF
CHAPTER 2 – Construction of vectors for IGF chimera expression

2.3.2 KITS FOR MOLECULAR BIOLOGY

The Mo Bio DNA purification kit for plasmid extraction from bacterial cultures and Mo Bio Gel Clean kit for gel purification of DNA was purchased from Geneworks (Adelaide, Australia). The QIAgen QIAquick PCR purification kit was purchased from QIAgen Pty. Ltd. (Victoria, Australia). The TA cloning vector system pGEM®-T-Easy Vector System I was purchased from Promega (Madison, U.S.A.). The ABI PRISM Dye terminator Cycle Sequencing kit with AmpliTaq DNA polymerase was purchased from Perkin Elmer (Norwalk, U.S.A.).

2.3.3 EXPRESSION VECTOR

The expression vector pGH(1-11) (King et al., 1992) was used for expression of the IGF chimeras (Figure 2.2). This vector has been optimized for the high level expression of IGF-I and IGF-II in E. coli (King et al., 1992) and has been routinely used for the expression of wildtype human IGF-I and IGF-II (Lien et al., 2001) and IGF mutants (Shooter et al., 1996; Magee et al., 1999).
Figure 2.2 Expression vector used to express human IGF-I, IGF-II and all IGF chimeras. The vector used to express IGF-I and IGF-II based chimeras in *E. coli* JM101 is similar to that used to express both wildtype and mutant IGFs previously (King et al., 1992; Shooter et al., 1996; Magee et al., 1999). All proteins cloned into the pGH(1-11) vector are expressed as a fusion to the first 11 amino acids of porcine growth hormone. Due to the different N-termini of IGF-I and IGF-II different cleavage processes are used. Hydroxylamine cleaves between asparagine in the linker and glycine which is the first residue of IGF-I. Prag A9, an α-lytic protease, cleaves between a methionine in the engineered linker and alanine at the first position in IGF-II (Lien et al., 2001). The expression is driven by an IPTG-inducible ptrc promoter. The vector also contains an optimized ribosome entry site, transcription termination sequence and β-lactamase gene conferring ampicillin resistance (amp'). *IGF-I or all chimeras containing the IGF-I B domain. #IGF-II or all chimeras containing the IGF-II B domain.
2.3.4 OLIGONUCLEOTIDES

The template primers and amplification primers used for the construction of the sequence coding the IGF chimeras are show in Table 2.1 and 2.2 respectively. All primers were designed using Oligo™ 4.0s and were synthesized by Geneworks Pty Ltd. (South Australia, Australia).

2.4 METHODS

2.4.1 AGAROSE GEL ELECTROPHORESIS OF DNA

DNA integrity was determined by separation on agarose gels using a TBE running buffer. The concentration of agarose gels varied from 1-2 % depending on the size of the DNA sample. Each DNA sample was mixed with a DNA loading dye to aid the addition of the sample to the gel and electrophoresed at 100 V for 30 minutes. The DNA was visualized by incubating the gel in 1 μg/ml ethidium bromide for 10 minutes before destaining in H2O for 5 minutes. Short wave UV was used to excite the intercalacted ethidium bromide and a photograph was taken.
## Table 2.1

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2.4.2 RESTRICTION ENZYME DIGESTION OF DNA

Restriction enzyme digestion of DNA was conducted using a protocol from the supplier of the enzyme. Each reaction was stopped by either storage at -20 °C or running on an agarose gel.

2.4.3 PURIFICATION OF DNA BY DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

Large template primers and digested TA cloned fragments were purified on a denaturing polyacrylamide gel that was prepared with 12 % (w/v) polyacrylamide, 1 X TBE, 0.1 % (v/v) TEMED, 0.2 % ammonium persulphate and 2 M Urea. Twenty micrograms of oligonucleotide or an entire digest was loaded onto the gel using 95 % (v/v) formamide as loading dye and electrophoresed at 100 V. The band of correct size, as compared with molecular weight markers, was excised and incubated in MQH₂O overnight at 4 °C. Two different methods for concentrating either the oligonucleotides or the digest were used. For the oligonucleotides: The following day the supernatant was removed and the DNA was concentrated by a butanol precipitation (Sawadogo and Van Dyke, 1991). Briefly 5 volumes of butanol was added to the supernatant and then the mixture was vortexed vigorously for 15 seconds. The mixture was then centrifuged for 3 minutes at 16,110 x g. The supernatant was then removed and the pellet was dried on a Speedy-Vac™ for 15 minutes. For the digests: The following day the supernatant was removed and to it was added 25 µl of 3 M sodium acetate pH 5.2, 2.5 volumes of 100 % RNase free ethanol and 20 µg glycogen. This mixture was incubated at -20 °C for 2 hours and then centrifuged at 16,110 x g for 15 minutes at 4 °C. The supernatant was removed and the pellet was washed in 70 % ethanol by vortexing briefly, before centrifugation.
once again at 16,110 x g for 10 minutes at 4 °C. The supernatant was removed and the pellet was dried on a speedy vac.

2.4.4 POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction was used to create the sequences coding for the IGF chimeras. Approximately 1μg of purified template primer (Table 2.1) was added to a PCR reaction that also included 100ng of appropriate amplification primer (Table 2.2), 1 x PCR reaction buffer and 1U of DyNAzyme EXT polymerase. Each reaction was incubated in a PTC-200 MJ Research Thermocycler using the following protocol: Step 1 (initial denaturation) – 92 °C for 1 minute: Step 2 (denaturation)– 92 °C for 1 minute: Step 3 (annealing)– 55 °C for 1 minute: Step 4 (extension)– 72 °C for 1.5 minutes. Steps 2-4 inclusive were repeated 29 times followed by a final extension step of 72 °C for 10 minutes. The reaction was terminated by lowering the incubation temperature to 12°C. The success of all PCR reactions was determined by visualizing the PCR products on a 2 % agarose gel.

2.4.5 PURIFICATION OF PCR PRODUCTS FROM AGAROSE GELS

For purification of PCR products from agarose gels, a similar protocol to that in section 2.4.1 was used to electrophorese the DNA. In this situation however DNA was visualized using long wave UV instead of short wave UV. Purification of DNA from agarose gels was by Mo Bio Gel Clean kit.
2.4.6 TA CLONING OF PCR FRAGMENTS

DyNAzyme EXT polymerase used in the PCR is a Taq based enzyme that leaves an adenosine overhang at the 3’ end of each amplified strand (Clark, 1988). This then allows the fragment to be cloned into a vector that has been digested with EcoRV which leaves a thymine overhang at the 5’ end of strand at the site of DNA cleavage. Each PCR fragment was gel purified using the QIAgen QIAquick gel purification kit and ligated into the pGEM®-T-Easy vector in a reaction containing the following; entire purified PCR product, 25 ng of pGEM-T-Easy vector, 1 X Rapid Ligation buffer and 3 U of T4 DNA ligase. The ligation reaction was incubated at 22°C for 2 hours before being mixed with 100μl of competent DH5α for transformation and plated on LB plates containing ampicillin selection. Several colonies were selected and grown overnight in LB media containing 100μg/ml ampicillin and then the plasmid was extracted using the Mo Bio DNA purification Kit. The purified plasmids were digested to determine presence of the cloned insert and if positive plasmids were sequenced using the ABI PRISM dye terminator cycle sequencing ready reaction kit to determine integrity of amplified fragment (section 2.4.7).

A selected plasmid that contained the correct insert (as determined by DNA sequencing) was digested with either Hpa I and Hinf I, for all BC fragments or Hinf I and Hind III, for all AD fragments and digests were purified on a 12% denaturing polyacrylamide gel. (see section 2.4.3). The digested fragments were then ligated into the expression vector pGH(1-11) (see section 2.4.9).
2.4.7 DYE TERMINATOR DNA SEQUENCING

The ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit involves amplification of the DNA to be sequenced with a 1x terminator concoction (fluorescently-labelled dideoxynucleotides (G-dye terminator, T-dye terminator, C-dye terminator, A-dye terminator), dGTP, dTTP, dCTP, dATP, Tris-HCl MgCl₂, thermal stable pyrophosphatase, AmpliTaq DNA polymerase, FS) and the appropriate sequencing primer. Incorporation of a dideoxynucleotide into the extending DNA strand results in chain termination resulting in DNA fragments of various lengths. If a dideoxynucleotide is incorporated at each base along the extending DNA strand, across the region of DNA to be sequenced, the fluorescent dideoxynucleotide will provide a means of address and the integrity of each base can then be determined. The terminal base in each fragment was identified using a model 373 Perkin Elmer sequencing machine operated by staff at the Adelaide Sequencing Centre.

2.4.8 REMOVAL OF 5'-PHOSPHATE FROM VECTOR

To promote DNA ligation efficiency by preventing religation of restricted vector, shrimp alkaline phosphatase (SAP) was used to remove the 5'-phosphate. In a reaction containing 20 mM Tris-HCl pH 8.0 and 10 mM MgCl₂, 2 units of SAP were used to treat 2 μg of Hpa I and Hind III digested vector, at 37°C for 2 hours. The SAP was inactivated by incubation of the reaction at 65°C for 15 minutes.
2.4.9 DNA LIGATION

The ligation of appropriately digested DNA fragments and vectors was completed using T4 DNA ligase. Prior to ligation, digested vector was treated with shrimp alkaline phosphatase (SAP) as per 2.4.8. Digested fragments and vector were added to a reaction containing 5mM ATP, 1X One for All buffer and 1U of T4 DNA ligase and incubated for 14 hours at 14°C. The ligation reaction was then transformed into bacteria and plated out on an agar plate containing ampicillin for selection.

2.4.10 PREPARATION OF COMPETENT CELLS

_E. coli_ DH5α or JM101 were prepared for transformation as described by CLONTECH laboratories Inc. (Palo Alto, CA, U.S.A.). LB (5 ml) was inoculated with a single colony and grown overnight at 37 °C. The following day an aliquot of this culture (330 µl) was subcultured into 10 ml of LB and incubated at 37 °C until _A_\textsubscript{600nm} reached 0.6 at which time another subculture of 5 ml into 100ml fresh LB was performed. Once this 100 ml culture reached _A_\textsubscript{600nm} of 0.6 the cells were pelleted at 4000 x g for 5 minutes at 4 °C and then resuspended in Transformation buffer 1 (section 2.2.1) and incubated on ice for 5 minutes. The cells were then pelleted at 4000 x g for 5 minutes at 4 °C before resuspending the cells in 4 ml of Transformation buffer 2 (section 2.2.1). The cells were incubated on ice for 15 minutes before aliquoting into 100 µl or 200 µl volumes and then stored at -80 °C.
2.4.11 BACTERIAL TRANSFORMATIONS

The entire ligation reaction (10 μl) was incubated with 200 μl of competent *E. coli* DH5α cells for 30 minutes on ice. The cells were then heat-shocked for 90 seconds at 42 °C and then the cells were placed on ice for 5 minutes. One ml of LB media was added to the cells prior to incubation at 37 °C for 30 minutes to allow expression of the β-lactamase gene conferring antibiotic resistance. The cells were then centrifuged at 1,500 x g for 2 minutes, 1 ml of supernatant was removed and the pellet was resuspended in the remaining liquid before plating onto LB plates containing ampicillin. The plates were incubated overnight at 37 °C. To determine the extent of spontaneous religation of digested vector, a ligation reaction with vector only was completed and transformed into the DH5α bacterial cells.

2.5 RESULTS AND DISCUSSION

To investigate the receptor binding specificity of IGF-I and IGF-II, chimeras exchanging the C and D domains were produced. Consequently, the construction of vectors coding IGF-I and IGF-II, where the C and D domains had been swapped either singly or together, is detailed in this section. To assess the binding of these chimeras to all the proteins that IGFs interact with required a substantial amount of protein i.e. >500 μg. The vector pGH(1-11) allowed high levels of expression of native human IGF-I, IGF-II and all IGF chimeras in *E. coli*.
2.5.1 Codon optimisation of sequences for expression

Construction of sequences for expression of IGF chimeras using a PCR method with totally synthetic oligonucleotides allowed total codon optimization for expression in bacteria (Figure 2.5 and 2.6). The 20 amino acids used by all living organisms are encoded by 61 codons. Of these 61 codons all organisms use a defined subset of these with which they produce most of their mRNA. Highly expressed genes usually contain "major codons", which are codons that have large pools of t-RNA containing their cognate anti-codon (Hatfield et al., 1992). Lowly expressed genes are generally comprised of "minor" or "rare codons", which do not have large ready amounts of t-RNA for efficient high-level translation (Hatfield et al., 1992). The subset of codons used frequently by human cells is different to that used by E. coli cells and therefore a major codon in humans may be a minor codon in E. coli (Nakamura et al., 2000). Such variation in the frequency of codons used can cause problems when expressing a human protein in bacteria. For example, arginine is encoded by four of the top six most rare codons in E. coli (Kane, 1995). Several arginine codons are used at a very low frequency in E. coli, particularly AGG/AGA (occurrence is 2.0/3.46 per 1000 codons (Nakamura et al., 2000)) and the presence of these codons in heterologous mRNA can cause frameshifts during the translation event (Spanjaard and van Duin, 1988). In particular, Seetharam et al., 1988 observed mistranslation of IGF-I during expression in E.coli where lysine was incorrectly incorporated at low frequency arginine codons (Seetharam et al., 1988). Leucine is another amino acid encoded by sequences in humans that are rarely used in E. coli (Goldman et al., 1995). Consequently for efficient translation and hence protein expression of human genes in E. coli all codons in the heterologous gene should be "optimized" to those that occur frequently in E. coli genes. Codon optimisation frequently yields between 2 to >1000-fold increases in
Figure 2.5 Codon usage by *E. coli* for IGF-I sequences prior to and after optimisation. The codon usage values for *E. coli* was taken from (Nakamura et al., 1996). Solid bars represent codon usage at each amino acid prior to optimisation. Open bars represent codon usage after optimisation. The codon usage of some codons decreased after optimisation to incorporate unique silent restriction sites to confirm integrity of sequence while cloning.
Figure 2.6 Codon usage by *E. coli* for IGF-II sequences prior to and after optimisation. The codon usage values for *E. coli* was taken from (Nakamura *et al.*, 1996). Solid bars represent codon usage at each amino acid prior to optimisation. Open bars represent codon usage after optimisation. The codon usage of some codons decreased after optimisation to incorporate unique silent restriction sites to confirm integrity of sequence while cloning.
protein expression level compared to the level achieved using unoptimised sequences (Gustafsson et al., 2004).

All codons in the hIGF-I sequence, used routinely in our laboratory, were already optimized, however the sequence of hIGF-II previously employed to express hIGF-II was rich in E. coli non-preferred or rare codons (Figure 2.6). The hIGF-II sequence contained 2 rare arginine codons. The one at the N-terminus arginine 3 is of particular note as rare codons at the N-terminal region of a transcript have been reported to be more detrimental to translation (Goldman et al., 1995). In addition rare proline, leucine, glycine, lysine and threonine codons were all optimized. When optimizing the codons of frequently occurring amino acids within the sequence, the most preferred codon was not used in each case, so as to prevent depletion of the intracellular pool of the cognate t-RNA. The phenomenon of t-RNA depletion by a commonly occurring codon is termed the “hungry codon syndrome” and can adversely affect protein expression (Kane, 1995; Kurland and Gallant, 1996). While a direct comparison of the expression level of IGF-II from coding regions before and after codon optimization was not performed, the level of recombinant protein yield in inclusion bodies from the IGF-II ferment was higher than previously obtained in the laboratory.

2.5.2 Strategy to construct coding sequence for IGF chimeras

IGF-I and IGF-II are small proteins of 70 and 67 amino acids respectively clearly defined into 4 domains: B and A domains named due to their sequence similarity to the B and A chains of insulin and the C domain named due to its similarity to the C peptide in the analogous position in insulin. The D domain has no counterpart in insulin and is classified in accordance with the alphabetical nature of the domain naming. Introducing silent restriction sites at domain junctions to facilitate
chimera construction was virtually impossible. For this reason the sequences encoding the chimeras were produced entirely from large overlapping synthetic oligonucleotides and the resultant template sequence amplified by shorter PCR primers.

Large template primers were generated following constraints imposed by the Oligo 4.05™ program. This program predicts several biochemical characteristics of the oligos such as Tm, hairpin loop formation and binding palindromes. The synthesis of oligonucleotides occurs from 3' to 5' and addition of each successive nucleotide occurs with an average of 99.25 % efficiency (Geneworks, 2001). As a result the percentage of full length product decreases proportionally at each coupling step, so that when a 90 mer oligonucleotide is produced, full length material only constitutes 53 % of the oligonucleotide synthesized. To purify full length oligonucleotide and therefore maximize the concentration of correct length template primer used in PCR, the oligonucleotides were run on a 12 % denaturing polyacrylamide gel. The band corresponding to the full length primer was excised, extracted from the gel and concentrated using a butanol precipitation method (section 2.4.3) (Sawadogo and Van Dyke, 1991). Non-purified template primer did not yield a successful PCR product.

![Figure 2.9 Example of PCR products generated using selected primers from Table 2.1 and 2.2 in the PCR method as shown in Figure 2.7. Lane 1, Hpa II cut pUC19 phage vector, Lanes 2-5, PCR products encoding BC domains for IGF-I, Lanes 6-9, PCR products encoding AD domains for IGF-I.](image)
The PCR method used to create the IGF chimera constructs is shown in Figure 2.7. To generate halves of each chimera both large template primers and both small PCR primers were all added to the same PCR reaction from cycle 1. Due to the nature of PCR only template primers that have correctly annealed and fully extended would be amplified by the small PCR primers. An example of the success of the PCR reactions is shown in Figure 2.9.

After each PCR reaction the product was purified on a 2.5 % agarose gel and gel purified using a QIAgen Gel purification kit. The large oligonucleotide primers shown in Table 2.1 allowed construction of wildtype human IGF-I and IGF-II and also all single and double chimeras in two separate halves: either the B and C domain or the A and D domain. Each BC fragment contained a Hpa I site at the 5' end and a Hinf I at the 3' end to facilitate cloning, while all AD fragments contained a Hinf I site at the 5' end and a Hind III site at the 3' end also to aid cloning (Figure 2.8). Fragments were then cloned into the pGEM®-T-Easy Vector system and sequenced to determine the fidelity of the insert. Once the integrity of the insert was confirmed the vector was digested with (regardless of whether the domains are IGF-I or IGF-II) either Hpa I and Hinf I for all BC fragment or Hinf I and Hind III for all AD fragments. The pGEM®-T-Easy vector contains the Hinf I restriction site eight times so the number of fragments that results for digestion with Hinf I and either Hpa I or Hind III is 9. The size of the BC and AD fragments are approximately 120bp or smaller and are poorly resolved on agarose gels so purification of the digests was completed by running on a 12 % denaturing polyacrylamide gel (Figure 2.10). The correct bands, as determined by comparison with molecular weight markers, were excised and allowed to diffuse into 1x TE overnight at 4 °C.
Figure 2.7 Construction of coding sequences for IGF-I, IGF-II and IGF chimeras – PCR and TA cloning. The template primers and amplification primers shown in Tables 2.1 and 2.2 respectively were used in a PCR reaction to create the coding sequences in two separate halves (section 2.4.4). These PCR products were then purified and cloned into the p-GEM-T-Easy vector (section 2.4.6).
**Figure 2.8** Cloning into pGH(1-11) expression vector. BC and AD fragments were digested from the p-GEM-T Easy TA cloning vector (section 2.4.6) and purified on a 12% denaturing polyacrylamide gel (section 2.4.3). The digest fragments were then cloned into the expression vector pGH(1-11) [King, 1992 #105] (section 2.4.9) The entire coding region was sequenced to confirm the integrity of the sequence.
Appropriate combinations of purified fragments were then cloned into *Hpa* I and *Hind* III digested expression vector pGH(1-11). The vectors were then sequenced to confirm the integrity of the coding region.

![Image](image_url)

**Figure 2.10 Purification of digested fragments for pGEM-T-Easy vectors containing either BC or AD fragments.** A) *Hpa* II cut pUC19 markers are shown in Lane 1. Digestion of p-GEM-T-Easy vectors containing either IGF-I BC fragment (lanes 2 and 3) or AD fragment (lanes 4 and 5). Vector was digested with either Hpa I and Hinf I (lanes 2 and 4) or only Hinf I (lanes 3 and 5). Comparing the results of the single and double digests allowed identification of the required bands, indicated by white arrows. B) Identical samples and loading to A) however run on a 12 % denaturing polyacrylamide gel. A scan of this preparative gel was taken after the bands were excised and therefore their position is denoted by white open boxes.

### 2.5.3 Summary

Using a PCR method sequences were produced encoding human IGF-I, IGF-II and all six IGF chimeras, where the C and D domains of IGF-I and IGF-II were exchanged singly or together. Construction using synthetic oligonucleotides allowed codon optimization for expression in *E. coli*. The expression and purification of IGF-I, IGF-II and all IGF chimeras could then be completed.
Expression and purification of IGF-I, IGF-II and novel IGF chimeras
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

3.1 INTRODUCTION

IGF-I and IGF-II were initially isolated from a Cohn fraction of human serum almost 30 years ago (Rinderknecht and Humbel, 1976) and early investigation into the biological roles of the IGFs was hampered by a lack of pure protein (Humbel, 1990). Various methods have been used to either purify IGF-I and IGF-II from biological fluids e.g. human serum (Schalch et al., 1984) or bovine colostrum (Francis et al., 1988), or to chemically synthesize native IGFs and IGF analogues (Ballard et al., 1987; Bagley et al., 1989; Bagley et al., 1990). These methods, however, are costly, laborious and yield low amounts of pure protein. As a result recombinant expression of the IGFs has been of major interest. Expression of recombinant IGF-I has been demonstrated in a number of heterologous expression systems such as yeast (Bayne et al., 1988; Cascieri et al., 1988; Maly and Luthi, 1988; Bayne et al., 1989; Cascieri et al., 1989; Bayne et al., 1990; Zhang et al., 1994), bacteria (Peters et al., 1985; Moks et al., 1987; Nishikawa et al., 1987; Francis et al., 1992; King et al., 1992), silk worm (Bombyx mori) (Sakano et al., 1991) and mammalian cells (Bayne et al., 1987; McKinnon et al., 1991). Recombinant production of IGF-I and IGF-II has allowed easy genetic manipulation of the IGF coding sequences, resulting in creation of numerous recombinant IGF deletion and point mutants (Bayne et al., 1988; Cascieri et al., 1988; Bayne et al., 1989; Cascieri et al., 1989; Bayne et al., 1990; Clemmons et al., 1992; Francis et al., 1992; Zhang et al., 1994; Hodgson et al., 1995; Hodgson et al., 1996; Shooter et al., 1996; Magee et al., 1999; Forbes et al., 2001).

Despite the advantages of potential high level of protein expression and low production cost, the production of recombinant proteins in E. coli does have several pitfalls. These including the inability to express certain recombinant proteins, the stability and effectiveness of mRNA translation, the low protein solubility, degradation by host cell proteases, toxicity of heterologous protein to host and major variations in
codon usage between the non-native gene to be expressed and E. coli (reviewed in (Makrides, 1996)). In addition high level expression can cause modification to the heterologous protein including non-efficient removal of the N-terminal initiator methionine (Ben-Bassat et al., 1987), N and C terminal truncations (Daumy et al., 1989; Tu et al., 1995) or extensions (Tu et al., 1995) and incorrect amino acid incorporation e.g. lysine for arginine (Seetharam et al., 1988) and norleucine for methionine (Bogosian et al., 1989).

The expression and purification of recombinant IGF-I and IGF-II in E. coli has been optimized by our laboratory and collaborators (Francis et al., 1992; King et al., 1992; Shooter et al., 1996; Magee et al., 1999). The vector used for high level expression of IGF-I and IGF-II in E. coli, pGH(l-11)-VN (Francis et al., 1992; King et al., 1992) was also used to express all IGF chimeras (Figure 2.2). The expression of the cloned IGF sequence is driven by a trc promoter (Brosius et al., 1985), with its activity being inhibited by the lac repressor until protein expression is induced by IPTG. This expression system produces recombinant proteins fused at the N-terminus to the first 11 amino acids of porcine growth hormone in inclusion bodies (King et al., 1992). The N-terminal fusion partner allows efficient expression, improves solubility during purification and increases recovery of correctly refolded IGF-I (Francis et al., 1992). Between the N-terminal fusion partner and recombinant IGF-I or IGF-II is a linker of either VN or VNPAPM, respectively (Figure 2.2). Hydroxylamine cleavage between the asparagine at P-1 and the N-terminal glycine at P+1 yields native human IGF-I (King et al., 1992; Antorini et al., 1997; Jansson et al., 1998), while cleavage between the methionine at P-1 and the P+1 alanine by the mutant α-lytic protease Prag A9 yields native human IGF-II (Lien et al., 2001). The cleavage procedure circumvents the problem of ineffective N-terminal initiator methionine processing.
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

High-level expression in bacteria generally results in intracellular accumulation of the recombinant protein in a denatured state in inclusion bodies (Marston, 1986; Hartley and Kane, 1988). Renaturation or refolding of proteins from inclusion bodies into their native state at high yields is a complex task (Humbel, 1990). However, our laboratory has developed an *in vitro* refolding protocol to efficiently renature human IGF-I and IGF-II and several IGF-I and IGF-II mutants from inclusion bodies (Francis et al., 1992; King et al., 1992). In Chapter 2 the codon optimization of all constructs encoding human IGF-I, IGF-II and all IGF chimeras, was reported, potentially increasing translational efficiency and preventing incorporation of incorrect amino acids into IGF-I as has been previously observed (Seetharam et al., 1988).

In the following chapter the expression and purification of human IGF-I, IGF-II and all IGF chimeras is detailed.
3.2 MATERIALS

Materials for bacterial transformation and fermentation are already described in section 2.2. All reagents were of analytical grade or higher. Hydroxylamine, 2-Hydroxyethyl disulphide, dithiothreitol (DTT), β-mercaptoethanol, urea, LiOH, tricine, bromphenol blue, Coomassie Brilliant Blue R250, sodium dodecyl sulphate (SDS), N,N,N,N’N’-tetramethylethylene-diamine (TEMED) and Tris-HCl-(hydroxymethyl)-aminomethane (Tris-HCl) were purchased from Sigma Chemical Co., (MO, U.S.A.). α-lytic protease Prag A9 was a kind gift from GroPep Pty Ltd. (Thebarton, South Australia, Australia). CM5 sensor chips, amine coupling reagents N-ethyl-N’-(dimethylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), ethanolamine hydrochloride pH 8.5 and surfactant P20 were purchased from BIACore (Melbourne, Australia). N-2-Hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES) was purchased from BDH laboratory Supplies (Poole, England). Acetic acid, HPLC grade acetonitrile and methanol were purchased from BDH Chemicals Australia Pty Ltd. (Melbourne, Australia). Sequencing grade trifluoroacetic acid (TFA) was purchased from Perkin Elmer, Applied Biosystems Division (Warrington, Great Britain). Bis-acrylamide and acrylamide was purchased from BioRad Pty Ltd. (North Ryde, Australia) as was the protein gel apparatus Mini Protean II Gel system. Gel filtration and ion exchange chromatography were performed using the High Precision Pump P-500 system from Pharmacia Biotech, (Uppsala, Sweden), with online detection using the Pharmacia UV-1 monitor at 280nm. HPLC was performed using Waters 510 HPLC pumps, an online Waters 486 Tunable Absorbance Detector and Millenium software from Waters (Sydney, Australia). The analytical HPLC columns were PE Brownlee Aquapore™ C4 (2.1 mm x 10 cm, 7 mm particle size, 300-A pore size) BU-300 with purchased from Rainin LC and Supplies, (Victoria, Australia). The
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

Delta-Pak Prep LC (25 X 100 mm) column and guard column was obtained from (Sydney, Australia). Mark-12 markers for Coomassie gels were purchased from NOVEX, Carlsbad, CA, U.S.A. BIAcore 2000 software and instrumentation was purchased from BIACORE™ (Uppsala, Sweden).

3.2.1 Expression and Purification buffers

Inclusion body wash buffer/ resuspension buffer

10 mM KH₂PO₄, 30 mM NaCl, 0.5 mM ZnCl₂ pH 7.8

Inclusion body dissolution buffer

8 M Urea, 0.1 M Tris, 40 mM Glycine, 0.5 mM ZnCl₂ pH 9.1 (filtered 1μm)

Refold dilution buffer

1 M Tris-HCl, 50 mM EDTA pH 9.1 (filtered 1μm)

Ion exchange buffer A

8 M Urea, 50 NaAcetate pH 4.8 (adjusted pH with acetic acid) (filtered 1μm)

Ion exchange buffer B

8 M Urea, 50 mM NaAcetate, 1M NaCl pH 4.8 (adjusted pH with acetic acid)

(filtered 1μm)

HPLC Buffer A

0.1 % Trifluoroacetic acid (filtered 0.22 μm)

HPLC buffer B

80 % Acetonitrile, 0.08 % Trifluoroacetic acid (filtered 0.22 μm)
CHAPTER 3 - Expression and Purification of IGF-I, IGF-II and IGF chimeras

3.2.2 Protein Electrophoresis buffers

Protein Gel running buffer

250 mM glycine, 25 mM Tris-HCl pH 8.3, 0.1 % (w/v) SDS

2 x Protein loading buffer

125 mM Tris-HCl, 4 % (w/v) SDS, 10 % (v/v) glycerol, 0.1 % Bromophenol Blue pH 6.8

Gel Fixing Solution

50 % (v/v) ethanol, 10 % (v/v) acetic acid

Gel Destain Solution

10 % acetic acid

Coomassie Blue

0.3 % (w/v) Coomassie Blue R-250, 10 % (v/v) acetic acid

Gel Drying Solution

5 % (v/v) glycerol, 30 % (v/v) ethanol

3.2.3 BIAcore buffers

HBS buffer

10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.05 % (v/v) surfactant P20 pH 7.4 and filter sterilized
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

3.3 METHODS

3.3.1 ANALYTICAL HPLC

At each step of the purification procedure the concentration or integrity of protein was determined by analytical reverse phase HPLC (rpHPLC). Each analysis was performed on a Brownlee Aquapore™ BU300, 7 µm particle size, 300-Å pore size, 2.1 mm x 100 mm column using a 20-50 % acetonitrile gradient over 30 minutes in the presence of 0.1 % TFA at a flow rate of 0.5 ml/min.

3.3.2 EXPRESSION OF PROTEINS IN E. coli

Expression vector containing the coding sequence for either human IGF-I, IGF-II or IGF chimera was transformed into 100 µl of E. coli JM101 (see section 2.4.11) and plated onto an LB agar plate containing 100 µg/ml ampicillin and incubated overnight at 37°C. The following day a single colony was picked and used to inoculate 5 ml of LB media, containing 100 µg/ml ampicillin, that was incubated for 8 hours at 37°C before 500 µl was subcultured into each of 4 x 50 ml of LB media containing 100 µg/ml ampicillin and incubated overnight at 37°C. Each 50 ml culture was used to inoculate one of 4 x 500 ml LB media containing 100 µg/ml ampicillin and incubated at 37°C until A660nm reached 0.6 at which time IPTG was added to a final concentration of 0.1 mM to induce protein expression. The induced cultures were incubated at 37°C, shaking vigorously for 5 hours before the cells were pelleted at 7000 x g for 10 minutes at 4°C. Successful expression was demonstrated by running a sample of the inclusion bodies on a 15 % tris-tricine SDS polyacrylamide gel and also on an analytical C4 Aquapore™ Brownlee HPLC column.
3.3.3 SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

At the end of the 5 hour fermentations the OD$_{600nm}$ of each of the four flasks was taken and a 1 ml sample of each ferment was collected. The 1 ml samples were centrifuged at 16,110 x g for 1 minutes and the supernatants removed. Each pellet was resuspended in a lysis buffer of 2 % SDS, 10 % β-mercaptoethanol at a volume of 50 µl per unit OD$_{600nm}$. This standardized the concentration of bacteria that were loaded onto the gels and subsequent visualization of the relative amount of protein expression in each flask could be made. To 5 µl of lysed cells, 5 µl of 2 X Loading buffer was added and the mixture was boiled at 100°C for 5 minutes, centrifuged at 16,110 x g for 1 minute and then the entire volume was loaded onto the gel. All samples were analysed on a 15 % polyacrylamide tricine gel (8 x 6 x 0.1 cm) as described by Schagger and von Jaspow (Schagger and von Jagow, 1987)(all buffers used detailed in section 3.2.2). A voltage of 40 V per gel was applied until the dye front had electrophoresed off the end of the gel.

3.3.4 LYSIS OF E. coli BY FRENCH PRESS

Pelleted bacterial cells were resuspended in 100 ml of inclusion body wash buffer (section 3.2.1) and inclusion bodies (IBs) were released using two passes through a French press at 3,500 lb/in$^2$. The IBs and cell debris were then spun at 10,000 x g for 15 minutes at 4°C and the supernatant removed. The pellet was resuspended with 5% (w/v) resuspension buffer and incubated at room temperature for 15 minutes before centrifugation at 7,000 x g for 15 minutes at 4°C. The supernatant was removed and then the pellet once again resuspended at 5 % (w/v) with inclusion
CHAPTER 3 - Expression and Purification of IGF-I, IGF-II and IGF chimeras

body wash buffer and finally spun at 5,000 x g for 10 minutes at 4°C. The final pellet was stored at -80°C until further use.

3.3.5 GEL FILTRATION

All inclusion bodies of proteins containing the IGF-II C domain e.g. IGF-II, IGF-II DI, IGF-I CII and IGF-I CIIDII, were initially purified by gel filtration. The inclusion bodies of proteins that contained the IGF-I C domain e.g. IGF-I, IGF-I DII, IGF-II CI and IGF-II CIDI did not require gel filtration. Previous purification of IGF-II and IGF-II analogues in our laboratory has shown that gel filtration is required to prevent proteolysis. The IGF-I C domain does not appear to be as susceptible as the IGF-II C domain to protease degradation.

A XK-26 column (Length: 100cm, internal diameter 26 mm) (Pharmacia, Uppsala, Sweden) packed with 400 ml of Sephacyr S-200 resin (Pharmacia, Uppsala, Sweden) was washed with 0.5 column volumes of MQ H2O, then 0.5 column volumes of 0.5 M NaOH, then 0.5 column volumes of MQ H2O, before finally being equilibrated in 1 column volume of inclusion body dissolution buffer. All washes, loading and running of the column was performed at a flow rate of 1 ml/min.

Inclusion bodies were dissolved in IB dissolution buffer (section 3.2.1) containing 20 mM DTT for 30 minutes at room temperature at a final concentration of 10 ml/g (wet weight) IBs and then loaded on to the gel filtration column. The inclusion body dissolution buffer was used as a running buffer and the elution profile was monitored using a detector measuring at a wave length of 280 nm. Fractions of 5 ml volume were collected and every other fraction was examined by SDS polyacrylamide gel electrophoresis (section 3.3.3).
3.3.6 REFOLDING OF FUSION PROTEINS

The inclusion bodies of proteins that contain the IGF-I C domain were dissolved in IB dissolution buffer + 20 mM DTT (section 3.2.1) prior to refolding while all proteins containing the IGF-II C domain were already in the dissolution buffer from the gel filtration step. Refolding of all IGF proteins occurred at a final protein concentration of 0.2 mg/ml in 2 M Urea, 0.1 M Tris, 10 mM glycine, 5 mM EDTA, 0.4 mM DTT at pH 9.1. Oxidizing conditions that promote disulphide bond formation and hence stabilize the refolded polypeptide were initiated by addition of 1 mM 2-Hydroxyethyldisulphide. The refolding reaction was incubated at room temperature for 180 minutes stirring and the status of the refolding isomers was examined at various time points by analytical rpHPLC. The refolding reaction was terminated by acidification with concentrated HCl to pH 2.5.

3.3.7 ION EXCHANGE CHROMATOGRAPHY

The refolded protein was purified by ion exchange chromatography in an XK-16 column (Length: 20cm, internal diameter 16 mm)(Pharmacia, Uppsala, Sweden) packed with Fast Flow S (FFS) cation exchange resin (Pharmacia-LKB Pty Ltd, North Ryde, NSW, Australia). Prior to loading the refolded protein, the column was sanitized with 0.5 M NaOH and H₂O and charged by 10 column volumes of 0.5 M acetic acid. The refold solution was filtered through a 1 μm filter and loaded onto the column at a flow rate of 8 ml/min. The column was then washed with Ion exchange buffer A (section 3.2.1) until all unbound protein was removed and a stable baseline on the chart recorder was reached. The bound protein was eluted with Ion exchange buffer B (section 3.2.1) containing 1M NaCl.
3.3.8 HYDROXYLAMINE CLEAVAGE

To produce native IGF-I using this expression system the fusion protein must by removed. The protein eluted off the Ion exchange column was diluted to 0.2 mg/ml in 2 M urea, 1 M hydroxylamine, 0.1 M Tris and using LiOH the pH was set to 8.65. The cleavage mixture was sparged with N₂ and incubated in a 37°C water bath for 22 hours. The reaction was terminated by acidification, while stirring on ice, to pH 2.5 with concentrated HCl.

3.3.9 α-LYTIC PROTEASE CLEAVAGE

Native IGF-II is liberated from its fusion partner by a mutant of α-lytic protease called Prag A9 (Lien et al., 2001). This enzyme can cleave between the methionine of the linker PAPM and alanine which is the first residue of wildtype human IGF-II. The cleavage reaction was performed at a protein concentration of 0.2 mg/ml in 2 M urea, 0.1 M Tris pH 8 at 37°C for 180 minutes. The enzyme was added at a ratio of 1:500 enzyme:fusion protein. Monitoring of the cleavage reaction was by rpHPLC and termination was by acidification to pH 2.5 by concentrated HCl.

3.3.10 PREPARATIVE REVERSE PHASE HPLC

The final purification step was preparative rpHPLC to remove the cleaved leader and other impurities. The acidified cleavage reaction (either hydroxylamine or α-lytic protease reactions) were filtered through a 0.22 μm filter and loaded onto a C4 Prep Pak column. A 20-50 % acetonitrile gradient over 180 minutes in the presence of 0.1 % TFA at a flow rate of 5 ml/min was used to separate the recombinant native
protein from other protein contaminants. All fractions collected corresponding to the pure IGF-I, IGF-II or IGF chimeras were pooled, lyophilized and quantified. Final purity was determined by N-terminal sequencing, and mass spectrometry was used to confirm the identity of the purified proteins.

### 3.3.11 FREEZE DRYING

The final concentration of purified protein in the pool from the preparative HPLC column was determined by analytical rpHPLC analysis. From this determination each protein was aliquoted at 20 μg and 100 μg in siliconised Eppendorf tubes. The samples were then lyophilized on a Virtis Benchtop 4K freeze-dryer (Virtis, Gardiner, N.Y., U.S.A).

### 3.3.12 QUANTITATION OF PURIFIED PROTEIN

To determine accurately the amount of protein after freeze drying, triplicate lyophilised aliquots were resuspended in 0.1 % TFA and analysed by rpHPLC on a 30 minute 20-50 % acetonitrile gradient. The area under the peak on the rpHPLC profile was compared to a reference standard of Long [Arg³]-IGF-I taking into account the extinction coefficients (relative absorbance at 215 nm) of each protein relative to Long [Arg³]-IGF-I shown in Table 3.1.
### Table 3.1 Extinction coefficients of all proteins relative to Long [Arg³]-IGF-I.

Extinction coefficients were used to estimate the protein concentration from analytical HPLC profiles. The extinction coefficient values were derived as in (Buck et al., 1989).

<table>
<thead>
<tr>
<th>Protein</th>
<th>Relative Extinction Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>0.9813</td>
</tr>
<tr>
<td>IGF-II</td>
<td>0.9486</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>0.9824</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>0.9363</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>0.9483</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>0.9927</td>
</tr>
<tr>
<td>IGF-I CII DII</td>
<td>0.9362</td>
</tr>
<tr>
<td>IGF-II CIDI</td>
<td>0.9915</td>
</tr>
</tbody>
</table>

#### 3.3.13 MASS SPECTROMETRY AND N-TERMINAL SEQUENCING

Aliquoted protein samples (20 µg) was analysed by mass spectrometry by Chris Cursaro at the Department of Chemistry, The University of Adelaide, Adelaide, South Australia. Sequencing the first 6 N-terminal amino acids was by Edman degradation using an Applied Biosystems 492 Procise Protein Sequencer, operated by Chris Cursaro at the School of Molecular and Biomedical Science, The University of Adelaide, Adelaide, South Australia.
3.3.14 BIACORE ANALYSES

**Surface preparation:** To confirm the integrity of the tertiary structure of all purified proteins, their ability to bind to IGFBP-3 was analysed by surface plasmon resonance. Human IGFBP-3 was amine-coupled to a CM5 chip and various concentrations of IGF-I, IGF-II or IGF chimera were injected across the surface in the HBS buffer. An uncoupled flow cell, acting as a reference surface was used to assess any change in refractive index due to change in buffers and to examine any non-specific binding of the analyte to the carboxymethylated dextran matrix. Briefly, human IGFBP-3 (2 µg) at 12.5 µg/ml in 50 mM sodium acetate pH 4.6 was coupled to a flow cell previously activated with EDC and NHS and blocked with ethanolamine, as described in (Lofas and Johnsson, 1990; Carrick et al., 2001). The resulting biosensor surface had 200 resonance units coupled as determined by the BIAcore Wizard program.

**Kinetic assays of IGF-I, IGF-II and IGF chimera binding to IGFBP-3:** IGF-I, IGF-II and IGF chimera were injected over the chip surfaces at the following concentrations: 200 nM, 100 nM, 50 nM, 25 nM and 12.5 nM in HBS running buffer for 5 min at a flow rate of 40 µl/min to minimize mass transfer effects. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by 60 µl of 10 mM HCl. Reference flow cell data was subtracted from all runs to account for changes in the bulk refractive index due to the buffer. All kinetic data was analysed using the BIAevaluation 3.2 software. Models were fitted globally across all concentrations. All IGFBP-3 interactions were fitted to a two state conformational change model which describes the 1:1 interaction of analyte with bound ligand (A + B ↔ AB ↔ AB*). The 1:1 stoichiometry of IGF:IGFBP binding was determined by Bourner et al. (Bourner et al., 1992).
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

3.4 RESULTS

Recombinant human IGF-I and IGF-II have been routinely expressed and purified by our laboratory, with the purification generally as described by (King et al., 1992; Shooter et al., 1996; Magee et al., 1999; Lien et al., 2001).

3.4.1 IGF-I

The expression plasmid pGH(1-11) containing the IGF-I coding sequence, optimized for E. coli expression, was transformed into E. coli JM101 cells and then expression was successfully induced by IPTG (section 3.3.2). A sample of the fermentation prior to and post induction was run on a 15% tris tricine gel and by comparing pre- and post-induction lanes the success of the fermentation was confirmed. The optical density of the fermentation at termination was 2.04 (Table 3.2). The recombinant fusion protein was produced in inclusion bodies in the cytoplasm of the E. coli and consequently the cells were disrupted under high pressure by French pressing which yielded 1.94 g (wet weight) of intracellular inclusion bodies (Table 3.2). A sample of the inclusion bodies was solubilized in inclusion body dissolution buffer and run on rpHPLC to further confirm that the inclusion bodies contained the recombinant human IGF-I as a fusion with the first 11 amino acids of porcine growth hormone (Figure 3.1 A).

Dissolution of the entire inclusion body pellet occurred at pH 9.1 in the presence of a chaotroph (8 M urea) and a thiol catalyst (20mM DTT) to reduce the protein. Refolding of the fusion protein was induced by addition of 2-Hydroxyethylisulphide and dilution of urea to 2 M and DTT to 0.4 mM. This created an oxidizing environment and caused formation of disulphide bonds and renaturation of the wildtype IGF structure. Analytical HPLC analysis of the refolded protein
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

Reduced inclusion bodies (A) 100mg

Refold (B) 60 mg

Ion Exchange

Hydroxylamine Cleavage (C) 15mg

Preparative HPLC (D) 9.1mg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry (Da)</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>7649</td>
<td>7648.2 ± 0.08</td>
<td>&gt;95%</td>
</tr>
</tbody>
</table>

Figure 3.1 Purification summary of human IGF-I.
The purification protocol for human IGF-I is shown as a flow diagram left, with protein yield given at each step. At the each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), hydroxylamine cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing human IGF-I, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
showed a decrease in retention time (28.5 min vs 21 min) characteristic of a change to a more hydrophilic structure (Figure 3.1 A vs. B).

Cation exchange chromatography of the refold was used to remove endotoxins and other co-eluting proteins while allowing the positively charged protein to elute as a unique species. After cation exchange chromatography the pooled fraction was cleaved with hydroxylamine. The major peak after cleavage had a reduced retention time reflecting the removal of the predominantly hydrophobic fusion leader sequence (14.5 min) (Figure 3.1 C). The complex cleavage mix was separated on a C4 DeltaPak™ preparative reverse phase HPLC column employing an acetonitrile gradient to yield pure IGF proteins (Figure 3.1 D). The purified protein was lyophilized and shown by mass spectrometry to be the correct mass and >95% pure as determined by N-terminal sequencing.

<table>
<thead>
<tr>
<th>Protein</th>
<th>OD A_{600nm}</th>
<th>IB wet weight (g)</th>
<th>Fusion protein (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>2.04</td>
<td>1.94</td>
<td>100</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>2.38</td>
<td>1.5</td>
<td>55</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>2.62</td>
<td>2.26</td>
<td>45</td>
</tr>
<tr>
<td>IGF-I CIIDII</td>
<td>3.33</td>
<td>2.11</td>
<td>24</td>
</tr>
<tr>
<td>IGF-II</td>
<td>2.56</td>
<td>2.43</td>
<td>60</td>
</tr>
<tr>
<td>IGF-II D1</td>
<td>1.96</td>
<td>2.33</td>
<td>6.6</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>2.57</td>
<td>2.15</td>
<td>60</td>
</tr>
<tr>
<td>IGF-II CIDI</td>
<td>2.69</td>
<td>2.0</td>
<td>87</td>
</tr>
</tbody>
</table>

Table 3.2 Summary of the fermentation of all expressed proteins. The absorbance OD A_{600nm} of each of the four fermentation flasks was determined at the end of the 5 hour induction and then averaged to give the value shown. The IB wet weight was determined after French pressing and inclusion body wash steps (section 3.3.4). The fusion protein amount in the inclusion bodies was determined by rPHPLC analysis.
3.4.2 IGF-II

The purification of recombinant human IGF-II was similar to that previously reported (Lien et al., 2001). The purification is also similar to that used for IGF-I production however the following steps are unique. After dissolution and prior to refolding, the protein was partially purified by gel filtration on a Sephacryl S-200 column (section 3.3.5) which removed much of the contaminating high molecular weight material, possibly also proteases, as shown by gel electrophoresis. Without the gel filtration step the IGF-II fusion protein cannot refold into the wildtype structure as determined by its ability to bind the IGF-1R. As briefly mentioned in the section 3.3.5 gel filtration prevents proteolysis of the IGF-II molecule, which if it occurs precludes native IGF-II refolding.

After the cation exchange step, the porcine growth hormone fusion partner was removed by enzymatic α-lytic protease cleavage (section 3.3.9) whereas the fusion protein is removed from IGF-I B domain containing proteins by chemical cleavage using hydroxylamine. The α-lytic protease linker, PAPM, has been optimized by substrate phage display to be digested by the α-lytic mutant Prag A9 (Lien et al., 2001). The cleavage was purified to > 95 % purity on a C4 DeltaPak™ preparative reverse phase HPLC column employing an acetonitrile gradient (Figure 3.2). Analytical rpHPLC profiles at various stages throughout the purification process and a summary of the final purification yield are shown in Figure 3.2.

3.4.3 IGF-I DII

The purification of IGF-I DII was essentially the same as IGF-I (section 3.4.1). Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.3.
Reduced inclusion bodies (A) 60mg

Gel Filtration

Refold (B) 18mg

Ion Exchange

α-lytic protease Cleavage (C) 10mg

Preparative HPLC (D) 4.5mg

<table>
<thead>
<tr>
<th></th>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Predicted</td>
<td>IGF-II 7469 Da</td>
<td>7468.2 ± 0.16 Da</td>
<td>&gt; 95 %</td>
<td>4.5 mg</td>
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</table>

Figure 3.2 Purification summary of human IGF-II.

The purification protocol for human IGF-II is shown as a flow diagram left. At each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), α-lytic protease cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing human IGF-II, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
Reduced inclusion (A) 55mg

Refold (B) 30mg

Ion Exchange

Hydroxylamine Cleavage (C) 10mg

Preparative HPLC (D) 7.5mg

<table>
<thead>
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<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I DII</td>
<td>7469 Da</td>
<td>&gt;95%</td>
<td>7.5 mg</td>
</tr>
<tr>
<td></td>
<td>7468.8 ± 0.06 Da</td>
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</tbody>
</table>

Figure 3.3 Purification summary of IGF-I DII.
The purification protocol for the chimera IGF-I DII is shown as a flow diagram left, with protein yield given at each step. At the each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), hydroxylamine cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-I DII, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
3.4.4 IGF-I CII

The purification of IGF-I CII was essentially the same as that for IGF-II (section 3.4.2) however, removal of the fusion protein was by chemical cleavage using hydroxylamine instead of enzymatic cleavage using α-lytic protease. Interestingly, without an initial gel filtration step prior to refolding, the chimera failed to fold into a structure that could bind to the IGF-1R as determined by a competition binding assay (data not shown). This suggests that the protease-sensitive site must reside in the IGF-II C domain. Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.4.

3.4.5 IGF-I CIIDII

The purification of IGF-I CIIDII was essentially the same as that for IGF-II (section 3.4.2), however fusion protein removal was by chemical cleavage using hydroxylamine instead of an enzymatic cleavage using α-lytic protease. Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.5.

3.4.6 IGF-II DI

The purification of the IGF-II DI was the same as IGF-II (section 3.4.2). Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.6.
Reduced inclusion bodies (A) 45mg

Gel Filtration

Refold (B) 26mg

Ion Exchange

Hydroxylamine Cleavage (C) 5.5mg

Preparative HPLC (D) 2.5mg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I CII</td>
<td>7386 Da</td>
<td>7384.7 ± 0.06 Da</td>
<td>&gt;95 %</td>
</tr>
</tbody>
</table>

Figure 3.4 Purification summary of IGF-I CII.
The purification protocol for IGF-I CII is shown as a flow diagram left, with protein yield given at each step. At each step the purification was monitored by reverse phase HPLC analysis on a 20-50% acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), α-hydroxylamine cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-I CII, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
Reduction inclusion bodies (A) 24mg

Gel Filtration

Refold (B) 5.5mg

Ion Exchange

Hydroxylamine Cleavage (C) 800μg

Preparative HPLC (D) 529μg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I CIIDII</td>
<td>7207 Da</td>
<td>&gt;95 %</td>
<td>529 μg</td>
</tr>
<tr>
<td></td>
<td>7205.6 ± 0.6 Da</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.5 Purification summary of IGF-I CIIDII.
The purification protocol for the chimera IGF-I CIIDII is shown as a flow diagram left. At each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (Section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), hydroxylamine cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-I CIIDII, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
Reduced inclusion bodies (A) 6.6mg

Gel Filtration

Refold (B) 4.3mg

Ion Exchange

α-lytic protease Cleavage (C) 3mg

Preparative HPLC (D) 2.65 mg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II DI</td>
<td>7648 Da</td>
<td>7647.9 ± 0.09 Da</td>
<td>&gt;95%</td>
</tr>
</tbody>
</table>

**Figure 3.6 Purification summary of IGF-II DI.**
The purification protocol for the chimera IGF-II DI is shown as a flow diagram left, with protein yield given at each step. At each step the purification was monitored by reverse phase HPLC analysis using a 20-50% acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), α-lytic protease cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-II DI, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
3.4.7 IGF-II CI

The purification of IGF-II CI was essentially the same as IGF-I (section 3.4.1), however fusion protein removal was by α-lytic protease instead of chemical cleavage by hydroxylamine. Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.7.

3.4.8 IGF-II CIDI

The purification of IGF-II CIDI was essentially the same as IGF-I (section 3.4.1), however fusion protein removal was by α-lytic protease instead of chemical cleavage by hydroxylamine. Analytical rpHPLC profiles at various stages throughout the purification process and summary of final purification yield are shown in Figure 3.8.

3.4.9 BIACORE ANALYSES

To confirm that the tertiary structure of the IGF chimeras had been maintained, their ability to bind human IGFBP-3 was measured by BIAcore analysis. Human IGFBP-3 was coupled to a CM5 chip as stated in section 3.3.14 and then various concentrations of IGF-I, IGF-II or IGF chimeras were passed over the surface. Representative BIAcore profiles from one experiment are shown in Figure 3.9. BIAcore profiles of all proteins at 50 nM binding to human IGFBP-3 is shown in Figure 3.10. All proteins had sub-nanomolar dissociation constants. IGF-I had a slightly poorer affinity for IGFBP-3 than IGF-II, with a dissociation constant 64 % that of IGF-II for binding IGFBP-3. All IGF chimeras had less than two fold decrease in
Reduced inclusion bodies (A) 60mg

\[ \text{Refold (B) 16mg} \]

\[ \text{Ion Exchange} \]

\[ \text{\( \alpha \)-lytic protease cleavage (C) 14mg} \]

\[ \text{Preparative HPLC (D) 12mg} \]

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II CI</td>
<td>7732 Da</td>
<td>7731 ± 0.19 Da</td>
<td>&gt;95 %</td>
</tr>
</tbody>
</table>

**Figure 3.7 Purification summary of IGF-II CI.**

The purification protocol for the chimera IGF-II CI is shown as a flow diagram left, with protein yield given at each step. At the each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes. Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), \( \alpha \)-lytic protease cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-II CI, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
Reduced inclusion bodies (A) 87mg

Refold (B) 76mg

Ion Exchange

α-lytic protease cleavage (C) 53mg

Preparative HPLC (D) 42.5mg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II CIDI</td>
<td>7912</td>
<td>7911.3 ± 0.001 Da</td>
<td>&gt;95 %</td>
</tr>
</tbody>
</table>

Figure 3.8 Purification summary of IGF-II CIDI.

The purification protocol for the chimera IGF-II CIDI is shown as a flow diagram left, with protein yield given at each step. At each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), α-lytic protease cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing IGF-II CIDI, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
affinity for IGFBP-3 relative to both IGF-I and IGF-II. The affinity of each chimera was not significantly different from either IGF-I or IGF-II. These results strongly suggest that the wildtype IGF structure was maintained despite exchanging the C and/or D domains.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{a1}$ x 10^5 1/ms</th>
<th>$k_{a2}$ x 10^3 1/Ms</th>
<th>$k_{d1}$ x 10^2 1/s</th>
<th>$k_{d2}$ x 10^2 1/s</th>
<th>$K_A$ x 10^9 1/Ms</th>
<th>Relative to IGF-I $K_A$</th>
<th>Relative to IGF-II $K_A$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II</td>
<td>15</td>
<td>1.86</td>
<td>1.26</td>
<td>4.42</td>
<td>5.03 ± 0.79</td>
<td>0.61</td>
<td>1.0</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>13.3</td>
<td>0.8</td>
<td>0.96</td>
<td>3.59</td>
<td>2.83 ± 0.35</td>
<td>1.09</td>
<td>1.78</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>19.1</td>
<td>0.95</td>
<td>1.25</td>
<td>4.6</td>
<td>2.9 ± 0.56</td>
<td>1.06</td>
<td>1.73</td>
</tr>
<tr>
<td>IGF-I CIDII</td>
<td>14.7</td>
<td>1.84</td>
<td>1.06</td>
<td>4.52</td>
<td>5.54 ± 0.33</td>
<td>0.56</td>
<td>0.91</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>11.9</td>
<td>2.34</td>
<td>1.25</td>
<td>6.22</td>
<td>4.25 ± 1.9</td>
<td>0.72</td>
<td>1.18</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>12.8</td>
<td>1.61</td>
<td>1.34</td>
<td>5.5</td>
<td>3.57 ± 0.78</td>
<td>0.86</td>
<td>1.41</td>
</tr>
<tr>
<td>IGF-II CIDI</td>
<td>12.4</td>
<td>1.46</td>
<td>1.52</td>
<td>2.76</td>
<td>6.4 ± 1.6</td>
<td>0.48</td>
<td>0.79</td>
</tr>
<tr>
<td>IGF-I</td>
<td>13.6</td>
<td>1.29</td>
<td>1.24</td>
<td>4.91</td>
<td>3.1 ± 0.47</td>
<td>1.0</td>
<td>1.64</td>
</tr>
</tbody>
</table>

Table 3.3 Summary of kinetic analysis of IGF-I, IGF-II and IGF chimeras binding to human IGFBP-3 as measured by BIACore. The two-state conformational change model was used to fit the kinetic data and ultimately derive the association constant. Methods as per section 3.3.14. The results generated are from two separate runs on two independently coupled CM5 chips. Association constants were derived using both association ($k_a$) and dissociation ($k_d$) rates ($K_A = (k_{a1}/k_{d1})(k_{a2}/k_{d2})$). Association constants of each protein relative to that of IGF-I and IGF-II are also shown.
Figure 3.9 Binding of all recombinant proteins to human IGFBP-3 on the BIACore.
Figure legend continued on next page.
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

Figure legend continued:

IGF-I, IGF-II or IGF chimera (200, 100, 50, 25, 12.5 nM) was passed over IGFBP-3 flow cells at a flow rate of 40 μl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by 60 μl of 10 mM HCl. Reference flow cell data was subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data was analysed using the BIAevaluation 3.2 software. The 2 state conformational change model was used to evaluate the binding all proteins to IGFBP-3. Kinetic analysis results are shown in Table 3.3.

![Graph](image)

**Figure 3.10 IGFBP-3 binding analysis of all recombinant proteins.** 50nM IGF-I, IGF-II or IGF chimeras over IGFBP-3 is shown. IGF-II CIDI and IGF-I CIIDII were analysed on a separate IGFBP-3 coupled chip than the single chimeras.

3.4 DISCUSSION

To investigate the binding specificity of the IGFs, correctly folded, pure recombinant chimeras of IGF-I and IGF-II were required. Most IGF chimeras expressed at high levels and all were purified to greater than 95 % purity as determined by N-terminal sequencing. While some chimeras expressed at a higher level than others, there was no obvious pattern relating expression level to type of chimera.
Gel filtration of all chimeras containing the IGF-II C domain was required to allow correct refolding to what appears, from IGFBP-3 binding studies, to approximate the wildtype tertiary structure. Small scale refolding trials of IGF-II C domain containing proteins failed to yield any native protein as determined by the ability of purified refolding peaks to compete with labeled IGF-I for binding to the IGF-1R (data not shown). The reason for the gel filtration step allowing correct refolding of these proteins is unknown, however it is possible that this procedure removes an endogenous protein that prevents correct folding. Previous studies have shown that renaturation of recombinant IGF-II is more complicated than IGF-I and that IGF-II is more likely to be proteolytically degraded than IGF-I (Humbel, 1990). While proteolysis was not observed in any of the purifications detailed here, the additional positive charges in the C domain of IGF-II make IGF-II more susceptible to protease cleavage (Kerrie McNeil, personal communication).

The in vitro refolding of IGF-I and IGF-II from intracellularly accumulated aggregates or inclusion bodies has been demonstrated in our laboratory previously (Francis et al., 1992; King et al., 1992; Francis et al., 1993; Shooter et al., 1996; Magee et al., 1999). Oxidative folding of IGF-I in vitro results in two major folding isomers with equal thermodynamic stability (Hober et al., 1992; Miller et al., 1993). One folding isomer is native IGF-I with the disulphide bond formation Cys6-Cys48, Cys18-Cys61, Cys47-Cys52 (Raschdorf et al., 1988; Iwai et al., 1989; Axelsson et al., 1992) while the second major isomer, “mismatched IGF-I”, contains incorrect disulphide pairing: Cys6-Cys47, Cys18-Cys61, Cys48-Cys52 (Raschdorf et al., 1988). This mismatched IGF-I folding isomer has an altered tertiary structure compared to wildtype IGF-I (Miller et al., 1993; Gill et al., 1999) and consequently has reduced IGF-1R binding (Milner et al., 1995; Gill et al., 1999). Interestingly an alternative isomer of IGF-II has not been reported. To determine which refolding peak on
rPHPLC analysis contained protein with wildtype disulphide bond orientation each major isomer was purified on rPHPLC and its ability to compete Eu-IGF-I for binding to solubilized IGF-1R was examined. Each of the refolded IGF-I, IGF-II and IGF chimeras yielded one isomer that had potent binding ability and all other purified refolding isomers were very poor (data not shown).

The non-enzymatic cleavage of Asn-Gly bonds by hydroxylamine was first shown in bovine ribonuclease over 30 years ago (Bornstein and Balian, 1970) and since then has been utilised to cleave fusion protein leader sequences from several recombinantly expressed proteins (Bornstein and Balian, 1977) including IGF-I (Nilsson et al., 1991; King et al., 1992). Cleavage with hydroxylamine can result in side reactions that cause hydroxyamation of asparagines and glutamine residues (Canova-Davis et al., 1992). Fortunately the cleavage of the porcine growth hormone leader from human IGF-I has been optimized to reduce formation of hydroxamic acid variants (Milner et al., 1996). Hydroxyamation of asparagine 26 does not alter the biological properties of the protein as determined by IGF-1R binding and induction of protein synthesis (Milner et al., 1996), however the effect of hydroxyamate formation on IR, IGFBP and IGF-2R binding in unknown and hence minimizing formation of modified protein is desirable. In all purification procedures the presence of hydroxyamated variants was low (≤10 %) as determined by mass spectrometry. The enzymatic cleavage of IGF-II and IGF chimeras with an IGF-II B domain was achieved using an α-lytic protease which does not modify any side chain moieties or digest the protein at sites other than the designated cleavage linker (Lien et al., 2001).

Several chimeras of IGF-I and insulin have been produced without any change in tertiary structure e.g. (Cara et al., 1990; Schaffer et al., 1993; Kristensen et al., 1995) however no chimeras of IGF-I or IGF-II have been made previously. To determine whether exchanging the domains had any change in the secondary or tertiary
CHAPTER 3 – Expression and Purification of IGF-I, IGF-II and IGF chimeras

structure of the IGF chimeras reported here, the ability of all proteins to bind to IGFBP-3 was examined.

It should be noted that confirmation of correct folding and three-dimensional structure of the engineered mutants has been shown to be essential as IGF-I is highly sensitive to mutation (Jansson et al., 1997). Point mutations in the IGF-I B domain caused only slight changes in the total α-helical content of IGF-I but this was enough to decrease binding affinity for IGFBP-1 and IGF-1R between 2 and 50-fold (Jansson et al., 1997). The reduction in affinity for IGFBP-1 was due to decreased on-rates of the IGF-I mutants. Furthermore, deletion of the IGF-I C domain did not change the total α helical content of IGF-I as determined by circular dichroism (Gill et al., 1996) but did affect the orientation of the α helices relative to each other, which was only realized by extensive NMR studies (De Wolf et al., 1996). There have been reported several other mutations or deletions that cause global structural perturbations, which in turn affects an understanding of which IGF-I residues are involved in IGF-1R binding (Gill et al., 1996) and in IR binding (Xu et al., 2002).

IGFBP binding is a method previously used to determine changes in tertiary structure as slight alterations in the three-dimensional fold of the IGFs can cause significant changes to the kinetic properties of IGFBP binding (Jansson et al., 1997). In addition it is essential to determine experimentally the effect of swapping domains in both the IGF-I and IGF-II backgrounds as it has been elegantly shown that two proteins with very similar structure can respond differently to engineered sequence changes such as mutation (Cota et al., 2000).

IGFBP-3 binds both IGF-I and IGF-II with almost equal affinity and therefore swapping the C and D domains between the IGFs would not be expected to alter any binding specificity, which in turn may conceal structural changes. In addition the major mispaired disulphide isomer of IGF-I ((Cys6-Cys47, Cys48-Cys52, Cys18-
Cys$^{61}$IGF-I), derived from a similar in vitro refolding protocol to that used in these studies, bound IGFBPs from conditioned media with almost 300 fold lower affinity than wildtype IGF-I (Milner et al., 1995). All IGF chimeras produced had relatively similar wildtype affinity for IGFBP-3, as determined by surface plasmon resonance (Table 3.3) suggesting no major change in structure. Both the association and dissociation of all IGF chimeras was similar to wildtype.

Several reports suggest that the global tertiary structure of the IGF chimeras would not change from the wildtype fold. Chimeras of Insulin and IGF-I have shown that the B domain contains all the information required for IGF-I to adopt its characteristic fold (Guo et al., 2002; Guo et al., 2002). In addition the C-domain does not affect the orientation or conformation of the neighbouring A domain (Brzozowski et al., 2002). However the C-peptide of insulin has been shown to facilitate the folding to the native structure and also regulates the kinetic folding pathway (Liu et al., 2003; Qiao et al., 2003; Min et al., 2004). Whether C domains of IGF-I and/or IGF-II are also involved in the folding process is unknown, however 4-Gly IGF-I folds correctly without the presence of any wildtype residues in the C domain, as its affinities for IR, IGF-2R and IGFBP are only slightly changed from native IGF-I (Bayne et al., 1989). Mini-IGF-I, where the C domain had been deleted, did however have an altered tertiary structure indicating that tethering of the B domain C-terminus and A domain N-terminus may be a absolute requirement for formation of the correct 3-dimensional fold, independent of the amino acid composition of the tether (De Wolf et al., 1996).

Therefore, with the successful production of human IGF-I, IGF-II and all IGF chimeras, the investigation into the receptor binding and activation specificity of the IGFs could be undertaken and these studies will be described in the following chapters.
Binding and activation of the IR-A and IR-B by insulin, IGF-II, IGF-I and IGF chimeras
CHAPTER 4 - Binding of Insulin, IGF-I, IGF-II and IGF chimeras to the IR-A and IR-B

4.1 INTRODUCTION

The general view, based on numerous studies with whole receptors or soluble ectodomains, is that while insulin and IGF-I bind their own receptors with high affinity, they bind the heterologous receptor poorly (<2%) (see (Adams et al., 2000)). In contrast IGF-II but not IGF-I has been reported to bind the insulin receptor A isoform with an affinity approaching that of insulin (Table 1.1) (Frasca et al., 1999). Interestingly, many years before IGF-II was shown to bind the IR-A with high affinity (Frasca et al., 1999), it was noted by several groups that IGF-II but not IGF-I could compete[^125I]-Insulin for binding purified human placental insulin receptors almost as well as insulin (Sakano et al., 1991; Hashimoto et al., 1995). Despite these observations the molecular basis for this differing affinity of IGF-I and IGF-II for the IR-A has not been explored. There are 26 sequence differences between IGF-I and IGF-II (Figure 2.1) with the greatest concentration occurring in the C and D domains, making them prime candidates for this difference in receptor interactions. The IGF-I C domain is four residues larger and differs at a further five positions when compared to the C domain of IGF-II. The D domain of IGF-I is two residues larger and differs in a further two residues from the D domain of IGF-II (Figure 2.1). The successful expression and purification of chimeras of IGF-I and IGF-II where the C and D domains were swapped either singularly or together was detailed in Chapter 3. These proteins now allow investigation into whether these exchanged domains regulate the difference in IR isoform binding affinity of IGF-I and IGF-II.

As shown in Tables 4.1 there is a variation in the IC₅₀ values for the binding of all ligands to the IR isoforms and not one study has accurately determined, in the one experiment, the binding affinities of insulin, IGF-II and IGF-I. For this reason the major aims of this study were not only to determine if the C and D domains of IGF-I and IGF-II regulate the IR binding specificity but also to complete for the first time a
CHAPTER 4 - Binding of Insulin, IGF-I, IGF-II and IGF chimeras to the IR-A and IR-B

comprehensive analysis of the ability of insulin, IGF-II and IGF-I to bind and activate both IR isoforms.

Traditional competition assays have used ligands that have been labelled with radioactive isotopes e.g. $^{125}$I or $^3$H. Drawbacks of radioactive ligands include short shelf life, high production expense, complex handling procedures when couriered and safety hazards during use (Pelizzola et al., 1995). As a result of these disadvantages non-radioactive labelling has become an attractive alternative for use in receptor binding assays. Lanthanide chelates such as europium ($\text{Eu}^{3+}$), samarium ($\text{Sm}^{3+}$) or terbium ($\text{Tb}^{3+}$) have long decay fluorescence properties that when used in conjunction with time-resolved detection, result in comparable sensitivity to radioactively labelled ligands. Other advantageous fluorescent properties of the lanthanides include large Stoke's shift, narrow emission peaks and optimal excitation and emission wavelengths for use with biological material e.g. serum (Dickson et al., 1995).

While fluorescent labelling is far from ubiquitous in binding assays, a variety of ligands have been labelled with europium e.g. IL-2 (Stenroos et al., 1997; Stenroos et al., 1998), IL-8 (Inglese et al., 1998), EGF (Mazor et al., 2002) and neurotensin (Mazor et al., 2002), as well as antibodies (Okada et al., 1998; Amir-Zaltsman et al., 2000; Maple et al., 2001; Chen et al., 2003; Gazi et al., 2003) and nanoparticles (Harma et al., 2000). In this chapter IR isoform binding assays using europium labelled insulin are detailed. The development of both the receptor binding assay and phosphorylation assay was by Dr. Colin Ward, Dr. Tim Adams and Mr. Peter Hoyne (CSIRO Health Science and Nutrition, Parkville, Australia) and is based on the assay previously described for the analysis of EGF and neurotensin binding (Mazor et al., 2002).

The stably transfected cells lines used in this chapter, were produced by Eric R. Bonython (The University of Adelaide, Adelaide, Australia). Embryonic fibroblasts
from a mouse with a targeted ablation of the IGF-1R, termed R' cells (kindly provided by Prof. Renato Baserga), were transfected to express either human IR-A or human IR-B, then designated R'IR-A or R'IR-B cells respectively. The parental R' cell line was chosen as it does not express IGF-1R (Sell et al., 1994) and expresses very low levels of the IR-A (Prisco et al., 1999; Wu et al., 2003), hence the IGF-1R, hybrids of IGF-1R/IR and hybrids of IR-A/IR-B would not affect our binding and phosphorylation results. Fluorescent activated cell sorting was used to isolate populations of cells that expressed similar numbers of IR-A and IR-B. Reverse transcriptase PCR was used to confirm the integrity of the isoforms.
## Table 4.1 Summary of the literature showing IR-A and IR-B IC\textsubscript{50} values for insulin, IGF-II, IGF-I and proinsulin.

All reports used [Tyr\textsuperscript{Ala}] moniodinated insulin as the labelled ligand. All cell lines are engineered to ectopically express either the human IR-A or IR-B.

<table>
<thead>
<tr>
<th>Protein</th>
<th>IR-A IC\textsubscript{50} (nM)</th>
<th>IR-B IC\textsubscript{50} (nM)</th>
<th>Receptor preparation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin</td>
<td>0.9</td>
<td>1.0</td>
<td>3T3 cells</td>
<td>(Frasca \textit{et al.}, 1999)</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>0.44</td>
<td>Rat 1 cells</td>
<td>(Mosthaf \textit{et al.}, 1990)</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>1.2</td>
<td>Rat 1 cells</td>
<td>(McClain, 1991)</td>
</tr>
<tr>
<td></td>
<td>2.5 ± 0.56</td>
<td>5.3 ± 1.1</td>
<td>CHO cells</td>
<td>(Yamaguchi \textit{et al.}, 1991)</td>
</tr>
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<td></td>
<td>0.2 ± 0.2</td>
<td>0.3 ± 0.4</td>
<td>Immunopurified</td>
<td>(Pandini \textit{et al.}, 2002)</td>
</tr>
<tr>
<td></td>
<td>0.11 ± 0.3</td>
<td>0.11 ± 0.3</td>
<td>Immunopurified</td>
<td>(Surinya \textit{et al.}, 2002)</td>
</tr>
<tr>
<td></td>
<td>2.1 ± 0.2</td>
<td>1.8 ± 0.2</td>
<td>Soluble ectodomain</td>
<td>(Whittaker \textit{et al.}, 2002)</td>
</tr>
<tr>
<td>IGF-II</td>
<td>2.5</td>
<td>&gt;20</td>
<td>3T3 cells</td>
<td>(Frasca \textit{et al.}, 1999)</td>
</tr>
<tr>
<td></td>
<td>0.9 ± 0.4</td>
<td>11 ± 5</td>
<td>Immunopurified</td>
<td>(Pandini \textit{et al.}, 2002)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>&gt;20</td>
<td>&gt;20</td>
<td>3T3 cells</td>
<td>(Frasca \textit{et al.}, 1999)</td>
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<td>&gt;30</td>
<td>&gt;30</td>
<td>Immunopurified</td>
<td>(Pandini \textit{et al.}, 2002)</td>
</tr>
<tr>
<td></td>
<td>4.6 ± 0.75</td>
<td>33 ± 8.9</td>
<td>Immunopurified</td>
<td>(Surinya \textit{et al.}, 2002)</td>
</tr>
<tr>
<td></td>
<td>41 ± 15</td>
<td>390 ± 50</td>
<td>CHO cells</td>
<td>(Yamaguchi \textit{et al.}, 1993)</td>
</tr>
<tr>
<td>Proinsulin</td>
<td>28</td>
<td>145</td>
<td>Rat 1 cells</td>
<td>(McClain, 1991)</td>
</tr>
</tbody>
</table>
4.2 MATERIALS

Long<sup>TM</sup>Arg<sup>3</sup>IGF-I and human IGF-I were purchased from GroPep Pty Ltd (Adelaide, South Australia). Greiner LumiTrac 600 96-well plates were from Omega scientific (Tarzana, USA). Human insulin was purchased from Novo Nordisk (Bagsvaerd, Denmark). DELFIA<sup>®</sup> Eu-labelling kit, DELFIA<sup>®</sup> enhancement solution and europium conjugated anti-phosphotyrosine antibody PY20 were purchased from Perkin Elmer (Turku, Finland). The anti-IR antibody 83-7 was a kind gift from Prof. K. Siddle, Cambridge, UK. R<sup>−</sup> cells (mouse 3T3-like cells with a targeted ablation of the IGF-1R gene) (Sell et al., 1994) were a kind gift from Prof. R. Baserga (Philadelphia, USA).

4.3 METHODS

4.3.1 CONSTRUCTION OF CELLS EXPRESSING THE HUMAN IR-A AND IR-B

The construction of stable cell lines expressing the human IR-A and IR-B was completed by Eric R. Bonython (School of Molecular and Biomedical Sciences, The University of Adelaide). The cDNAs encoding the human IR-A and IR-B isoforms were previously generated as described (Ellis et al., 1986; Hoyne et al., 2000). The pECE:hIR-A and hIR-B plasmids were restricted with SalI and XbaI to release a 2.9kb fragment containing the insulin receptor and ligated to XhoI/XbaI cut pEFIRESneo (Hobbs et al., 1998). The exon 11 status of the constructs was confirmed by PCR analysis. R<sup>−</sup> cells were transfected with the constructs using Lipofectamine<sup>TM</sup> (Gibco/BRL Life Technologies) and stably transfected cells were screened for the IR cDNA by PCR analysis and for IR expression by FACS analysis using the monoclonal
anti-IR antibody 83-7. Cells expressing human IR underwent single-cell sorting to isolate cells expressing similar levels of receptors. These clonal cell lines were used in all subsequent experiments. R’ cells expressing the human IR-A are designated, R’IR-A and R’ cells expressing the human IR-B are designated R’IR-B.

### 4.3.2 BINDING ANALYSES OF CHIMERAS TO INSULIN RECEPTOR ISOFORMS

Receptor binding affinities were measured using an assay similar to that measuring EGF binding to the EGF receptor (Mazor et al., 2002). R’IR-A and R’IR-B cells were used as sources of IR-A and IR-B respectively. Cells were lysed with lysis buffer (20 mM HEPES, 150 mM NaCl, 1.5 mM MgCl₂, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM EGTA pH 7.5) for 1 hour at 4°C. Lysates were centrifuged for 10 minutes at 1,100 x g and then 100 µl was added per well to a white Greiner Lumitrac 600 plate previously coated with anti-insulin receptor antibody 83-7 (Soos et al., 1989). The capture antibody does not interfere with receptor binding by insulin, IGF-I (Soos et al., 1989; Soos et al., 1992) or IGF-II (Leah J. Cosgrove, unpublished results).

Europium-labelled receptor grade human insulin was prepared as instructed by the manufacturer (DELFIA® Eu-labelling kit, Perkin Elmer, Turku, Finland). Briefly, 0.43 mM peptide was incubated with 2 mM labelling reagent in a 30µl reaction (0.1 M Na₂CO₃ pH 8.5), 4°C for 2 days. The reaction was terminated with 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5 and unbound europium was removed by size exclusion chromatography in the termination buffer (Superdex 75, Pharmacia, Sweden). Approximately 100,000 fluorescent counts of europium-labelled insulin were added to each well along with various amounts of unlabelled competitor and incubated for 16 hours at 4°C. Wells were washed with 20 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween
20 (TBST), and then DELFIA® enhancement solution (100 µl/well) was added. Time-resolved fluorescence was measured using 340 nm excitation and 612 nm emission filters with a BMG Lab Technologies Polarstar™ Fluorimeter. IC₅₀ values were calculated, using Prism 3.03, by curve-fitting with a one-site competition model. The baseline used to calculate all IC₅₀ values was set at the % bound/total value of the highest competing insulin concentration. As a negative control R- cells where lysed as above and added to an 83-7 coated plate. No binding of Eu-insulin was detected suggesting that the presence of any low level of endogenous mouse IR was not affecting the results (performed by Eric Bonython - data not shown).

4.3.3 INSULIN RECEPTOR PHOSPHORYLATION ASSAYS

Receptor phosphorylation was detected essentially as described by Chen et al. (Chen et al., 2003). RIR-A or RIR-B cells were plated in a Falcon 96-well flat-bottom plate at 2.5 x 10⁴ cells/well and grown overnight at 37°C, 5% CO₂. Cells were washed for 4 hours in serum-free medium before being treated with various concentrations of insulin, IGF-II, IGF-I or IGF chimera in 100 µl DMEM with 1% BSA for 10 minutes at 37°C, 5% CO₂. Lysis buffer containing 2mM Na₃VO₄ and 1 mg/ml NaF was added to the treated cells, and receptors from lysates were captured on 96-well plates precoated with antibody 83-7 and blocked with 1x TBST/0.5% BSA. After overnight incubation at 4°C, the plates were washed with 1 x TBST. Phosphorylated receptor was detected with europium-labelled antiphosphotyrosine antibody PY20 (130 ng/well, room temperature, 2 hours). DELFIA® enhancement solution (100 µl/well) was added and time-resolved fluorescence was detected as described above.
CHAPTER 4 - Binding of Insulin, IGF-I, IGF-II and IGF chimeras to the IR-A and IR-B

4.4 RESULTS

4.4.1 Binding analyses of insulin, IGF-II, IGF-I and IGF chimeras to the IR-A

The competition binding curves for insulin, IGF-I, IGF-II and the six chimeras with the IR-A are shown in Figure 4.1 A and C with the IC₅₀ values and relative binding affinities compared to IGF-II listed in Table 4.2. The results show that the C and D domains of IGF-II allow high affinity binding to the IR-A while the IGF-I C and D domains do not. The affinity of IGF-II for IR-A is almost 7 times higher than that of IGF-I. The binding characteristics of the chimeras indicates that this difference is due to the IGF-II C and D domains. The IGF-I CII chimera has a 1.9 fold higher binding affinity than IGF-I for IR-A, while the IGF-I DII chimera has a 1.5 fold increase in affinity. These contributions are additive as the double chimera binds IR-A almost as well as IGF-II (Table 4.2). In the converse constructs, exchanging the C or D domains, or both, of IGF-II with those of IGF-I made the chimeras more IGF-I like; their relative IR-A binding affinities being 27%, 37% and 17% respectively, that of IGF-II. The double chimera IGF-II CIDI has the same affinity for the IR-A as IGF-I.
Figure 4.1. Competition binding curves of Eu-Insulin binding to immunopurified human IR-A or IR-B.

Immunocaptured IR-As or IR-Bs were incubated with Eu-Insulin in the presence or absence of increasing concentrations of insulin, IGF-I, IGF-II or IGF chimeras as described under section 4.3.2. The graphs shown are representative of three independent experiments. A and C, competition for binding to IR-A; B and D, competition for binding to the IR-B. Results are expressed as a percentage of Eu-Insulin bound in the absence of competing ligand and the data points are means ± S.E.M. of triplicate samples. Errors are shown when greater than the size of the symbols. The ligands are as follows in A and B, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); IGF-II DI (◆). C and D: Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); IGF-II CIDI (■).
4.4.2 Binding analyses of insulin, IGF-II, IGF-I and IGF chimeras to the IR-B

The competition binding curves for insulin, IGF-I, IGF-II and the six chimeras with the IR-B are shown in Figure 4.1 B and D with the IC\textsubscript{50} values and relative binding affinities compared to IGF-II listed in Table 4.2. The data show that insulin binds IR-B with 2 fold higher affinity than IR-A while IGF-I (3 fold), IGF-II (3.7 fold) and the IGF chimeras (2-5 fold) all bind IR-A better than IR-B. Previous reports on the relative affinities of insulin for the two IR isoforms range from IR-B having higher affinity (Whittaker et al., 2002) as reported here; to no difference in affinity of insulin for either isoform (Frasca et al., 1999; Surinya et al., 2002); or to the IR-A isoform having the higher insulin binding affinity (Mosthaf et al., 1990; Yamaguchi et al., 1991). Different binding assays and assay conditions could have contributed to the variation between these studies. The presence of the exon I1 encoded residues had more of a negative effect on IGF-II binding (IC\textsubscript{50} IR-A: 18.2 nM vs IC\textsubscript{50} IR-B: 68 nM) than on IGF-I binding (IC\textsubscript{50} IR-A: 120.4 nM vs IC\textsubscript{50} IR-B: 366 nM) (Table 4.2).

While the absolute binding affinities of IGF-I, IGF-II and the four single chimeras are lower for IR-B compared to IR-A, their relative affinities are similar (Figure 4.1B). As summarised in Table 4.2, the relative order of binding affinity with the IR-A isoform is IGF-II followed by IGF-I CII DII, IGF-II DI, IGF-I CII, IGF-II CI, IGF-I DII, IGF-II CI DI and IGF-I. The relative order of binding affinity for the IR-B isoform is similar but not identical. In both cases the four highest affinity ligands contain the IGF-II C domain and the four lowest binders contain the IGF-I CI domain. Minor differences between the two IR isoforms are the reversal in the relative positions of the IGF-II DI and the IGF-I CII ligands with IR-B and the equal binding of the IGF-II CI and IGF-I DII ligands on IR-B (equal 5\textsuperscript{th}) compared to their consecutive ranking (5\textsuperscript{th} and 6\textsuperscript{th}) on IR-A. In contrast to IR-A, the relative affinities of the double chimeras with IR-B fall just outside the range seen with IGF-I and IGF-II. The IGF-I
CIIDII chimera, the smallest of the constructs (64 residues), has a higher affinity than IGF-II for binding the IR-B while the IGF-II CIDI chimera, the largest of the constructs (73 residues), has a slightly lower affinity than IGF-I for binding the IR-B. The results presented here show that the C and D domains are responsible for the higher affinity of IGF-II for IR-B, compared to IGF-I.

<table>
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<tr>
<th>Ligand</th>
<th>IR-A</th>
<th>IR-B</th>
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<tbody>
<tr>
<td></td>
<td>IC$_{50}$ (nM)</td>
<td>IC$_{50}$ (nM)</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.8 ± 0.3</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>IGF-II</td>
<td>18.2 ± 2.4</td>
<td>68 ± 11</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>49.3 ± 12.7</td>
<td>194 ± 78</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>66.3 ± 11.2</td>
<td>310 ± 120</td>
</tr>
<tr>
<td>IGF-II CIDI</td>
<td>106.0 ± 41.3</td>
<td>405 ± 98</td>
</tr>
<tr>
<td>IGF-I</td>
<td>120.4 ± 34.1</td>
<td>366 ± 15</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>83.2 ± 3.0</td>
<td>295 ± 25</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>64.0 ± 18.4</td>
<td>179 ± 12</td>
</tr>
<tr>
<td>IGF-I CIIDII</td>
<td>19.5 ± 8.4</td>
<td>44 ± 5</td>
</tr>
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<thead>
<tr>
<th></th>
<th>IC$_{50}$ Rel. IGF-II (%)</th>
<th>IC$_{50}$ Rel. IGF-II (%)</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>654</td>
<td>1300</td>
</tr>
<tr>
<td>IGF-II</td>
<td>100</td>
<td>27</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>37</td>
<td>9</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>27</td>
<td>6</td>
</tr>
<tr>
<td>IGF-II CIDI</td>
<td>17</td>
<td>4</td>
</tr>
<tr>
<td>IGF-I</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>22</td>
<td>6</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>28</td>
<td>10</td>
</tr>
<tr>
<td>IGF-I CIIDII</td>
<td>93</td>
<td>41</td>
</tr>
</tbody>
</table>

Table 4.2 Inhibition of binding of europium-labelled insulin to the IR-A and IR-B by insulin, IGF-I, IGF-II and IGF chimeras. The IC$_{50}$ relative to that of IGF-II binding to the IR-A is also shown. Values are the means and ± S.E.M. from three independent experiments.
4.4.3 Phosphorylation of the IR-A and IR-B by stimulation with chimeric IGFs

The data showing the activation of R'IR-A or R'IR-B cells by insulin, IGF-I, IGF-II and the two double chimeras, IGF-I CIIDII or IGF-II CIDI are presented in Figure 4.2. As seen with the binding studies (Table 4.2), insulin was more potent at inducing phosphorylation of IR-B (IC$_{50}$: 4.1 ± 0.56 nM) than IR-A (IC$_{50}$: 18.9 ± 5.1 nM) although in this case the relative potency was 4 fold higher not 2 fold. Relative to insulin, IGF-II was capable of activating both the IR-A and IR-B to only 40% and 7.2% respectively. This reflected the IGF-II binding affinity for the IR-A and IR-B relative to insulin. IGF-I showed only a modest ability to stimulate autophosphorylation of either isoform (Figure 4.2). Replacing the C and D domains of IGF-II with those of IGF-I reduced its capacity to activate either IR isoform. The ability of IGF-I and the IGF-II CIDI chimera to phosphorylate the IR-A and IR-B relative to insulin is considerably less than their ability to bind the IR isoforms relative to insulin (Table 4.2). Conversely, replacing the C and D domains of IGF-I with those of IGF-II resulted in an IGF-I based chimera that was slightly more active than IGF-II on both IR-A and IR-B. In line with the binding studies, the ability of IGF-II to potently activate the IR-A is due to its C and D domains.
Figure 4.2 Activation of the human IR isoforms by insulin, IGF-II, IGF-I or IGF chimeras.

R- cells overexpressing the human IR isoforms were serum starved for 4 hours followed by stimulation with various concentrations of either insulin, IGF-II, IGF-I or IGF chimeras for 10 minutes. Cells were lysed with ice-cold lysis buffer containing phosphatase inhibitors, and the activated receptors were immunocaptured with the anti-IR antibody 83-7 as described in section 4.3.3. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY2O to detect phosphorylated tyrosines. A, IR-A activation by Insulin, IGF-I, IGF-II and IGF double chimeras. B, IR-B activation by insulin, IGF-I, IGF-II and IGF double chimeras. The graphs shown are a representative of three experiments and data points are means ± S.E.M. of triplicate points. Errors are shown when greater than the size of symbols. The ligands are as follows in A and B, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIIDII (□); IGF-II CIDI (■).
In this chapter the structural determinants that allow IGF-II to bind to and potently activate the IR-A are described. IR isoform binding analysis of the IGF chimeras revealed that the IGF-II C and D domains allow an IR-A binding affinity near that of insulin. The IGF-I C and D domains prevent high affinity binding and do not allow potent activation of the IR-A. In addition the C and D domains are also responsible for the higher affinity of IGF-II for the IR-B compared to IGF-I, although the affinities seen with IR-B are lower than those seen with IR-A.

Figure 4.3 Summary of relative IR-A and IR-B binding of Insulin, IGF-I, IGF-II and IGF chimeras.
Affinities of all ligands for the IR-A as a relative % of IGF-II binding are shown in dark grey bars. Affinities of ligands binding to the IR-B relative to IGF-II binding to the IR-A are shown in open bars. Standard errors are not shown, however for ligands binding to IR-A standard errors are between 3.6 % and 43 % and for IR-B between 4 % and 40 %. Absolute values are listed in Table 4.2.

In chapter 3 the generation of the first whole domain chimeras of IGF-I and IGF-II, was described which allowed investigation into the roles of the C and D
domains of IGF-I and IGF-II in receptor binding specificity. As shown in Figure 4.3 the C domains, and to a lesser extent the D domains of the IGFs make major contributions to the IGF binding specificity to the IR isoforms. The binding of all of these chimeras to the IR-B isoform is lower than to the IR-A, but the relative trends are similar. The four best binders to both IR isoforms contain the C domain from IGF-II while the four ligands that contain the C domain from IGF-I have the lowest affinities for both isoforms (Figure 4.3).

While the B and A domains of IGF-I and IGF-II do not appear to be as important in regulating receptor binding specificity they do make critical contributions to the free energy of receptor binding (Bayne et al., 1990; Hodgson et al., 1996). Moreover, the determining the molecular basis for why IGF-II binds with a higher affinity (IC$_{50}$: 18 nM) than IGF-I (IC$_{50}$: 120 nM) for the IR-A only accounts for only a small percentage of the total free energy of ligand binding. Removal of the entire IGF-I C domain increased IR binding suggesting that the mere presence of the C domain is not an absolute requirement for high affinity binding to the IR (Bayne et al., 1989). Collectively this suggests that the regions that determine IR binding specificity have evolved in different regions of the protein to those that contribute most to the free energy of IR binding.

Interestingly in this study insulin has a 2-fold lower IC$_{50}$ for the IR-B than the IR-A. This result is consistently seen when these binding assays are conducted in several other laboratories at this University. In addition this result in also obtained when using $^{125}$I-Insulin as a tracer in competition binding assays suggesting that our result presented here is not due to the europium label (Eric R. Bonython, personal communication). There is no consensus in the literature as to the relative affinities for insulin binding to the IR-A and IR-B, as summarized in Table 4.1 and our data is within the range of this variation.
The receptor binding IC₅₀ values reported here are slightly higher than previously published (Frasca et al., 1999; Pandini et al., 2002). Several reasons may account for the differences: many studies have used TyrA₁₄-[¹²⁵I]-insulin as a label and here we have used europium. The europium ion and chelating cage that houses it are covalently attached to amino groups such as the N-terminus and lysine side chains. Therefore the label has the possibility of attaching itself to 3 sites in insulin (B and A chain N-termini and A₂⁹ lysine). This may affect the affinity of insulin for the insulin receptor and as a result influence our IC₅₀ values. Deletion of four residues of the insulin B-chain N-terminus resulted in only a slight change in biological activity (Schwartz and Katsoyannis, 1978), as did mutation at Lysine A₂⁹ (Mirmira and Tager, 1991), suggesting that a europium label at these positions may not affect IR binding. However, residues at the N-terminus of the A chain have been implicated in IR binding (Hua et al., 1991; Xu et al., 2002; Xu et al., 2002; Wan et al., 2003; Xu et al., 2004) and therefore a europium label at the A-chain N-terminus may disrupt the interaction with the IR.

Alternatively, the concentration of the labelled ligand can influence the IC₅₀ values obtained in competition experiments (Schaffer, 1994; Surinya et al., 2002). In a similar study of insulin receptor binding Surinya and colleagues reported different IC₅₀ values at a range of different concentrations of radioligand. Specifically, a lower level of radioligand resulted in a decrease in the IC₅₀ values obtained (Surinya et al., 2002). Here a higher amount of europium-labelled insulin was added compared to other studies using radiolabel. Insulin binding to the insulin receptor is affected by changes in pH and slight variation could change the IC₅₀ values (De Meyts et al., 1976; Waelbroeck, 1982; Wang et al., 1988). While the absolute affinities are slightly higher the relative binding affinities are in good agreement with previously published work (Pandini et al., 2002). The binding of IGF-II to the IR-A is 6.5 fold lower relative to
insulin (Table 4.2). The initial report showing high affinity binding of IGF-II to the IR-A showed that IGF-II had only a 2.5 fold lower affinity for the IR-A than insulin (Frasca et al., 1999), however the same group in a subsequent study reported that IGF-II had a 5 fold lower affinity for the IR-A than insulin (Pandini et al., 2002) (Table 4.1).

It has been reported that the presence of the exon 11 encoded amino acids has little (Mosthaf et al., 1990; Yamaguchi et al., 1991) or no effect (Frasca et al., 1999; Surinya et al., 2002) on insulin binding. Despite this relatively small effect on insulin binding affinity alanine scanning of the proposed insulin binding-site on both IR isoforms has revealed differences in the energetic contribution of common receptor side chains in the two receptor isoforms (Whittaker et al., 2002). This suggests subtly different modes of insulin binding and also that there is significant accommodation for structural differences induced by the extra 12 amino acids to allow almost equal binding affinities for insulin. In this report we show that the presence of the exon 11 encoded sequence does have a significant influence on the binding of IGF-II c.f. (Frasca et al., 1999; Pandini et al., 2002), the single and double chimeras and IGF-I (Figure 4.3). The results here show for the first time that the presence of the exon 11 peptide has a greater negative effect on IGF-II binding (3.7 fold reduction) than on IGF-I binding (3 fold reduction). However, the absolute affinity of IR-B for IGF-II is still higher than that of IR-A for IGF-I (Table 4.2).

The 16 amino acids at the C-terminus of the IR α-subunits, residues 704-719 in IR-A, are essential for ligand binding as shown by chemical cross-linking (Kurose et al., 1994), mutagenesis and receptor minimization studies (Mynarcik et al., 1996; Mynarcik et al., 1997; Brandt et al., 2001; Kristensen et al., 2002). In the IR-B the exon 11 encoded region, which has a negative effect on IGF binding but not on insulin binding, is directly C-terminal of these 16 amino acids and may exert its effects by
sterically interfering with residues/regions of the IGF molecule that are not present in insulin. Further studies are needed to determine whether IGF-I or IGF-II directly interacts with the exon 11 encoded amino acids. The larger size of the C and D domains of IGF-I may be important in regulating IR binding specificity. The C domain of IGF-I forms a large wedge shape (Brzozowski et al., 2002) and is 4 amino acids longer than the IGF-II C domain. The D domain of IGF-I contains two more amino acids, compared to the IGF-II D domain. To determine the nature of these size differences molecular threading was used to model the structure of IGF-II C1. This allows a comparison of the IGF-II and IGF-I C domains to be made (Figure 4.4). The most striking difference is the increased volume of the IGF-I C domain reflecting the presence of the four extra amino acids not present in the IGF-II C domain. Specifically annotated in the IGF-I C domain are Tyr 31 and Pro 39 with no equivalent residues in the IGF-II C domain. These residues may be sterically hindering the IGF-I C domain interaction with the IR. Supporting our hypothesis is the observation that when the IGF-I C domain was shortened from the native 12 amino acids in two amino acid decrements to an ultimate length of 6 amino acids, in a two chain IGF, the IR binding affinity increased (DiMarchi, 1997).

Yip and Ottensmeyer have used electron cryomicroscopy and molecular replacement to yield an atomic model of the insulin-IR complex (Luo et al., 1999; Ottensmeyer et al., 2000). In a subsequent report, when replacement of the insulin molecule with that of IGF-I occurred, modelling then the interaction of IGF-I binding to the IR, the IGF-I D domain exhibited serious steric clash with the L2 domain of the IR (Yip and Ottensmeyer, 2003). Hence to prevent this interaction the ligand had to be rotated and the predicted side chain interactions of the IGF-I with the IR were substantially less than that for insulin and the IR, providing a molecular basis for lower

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affinity of IGF-I : IR interaction. Modelling of IGF-II binding to the IR has not been reported.

![Comparison of the C domains of IGF-II and IGF-I](image)

Figure 4.4 Comparison of the C domains of IGF-II and IGF-I. A ribbon representation of the NMR structure of IGF-II with the C domain in surface mode is shown on the left. A model of IGF-II CI is shown on the right. The sequence of IGF-II CI was entered into SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html) and threaded through the backbone of IGF-II using coordinates from the NMR structure (Torres et al., 1995). The C domain of IGF-II CI is highlighted in surface mode.

There are some contradictions in the literature that would suggest that size alone might not be the only determinant of IR binding specificity. Kristensen et al. (Kristensen et al., 1995) demonstrated that inserting the 12 amino acid C domain of IGF-I into insulin to form a single chain hybrid did not affect IR binding. However, Chang et al. (Chang et al., 1998) showed proinsulin, with a 31 amino acid C peptide, binds poorly to the IR. Interestingly proinsulin had an IC<sub>50</sub> of 28 nM for binding to the IR-A but 145 nM for binding to the IR-B (McClain, 1991). Proinsulin contains a C peptide analogous to the C domain in the IGFs, although larger and the presence of exon 11 appears also to affect its binding affinity. Replacement of the C peptide in proinsulin with a short turn forming pentapeptide sequence caused a 25-fold increase in IR binding affinity (most likely the receptor used in this study was IR-A as lymphocytes were used as a source of IR (Moller et al., 1989)), again suggesting that steric restrictions may be regulating IR binding (Chang et al., 1998).
A sequence comparison between IGF-I and IGF-II reveals several charge differences between the IGF-I and IGF-II C and D domains that may influence binding. In particular the IGF-II C domain contains two extra positive arginine residues compared to the IGF-I C domain. Also the IGF-II D domain has a negative glutamate residue located at its C-terminus while the IGF-I D domain contains two positive arginine charges. Charge interactions have been shown to mediate in part insulin binding to the insulin receptor (Rafaeloff et al., 1992). It has also been shown in various other situations that charged residues at the periphery of a protein-protein interface can affect association rates, while not affecting dissociation rates, thereby increasing the equilibrium binding affinity (Schreiber and Fersht, 1993; Schreiber et al., 1994; Schreiber and Fersht, 1995; Marvin and Lowman, 2003). While we have not determined the kinetics of IGF-II, IGF-I and IGF chimera binding, it could be postulated that the extra charges in the IGF-II C domain, may increase the association rate of IGF-II for the IR-A compared to IGF-I.

Sequencing of insulin from *Amphiuma tridactylum* (three toed salamander) revealed an extension at the N terminus of the A chain of Ala-Arg which enhanced its binding to the human insulin receptor relative to porcine insulin (Conlon et al., 1996). A model of insulin binding to the IR has suggested that this additional arginine can interact with glutamate 287 in the cysteine rich domain of the receptor (Ottensmeyer et al., 2000). Human IGF-II has Ser-Arg at the same position in the C domain possibly conserving the high affinity contacts with the IR while IGF-I has uncharged Gln-Thr at the equivalent positions.

The results of the IR phosphorylation assay shows all proteins exhibit a higher potency for IR-B phosphorylation than IR-A. It has been reported previously in the literature that the IR-B has a higher insulin-stimulated kinase activity than the IR-A (Kellerer et al., 1992; Kosaki et al., 1995). Furthermore, maximal insulin stimulation
of the IR-B was higher than the maximal insulin stimulation of the IR-A (Kosaki et al., 1995). Furthermore, the physiological consequence of this differential kinase activity has been postulated in a number of reports, where an increase in prevalence of the lower kinase active IR-A in the muscle of myotonic dystrophy patients has been associated with the incidence of insulin resistance (Savkur et al., 2001; Savkur et al., 2004). In addition, tryptic peptide mapping identified a novel phosphopeptide in the IR-B not present in the IR-A when activated by insulin (Kosaki et al., 1995). Another study found that a switch in expression from the IR-A to the IR-B isoform on HepG2 cells correlates with an increase in sensitivity to insulin’s metabolic effects, as measured by glucose incorporation into glycogen and 2-deoxyglucose transport (Kosaki and Webster, 1993). In all these cases the IR-B is more responsive to insulin than the IR-A, which is in agreement with the data presented here.

Binding studies of C domain mutants of IGF-I to IR/IGF-1R chimera have suggested that the N-terminus of the IR α subunit may make negative contacts with the IGF-I C domain (Zhang et al., 1994). Analysis of the binding of the IGF-I and IGF-II chimeras reported here to alanine mutants of the IR-A (Whittaker et al., 2002) will allow a more specific delineation of the receptor sites that contact the IGF-I and IGF-II C and D domains. While these proposed studies would not unequivocally prove that the C or D domains contact the alanine scanned sites it would provide clues as to the orientation of the ligand in the receptor binding pocket.

A change in receptor binding affinity due to mutation in a ligand does not always denote that the affected residues make contact with the receptor, as illustrated by the following example.

Analysis of the structure of growth hormone (GH) uncomplexed (Abdel-Meguid et al., 1987), complexed with one growth hormone receptor (GHR) (Ultsch et al., 1994; Sundstrom et al., 1996) and complexed with two GHRs (de Vos et al., 1992)
has revealed that GH, upon interaction of its first binding site with a GHR, undergoes structural rearrangements that orientate residues on a second site for high affinity binding of a second GHR. Residues that mediate the site 1 induced changes to the second receptor binding site on GH have been identified (Duda and Brooks, 2003). In addition, mutation of these residues that do not contact the receptor and do not affect the structure of the unbound GH resulted in reduced lactogenic activity of the mutant GH (Duda and Brooks, 2003). The IR is a covalently bonded dimer prior to ligand binding and it is unlike monomeric GHRs in that it does not require ligand binding for dimerisation. However, a model for insulin binding, and possibly IGF-I/II binding, proposed that IR activation involved distinct sites on the ligand crosslinking two opposite sites on distinct IR α subunits (De Meyts, 1994a; Schaffer, 1994)(see section 1.4.9). Therefore in a similar manner to the GH dimerising two GHRs, insulin may in fact orientate the two halves of the IR by a similar crosslinking mechanism, and thus binding at one site on insulin may result in an altered conformation at its second receptor binding site. Indeed, the binding of insulin to the first site on the receptor is postulated to cause a conformational change in the C-terminus of the insulin B chain that reveals critical receptor binding residues in the N-terminus of the A chain (Hua et al., 1991). It is unknown if a similar mechanism exists for IGF binding to the IR. Due to the flexible nature of the C and D domains it is unlikely that exchanging them alters the communication between the initial receptor binding site and a second receptor binding site, however it cannot be entirely dismissed. As no structure of an IGF ligand with the IR exists, the exact conformation of the IGF once bound to the IR is unknown.

In conclusion, the domains of IGF-II that allow it to bind and activate the IR-A with high affinity have been determined. Clearly, a structure of the receptor/ligand complex will ultimately be needed to reveal the molecular details of the high affinity IGF-II/IR-A interaction.
Insulin, IGF-II, IGF-I and IGF chimeras signaling through the IR-A and IR-B
CHAPTER 5 – Insulin, IGF-II, IGF-I and IGF chimera signalling through the IR isoforms

5.1 INTRODUCTION

Membrane spanning receptor tyrosine kinases provide an avenue for the traffic of biological information from the extracellular space into the cytoplasm of the cell. The information once in the cell is passed to specific subcellular compartments within the cell, e.g. the nucleus, via successive molecular recognition events.

Generally, receptor tyrosine kinases, e.g. FGFR, EGFR, GHR, EPOR, VEGFR and PDGFR, exist as monomers at the cell surface and are dimerised by ligand which induces autophosphorylation of their intracellular domains (de Vos et al., 1992; Wiesmann et al., 1997; Kossiakoff and De Vos, 1998; Jiang and Hunter, 1999; Plotnikov et al., 1999; Schlessinger, 2000; Garrett et al., 2002; Burgess et al., 2003; Garrett et al., 2003). The insulin/IGF receptor family of receptor tyrosine kinases are a unique family in that they exist as preformed homodimers of two disulphide bonded αβ monomers and that ligand binding to the extracellular regions of the receptors causes rearrangement of the quaternary structure resulting in autophosphorylation of the intracellular regions (Czech, 1982; Czech and Massague, 1982; Boni-Schnetzler et al., 1986; Bajaj et al., 1987). The Met receptor (hepatocyte growth factor receptor) and its family members also exist as a disulphide bonded receptor, of a short entirely extracellular α chain bonded to a membrane traversing β subunit (Hubbard and Till, 2000).

Autophosphorylation of the insulin receptor occurs rapidly, first on tyrosine 1162, then 1158 and finally 1163 in the activation loop of the kinase domain allowing unobstructed access of ATP and substrate to the kinase active site (Dickens and Tavare, 1992; Hubbard et al., 1994; Wei et al., 1995; Hubbard, 1997). The activated catalytic core then phosphorylates tyrosine residues outside the kinase domain on the receptor e.g. tyrosines 1316 and 1322 (White et al., 1988) in the c-terminal tail and tyrosine 960 in the juxtamembrane domain (Tavare and Denton, 1988; Tavare et al.,
Phosphorylation of the IR and IGF-1R provide docking sites for adaptor molecules that then recruit other signaling proteins to the receptor kinase. IR and IGF-1R signaling relies heavily on the phosphorylation of docking proteins rather than autophosphorylation to enable recruitment and activation of downstream signaling molecules (Schlessinger, 2000; Siddle et al., 2001), therefore docking of the adaptor molecules is the initial stage of many signaling cascades.

The most commonly studied adaptor molecules recruited to the activated IR are the insulin receptor substrate family of proteins (IRS 1-4) and the Src homology/collagen proteins (Shc – p46/p52/p66 isoforms). Tyrosine 960 in the juxtamebrane domain, when phosphorylated provides a docking site for IRS-1(White et al., 1988; Backer et al., 1991), IRS-2 (Sun et al., 1995) and Shc (Wolf et al., 1995). The pathways initiated by ligand binding to the IR through the IRS proteins and Shc is shown in Figure 1.4.

The IRS proteins link the IR to the PI3K pathway which is responsible for mediating much of insulin’s ability to mediate glucose uptake (Hara et al., 1994), cell-cycle progression, protection from apoptosis and to regulate transcription (Bevan, 2001; Brazil et al., 2004). The autophosphorylated sites on the receptor provide docking sites for IRS proteins which are then themselves phosphorylated by the receptor tyrosine kinase (Backer et al., 1991; Shoelson et al., 1992). Certain phosphotyrosine sites on the IRS proteins create binding sites for SH2 domain containing proteins, of which the p85 regulatory subunit of PI3K is one (Myers et al., 1992). Interaction of both SH2 domains of the p85 subunit with IRS proteins causes a conformational change in p85 that is transmitted to the catalytic subunit p110 resulting in an increase in PI3K activity (Shoelson et al., 1993; Rordorf-Nikolic et al., 1995). The binding of p85 to the IRS also serves a second function which is to recruit the p110 catalytic subunit to the plasma membrane, where it catalyses the addition of a
phosphate group to the 3' position on the inositol ring of the lipid substrates PtdIns(4,5)P_2 converting it to PtdIns(3,4,5)P_3. PtdIns(3,4,5)P_3 is recognized by the Pleckstrin homology (PH) domain of PDK-1 and PKB/Akt, thereby colocalizing both these proteins to the membrane facilitating the phosphorylation and subsequent activation of PKB/Akt by PDK-1 (Stokoe et al., 1997; Stephens et al., 1998). The serine/threonine kinase PKB/Akt, one of the major effector proteins of the PI3K pathway, binds and phosphorylates a variety of substrates involved in the protection against apoptosis (e.g. BAD (Datta et al., 1997)) and in metabolism (e.g. GSK-3 (Cross et al., 1995)). Its activity is also regulated by a wide variety of interacting proteins (e.g. Grb10 (Jahn et al., 2002), TCL1 (Laine et al., 2000; Pekarsky et al., 2000) and Hsp90 (Sato et al., 2000)). PKB/Akt acts both in the cytoplasm and the nucleus (Lawlor and Alessi, 2001; Brazil et al., 2002).

Both IRS-1 and Shc connect the IR with the MAPK pathways. The MAPKs are grouped into several distinctly regulated groups, however the extracellular signal-related kinases (Erk)-1/2 are the most well studied group relating to IR signaling (for reviews of all MAPK groups see (Chang and Karin, 2001; Johnson and Lapadat, 2002; Yang et al., 2003; Roux and Blenis, 2004)). Shc, once recruited and phosphorylated by the insulin receptor, can dock with Grb2 which is constitutively associated with Sos. These interactions can then activate the Ras/Erk1/2 pathway (Pelicci et al., 1992).

While MAPK and PI3K are the most well characterized pathways, coupled to the IR by Shc and IRS-1 respectively, the insulin receptor also phosphorylates Stat5b (Chen et al., 1997), inducets Janus kinases (JAKs) to phosphorylate STAT5b (Le et al., 2002) and interacts with G-proteins including the α subunit of G_i2 (Krieger-Brauer et al., 1997). The pathways downstream of these receptor-proximal events are not as well characterized as those of the MAPK and PI3K pathways. In addition, the phosphorylation of the three tyrosines in the activation loop also provides docking sites.
for the adaptor molecule APS (Moodie et al., 1999) which connects the IR with the CAP/Cbl pathway, important for glucose uptake in adipocytes (Liu et al., 2002).

A number of studies have shown that IGF-II potently induces autophosphorylation of the IR-A while IGF-I does not (Chapter 4)(Frasca et al., 1999; Denley et al., 2004). As mentioned the autophosphorylation of the intracellular domains of the IR occurs on a variety of tyrosine residues, however the two studies that show differential IR activation by IGF-I and IGF-II (Frasca et al., 1999; Denley et al., 2004)(Chapter 4) did not determine the phosphorylation status of specific tyrosine residues, but rather reported an average phosphorylation state of the entire intracellular β subunits. As the phosphorylation of tyrosines in the intracellular region follows a sequential order and as each phosphotyrosine creates a site for signaling molecule interaction, the ability of insulin, IGF-II, IGF-I and all IGF chimeras to induce phosphorylation of specific tyrosine residues was investigated and is reported in this chapter. Differential phosphorylation of sites in the β subunits of the IR by either insulin, IGF-II or IGF-I, may provide a novel mechanism for specific activation of signaling pathways and may explain differences in their ability to stimulate certain biological responses. Indeed, certain domains of the IGF-IR β subunit have been shown to be differentially involved in apoptosis protection (O'Connor et al., 1997). In addition the ability of insulin, IGF-II, IGF-I and IGF chimeras to induce phosphorylation of several downstream signaling molecules via the IR-A and IR-B was also studied.
5.2 MATERIALS

The following antibodies were purchased: IRS-1, phospho-Erk 1/2, Erk 1/2, Akt from Cell Signalling Technology (MA, U.S.A.). Phospho-Akt [pS\textsuperscript{473}], Phospho-IR/IGF-1R [pYpY\textsuperscript{1158/1162/1163}], phospho IR [pY\textsuperscript{972}] from the Biosource International (CA, U.S.A). Rat carboxy-terminal IRS-1 and IRS-2 were purchased from Upstate Biotechnology (N.Y, U.S.A). IR β subunit antibody was purchased from Santa Cruz (CA, U.S.A.). IR subunit antibody was purchased from Santa Cruz (CA, U.S.A.). Pepstatin and sodium orthovanadate was obtained from Sigma Chemical Co., (MO, U.S.A.). 10% Bis-tris acrylamide Criterion\textsuperscript{TM} gels and 7% tris-acetate acrylamide gel Criterion\textsuperscript{TM} gels were purchased from Biorad (CA, U.S.A.). Human insulin was purchased from Novo Nordisk (Bagsværd, Denmark). R\textsuperscript{-} cells (mouse 3T3-like cells with a targeted ablation of the IGF-1R gene) (Sell et al., 1994) were a kind gift from Prof. R. Baserga (Philadelphia, USA). The production of 3T3-IR-A cells is reported in (Faria et al., 1994). The construction, expression, and purification of chimeras of IGF-I and IGF-II has been previously reported in chapters 2 and 3.

5.3 METHODS

5.3.1 CONSTRUCTION OF CELLS EXPRESSING THE IR ISOFORMS

The transfection, selection and characterization of R\textsuperscript{-}IR-A and R\textsuperscript{-}IR-B cells is detailed in chapter 4 (section 4.3.1)

5.3.2 IR-A AND IR-B PHOSPHORYLATION AND ACTIVATION OF INTRACELLULAR SIGNALLING MOLECULES IN RESPONSE TO INSULIN, IGF-I, IGF-II and IGF CHIMERAS

a) Cell stimulation and preparation of whole-cell lysates

R\textsuperscript{-}IR-A, R\textsuperscript{-}IR-B cells or 3T3-IR-A cells were grown to 80% confluency and serum-starved overnight at 37°C, 5% CO\textsubscript{2}. The cells were then treated with 10 nM
ligand for either 5 minutes or a time course of 2, 5, 10 and 60 minutes. Stimulation was terminated by two washes with ice-cold PBS (pH 7.4) and addition of lysis buffer (50 mM Tris [pH 6.8], 1 % (w/v) sodium dodecyl sulphate and 10 % (v/v) glycerol). After scraping the cells, the lysates were boiled immediately to inhibit protease and phosphatase action and centrifuged at 13,200 rpm for 1 minute. The protein concentration was determined with a DC protein assay kit (Bio-Rad, Hercules, CA).

b) IRS-1 immunoprecipitation

Cells were stimulated as above, but were lysed in a different lysis buffer (150 mM NaCl, 10 % (v/v) glycerol, 20 mM Tris [pH 8], 1 mM EDTA, 0.2 % SDS, 1 tablet complete protease inhibitors, 2 mM sodium orthovanadate and 2 µg/ml pepstatin). Lysates (500 µg) were pre-cleared with 25 µl protein A-agarose beads for 30 min rocking at 4°C, before addition of 5 µg of anti-IRS-1 antibody and incubation overnight at 4°C rocking. Protein A-agarose beads were added for 3 hours at 4°C and then the immunoprecipitates were eluted and subjected to SDS polyacrylamide gel electrophoresis.

5.3.3 WESTERN BLOT ANALYSIS

Immunoprecipitates or whole-cell lysates (20 µg) were subjected to reducing SDS-polyacrylamide gel electrophoresis on 10 % Bis-tris acrylamide Criterion™ gels (for IR, PKB/Akt, and Erk1/2) or 7 % tris-acetate acrylamide gel Criterion™ gels (IRS-1). After separation, the electrophoresed proteins were transferred to nitrocellulose membranes and immunoblotted with phospho-specific antibodies or, in the case of IRS-1 and IRS-2, immunoprecipitates were probed with the antiphosphotyrosine antibody PY20. In all cases, after probing with the phospho-specific antibody, the nitrocellulose was stripped (100 mM Tris-HCl [pH 6.8], 10 %
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SDS and 100 mM β-mercaptoethanol for 30 minutes at 60 °C) and reprobed with an antibody against the non-phosphorylated form of the protein. Immunoreactive bands were detected using an enhanced chemiluminescence (ECL) Western blotting protocol (Amersham, Densitometry was performed to quantitate the ECL visualized bands. Statistical analysis to determine significance was by paired Students t-test using Prism 4.0.

5.3.4 MIGRATION ASSAYS

The migration and cell survival assays were performed by Gemma V. Brierley (The University of Adelaide, Australia).

The lower wells of an AC96 NeuroProbe A Series 96 Well Chamber (NeuroProbe, USA) were loaded with 25ul DMEM containing 0.5% BSA and different concentrations of either IGF-I, IGF-II, Insulin, or IGF chimeras. Framed polycarbonate filters with a pore size of 12um were pretreated overnight at 4°C in 10mM acetic acid containing 25ug/ml type I Collagen (Sigma, USA). Filters were rinsed in PBS and slotted against the rubber gasket of the top plate of the chamber. The top plate of the chamber was then gently lowered onto the lower wells containing chemoattractant and fastened with screws. R-IRA cells from approximately 60 -80% confluent monolayers were trypsinised and washed two times in DMEM containing 0.5% BSA. The cells were diluted to a concentration of 4x10^6 cells/ml in DMEM, 0.5% BSA and incubated at 37°C, 5% CO₂ for 30 minutes with 2.2ug/ml Calcein-AM (Molecular Probes, USA). The cells are washed a further two times and then re-suspended to 4x10^6 cells/ml in DMEM, 0.5% BSA. 200,000 cells/well are loaded to the top wells of the chamber and were allowed to migrate for 5 ½ hours at 37°C, 5% CO₂. Following the incubation the chamber was disassembled and any non-migrating cells remaining on the upper surface of the polycarbonate filter were wiped away using a PBS damp Terriwipe. The filters
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were then allowed to dry and the fluorescence of the calcine-labeled migrated cells on the underside of the filters were quantified using 485nm excitation and 535nm emission filters with a Victor 3V 1420 Multilable Counter (Wallac, PerkinElmer).

5.3.5 CELL SURVIVAL ASSAYS

R-IRA cells were grown to 80% confluency, trypsinised, plated into opaque white 96-well flat-bottom plate (Nunc, Roskilde, Denmark) at 2,500 cells/well in DMEM containing 10% FCS, and allowed to proliferate for 48 hours at 37°C, 5% CO₂. Cells were serum-starved for 5 hours at 37°C, 5% CO₂ before being treated with either IGF-I, IGF-II, Insulin, or IGF chimera in 100μl DMEM with 0.1% BSA, 5mM sodium butyrate. Following a further 48 hour incubation at 37°C, 5% CO₂, cell viability was determined by measuring ATP metabolism utilising the Cell-Titer Glo Cell-Viability Luminescent Assay according to manufacturers instructions (Promega, Madison, WI, USA). Luminescence was measured using a 572nm emission filter with a Victor 3V 1420 Multilable Counter (Wallac, PerkinElmer).

5.4 RESULTS

5.4.1 A timecourse of phosphorylation of Y960 on the IR-A stimulated by insulin.

IGF-II, IGF-I and IGF C domain chimeras

To examine in more detail the activation of Y960 of the IR-A by insulin, IGF-II, and IGF-I, a time-course of Y960 phosphorylation was examined. In this experiment whether exchanging the C domains between IGF-I and IGF-II changed the kinetics of receptor phosphorylation was investigated. As shown in Figure 5.1, insulin induced a 27-fold increase in phosphorylation of Y960 over basal after 5 minutes in 3T3-IR-A cells and maintained a high level of phosphorylation even after 60 minutes
(20-fold over basal levels). IGF-II increased Y960 phosphorylation to a maximum of 8-fold over basal levels after 5 minutes, which slowly decreased to 4-fold over basal by 60 minutes. IGF-I was extremely poor at inducing Y960 phosphorylation at each time tested. Interestingly, over the entire time-course, IGF-I CII stimulated Y960 phosphorylation to the same extent as IGF-II, with a maximum induction of 9-fold over basal levels and a slow decline to 6-fold over basal levels by 60 minutes. IGF-II CII was equally as poor as IGF-I at inducing phosphorylation of Y960. These results suggest that IGF-II is substantially more potent than IGF-I at inducing Y960 phosphorylation on the IR-A, and that this is due to elements within its C domain. As maximal phosphorylation was observed 5 minutes after stimulation with all ligands, this time point was used for all subsequent experiments. Although the Y960 phosphorylation induced by insulin, IGF-II and IGF-I was as expected for the IR-A the presence of low levels of endogenous mouse IGF-1R in the 3T3-IR-A cells could influence further signalling results. For this reason the R'IR-A and R'IR-B cells were used for all subsequent experiments.
**Figure 5.1 Time-course of Y960 activation of IR-A by insulin, IGF-II, IGF-I, and IGF C domain chimeras.**

Serum-starved NIH 3T3 cells expressing a human IR cDNA were treated with 10 nM ligand for 2, 5, 10 or 60 minutes. Whole-cell lysates were prepared and subjected to SDS-PAGE and then immunoblotted for phosphorylated Y960 as described in sections 5.3.2 and 5.3.3. *A*, densitometry results of the three independent experiments ± S.E.M (phospho-Y960/total IR-β subunit). The ligands are as follows in *A*, insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-II CI (●). *B*, Upper panel, anti-phospho Y960 antibody blot showing a representative result of three independent experiments. *Lower panel*, reblotting with anti-insulin receptor β subunit antibody.
5.4.2 Characterization of RIR-A and RIR-B cell lines

R cell lines, which are embryonic fibroblasts from a mouse with a knockout of the IGF-IR gene, were transfected to express IR-A and IR-B (section 4.3.1)(Denley et al., 2004). These transfections were performed by Eric R. Bonython (Molecular and Biomedical Science, The University of Adelaide). The R cells provide an IGF-IR null background with which the signalling and biological response of ligands through the IR-A and IR-B can be investigated without interference from IGF-IR or IGF-IR/IR hybrid receptors. Previous work has shown that both insulin and IGF-II do not stimulate R cells to proliferate or induce DNA synthesis in these cells (Morrione et al., 1997). Also these cells do not grow in serum-free media supplemented with PDGF, EGF and IGF-I (Sell et al., 1993). In the biological assays described in this chapter the R cells were not responsive to any ligand tested (data not shown). Therefore, all the signaling and biological responses resulting from treatment of the RIR-A and RIR-B cells with growth factor is then a result of the presence of the human IR-A and IR-B.

In addition, both cell lines were sorted by FACS analysis to generate cell lines that express equivalent levels of receptor (75,000 receptors/cell - data not shown). Of note, this is an order of magnitude lower than those used in previous studies (Frasca et al., 1999; Pandini et al., 2002) and reflect a more physiological cell-surface receptor density.

5.4.3 Induction of autophosphorylation of tyrosines in the activation loop of the IR-A and IR-B by insulin, IGF-II, IGF-I, and IGF chimeras

Ligand binding to the IR causes an increase in kinase activity due to phosphorylation of residues Y1158, Y1162, Y1163 in the activation loop, which then allows unobstructed access of peptide substrate and ATP to the kinase active site.
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(Hubbard, 1997). Phosphorylation of these three tyrosine residues is the first detectable event after ligand binding, and we examined the ability of 10 nM insulin, IGF-II, IGF-I, and IGF chimeras to induce phosphorylation of Y1158, Y1162, and Y1163 in the IR-A (Y1170, Y1174 and Y1175 in the IR-B; abbreviated as 3Y)(Figure 5.2 A). Insulin caused a 22-fold increase in phosphorylation of 3Y over basal, whereas IGF-II was 4-fold less potent, and IGF-I only slightly activated phosphorylation of 3Y over basal levels (IGF-II vs IGF-I: p<0.01). IGF-I chimeras containing the C and D domains of IGF-II (IGF-I CIIDII) or only the IGF-II C domain (IGF-I CII) were equally as potent as IGF-II at stimulating phosphorylation of 3Y in the R'IR-A cells (IGF-II vs IGF-I CII: p>0.5). IGF-I DII was slightly more potent than IGF-I. IGF-II CIDI and IGF-II CI were not statistically significantly different from IGF-I in their ability to stimulate 3Y phosphorylation (IGF-I vs IGF-II CI: p>0.5), and IGF-II DI was slightly poorer than IGF-II at activating 3Y.

Stimulation of IR-B by insulin caused a 45-fold increase in 3Y phosphorylation. This level was greater than the fold over basal stimulation of IR-A by insulin (Figure 5.2 B). This is surprising considering both cell lines express a similar number of receptors. IGF-II stimulated 3Y phosphorylation to only 1/8th the level that insulin did and was able to induce only 1.9-fold higher 3Y phosphorylation than IGF-I did. Exchanging the C or D domain or both between IGF-I and IGF-II had the same relative effect on 3Y phosphorylation on the IR-B as on the IR-A, however all treatments were not statistically significantly different from each other i.e p > 0.05.

These results suggest that both insulin and IGF-II are potent at inducing phosphorylation of 3Y on the IR-A, whereas IGF-I is extremely poor, and that the potency of IGF-II in activating 3Y is due to its C domain.
Figure 5.2 Induction of autophosphorylation of Y1158, Y1162 and Y1163 on the IR-A and IR-B by insulin, IGF-II, IGF-I, and IGF chimeras.

Serum-starved RIR-A (A) or RIR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and subjected to SDS-PAGE and then immunoblotted and assayed for phosphorylated Y1158, Y1162, and Y1163 (phospho-3Y) as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of the three independent experiments ± S.E.M (phospho-3Y/total IR-β subunit). Middle panel, anti-phospho Y1158, Y1162, Y1163 antibody blot showing a representative result of three independent experiments. Lower panel, reblotting with anti-insulin receptor β subunit antibody. (A) IR-A : *IGF-II vs IGF-I p <0.01,
5.4.4 Activation of Y960 on IR-A and Y972 on IR-B by insulin, IGF-II, IGF-I, and IGF chimeras

Following activation of the IR kinase, both exogenous (signalling molecules) or endogenous (the receptor itself) peptide substrates can become phosphorylated. Induction of tyrosine 960 phosphorylation in the juxtamembrane domain of the IR-A (Y972 in the IR-B) provides a docking site for binding of the adaptor molecules Shc, IRS-1 and IRS-2, as well as other receptor substrates such as Stat5b (Le et al., 2002). As a result the ability of 10 nM insulin, IGF-II, IGF-I, and IGF chimeras to induce phosphorylation of this site in both IR-A and IR-B was examined. The results of these experiments are shown in Figure 5.3. After a 5-minute stimulation with 10 nM ligand, Y960 was phosphorylated 10-fold over basal levels by insulin and 4-fold over basal levels by IGF-II. IGF-I stimulation of Y960 phosphorylation was only 1.6-fold over basal levels on the R'IR-A (IGF-II vs IGF-I: p<0.05). IGF-I CIIDII and IGF-I CII both stimulated Y960 phosphorylation of the IR-A to the same extent as IGF-II (IGF-II vs IGF-I CII: p>0.5), while IGF-II CIDI and IGF-II CI were both as poor as IGF-I (IGF-I vs IGF-II CI: p>0.5). Swapping the D domains had a small effect on the ability to activate Y960 of the IR-A.

Insulin induced a 16-fold increase in IR-B Y960 phosphorylation over basal. This was slightly higher than that seen with the IR-A. The basal level of Y960 phosphorylation was similar in R'IR-A and R'IR-B cells. IGF-II only induced a 3-fold increase in Y960 phosphorylation over basal levels, while IGF-I did not cause a significant increase in Y960 phosphorylation over basal levels. The introduction of the IGF-II C domain into IGF-I produced a protein that stimulated IR-B Y960 phosphorylation over basal (IGF-II vs IGF-I CII: p>0.5); conversely, putting the IGF-I C domain into IGF-II ablated its ability to stimulate activation of this site (IGF-I vs IGF-II CI: p>0.5).
Figure 5.3 Autophosphorylation of Y960 on the IR-A and IR-B by insulin, IGF-II, IGF-I and IGF chimeras.
Serum-starved RIR-A (A) or RIR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and subjected to SDS-PAGE and then immunoblotted for phosphorylated Y960 as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of the three independent experiments ± S.E.M (phospho-Y960/total IR-β subunit). Middle panel, anti-phospho Y960 antibody blot showing a representative result of three independent experiments. Lower panel, reblotting with anti-insulin receptor β subunit antibody. (A) IR-A: *IGF-II vs IGF-I; p<0.05.
5.4.5 Phosphorylation of IRS-1 by IR-A and IR-B activated by insulin, IGF-I, IGF-II, and IGF chimeras

Phosphorylation of residue Y960 provides a docking site for IRS-1, which then allows IRS-1 to be phosphorylated by the IR kinase domain. Here described is the ability of insulin, IGF-II, IGF-I, IGF-I CII, and IGF-II CI to stimulate IRS-1 phosphorylation in R’IR-A and R’IR-B cells (Figure 5.4). Five minutes after stimulation with insulin in the R’IR-A cells, IRS-1 was phosphorylated. IGF-II stimulated a 4-fold lower level of IRS-1 phosphorylation relative to insulin, while IGF-I induced 3.5-fold less tyrosine phosphorylation of IRS-1 compared to IGF-II (IGF-II vs IGF-I: p<0.05). IGF-I CII stimulated tyrosine phosphorylation of IRS-1 to the same level as IGF-II (IGF-II vs IGF-I CII:p>0.5), while IGF-II CI was as ineffective as IGF-I at inducing IRS-1 phosphorylation in R’IR-A cells (IGF-I vs IGF-II CI: p>0.5). Interestingly, in R’IR-B cells, the ability of IGF-II to activate IRS-1 tyrosine phosphorylation relative to insulin was similar to that in the R’IR-A cells (Figure 5.4). Surprisingly, IGF-I was more potent at inducing IRS-1 phosphorylation in R’IR-B cells than in the R’IR-A cells. The difference between IGF-II and IGF-I-induced phosphorylation of IRS-1 was only 1.4-fold in R-IR-B cells. This trend is in line with their relative binding affinities, where the difference in binding affinity between IGF-II and IGF-I for the IR-B is smaller that the difference in binding affinity of IGF-II and IGF-I for the IR-A (chapter 4)(Denley et al., 2004). The ability of IGF-I, IGF-II and both IGF C domain chimeras to induce phosphorylation of IRS-1 in R’IR-B cells was not significantly different (Figure 5.4).
Figure 5.4 IRS-1 phosphorylation in IR-A and IR-B-expressing cells.
Serum-starved R IR-A (A) or R IR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and immunoprecipitated with an anti-IRS-1 antibody as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of three independent experiments ± S.E.M (IB: PY20/total IRS-1). Middle panel, antiphosphotyrosine blot (PY20 antibody), representative result of three independent experiments. Lower panel, reblotting with anti-IRS-1 antibody. (A) IR-A: IGF-II vs IGF-I p<0.05.
5.4.6 Phosphorylation of IRS-2 by IR-A and IR-B activated by insulin, IGF-I, IGF-II, and IGF chimeras

The phosphorylation of Y960 also provides a docking site for another IRS protein, IRS-2, via its SH2 domain (Sun et al., 1995). However, IRS-2 has also been shown to bind to the insulin receptor via a non-SH2 mediated mechanism. The region of IRS-2 that mediates this novel receptor interaction includes residues 591-786 and this region is not conserved in IRS-1 (Sawka-Verhelle et al., 1996). The interaction of this region with the insulin receptor requires a functional receptor kinase and the phosphorylation of the three tyrosines in the catalytic loop (Sawka-Verhelle et al., 1996). The ability of insulin, IGF-II, IGF-I and IGF C domain chimeras to induce IRS-2 phosphorylation in R'IR-A and R'IR-B cells was investigated.

Insulin stimulated robust phosphorylation of IRS-2 after 5 minutes, however IGF-II was 2-fold more potent than insulin at inducing IRS-2 after 5 minutes in R'IR-A cells (Figure 5.5 A). This is contrary to the relative potencies of insulin and IGF-II for inducing 3Y, Y960 and IRS-1 phosphorylation. Strikingly, despite its poor activation of the IR-A, IGF-I was as potent as insulin at inducing IRS-2 phosphorylation after 5 minutes. While, these results represent stimulation only after 5 minutes, similar relative IRS-2 phosphorylation was induced by insulin, IGF-II and IGF-I over a time course of 60 minutes (C.T. Roberts Jr. personal communication). IGF-I CII, unlike for IRS-1 phosphorylation, did not exhibit the same ability as IGF-II to activate IRS-2 phosphorylation (although the difference was not statistically significant, IGF-II vs IGF-I CII: p=0.056). Interestingly, IGF-II CII was not significantly different from IGF-I at inducing IRS-2 phosphorylation in R'IR-A cells (IGF-I vs IGF-II CII: p>0.5).
A similar trend was observed in the relative abilities of insulin, IGF-II and IGF-I to induce IRS-2 phosphorylation in R'IR-B cells. However, in RIR-B cells IGF-I CII was equipotent as IGF-II at inducing IRS-2 phosphorylation (Figure 5.5 B).

Figure 5.5 IRS-2 phosphorylation in IR-A and IR-B-expressing cells.
Serum-starved RIR-A (A) or RIR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and immunoprecipitated with an anti-IRS-2 antobody as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of three independent experiments ± S.E.M (IB: PY20/total IRS-2). Middle panel, antiphosphotyrosine blot (PY20 antibody), representative result of three independent experiments. Lower panel, reblotting with anti-IRS-2 antibody.
5.4.7 Activation of Akt/PKB by insulin, IGF-II, IGF-I, and IGF chimeras in R1R-A and R1R-B cells

As shown in Figure 5.6, insulin strongly activated Akt/PKB, whereas equal concentrations of IGF-II and IGF-I induced Akt/PKB phosphorylation to only 30% and 11% that of insulin, respectively. Interestingly, all chimeras containing the IGF-II C domain (IGF-I CIIIDII, IGF-I CII, and IGF-II DI) stimulated Akt/PKB phosphorylation to the same level as IGF-II, while all chimeras containing the IGF-I C domain (IGF-II CIDI, IGF-II CI, and IGF-I DII) were all as poor as IGF-I at stimulating Akt/PKB phosphorylation in R1R-A cells. These results highlight the importance of the IGF-I and IGF-II C domain in determining the signalling properties of the IGFs.

In R1R-B cells, insulin also stimulated Akt/PKB phosphorylation, and IGF-II and IGF-I were 30% and 15% as potent as insulin, respectively. The IGF-I CIIIDII and IGF-I CII chimeras both induced slightly higher phosphorylation of Akt/PKB over that induced by IGF-II, whereas IGF-IDII was equally as potent as IGF-II, unlike the case in R1R-A cells. IGF-II CIDI and IGF-II CI stimulated Akt/PKB phosphorylation to the same level as IGF-I in R1R-B cells.
Figure 5.6 PKB/Akt phosphorylation in RIR-A and RIR-B cells stimulated with insulin, IGF-II, IGF-I, or IGF chimeras.

Serum-starved RIR-A (A) or RIR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and subjected to SDS-PAGE and then immunoblotted for phosphorylated Akt (Ser473) as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of the three independent experiments ± S.E.M (phospho-Akt/total Akt). Middle panel, anti-phospho Akt (Ser473) antibody blot showing a representative result of three independent experiments. Lower panel, reblotting with anti-PKB/Akt antibody.
5.4.8 Activation of Erk1/2 by insulin, IGF-II, IGF-I, and IGF chimeras in R’IR-A and R’IR-B cells

As shown in Figure 5.7, in R-IR-A cells, insulin was the only ligand that caused a significant increase in Erk1/2 phosphorylation over basal. No ligand in the R’IR-B cells induced Erk1/2 phosphorylation over basal. After stimulation of R’IR-B cells with any ligand the level of Erk1/2 phosphorylation had decreased below basal levels.
Figure 5.7 Erk1/2 phosphorylation in RIR-A and RIR-B cells stimulated by insulin, IGF-II, IGF-I, and IGF chimeras.

Serum-starved RIR-A (A) or RIR-B (B) cells were treated with 10 nM ligand for 5 minutes. Whole-cell lysates were prepared and subjected to SDS-PAGE and then immunoblotted for phosphorylated Erk1/2 as described in sections 5.3.2 and 5.3.3. In both (A) and (B): Upper panel, densitometry results of three independent experiments ± S.E.M (phospho-Erk1/2/ total Erk1/2). Middle panel, anti-phospho Erk1/2 antibody blot showing a representative result of three independent experiments. Lower panel, reblotting with anti-Erk1/2 antibody.
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5.4.9 R'IR-A cell protection from sodium butyrate-induced apoptosis by Insulin, IGF-II, IGF-I, and IGF chimeras.

The ability of IGF-I, IGF-II, insulin, and IGF chimeras to rescue R'IR-A cells from sodium butyrate induced apoptosis is presented in Figure 5.8. The ability of IGF-I and insulin to promote R'IR-A cell survival not only reflected the ability of these ligands to bind and activate the IR-A (chapter 4)(Denley et al., 2004), but also their ability to activate downstream signaling molecules. Interestingly, despite IGF-II's decreased ability to stimulate downstream signaling molecules relative to insulin, IGF-II was as potent as insulin at stimulating R'IR-A cell survival (p>0.5). IGF-II CI was as potent as IGF-I at promoting R'IR-A cell survival resulted (p>0.5). Likewise, IGF-I CII was as potent as IGF-II at stimulating R'IR-A cell survival (p>0.05). The results shown here suggest that the C-domain of the IGFs not only account for the differential recruitment and stimulation of downstream signaling molecules by IGF-I and IGF-II via the IR-A but also account for their differential ability to promote cell survival through the IR-A.
Figure 5.8 Effect of insulin, IGF-II, IGF-I, and IGF C domain chimeras on survival of butyrate-treated RIR-A cells. (A) Butyrate (5 mM) treated RIR-A cells were incubated in the presence or absence of increasing concentrations of insulin, IGF-I, IGF-II, or IGF chimeras as described in Materials and Methods. Results are expressed as a percentage of cell survival to 200 nM insulin, and the data points are means ± SEM of duplicate samples from three independent experiments conducted on separate occasions. Errors are shown when greater than the size of the symbols. In panel A, the ligands are as follows: insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (⊙); IGF-II CI (●). (B) Depicts a comparison of the ability of 10 nM ligand to stimulate RIR-A cell survival relative to serum-free media and 5 mM butyrate controls.
5.4.10 Insulin, IGF-I, IGF-II, and IGF chimera stimulated chemotaxis of RIR-A cells.

The data showing the ability of insulin, IGF-I, IGF-II, and IGF chimeras to stimulate chemotaxis of R-IRA cells are presented in Figure 5.9. IGF-II stimulated RIR-A chemotaxis to a greater extent than IGF-I. Exchange of the IGF C domains demonstrated that the C domain alone was sufficient to account for the differential ability of the IGFs to stimulate chemotaxis via the IR-A.

Figure 5.9 Insulin, IGF-I, IGF-II, and IGF chimera stimulated chemotaxis of R-IR-A cells
RIR-A cell chemotaxis in the presence of increasing concentrations of insulin, IGF-I, IGF-II, or IGF chimeras as described in Materials and Methods. Results are expressed as a percentage maximal cell migration to 10 nM insulin above that of basal, and the data points are means ± SEM of triplicate samples from three independent experiments conducted on separate occasions.
5.5 DISCUSSION

The binding, activation, and signaling of IGF-II via the IR-A is an important growth and migratory stimulus. Thus, the design of therapeutics for cancer treatment based on inhibiting IGF signaling through the IGF-IR may not provide a complete ablation of all IGF action if the IR-A is present. In addition, in a cell expressing both the IGF-IR and IR-A, inhibition of IGF signalling through the IGF-IR may select for cells that can gain a survival advantage through IR-A signaling. In this study, we have investigated signaling and biological outcomes in cells expressing the IR-A or the IR-B following activation by either insulin, IGF-II, IGF-I and IGF chimeras.

The relative abilities of insulin, IGF-II, and IGF-I to induce autophosphorylation of tyrosines 1158, 1162, 1163 in the activation loop of the kinase domain and tyrosine 960 in the juxtamembrane domain on the IR-A is proportional to their relative receptor binding affinities. Phosphorylation of the three tyrosines in the activation loop causes the loop to undergo a conformational change thereby allowing access of both ATP and protein substrates to the kinase catalytic site. The phosphorylation of the three tyrosines in the activation loop not only relieves autoinhibitory pressure on the kinase domain, but also provides docking sites for the adaptor molecules APS and SH2B (Moodie et al., 1999), which connect the IR with the CAP/Cbl pathway important for glucose uptake in adipocytes (Liu et al., 2002). Whether IGF-II or IGF-I activates APS recruitment to the IR-A or IR-B and consequently results in GLUT-4 translocation and glucose uptake is not known; however, it would be expected, based on receptor binding affinities, that IGF-II would be substantially more potent than IGF-I at triggering APS recruitment.

The ability of IGF-I CII and IGF-II CI chimeras to induce autophosphorylation of Y960 was investigated over a 60-minute time course. At each time point throughout the 60 minutes, IGF-I CII was equal to IGF-II in stimulating phosphorylation of Y960,
while IGF-II CI stimulated Y960 phosphorylation to the same extent as IGF-I at each time point. These results show that the kinetics of Y960 phosphorylation by IGF-I CII and IGF-II are the same, and that those with IGF-II CI and IGF-I are the same. Several reports have shown that certain mutations in insulin can decrease the dissociation rates of receptor binding, which can prolong receptor activation and alter biological outcomes (Hansen et al., 1996). As exchanging the C domains completely exchanged the Y960 phosphorylation kinetics, this further validates that creation of the chimeras has not changed the overall tertiary structure and also confirms that they are appropriate tools in probing the characteristic biological outcomes of IGF-I and IGF-II via the IR-A and IR-B. Most importantly, these results demonstrate that the C domain alone is sufficient to confer the differential abilities of IGF-I and IGF-II to activate IR-A.

Autophosphorylation of the IR on other sites provide avenues for signalling molecule recruitment. The IR and the IGF-IR rely heavily on the phosphorylation of docking proteins rather than autophosphorylation to enable recruitment and activation of downstream signalling molecules (Schlessinger, 2000; Siddle et al., 2001). One such docking protein, IRS-1, is a major adaptor molecule phosphorylated by the IR, and phosphorylated tyrosines on IRS-1 can then provide binding sites for a number of SH2 domain-containing proteins, including Grb-2 (Skolnik et al., 1993), which links the IR to ras (Skolnik et al., 1993) and the p85 regulatory subunit of PI3K (Myers et al., 1992), which then links the IR to the serine threonine kinase Akt/PKB (Cross et al., 1995). For that reason, the ability of insulin, IGF-II, IGF-I, and IGF chimeras to induce phosphorylation of IRS-1 was compared. IRS-1 was potently phosphorylated by insulin activated IR-A and IR-B. IGF-II was less potent than insulin at inducing IRS-1 phosphorylation in R'IR-A cells, in line with previous results (Frasca et al., 1999), but substantially better than IGF-I. In R'IR-B cells, IGF-II and IGF-I were
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almost as potent as each other in inducing IRS-1 phosphorylation. Stimulation of R'IR-A cells with IGF-I CII resulted in the same level of IRS-1 phosphorylation as IGF-II, while IGF-II CI stimulation caused the phosphorylation of IRS-1 to the same extent as IGF-I. These results suggest that the C domain not only mediates to a large extent the differential ability of IGF-II and IGF-I to bind and completely accounts for the differential ability to induce autophosphorylation on specific tyrosines in the cytoplasmic domain of the IR-A, but also accounts for each IGFs characteristic recruitment and stimulation of IRS-1 by the IR-A.

The interaction of IRS-2 with the IR is different to the interaction of IRS-1 with the IR. While IRS-2 interacts with the IR via an interaction with its SH2 domain and the phosphorylated Y960 in the IR juxtamembrane region (Sun et al., 1995), in an analogous mechanism to IRS-1, a novel interaction between residues 591-786 of IRS-2 and the IR has been identified using yeast two-hybrid analysis. This non-SH2 mediated interaction with the IR appeared more critical for the interaction with the IR (Sawka-Verhelle et al., 1996). In a separate study, mutation of Y960 to alanine did not affect the ability of the IR to phosphorylate IRS-2, but severely impaired its ability to phosphorylate IRS-1 (Chaika et al., 1999). In light of these studies, the level of IRS-2 phosphorylation was investigated when the IR-A and IR-B was activated by insulin, IGF-II, IGF-I and IGF C domain chimeras. The results showed that even though both IGF-I and IGF-II CI do not induce high levels of Y960 phosphorylation in the IR-A or Y972 in the IR-B, they both induced IRS-2 phosphorylation to a level equivalent to that stimulated by insulin. The binding of IGF-I and IGF-II CI to the IR may induce a conformational change that does not cause high levels of Y960 phosphorylation but does recruit IRS-2 via the non-SH-2 mechanism, allowing it to be phosphorylated.

The phosphorylation of Akt/PKB was robust with insulin, IGF-II, and every IGF chimera containing the IGF-II C domain. We show that IGF-II, due to its C
domain, can potently regulate the kinase that phosphorylates Ser473 on Akt via both the IR-A and IR-B. Interestingly, in both the RIR-A and RIR-B cells, Erk1/2 phosphorylation was not increased over basal by any ligand other than insulin, which itself only induced a small 3-fold increase. Previous studies into IGF activation of Erk via the IR-A have shown that IGF-II, but not IGF-I, can induce Erk1/2 activation above basal (Frasca et al., 1999; Pandini et al., 2002). In these studies, the IR-A was over-expressed to an approximate level of 500,000 receptors per cell (Frasca et al., 1999; Pandini et al., 2002), whereas the cells used here express only 75,000 receptors per cell. This could account for the difference in previous work and our study of the ability of IGF-II to stimulate Erk1/2 phosphorylation via the IR-A. Interestingly, in RIR-B cells Erk1/2 was still not activated even though IGF-II as in RIR-A cells, can induce autophosphorylation of IR and activate IRS-1.

In all the studies reported here the anti-phosphotyrosine antibody PY20 was used to detect the phosphorylation state of IRS-1 and IRS-2. The affinity of PY20 is most likely not the same for each phosphorylated tyrosine, due to the influence of the surrounding amino acids. Hence, if a receptor ligand for example, induces potent phosphorylation of a tyrosine residue in an intracellular signaling protein that is surrounded by amino acids that reduce the affinity of the PY20, the overall effect is that it appears that the ligand does not induce phosphorylation of the signaling molecule. Given that IRS-1 and IRS-2 have over 20 potential phosphorylation sites the overall effect of the differential affinity of PY20 for certain phospho-tyrosine would be small.

The ability of IGFs to signal responses in two biological processes important to cancer progression, namely survival from apoptosis and cellular migration was examined. Shown here for the first time is that IGF signaling through the IR-A can protect cells from butyrate-induced apoptosis. Butyrate is a potent pro-apoptotic
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compound whose mechanism of action is not definitively determined, but which may reflect its ability to inhibit histone deacetylase activity. Previous studies have shown that treatment with IGF-II protected SKUT-1 cells (no IGF-1R and >95 % IR-A) from staurosporin induced apoptosis (Sciacca et al., 2002). This study along with the results presented here suggest that IGF-II signalling through the IR-A can protect cells from various apoptotic agents whose mechanism of action are different. IGF-II was able to promote R'IR-A cell survival from butyrate-induced apoptosis to a significantly greater extent than IGF-I. This trend was also observed in the R'IR-A cell migratory response to these ligands. In both cell survival and migration, the exchange of the IGF C domains accounted for the differential ability of the IGFs to stimulate biological responses via the IR-A.

Interestingly despite IGF-II having a lower affinity for the IR-A relative to insulin and being substantially poorer at inducing phosphorylation of IRS-1 and Akt relative to insulin, IGF-II is as potent as insulin at protecting R'IR-A cells from butyrate induced apoptosis. Both insulin and IGF-II were equipotent at protecting SKUT-1 cells from staurosporin induced apoptosis (Sciacca et al., 2002). This highlights the importance of delineating exactly what signalling pathways are activated by either IGF-II or insulin via the IR-A. The ability of IGF-II to mediate cell survival and migration via the IR-A suggests a possible mechanism whereby cells can escape the growth restricting effects of IGF-1R inhibitors.

Results presented here provide novel insights into the mechanism of IGF action via the IR and more generally the dissociation between ligand binding and receptor signalling. In the binding studies detailed in chapter 4, exchanging the C domain alone between IGF-I and IGF-II was insufficient to completely exchange the IR-A or IR-B binding affinities of the IGFs. Swapping both the C and D domain was a requirement for complete exchange of the binding specificity for the IR-A, as well as the IR-B.
However, shown in this chapter is that substitution of only the IGF-I C domain for that of IGF-II now allows the chimera to activate specific tyrosines on the IR, IRS-1 and PKB/Akt to the same extent as IGF-II. It is possible that the C domain of IGF-II, while not accounting for the entire difference in the free energy of IR-A binding by IGF-II compared to IGF-I, can induce a conformational change in the receptor characteristic of authentic IGF-II. The C domain of the IGFs that encompass a relatively small number of amino acids compared to the whole molecule can determine the relative IR-A activation, signalling, and, ultimately, the biological response of the IGFs.

Inhibition of the IGF-1R signalling is being investigated as a potential target for cancer therapy (Zhang and Yee, 2004). Small molecule inhibitors of the IGF-1R that do not inhibit the IR kinase have been shown in mouse models to be effective at reducing tumour formation and growth (Garcia-Echeverria et al., 2004; Mitsiades et al., 2004). The presence of the IR-A on tumour cells may reduce the efficacy specific IGF-1R therapies, by providing an avenue for cell survival and proliferation, especially as many tumour cells overexpress IGF-II (Gicquel et al., 1994; Quinn et al., 1996; Renehan et al., 2000). Overcoming this potential problem by inhibiting the IR is also not advisable as reducing IR signalling may affect glucose metabolism. Specifically inhibiting IGF-II action is an attractive strategy that would prevent its action via both the IGF-1R and IR-A but allowing insulin to signal unaffected through the IR. Supporting the validity of this approach is the finding that a phage-displayed peptide, isolated against IGF-I, inhibits IGF-I binding to both the IGF-1R and IR, although the effect on insulin signalling via the IR in the presence of the peptide was not reported (Deshayes et al., 2002). The results presented in this chapter show that the IGF-II C domain, of which there is no analogous region in insulin, is critical for signalling, cell survival and migration induced by IGF-II via the IR-A. The IGF-II C domain therefore
provides a potential site for design of specific inhibitors of IGF-II, and possibly IGF-I, binding to the IR and IGF-1R.

This work presented here provides novel insights into the biological response of IGF ligand-receptor interactions and has ramifications for the production inhibitors of IGF-1R as therapies.
Binding and activation of the human IGF-1R by insulin, IGF-II, IGF-I and IGF chimeras
6.1 INTRODUCTION

In Chapter 4 IGF chimeras revealed that the difference in the binding affinity of IGF-II and IGF-I for the IR-A and IR-B was due to their C and D domains. While the complete structures of the IR and the IGF type 1 receptor (IGF-1R) have not been determined, the structure of the first three domains (L1-cys-rich-L2) of the IGF-1R has been solved (Garrett et al., 1998). The L domains resemble other leucine-rich repeat proteins and consist of a single-stranded, right-hand parallel β helix (Ward and Garrett, 2001), while the cys-rich region is composed of eight disulphide-bonded modules and resembles the cys-rich repeats in laminin. These three domains surround a cavity large enough to accommodate IGF-I (Garrett et al., 1998). Sequence analysis and limited structural information suggests that both IR and IGF-1R are very similar. It is therefore not surprising that all three growth factors, insulin, IGF-I and IGF-II, can interact with both IR and IGF-1R albeit with different affinities to initiate signalling cascades. Therefore in this chapter the question was asked: If the C and D domains regulate the IR isoform binding specificity of the IGFs, do these same regions account for the difference in IGF-1R binding affinity of IGF-I and IGF-II?

The insulin-like growth factor I receptor (IGF-1R) is a transmembrane tyrosine kinase receptor that mediates the growth promoting, differentiating and migratory properties of IGF-I and IGF-II (Adams et al., 2000). Knockout of the IGF-1R in mice results in a severe growth restricted phenotype and death immediately after birth due to respiratory failure (Liu et al., 1993). Moreover, double knockout studies in mice have shown that during development the growth promoting effects of IGF-I are solely mediated by the IGF-1R (Baker et al., 1993; Liu et al., 1993).

Mutational studies have highlighted residues Ala8, Val11, Phe23, Tyr24, Tyr31, Arg36, Arg37, Arg56, Met59, Tyr60, Lys65 and Lys68 as critical for IGF-1R binding (Figure 1.9). Most studies have involved either mutating a particular residue in
IGF-I to alanine or the amino acid found in the corresponding position in insulin. To date there are no reports of IGF-I mutants incorporating unique residues from IGF-II, with a view to determining which amino acids underlie the affinity difference between IGF-I and IGF-II binding to the IGF-1R.

IGF-I binds to the IGF-1R with a 4 fold higher affinity than IGF-II (Hodgson et al., 1995). Given the high degree of sequence and structural homology between the IR and IGF-1R it was hypothesized that the C and D domains, while determining the IR binding specificity of the IGFs, may also determine the IGF-1R binding specificity of IGF-I and IGF-II.

Recently alanine scanning mutagenesis of the IGF-1R revealed that the cys rich domain, critical for IGF-I binding, appeared to make a minimal contribution to the binding of IGF-II (Sorensen et al., 2004). This finding suggested that IGF-I and IGF-II use different mechanisms of receptor binding.
6.2 MATERIALS

P6 cells (BALB/c3T3 cells overexpressing the human IGF-1R) (Pietrzkowski et al., 1992) were a kind gift from Prof. R. Baserga (Philadelphia, USA). The anti-IGF-1R antibody 24-31 was a kind gift from Prof. K. Siddle (Cambridge, U.K.). All other materials used are detailed in the Materials Section of Chapter 4.

6.3 METHODS

All methods are reported in the Methods section of Chapter 4, however the following changes apply: Eu-IGF-I was used instead of Eu-Insulin and P6 cells were used as a source of IGF-1R for binding and phosphorylation studies. Eu-IGF-I was prepared under identical conditions to those used to create Eu-insulin and 24-31 was used to capture the solubilized IGF-1R (section 4.3.2).
6.4 RESULTS

6.4.1 Binding of insulin, IGF-II, IGF-I and IGF chimeras to the IGF-1R

The competition binding curves for insulin, IGF-I, IGF-II and the six chimeras with IGF-1R are shown in Figure 6.1 with the IC₅₀ values and relative binding affinities compared to IGF-I listed in Table 6.1. The data show that IGF-I has the highest affinity for the IGF-1R while insulin bound very poorly (1000 fold lower affinity). The affinity of IGF-II for the IGF-1R was only 18% that of IGF-I and the single chimeras all fell within that range (Table 6.1). Replacing the C domain of IGF-I with that of IGF-II reduced the binding affinity for IGF-1R by 75% to a value only slightly higher than that of IGF-II, whereas replacing the D domain of IGF-I caused no reduction in affinity (Table 6.1). Replacing both the C and D domains of IGF-I with those of IGF-II resulted in a chimera that had a lower binding affinity for the IGF-1R than IGF-II (Table 6.1). Conversely incorporating either the C or D domains of IGF-I into IGF-II resulted in proteins that were more IGF-I like. In these chimeras (IGF-II CI and IGF-II CIDI), however, the effects of exchanging either the C domain or the D domain were not significantly different, their binding affinities for IGF-1R being 2.9 and 2.6 fold higher than that of IGF-II respectively (Table 6.1). The binding affinity of the double chimera, IGF-II CIDI was further improved with an affinity that was closer to that of IGF-I (Figure 6.1B). A summary of IGF-1R binding by all IGF chimeras is shown in Figure 6.3.
Figure 6.1 Competition binding curves of Eu-IGF-I binding to immunopurified human IGF-1R.

Immunocaptured IGF-1R was incubated with Eu-IGF-I in the presence or absence of increasing concentrations of IGF-I, IGF-II, insulin or IGF chimeras as described in section 6.3 (see also section 4.3.2). The graphs shown are a representative of three experiments. A, competition for binding to IGF-1R by IGF-I, IGF-II, insulin and IGF single chimeras; B, competition for binding to the IGF-1R by IGF-I, IGF-II, Insulin and IGF double chimeras. Results are expressed as a percentage of Eu-IGF-I bound in the absence of competing ligand and the data points are means ± S.E.M. of triplicate samples. Errors are shown when greater than the size of the symbols. The ligands are as follows in A, insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); IGF-II DI (◆). Ligands in B, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); IGF-II CIDI (■).
CHAPTER 6 – Binding of IGF-I, IGF-II, insulin and IGF chimeras to the human IGF-1R

<table>
<thead>
<tr>
<th>Ligand</th>
<th>IC$_{50}$ (nM)</th>
<th>IC$_{50}$ Rel. IGF-I (%)</th>
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<tbody>
<tr>
<td>Insulin</td>
<td>&gt;100</td>
<td>&lt;1</td>
</tr>
<tr>
<td>IGF-I</td>
<td>0.8 ± 0.2</td>
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</tr>
<tr>
<td>IGF-I DII</td>
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<tr>
<td>IGF-I CII</td>
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<tr>
<td>IGF-I CIIDII</td>
<td>7.4 ± 2.6</td>
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<tr>
<td>IGF-II</td>
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</tr>
<tr>
<td>IGF-II DI</td>
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<td>47</td>
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<tr>
<td>IGF-II CI</td>
<td>1.5 ± 0.5</td>
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</tr>
<tr>
<td>IGF-II CIDI</td>
<td>1.1 ± 0.3</td>
<td>73</td>
</tr>
</tbody>
</table>

Table 6.1 Inhibition of europium-labelled IGF-I for binding to the IGF-1R by insulin, IGF-I, IGF-II and IGF chimeras. The IC$_{50}$ relative to that of IGF-I is also shown. Values are the means and ± S.E.M. from three independent experiments.

6.4.2 Phosphorylation of the IGF-1R by stimulation with chimeric IGFs

The activation of the human IGF-1R by insulin, IGF-I, IGF-II and the six chimeras (Figures 6.2A and 6.2B) mirrored the relative binding affinities. The EC$_{50}$ for IGF-I activation of the IGF-1R was 3.9 nM ± 0.43 nM. At that same concentration, IGF-II induced phosphorylation to only 35 % that of IGF-I (Figure 6.2A). Replacing the D domain of IGF-I with that of IGF-II had a negligible effect on IGF-1R phosphorylation (IGF-I DII: 95 % relative to IGF-I), whereas replacing the C domain had a dramatic effect and reduced the potency of this IGF-I based chimera to that of IGF-II (IGF-I CII: 46 % relative of IGF-I). The double chimera, where both the C and D domains of IGF-I were replaced by those of IGF-II was even poorer than IGF-II at inducing IGF-1R phosphorylation (IGF-I CIIDII: 30 % relative to IGF-I), reflecting the additive effects of these substitutions on IGF-1R binding (Table 6.1). In contrast, in the IGF-II based chimeras, the differential effects on
Figure 6.2 Activation of the human IGF-1R by IGF-I, IGF-II, insulin or IGF chimeras.

P6 cells overexpressing the human IGF-1R were serum starved for 4 hours followed by stimulation with various concentrations of either IGF-I, IGF-II, insulin or IGF chimeras for 10 minutes. Cells were lysed with ice cold lysis buffer containing phosphatase inhibitors and activated receptors were immunocaptured with the anti-IGF-1R antibody 24-31 as described in section 4.3.3. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY20 to detect phosphorylated tyrosines. A, IGF-1R phosphorylation by insulin, IGF-I, IGF-II and IGF single chimeras. B, IGF-1R phosphorylation by insulin, IGF-I, IGF-II and IGF double chimeras. The graphs shown are a representative of three experiments and data points are means ± S.E.M. of triplicate points. Errors are shown when greater than the size of symbols. The ligands are as follows in A, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CII (○); IGF-I DII (◇); IGF-II CI (●); IGF-II DI (◆). B, Insulin (▼); IGF-II (▲); IGF-I (△); IGF-I CIIDII (□); IGF-II CIDI (■).
phosphorylation were greater than those seen in the binding studies. There was little difference in the increase in IGF-1R binding affinity, relative to IGF-I, between the IGF-II CI (53%) and IGF-II DI (47%) chimeras (Table 6.1), however the difference was larger in their ability to stimulate phosphorylation relative to IGF-I (IGF-II CI: 72% vs IGF-II DI: 40% at EC₅₀ concentration of IGF-I). The importance of the IGF-I C domain is further illustrated by the fact that the double chimera IGF-II CIDI was only slightly more potent than the single IGF-II CI chimera (IGF-II CIDI: 73% relative to IGF-I). These results illustrate the dominant contribution of the IGF-I C domain to IGF-1R activation.
In this chapter the IGF-1R binding specificity of the IGFs was investigated. As described in the IR study in chapter 4, the B and A domains make critical contributions to the free energy of IGF-1R binding (Hodgson et al., 1995; Hodgson et al., 1996) and are also involved in determining in part the different affinities of IGF-I for the IGF-1R and IR (Hodgson et al., 1995; Shooter et al., 1996). However, the results show that the C and D domains represent the principle determinants of the difference in IGF-1R binding affinity between IGF-I and IGF-II.

There have been extensive mutagenesis and deletion studies of IGF-I and IGF-II with interest in determining what residues are critical for IGF-1R (Cascieri et al., 1988; Bayne et al., 1989; Bayne et al., 1990; Cara et al., 1990; Roth et al., 1991; King et al., 1992; Zhang et al., 1994; Hodgson et al., 1995; Bryant et al., 1996; Hodgson et al., 1996; Shooter et al., 1996; Jansson et al., 1997; Jansson et al., 1997; Jansson et al., 1998) and IGFBP binding (Bayne et al., 1988; Cascieri et al., 1989; Clemmons et al., 1992; Oh et al., 1993; Jansson et al., 1997; Jansson et al., 1998; Dubaquie and Lowman, 1999; Magee et al., 1999). In addition using phage display, alanine scanning mutagenesis of the entire IGF-I protein has been completed revealing residues important in IGFBP-1 and IGFBP-3 binding (Dubaquie and Lowman, 1999). The IGF-1R binding ability of these phage displayed IGF-I alanine mutants has not been reported. Despite all these studies the molecular basis for the difference in IGF-1R affinity between IGF-I and IGF-II is unknown.

As shown in Figure 6.1 and Table 6.1 in the results section the removal of the IGF-I C domain and its replacement with the IGF-II C domains causes a loss in binding that accounts for the vast majority of the affinity difference between IGF-I and IGF-II. Conversely, removal of the IGF-II C domain and its replacement with the IGF-I C domain results in a binding affinity only 2-fold lower than IGF-I. This
suggests that the B and A domains of IGF-I and IGF-II also make different contacts with the IGF-1R, as the changes in affinity resulting from the exchanges of the C and D domains depend on the B and A domain context of either IGF-I or IGF-II.

When exchanged together, the IGF-II C and D domains (IGF-I CIIDII) cause a more discordant change in IGF-1R binding affinity than would be expected from their singular domain exchanges (IGF-I DII and IGF-I CII). The same can be said for the effect of swapping the IGF-I C and D domains together, which results in an affinity only slightly higher than swapping either the C or D domains individually. When the IGF-I C domain is present neither the IGF-I or IGF-II D domains appear to influence the IGF-1R affinity (IGF-I 0.8 ± 0.2 nM vs IGF-I DII 0.7 ± 0.2 nM, IGF-II CIDI 1.1 ± 0.3 nM vs IGF-II CI 1.5 ± 0.5 nM). However, if the IGF-II C domain is present then the D domain does play a role (IGF-II DI 1.7 ± 0.5 nM vs IGF-II 4.4 ± 1.1 nM and IGF-I CIIDII 7.4 ± 2.6 nM vs IGF-I CII 3.2 ± 1.4 nM). The IGF C domains may put constraints on productive IGF-1R binding and heavily influence not only the differences in IGF-1R binding affinity between IGF-I and IGF-II but also the mechanism that IGF-I and IGF-II use to interact with the IGF-1R. Supporting this observation are the reports that the deletion of the IGF-I D domain has no effect on IGF-1R binding (Bayne et al., 1989) while removal of the IGF-II D domain causes a 5.6 fold reduction in IGF-1R binding (Roth et al., 1991). Alanine scanning mutagenesis studies of the IGF-1R show that certain receptor side chains are differentially involved in IGF-I and IGF-II binding (Whittaker et al., 2001; Sorensen et al., 2004), further supporting the notion that IGF-I and IGF-II use different mechanisms to bind to the IGF-1R.
Figure 6.3 Summary of relative IR-A, IR-B and IGF-1R binding of Insulin, IGF-I, IGF-II and IGF chimeras.

In the upper section of the histogram, affinities of all ligands for the IR-A as a relative % of IGF-II binding are shown in dark grey bars. Affinities of ligands binding to the IR-B relative to IGF-II binding to the IR-A is shown in open bars. In the lower section affinities of all ligands for the IGF-1R as a relative % IGF-I binding are shown in hatched bars. Standard errors are not shown, however for ligands binding to IR-A standard errors are between 3.6% and 43%, for IR-B between 4% and 40% and for ligands binding to the IGF-1R are between 25% and 44%, absolute values are listed in Tables 4.2 and 6.1.

The role that the C and D domains of IGF-I and IGF-II play in IGF-1R binding is opposite to that seen in IR binding. Why the C and D domains of IGF-I allow higher IGF-1R affinity compared to the same domains of IGF-II is not known. Residues 253-266 in the Cys-rich domain, of the IGF-1R comprise a loop that protrudes into the putative ligand binding region, and when substituted for the analogous region in the IR, allows IGF-I to displace $^{125}$I-insulin with 10 fold higher affinity (Hoyne et al., 2000). Compared to the IGF-1R loop, the IR loop is four residues larger and contains an additional disulphide bond (Garrett et al., 1998). The IR loop contains five basic
residues and one acidic residue (Garrett et al., 1998), whereas the corresponding IGF-1R loop has six acidic and one basic residue. Thus the increased affinity of IGF-I for the IR with the IGF-1R loop could be due to this smaller loop allowing the large IGF-I molecule to fit into the putative ligand binding pocket. Alternatively the change in net charge of the loop may remove electrostatic constraints on the binding event. Another receptor chimera study showed that the IGF-I C domain makes contact with the N-terminal 217-284 residues of the IGF-1R, which also contains the 253-266 loop, (Zhang et al., 1994) suggesting that the affinity conferred by the C domain of IGF-I and possibly of IGF-II is influenced by this loop. Interestingly IGF-1R residues phenylalanine 240, phenylalanine 241, glutamate 242 and phenylalanine 251 when mutated to alanine cause between a 2 and 6 fold reduction in IGF-I binding affinity (Whittaker et al., 2001), whereas they cause either no change or a slight increase (F241) in IGF-II binding affinity (Sorensen et al., 2004). This alanine-scanned region is directly adjacent to the 253-266 loop in three dimensional space, further suggesting there are differential interactions with IGF-I and IGF-II, possibly by their C domains, with the Cys-rich domain of the IGF-1R. Cross-linking studies could be used to explore the interaction of the IGF-I C domain with the IGF-1R Cys-rich domain and apparent lack of contact between the IGF-II C domains and the IGF-1R Cys-rich domain.

In our laboratory antibodies against the IGF-1R have been generated by Mehrnaz Keyhanfar. The binding epitope of two of these antibodies have been mapped to the cysteine-rich domain using IGF-1R/IR chimeric receptors (Schumacher et al., 1993). Both of these antibodies have been shown to inhibit IGF-I but not IGF-II binding to the IGF-1R (M. Keyhanfar, personal communication). This result confirms the finding that alanine scanning mutagenesis of the IGF-1R cysteine rich domain affects IGF-I binding but not IGF-II binding. Interestingly, IGF-II CI binding to the
CHAPTER 6 - Binding of IGF-I, IGF-II, insulin and IGF chimeras to the human IGF-1R

IGF-1R is inhibited by both antibodies whereas IGF-I CII binding is not (M. Keyhanfar, personal communication). These results suggest that the IGF-I C domain may make contact with the IGF-1R cysteine rich domain and that regardless of the background (either IGF-I or IGF-II) the C domain directs the mechanism of IGF-1R binding.

Further evidence that the C and D domains are differentially involved in IR and IGF-1R binding is the finding that positively charged residues arginine 36, arginine 37, lysine 65 and lysine 68 that reside in the C or D domains of IGF-I respectively, when mutated to alanine exhibit a decrease in IGF-1R binding affinity but an increase in IR binding affinity (Zhang et al., 1994). Furthermore deletion of the IGF-I C domain and replacement with a four glycine linker resulted in a 40-100 fold reduction in IGF-1R affinity (Bayne et al., 1989; Gill et al., 1996) but a small increase in IR affinity (Bayne et al., 1989). As already mentioned using receptor chimeras Zhang et al. 1994 were able to show that the N-terminal 286 amino acids of the IGF-1R were responsible for favouring binding of the charged arginine 36 and arginine 37 in the IGF-I C domain (Zhang et al., 1994). Furthermore a two chain hybrid of des-octapeptide (B23-B30)-insulin with an extension from ArgB22 of the corresponding portion of the IGF-I B domain and the entire IGF-I C domain was analysed for its binding to IR/IGF-1R chimeras (Schaffer et al., 1993). The results showed that the hybrid ligand bound with higher affinity to a chimeric IR that contained residues 191-290 of the IGF-1R than to wildtype IR. The chimeras of IGF-I and IGF-II reported here provide the necessary ligands that along with receptor chimeras could complement these previous reports (Schaffer et al., 1993; Zhang et al., 1994). More specifically the binding of these IGF-I and IGF-II chimeras to alanine mutants of the IGF-1R (Whittaker et al., 2001) could define more accurately the receptor residues that make positive or negative contacts with the IGF-I and IGF-II C domain. In collaboration with Dr. Jonathon Whittaker
(Case Western Reserve School of Medicine, Cleveland), the ability of these IGF chimeras to bind to alanine mutants of the IGF-1R will be investigated in the future.

In summary, this chapter describes the structural basis that accounts for a large portion of the difference in affinity between IGF-I and IGF-II for the IGF-1R. The C domain is responsible for most of the IGF-1R affinity difference between IGF-I and IGF-II and totally accounts for the differential ability of the IGFs to activate the IGF-1R.
Binding of IGF-I, IGF-II and IGF chimeras to the IGF-2R and IGFBPs
Previously in Chapter 4 the 7-fold difference in binding affinity between IGF-II and IGF-I for the IR-A was shown to be due to their C and D domains. Also in that same Chapter, the 5-fold difference in binding affinity of IGF-II and IGF-I for the IR-B was shown to be also due to their C and D domains. As described in Chapter 6 the 5-fold difference in binding affinity between IGF-I and IGF-II for the IGF-1R was in part due to their C and D domains. In this chapter interactions with the type II IGF receptor (IGF-2R) and IGF-II preferring binding proteins are investigated.

7.1.1 Type II IGF receptor (IGF-2R)

The IGF-2R or cation independent mannose-6-phosphate receptor (CI-MPR) is a multi-functional receptor that binds a diverse array of ligands including mannose-6-phosphate containing proteins (Oshima et al., 1988), IGF-II (Morgan et al., 1987), TGF-β1 (Dennis and Rifkin, 1991), granzyme B (Motyka et al., 2000), urokinase-type plasminogen activator receptor (uPAR) (Kreiling et al., 2003), plasminogen (Godar et al., 1999) and retinoic acid (Kang et al., 1997). The 300 kDa receptor is made up of 15 extracellular repeating domains, with no sequence or structural homology to either the IR or the IGF-1R and an intracellular region with no intrinsic tyrosine kinase activity (Kornfeld, 1992). The function of the IGF-2R in the IGF system is suggested to be simply removal of IGF-II from the extracellular space. Supporting this hypothesis is that mice with a knockout of the IGF-2R have 135 % higher concentrations of circulating and tissue IGF-II than wildtype littermates (Ludwig et al., 1996) and consequently these mice suffer from a fetal overgrowth syndrome (Lau et al., 1994; Wang et al., 1994; Ludwig et al., 1996). Generally these knockout mice die perinatally but can be rescued by a knockout of either the IGF-1R or IGF-II (Ludwig et al., 1996).
CHAPTER 7 – Binding of IGF-I, IGF-II and IGF chimeras to the IGF-2R and IGFBPs

The crystal structure of domains 1-3 and domain 11 of the IGF-2R have been solved (Brown et al., 2002; Olson et al., 2004), while the extracellular portion of the closely related cation dependent mannose-6-phosphate receptor (CD-MPR) has been solved with and without bound carbohydrate (Roberts et al., 1998; Olson et al., 1999; Olson et al., 2002). The IGF-2R domain 11 is the primary binding site for IGF-II (Dahms et al., 1994; Garmroudi and MacDonald, 1994; Schmidt et al., 1995; Garmroudi et al., 1996; Byrd et al., 1999) with domain 13 serving to enhance the affinity (Devi et al., 1998). Mutation of isoleucine 1572 in domain 11 completely abolished IGF-II binding (Garmroudi et al., 1996). Domains 1-3 and 7-9 are the sites of interaction with mannosylated proteins (Westlund et al., 1991; Dahms et al., 1993). The IGF-2R binds IGF-I extremely poorly if at all (Lee et al., 1986; Ewton et al., 1987; Tong et al., 1988).

Until recently (Linnell et al., 2001; Brown et al., 2002) the interaction of IGF-II with the IGF-2R was investigated using IGF-2R competition studies with [125I]-IGF-II. This method does not allow real-time binding analysis and requires the used of radioactively labeled growth factor. In addition many of the early studies into IGF-2R binding utilized purified IGF-2R from placenta of other species e.g. rat (Sakano et al., 1991; Hashimoto et al., 1995) or bovine (Hashimoto et al., 1995).

The affinity of IGF-II for the IGF-2R has been determined using various fragments of the receptor (Devi et al., 1998; Linnell et al., 2001) and various biochemical techniques. The IC50 of IGF-II displacing [125I]IGF-II from domain 11-13 of the IGF-2R is 2.5 ± 0.6 nM (Devi et al., 1998). However, a real time kinetic study on the BIAcore of IGF-II binding to dom 11-13 gave a Kd 100 nM (Linnell et al., 2001). The affinity of domain 11 for IGF-II, determined by BIAcore analysis, has also varied in the literature with values of 450 nM (Linnell et al., 2001) and 41 nM reported (Brown et al., 2002). The discrepancies in the literature have prompted the current
kinetic analysis of IGF-II binding to the IGF-2R fragments of domain 11 and domain 11-13.

The aim of this work is to determine whether the C and D domains of IGF-I and IGF-II are involved in the binding of IGF-II to the IGF-2R. Given that IGF-I has almost no detectable affinity for the IGF-2R (Braulke, 1999) any gain or loss of binding seen when swapping a domain relates directly to a large part of the free energy of receptor binding.

The IGFBP binding site on IGF-II has not been fully characterised, however Phe\textsuperscript{48}, Arg\textsuperscript{49}, Ser\textsuperscript{50} and Ala\textsuperscript{54}, Leu\textsuperscript{55} have been shown by mutagenesis to be important for the interaction (Sakano et al., 1991; Forbes et al., 2001) (summarised in section 1.6.1). Modelling of the IGF-II/IGF2R interaction has suggested residues within the IGF-II C domain could also contact the IGFBP (Roche et al., 2004, manuscript in preparation). The IGFBP binding site on IGF-II partially overlaps with the site of IGFBP interaction (see introduction, Figure 1.27).

### 7.1.2 Insulin-like growth factor binding proteins (IGFBPs)

The IGFBPs are a family of proteins that share highly conserved sequences and structural similarity (reviewed in (Firth and Baxter, 2002)). They are multifunctional, but primarily act to regulate the bioavailability of the IGFs to the IGF-1R and IR. Some IGFBPs have preferences for either IGF-I or IGF-II, with IGFBP-6 having the largest IGF-II preference: a 60-100 fold higher affinity for IGF-II than IGF-I. To date it is not understood which residues of the IGFBP-6 and IGF-II are involved in this marked preference for IGF-II compared to IGF-I. IGFBP-2 exhibits a slight 2-fold higher affinity for IGF-II compared to IGF-I (Bach et al., 1993; Carrick et al., 2001). IGFBP-1 and -3 show similar affinities for both IGF-II and IGF-I (Kiefer et al., 1992; Bach et al., 1993; Oh et al., 1993).
Here we report the investigation of the IGF-2R and IGFBP binding properties of these IGF chimeras, with an interest in determining whether the same regions that determine “signal mediating” receptor (i.e. IGF-1R and IR) specificity also determine “regulating” receptor specificity (IGF-2R and IGFBPs).

Our results show a previously unexpected role for the IGF C domains in IGF-2R interactions. This C domain has traditionally been thought of as a non-canonical IGF-2R binding domain. However, we now show it is involved to a certain extent in regulating IGF binding specificity. In addition we show that the C domains of IGF-I and IGF-II play a small role in determining the large difference between the IGFs in binding to IGFBP-6.
7.2 MATERIALS

IGFBP-1, -2, -3 and -6 were purchased from GroPep Pty Ltd (Adelaide, Australia) or R & D systems (Minneapolis, U.S.A.). All materials, buffers and equipment used in the BIAcore analysis of IGF-2R and IGFBP interactions are already stated in section 3.2.

7.3 METHODS

7.3.1 PRODUCTION OF IGF-2R FRAGMENTS

Both the IGF-2R fragments, domain 11 (dom 11) and domain 11-13 (dom 11-13), were engineered and purified by Dr. James Brown (Structural Biology, Oxford University). IGF-2R dom 11 was prepared as described in (Brown et al., 2002). For IGF-2R dom 11-13, the required region of igf2r cDNA was amplified by PCR using appropriate primers and introducing a C-terminal carboxypeptidase A-cleavable histidine tag [Lys-(His)_6-STOP]. After cloning into the mammalian expression vector pEE14 (Davis et al., 1990), the construct was verified by sequencing and transfected into Lec3.2.8.1 Chinese hamster ovary cells (Stanley, 1981) using Pfx-8 lipids (Invitrogen, Paisley, U.K.). Following expression under methionine sulfoxamine selection, IGF-2R dom 11-13 was purified by immobilised metal ion affinity chromatography and gel filtration (Chelating Sepharose Fast Flow and HiLoad 16/60 Superdex 200; Amersham Biosciences).

7.3.2 BIACORE ANALYSES OF IGF-2R INTERACTIONS

Surface preparation – IGF-2R was coupled to the CM5 biosensor chip using a method similar to that previously described (Carrick et al., 2001). Briefly, the IGF-2R fragment to be coupled was prepared in 50 mM sodium acetate pH 4.0 at 2.5 µg/ml and injected over the activated chip surface in HBS running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4). The surfaces were
then deactivated by 1 M ethanolamine. A deactivated flow cell was left uncoupled as a reference on all chips. The IGF-2R domain 11-13 fragment was coupled to the biosensor surface to give a final resonance value of 2000 response units (RU) while IGF-2R domain 11 was coupled to a final value of ~250 RU and 85% of the IGF-2R domain 11-13 surface was active while 51% of the domain 11 surface was active.

**Kinetic assays of IGF-I, IGF-II and IGF chimera binding to IGF-2R fragments** – IGF-I, IGF-II and IGF chimeras were injected over the chip surfaces at the following concentrations: IGF-II, IGF-I, IGF-II DI, IGF-II CI at concentrations of 500 nM, 250 nM, 200 nM, 100 nM, 50 nM, 25 nM and 12.5 nM; IGF-I CII at 8 μM, 4 μM, 2 μM, 1 μM, 500 nM, 250 nM, 200 nM, 100 nM, 50 nM, 25 nM. Samples were injected in HBS running buffer for 5 min at a flow rate of 40 μl/min to minimize mass transfer effects. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by injection of 60 μl of 10 mM HCl. Reference flow cell data was subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data was analysed using the BIAevaluation 3.2 software. Models were fitted globally across all concentrations. IGF-2R domain 11 curves were fitted to a steady state affinity model whereas the IGF-2R domain 11-13 curves were fitted either to a steady state affinity model (IGF-II CI and IGF-I CII) or 1:1 Langmuir binding model (IGF-II and IGF-II DI). The Steady State model determines the affinity of the interaction at equilibrium. The 1:1 Langmuir binding model describes a simple reversible interaction of two molecules in a 1:1 complex.

**7.3.3 BIACORE ANALYSES OF IGFBP INTERACTIONS**

All methods were the same whether IGFBP-1, -2, -3 or -6 was used.
**Surface preparation:** Human IGFBP-1, -2, -3 or -6 was amine coupled to a CM5 chip and various concentrations of IGF-I, IGF-II or IGF chimera were injected across the surface in the HBS buffer. An uncoupled flow cell, acting as a reference surface was used to assess any change in refractive index due to change in buffers and to examine any non-specific binding of the analyte to the carboxymethylated dextran matrix. Briefly, each human IGFBP (2 μg) at 12.5 μg/ml in 50 mM sodium acetate pH 4.6 was coupled to a flow cell previously activated with EDC and NHS and blocked with ethanolamine, as described in (Lofas and Johnsson, 1990; Carrick et al., 2001). The resulting biosensor surface had 200 resonance units coupled as determined by the BIAcore surface preparation Wizard program.

**Kinetic assays of IGF-I, IGF-II and IGF chimera binding to either IGFBP-1, -2, -3 or -6:** IGF-I, IGF-II and IGF chimera were injected over the chip surfaces at the following concentrations: 200 nM, 100 nM, 50 nM, 25 nM and 12.5 nM in HBS running buffer for 5 min at a flow rate of 40 μl/min to minimize mass transfer effects. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by 60 μl of 10 mM HCl. Reference flow cell data was subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data was analysed using the BIAevaluation 3.2 software. Models were fitted globally across all concentrations. All IGFBP interactions were fitted to a two state conformational change model which describes the 1:1 interaction of analyte with bound ligand (A + B ↔ AB ↔ AB*). The 1:1 stoichiometry of IGF:IGFBP binding was determined by Bourner et al. (Bourner et al., 1992). While IGF binding to an IGFBP has not been experimentally shown to cause a conformational change several lines of evidence suggest the two state conformational change model is the most appropriate to analyse IGF-IGFBP kinetic interactions. Several previous studies
have used the two state conformational change model to analyse IGF-IGFBP interactions and this model yields the best fit to the experimental data shown by the lowest $\chi^2$ values. The IGFBPs have been shown to contain two IGF binding sites, one sites in each of the N- and C-terminal domains and both sites are required in distinct orientations for wildtype high affinity binding (Carrick et al., 2001; Payet et al., 2003; Yan et al., 2004).

7.4 RESULTS

7.4.1 Kinetic analysis of IGF-I, IGF-II and IGF chimeras binding to IGF-2R fragments

The binding affinities of human IGF-II to IGF-2R fragments domain 11 (3.8 x $10^6$ M$^{-1}$) and domain 11-13 (89.2 x $10^6$ M$^{-1}$) (Table 7.1) were similar to those previously described (Linnell et al., 2001). Strikingly, while IGF-I could not bind to the IGF-2R domain 11-13 at the concentrations tested, IGF-I CII could bind the IGF-2R with micromolar affinity (Figure 7.1 and Table 7.1). At the concentrations tested the binding of IGF-I CII to domain 11 was undetectable.

Substituting the IGF-II C domain with that of IGF-I, reduced IGF-2R binding to domain 11-13 to an affinity only 0.17 that of wildtype IGF-II (Figure 7.2). The binding of IGF-II CI to domain 11 alone was also reduced to an affinity only 0.3 relative to that of human IGF-II (Figure 7.2). Replacing the IGF-II D domain with that segment of IGF-I did not significantly affect binding to either IGF-2R fragment.
Figure 7.1 BIACore analysis of IGF-II and IGF-I CII binding to IGF-2R dom 11-13. Each protein was passed over an IGF-2R domain 11-13 flow cell at a flow rate of 40 μl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by 60 μl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data were analysed using the BIACore 3.2 software using either a 1:1 Langmuir binding model for IGF-II or a Steady State binding model for IGF-I CII. A representative experiment is shown. Kinetic analysis results are shown in Table 7.1. IGF-II (100 nM, dark green), IGF-I CII (500 nM, dark blue; 250 nM, pink; 200 nM, light green; 100 nM, light blue; 50 nM, grey and 25 nM, orange) or IGF-I (250 nM, black).
Figure 7.2 BIAcore analysis of IGF-II, IGF-II DI and IGF-II CI binding to IGF-2R dom 11-13 and IGF-2R dom 11. 100 nM of either IGF-II (green), IGF-II DI (red) or IGF-II CI (blue) was passed over IGF-2R dom 11-13 (A) or dom 11 (B) at a flow rate of 40 μl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated with 60 μl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data were analysed using the BIACore evaluation 3.2 software. The 1:1 Langmuir binding model was used to evaluate the binding of IGF-II and IGF-II DI to IGF-2R domain 11-13. The steady state binding model was used for all other evaluations. A representative experiment is shown. Kinetic analysis results are shown in Table 7.1.
CHAPTER 7 – Binding of IGF-I, IGF-II and IGF chimeras to the IGF-2R and IGFBPs

Table 7.1. Summary of kinetic analysis of IGF-2R fragment binding. The 1:1 Langmuir binding model was used to fit kinetic data and determine an association constant for IGF-II and IGF-II DI binding to IGF-2R dom 11-13. All other interactions were evaluated using the steady state binding model. The association constants shown are calculated from at least three independent experiments. All interactions were verified by analysis on two independently coupled chips. NB = no binding could be detected.

7.4.2 Kinetic analysis of IGF-I, IGF-II and IGF chimeras binding to IGFBP-1, -2, -3 and -6

The binding of IGF-I, IGF-II and IGF chimeras to human IGFBP-1, -2, -3 and -6 was investigated using BIAcore technology by coupling the BPs to the sensor surface. IGF-I and IGF-II had almost identical affinity for IGFBP-1 confirming previous reports (Oh et al., 1993)(Table 7.2 and Figure 7.3). All chimeras except IGF-II CI had affinities that were not statistically significantly different from either IGF-I or IGF-II. IGF-II CI had an affinity 70 % that of IGF-II. These results suggest that exchanging the C and D domains between IGF-I and IGF-II have not disrupted the
overall tertiary structure confirming the conclusion from the binding analysis of all chimeras to IGFBP-3 (section 3.4.9).

IGF-II bound with 2.2-fold higher affinity than IGF-I to IGFBP-2 (Table 7.3 and Figure 7.4). IGF-II DI had an identical affinity to IGF-II for IGFBP-2 while IGF-II CI had a 2-fold lower binding affinity for IGFBP-2 compared to IGF-II. The affinity of IGF-I CII for IGFBP-2 was similar to that of IGF-I, however IGF-I DII exhibited a 2-fold decrease in IGFBP-2 binding relative to IGF-I.

IGF-II bound to human IGFBP-6 with a 8-fold higher affinity than IGF-I (Table 7.4 and Figure 7.5). The affinity of IGF-II DI for IGFBP-6 was not different from that of IGF-II, while the affinity of IGF-II CI for IGFBP-6 was 2-fold lower than IGF-II. Similarly for chimeras in an IGF-I background, substitution of the IGF-II D domain did not affect the binding affinity while substituting the IGF-II C domain increased the affinity for binding IGFBP-6 by 2-fold. These results suggest that while the C domain does not completely account for the large difference in affinity of IGF-II and IGF-I for IGFBP-6, it may be playing a small role.
Figure 7.3 IGF-I, IGF-II and IGF chimeras binding to IGFBP-1. 50nM ligand (IGF-I, IGF-II or IGF chimera) was passed over a IGFBP-1 flow cell at a flow rate of 40 µl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated by 60 µl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data were analysed using the BIAevaluation 3.2 software. The 2 state conformational change model was used to evaluate the binding of all ligands to IGFBP-1. A representative experiment is shown. Kinetic analysis results are shown in Table 7.2.

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<th>$k_{d1}$ x 10^-7 1/s</th>
<th>$k_{a2}$ x 10^8 1/s</th>
<th>$k_{d2}$ x 10^-8 1/s</th>
<th>$K_A$ x 10^8 1/Ms</th>
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<th>Relative to IGF-II</th>
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<td>IGF-II</td>
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<td>1.39</td>
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Table 7.2 Summary of kinetic analysis of IGFBP-1 binding. The 2 state conformational change model was used to fit kinetic data and determine association constants for all proteins binding to IGFBP-1. The association constants shown are calculated from two separate runs on two independently coupled IGFBP-1 chips.
Figure 7.4 IGF-I, IGF-II and IGF chimeras binding to IGFBP-2. 50nM ligand (IGF-I, IGF-II or IGF chimera) was passed over a IGFBP-2 flow cell at a flow rate of 40 µl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated with 60 µl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data were analysed using the BIAevaluation 3.2 software. The 2 state conformational change model was used to evaluate the binding of all ligands to IGFBP-2. A representative experiment is shown. Kinetic analysis results are shown in Table 7.3.

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<th>$k_{d2}$ x 10^-4 1/s</th>
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<td>11.95 ± 1.6</td>
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<td>0.81</td>
<td>8.56</td>
<td>3.73 ± 0.3</td>
<td>0.68</td>
<td>0.31</td>
</tr>
<tr>
<td>IGF-I CI</td>
<td>10.0</td>
<td>4.73</td>
<td>1.2</td>
<td>6.67</td>
<td>5.86 ± 0.4</td>
<td>1.07</td>
<td>0.49</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>8.00</td>
<td>4.81</td>
<td>0.85</td>
<td>4.48</td>
<td>10.62 ± 1.4</td>
<td>1.94</td>
<td>0.89</td>
</tr>
<tr>
<td>IGF-II CI</td>
<td>5.47</td>
<td>5.45</td>
<td>0.78</td>
<td>6.15</td>
<td>6.55 ± 1.1</td>
<td>1.20</td>
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<tr>
<td>IGF-I</td>
<td>6.44</td>
<td>5.36</td>
<td>0.9</td>
<td>7.14</td>
<td>5.48 ± 0.2</td>
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<td>0.46</td>
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</table>

Table 7.3 Summary of kinetic analysis of IGFBP-2 binding. The 2 state conformational change model was used to fit kinetic data and determine association constants for all proteins binding to IGFBP-2. The association constants shown are calculated from two separate runs on two independently coupled IGFBP-2 chips.
CHAPTER 7 - Binding of IGF-I, IGF-II and IGF chimeras to the IGF-2R and IGFBPs

Figure 7.5 IGF-I, IGF-II and IGF chimeras binding to IGFBP-6. 50nM ligand (IGF-I, IGF-II or IGF chimera) was passed over a IGFBP-6 flow cell at a flow rate of 40 µl/min for 5 minutes. Dissociation of bound analyte in HBS buffer alone was measured at the same flow rate for 15 minutes. All flow cells were regenerated with 60 µl of 10 mM HCl. Reference flow cell data were subtracted from all runs to account for bulk refractive index due to the buffer. All kinetic data was analysed using the BIAevaluation 3.2 software. The 2 state conformational change model was used to evaluate the binding of all ligands to IGFBP-6. A representative experiment is shown. Kinetic analysis results are shown in Table 7.4.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{a1}$ x 10^3 1/ms</th>
<th>$k_{d1}$ x 10^2 1/s</th>
<th>$k_{a2}$ x 10^2 1/s</th>
<th>$k_{d2}$ x 10^2 1/s</th>
<th>$K_a$ x 10^4 1/Ms</th>
<th>Relative to IGF-I $K_a$</th>
<th>Relative to IGF-II $K_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-II</td>
<td>8.73</td>
<td>3.96</td>
<td>0.86</td>
<td>6.4</td>
<td>6.54 ± 0.57</td>
<td>7.78</td>
<td>1.00</td>
</tr>
<tr>
<td>IGF-I DII</td>
<td>6.80</td>
<td>1.86</td>
<td>1.8</td>
<td>13.2</td>
<td>0.41 ± 0.04</td>
<td>0.48</td>
<td>0.06</td>
</tr>
<tr>
<td>IGF-I CII</td>
<td>9.18</td>
<td>3.27</td>
<td>1.6</td>
<td>11.4</td>
<td>1.98 ± 0.45</td>
<td>2.36</td>
<td>0.30</td>
</tr>
<tr>
<td>IGF-II DI</td>
<td>7.46</td>
<td>3.61</td>
<td>0.8</td>
<td>6.0</td>
<td>6.41 ± 1.45</td>
<td>7.63</td>
<td>0.98</td>
</tr>
<tr>
<td>IGF-II C1</td>
<td>7.10</td>
<td>2.82</td>
<td>0.94</td>
<td>6.5</td>
<td>2.61 ± 0.59</td>
<td>3.10</td>
<td>0.40</td>
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<tr>
<td>IGF-I</td>
<td>7.5</td>
<td>2.78</td>
<td>1.9</td>
<td>13.5</td>
<td>0.84 ± 0.2</td>
<td>1</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Table 7.4 Summary of kinetic analysis of IGFBP-6 binding. The 2 state conformational change model was used to fit kinetic data and determine association constants for all proteins binding to IGFBP-6. The association constants shown are calculated from two separate runs on two independently coupled IGFBP-6 chips.
7.5 DISCUSSION

To date little is known about the IGF-2R binding site on IGF-II and this presents a large hole in our understanding of ligand:receptor interactions in the IGF system. Here in this chapter, the binding specificity of the IGFs for the IGF-2R and IGFBPs was investigated. The results of these studies show that the IGF-II C domain is involved in IGF-2R binding whereas the D domain of IGF-II is not. In addition the C domains of IGF-I and IGF-II may play a small role in conferring the differences in IGFBP-6 binding affinity of the IGFs.

As summarized in section 1.6.1, hitherto only B and A domain residues of IGF-II have been shown to be involved in IGF-2R binding. This is the first time the C domain of IGF-II has been shown to be necessary for wildtype IGF-II binding. Despite IGF-I not exhibiting any detectable binding to IGF-2R dom 11-13 on the BIAcore, IGF-I CII did bind to the IGF-2R, albeit at a very low affinity. This suggested that the replacement of the IGF-I C domain by the IGF-II C domain has provided several positive contacts that confer a higher and therefore detectable IGF-2R binding affinity. Interestingly, when the IGF-I C domain was substituted into IGF-II the binding affinity for IGF-2R dom 11 was decreased 3.3-fold relative to the binding affinity of wildtype human IGF-II. In addition, IGF-II CI binding to IGF-2R dom 11-13 was decreased by 6-fold relative to wildtype IGF-II binding suggesting that the IGF-I C domain makes negative contacts or lacks positive contacts to both domain 11 and domain 13 of the IGF-2R. Exchanging the D domains between IGF-I and IGF-II did not affect IGF-2R binding affinities (Table 7.1).

In a study modeling the docking of IGF-II to domain 11 of the IGF-2R, Roche et al. described a favorable model where the IGF-II C domain residues serine 36, arginine 37, arginine 38, serine 39 and arginine 40 make positive contacts with domain 11 (Roche et al., manuscript in preparation). While the exact three dimensional position...
of the IGF-II C domain residues serine 36, arginine 37 and arginine 38 may not be analogous in IGF-I, they are present in IGF-I. This leaves only serine 39 and arginine 40 as the only unique C domain residues in IGF-II. These two amino acids may possibly confer higher affinity binding to the IGF-2R on IGF-II, while IGF-I with alanine, proline, glutamine and threonine in this position may not. The results presented here confirm the modeling predictions that the IGF-II C domain is involved in binding to IGF-2R.

BIAcore analysis revealed no difference between the affinity of IGF-I and IGF-II for IGFBP-1, supporting previous observations (Oh et al., 1993). The affinity of IGF-I for IGFBP-1 was similar to that previously reported using the BIAcore (K_a = 1.76 X 10^9 M^-1) (Jansson et al., 1997; Jansson et al., 1998). All chimeras except IGF-II CI had affinities not different from that of either IGF-I or IGF-II. The affinity of IGF-II CI was 70% that of the affinity of IGF-II for IGFBP-1. These results are similar to the IGFBP-3 binding analyses in chapter 3 (section 3.4.9, Table 3.3 and Figures 3.9 and 3.10). IGFBP-3 also has equal affinities for IGF-I and IGF-II and exchanging the C or D domain between IGF-I and IGF-II does not affect binding affinities. Both sets of data provide evidence that the chimeras maintain the overall tertiary structure.

IGF-II showed a 2-fold higher affinity for IGFBP-2 than IGF-I. The higher affinity of IGF-II for IGFBP-2 has been reported previously using the BIAcore (Carrick et al., 2001) and solution competition binding assays (Clemmons et al., 1992). As observed for IGFBP-1 all chimeras except one, this time IGF-I DII, had near equivalent affinities for IGFBP-2 as either IGF-I or IGF-II. The IGFBP-2 binding affinity of IGF-I DII was decreased 2-fold compared to that of IGF-I. Interestingly the affinity of IGF-II CI for IGFBP-2 was decreased almost 2-fold relative to IGF-II, resulting in an affinity that was similar to IGF-I. The affinity of IGF-I CII however, was not increased relative to IGF-I. Hence the presence of the IGF-I C domain
decreases IGFBP-2 affinity but the presence of the IGF-II C domain does not increase IGFBP-2 affinity.

IGFBP-6 has the largest binding affinity difference for the IGFs, as it binds IGF-II with 10-60 fold higher affinity compared to IGF-I (Kiefer et al., 1992; Marinaro et al., 1999). The affinity presented here for IGF-II binding to IGFBP-6 is similar to that reported previously (K_A = 1.88 x 10^9 M^-1) (Marinaro et al., 1999). However, the affinity of IGF-I for IGFBP-6 shown in Table 7.4 is slightly higher than those currently reported in the literature (Marinaro et al., 1999). As a result of this higher affinity for IGF-I the 8-fold difference in affinity between IGF-I and IGF-II reported here is smaller than the 12-fold reported previously (Marinaro et al., 1999). The molecular basis for the difference in affinity of IGFBP-6 for IGF-I and IGF-II is unknown. However, mutations in the C-terminal domain of IGFBP-6 have been shown to reduce IGF-II binding while IGF-I binding is unaffected (Headey et al., 2004). Previous reports have shown that the affinity difference between IGF-I and IGF-II for IGFBP-6 is due to differential dissociation kinetics (Marinaro et al., 1999). The results shown in Table 7.4 also show that a faster dissociation rate is the reason for the lower affinity of IGF-I for IGFBP-6 compared to IGF-II.

Swapping the D domains between IGF-I and IGF-II did not affect IGFBP-6 affinity suggesting this region is not involved in IGFBP-6 binding or that either the IGF-I or IGF-II D domain play an equal role in IGFBP-6 binding. Exchanging the C domain did however affect IGFBP-6 binding. Inserting the IGF-I C domain into IGF-II reduced binding by 2.5-fold relative to IGF-II, while substituting the IGF-I C domain for IGF-II improved binding by 2.4-fold relative to IGF-I. Exchanging the C domains between IGF-I and IGF-II appeared to affect the association rates to a slightly larger extent than the dissociation rates (Table 7.4).
CHAPTER 7 – Binding of IGF-I, IGF-II and IGF chimeras to the IGF-2R and IGFBPs

The BIAcore allows real-time kinetic measurement of a binding interaction, providing much more information (both association and dissociation phases) compared to solution binding assays. However, the BIAcore does have several limitations that should be addressed (for an extensive discussion see (Morton and Myszka, 1998; Myszka et al., 1998; Yan et al., 2004)). One limitation that may affect binding kinetics is the tethering of the ligand to the sensor chip. In the situation described in this chapter the ligand is either IGFBP-1, -2 or -6 (or IGFBP-3 in chapter 3). The coupling any of the IGFBPs to the sensor chip may restrict its movement in solution and possible conformational change upon binding, which in turn may alter the binding kinetics.

Recent NMR experiments have revealed residues of IGF-II which interact with the C-terminal domain of IGFBP-6 (Headey et al., 2004). Interestingly, this study did not identify any residues in the IGF-II C domain that interact with the IGFBP-6 C-terminal domain. Similarly a portion of the N-terminal domain of BP-5, termed mini-BP-5, was co-crystallized with IGF-I and no IGF-I C domain residues made contact with this BP-5 fragment (Zeslawski et al., 2001). The N-terminal domain of IGFBP-6 may interact with different IGF residues than the N-terminal domain of IGFBP-5 and hence interact with IGF-II C domain residues but this has not been investigated. Whether the N-terminal domain of IGFBP-6 does interact with IGF-II C domain residues awaits confirmation by structural analyses.

These BIAcore studies have shown that the C or D domains do not regulate the small affinity difference between IGF-I and IGF-II for IGFBP-2. The C domains of IGF-I and IGF-II may play a minor role in determining the different affinities that IGF-I and IGF-II exhibit for IGFBP-6.
Structural and functional analysis of Val44Met IGF-I
8.1 INTRODUCTION

As discussed in chapter 1 the insulin-like growth factor (IGF) system plays an important role in normal growth and development. Activation of the type-1 IGF receptor (IGF-1R) by IGF-I or IGF-II results in potentiation of growth, survival and differentiation. The action of IGFs is modulated by IGF binding proteins (IGFBPs), which regulate the availability to bind to the IGF-1R.

The importance of IGF-I in normal growth has been demonstrated experimentally in mice with an IGF-I knockout (Liu et al., 1993). Those mice exhibit a deficiency in intrauterine growth, and those that survive continue to show restricted growth; at birth they are 60% of normal weight but fall to 30% of normal weight at adulthood (Baker et al., 1993; Liu et al., 1993). The significance of IGF-I in normal growth was also demonstrated by disease states where a disruption in circulating IGF-I levels occurs. Overexpression of IGF-I resulting from overproduction of growth hormone leads to acromegaly, whereas low IGF-I levels resulting from an inactive growth hormone receptor leads to Laron dwarfism (Baumann, 2002; Paisley and Trainer, 2003).

Recently the phenotype resulting from a homozygous missense mutation in the human IGF-I gene was described (Walenkamp et al., 2004). The mutation (G274A) lead to the expression of IGF-I with a methionine instead of a valine at residue 44 (Val44Met IGF-I). This was the first description of the effect of IGF-I deficiency in adulthood, as the individual carrying the homozygous mutation is now 55 years old. Observed were several similarities between this individual and an earlier report of an IGF-I gene deletion described in a young male (Woods et al., 1997). Both patients suffered severe pre and post-natal growth retardation, deafness and mental retardation. In adulthood, however, the lack of functional IGF-I is well tolerated, with effects mainly on bone mass and gonadal function (Walenkamp et al., 2004).
In this chapter the biochemical and structural analysis of Val44Met IGF-I is reported. These results provide an explanation for the growth and developmental abnormalities observed (Walenkamp et al., 2004). The ability of Val44Met IGF-I to bind to the IGF-1R, the IR isoforms and IGFBP-2, 3 and 6 was studied.

This work has arisen from an international collaboration with Prof. Jan-Maarten Wit, Dr. Marcel Karperien and Dr. Marie-Jose E. Walenkamp (Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands). The NMR analyses of Val44Met IGF-I were performed by Prof. Ray Norton and Ms. Chunxiao C. Wang (The Walter and Eliza Hall Institute, Parkville, Victoria, Australia).

The results presented have been accepted for publication in Molecular Endocrinology.
8.2 MATERIALS

Oligonucleotides were purchased from Geneworks Pty Ltd. (Adelaide, South Australia). Restriction enzymes were from New England Biolabs (Hitchin, UK) or Geneworks Pty Ltd. (Adelaide, South Australia). $^{15}$N-labelled NH$_4$Cl was from Sigma (Castle Hill, Australia). Human IGF-I for europium labelling and human IGFBP-2 were purchased from GroPep Pty Ltd (Adelaide, South Australia). Human IGFBP-3 and IGFBP-6 were from R&D systems (Minneapolis, USA). Human insulin was purchased from Novo Nordisk (Bagsværd, Denmark). Greiner Lumitrac 600 96-well plates were from Omega Scientific (Tarzana, USA). DELFIA Eu-labelling kit, DELFIA enhancement solution and europium-conjugated anti-phosphotyrosine antibody PY20 were purchased from Perkin Elmer (Turku, Finland). Europium-labelled IGF-I (Eu-IGF-I) and insulin (Eu-insulin) were produced as described in chapter 4 (section 4.3.2) as per the manufacturer’s instructions. Antibodies 83-7 and 24-31 were kind gifts from Prof. K. Siddle, Cambridge, UK. P6 cells (BALB/c3T3 cells overexpressing the human IGF-1R) (Pietrzkowski et al., 1992) and R' cells (mouse 3T3-like cells with a targeted ablation of the IGF-1R gene) (Sell et al., 1994) were a kind gift from Prof. R. Baserga (Philadelphia, USA). Cells overexpressing the exon 11-/IR-A and exon 11+/IR-B isoforms of the insulin receptor (R'IR-A and R'IR-B cells, respectively) were created as previously described in chapter 4 (section 4.3.1).
8.3 METHODS

8.3.1 CONSTRUCTION OF EXPRESSION PLASMIDS ENCODING HUMAN IGF-I AND VAL44MET IGF-I

The IGF expression vector was developed by King et al. (King et al., 1992) (Figure 2.2). Quikchange™ site-directed mutagenesis kit was used to incorporate a G to A mutation in the IGF-I coding sequence (same IGF-I sequence used to produce human IGF-I section 3.4.1) at position 130 using the following oligonucleotides: Val44Met forward 5' CCG CAG ACC GGA ATC ATG GAT GAA TGC TGC 3', Val44Met reverse 5' GCA GCA TCC ATC CAT GAT TCC GGT CTG CGG 3'. The Val44Met IGF-I coding sequence was then subcloned using HpaI and HindIII restriction enzymes into the pGH(I-11) expression vector (King et al., 1992).

8.3.2 RECOMBINANT IGF-I AND VAL44MET IGF-I PRODUCTION

IGF-I and Val44Met IGF-I were expressed and purified essentially as described by Shooter et al. (Shooter et al., 1996) and described in chapter 3 (section 3.3.2). The purification of Val44Met IGF-I was identical to that of IGF-I (section 3.4.1). $^{15}$N labelled Val44Met IGF-I was expressed in minimal medium supplemented with $^{15}$NH$_4$Cl essentially as described previously (Torres et al., 1995). The purified proteins were analysed by mass spectroscopy and N-terminal sequencing and were shown to have the correct masses (93% incorporation of $^{15}$N) and to be greater than 95% pure. Quantitation of proteins was performed by comparing analytical C4 HPLC profiles with profiles of standard Long-[Arg$^3$] IGF-I preparations (section 3.3.12) (extinction coefficient relative to Long-[Arg$^3$] IGF-I = 0.9773) (Milner et al., 1995).
8.3.3 BINDING ANALYSIS OF VAL44MET IGF-I TO THE IGF-1R AND IR ISOFORMS

Receptor binding affinities were measured using an assay similar to that described for analysing EGF binding to the EGF receptor (Mazor et al., 2002) and outlined by Denley et al. (Denley et al., 2004) and detailed in chapter 4 (section 4.3.2 and 6.1).

8.3.4 IR AND IGF-1R PHOSPHORYLATION ASSAYS

Receptor phosphorylation was detected essentially as described by Denley et al. (Denley et al., 2004) and in chapter 4 (section 4.3.3 and 6.1).

8.3.5 BIACORE ANALYSES OF IGFBP BINDING

Coupling of IGFBPs to CM5 BIAsensor chips via amine group linkage was achieved using standard coupling procedures (Lofas and Johnsson, 1990; Carrick et al., 2001; Forbes et al., 2002). Briefly, IGFBPs were coupled to activated surfaces (2μg IGFBP/210μl in 10 mM sodium acetate, pH 4.5) at 5 μl/min. Unreacted groups were inactivated with 35 μl 1 M ethanolamine-HCl, pH 8.5. A sensor surface with 600 response units (RU) of coupled IGFBP-2 would routinely result in a response of approximately 100 RU with 100 nM IGF-I. In addition, a surface with 470 RU IGFBP-6 would result in a response of 70 RU and a surface with 400 RU IGFBP-3 would result in a response of 45 RU with 100nM IGF-I. Kinetic studies with 6.25, 12.5, 25, 50 and 100 nM IGF-I or Val44Met IGF-I were determined at a flow rate of
40 μl/min to minimize mass transfer effects, and by allowing 300s for association and
900s for dissociation. IGFBP biosensor surfaces were regenerated with 10 mM HCl for
1 minute. Analysis of kinetic data was performed with BIAevaluation 3.2 software. For
each binding curve the response obtained using control surfaces (no protein coupled)
was subtracted. Analysis of all binding data yielded similar affinity constants whether
a 2-state conformational change model was used or a 1:1 Langmuir binding model.
Shown in this chapter are the analyses using a 1:1 Langmuir binding model. IGF-I
binding fitted a 1:1 Langmuir binding model using global fitting. This model describes
a simple reversible interaction of two molecules in a 1:1 complex. Goodness of fit
measured as a Chi-squared value was not greater than 5 for all experiments. All
binding experiments were repeated at least in duplicate and biosensor chips coupled at
different times yielded surfaces with identical binding affinities. The binding affinities
of IGF-I to IGFBP-2 (K_D=0.7nM), IGFBP-3 (K_D=0.75 nM) and IGFBP-6 (K_D=6.6
nM) were comparable to the binding affinities reported by Hobba et al. (Hobba et al.,
1998) and Wong et al. (Wong et al., 1999) for hIGFBP-2 (K_D=0.3nM) and hIGFBP-2
(K_D=0.45nM) respectively, Heding et al. (Heding et al., 1996) for IGFBP-3
(K_D=0.23nM) and Marinaro et al. (Marinaro et al., 1999) for IGFBP-6
(K_A=0.028nM^-1) using BIAcore technology.
8.4 RESULTS

8.4.1 Purification of Val44Met IGF-I

A summary of the purification of Val44Met IGF-I is shown in Figure 8.1. The purification procedure was identical to that used to purify IGF-I (section 3.4.1).

8.4.2 IGF-1R binding and activation

Purified IGF-I and Val44Met IGF-I were analysed for their relative abilities to bind and activate the IGF-1R and both isoforms of the insulin receptor (IR-A and IR-B). Competition binding curves for binding to the IGF-1R are shown in Figure 8.2A and IC₅₀ values are summarised in Table 8.1. The affinity of Val44Met IGF-I was approximately 90-fold lower than IGF-I for the IGF-1R. IGF-1R activation on P6 cells was assessed using IGF-I, IGF-II, insulin and Val44Met IGF-I (Figure 8.2B). While IGF-I activated the IGF-1R with an IC₅₀ of 3.9 nM ± 0.43 nM, IGF-II at the same concentration was only able to induce IGF-1R phosphorylation equal to 35% that of IGF-I. In addition, activation by insulin can only be detected at concentrations greater than 50 nM. Val44Met IGF-I is only slightly more potent than insulin in IGF-1R activation as a result of decreased receptor binding affinity.
CHAPTER 8 – Structural and Functional analysis of Val44Met IGF-I

Reduced inclusion bodies (A) 75mg

Refold (B) 47mg

Ion Exchange

Hydroxylamine cleavage (C) 20mg

Preparative HPLC (D) 12.5 mg

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mass Spectrometry</th>
<th>Purity</th>
<th>Final Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Val44Met IGF-I</td>
<td>7681 Da</td>
<td>7680 ± 0.09 Da</td>
<td>&gt;95 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>12.5 mg</td>
</tr>
</tbody>
</table>

Figure 8.1 Purification summary of Val44Met IGF-I.
The purification protocol for Val44Met IGF-I is shown as a flow diagram left, with protein yields given at each step. At the each step the purification was monitored by reverse phase HPLC analysis using a 20-50 % acetonitrile gradient over 30 minutes (section 3.3.1). Shown are the analytical HPLC profiles of reduced inclusion bodies (A), refold at termination (B), hydroxylamine cleavage at termination (C) and final analytical profile after preparative HPLC (D). In each HPLC profile the peak containing human Val44Met IGF-I, at the various stages of purification, is shown by an arrow. The final yield and confirmation of mass and purity are shown in the table above.
A, Immunocaptured IGF-1R was incubated with Eu-IGF-I in the presence or absence of increasing concentrations of IGF-I or Val44Met IGF-I as described in section 8.3.3. The graph shown is a representative of two independent experiments. Results are expressed as a percentage of Eu-IGF-I bound in the absence of competing ligand and the data points are means ± S.E.M. of triplicate samples. Errors are shown when greater than the size of the symbols. The ligands are as follows in A, IGF-I (△); Val44Met IGF-I (■). B, IGF-1R phosphorylation by IGF-I, IGF-II, insulin and Val44Met IGF-I. P6 cells overexpressing the human IGF-1R were serum starved for 4 h followed by stimulation with various concentrations of ligand for 10 min. Cells were lysed with ice cold lysis buffer containing phosphatase inhibitors and activated receptors were immunocaptured with the anti-IGF-1R antibody 24-31 as described in section 8.3.4. Receptor autophosphorylation was measured by time-resolved fluorescence using Eu-PY20 to detect phosphorylated tyrosines. The graph shown is a representative of three experiments and data points are means ± S.E.M. of triplicate points. Errors are shown when greater than the size of symbols. The ligands are as follows in B, IGF-I (△); IGF-II (▲); Insulin (●); Val44Met-IGF-I (■).
<table>
<thead>
<tr>
<th>Ligand</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; Rel. IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>1.7 ± 0.09</td>
<td>1</td>
</tr>
<tr>
<td>Val44Met IGF-I</td>
<td>142 ± 43</td>
<td>83.8</td>
</tr>
</tbody>
</table>

Table 8.1 Inhibition of europium-labelled IGF-I binding to the IGF-1R by IGF-I and Val44Met IGF-I. The IC<sub>50</sub> relative to that of IGF-I is also shown. Values are the means and ± S.E.M. from two independent experiments.

8.4.3 IR binding and activation

Competition binding curves for binding to the two isoforms of the insulin receptor (IR-A and IR-B) are shown in Figure 8.3A and 8.3B and IC<sub>50</sub> values are summarised in Table 8.2. No competition by Val44Met-IGF-I for europium-labelled insulin (Eu-insulin) binding is detected using either IR isoform –A or -B, even at micromolar concentrations. IGF-I is a relatively poor binder to both IR isoforms and binds with a 3-fold higher affinity to the IR-A (IC<sub>50</sub>= 120nM) than the IR-B (IC<sub>50</sub>= 366nM). In contrast, insulin binds with high affinity to both insulin receptor isoforms, with a slightly higher affinity to the IR-B isoform in our assay (IR-A, IC<sub>50</sub>= 1.4nM versus IR-B, IC<sub>50</sub>=2.8nM) (Denley et al., 2004). IGF-II also competes with high affinity (IC<sub>50</sub>=18nM) for Eu-insulin binding to the IR-A, and has a 3.7-fold lower affinity for the IR-B. In addition, activation of the insulin receptor by concentrations of up to 1 µM Val44Met IGF-I is not detectable (data not shown), whereas activation of insulin receptor phosphorylation by the other ligands correlates with receptor binding affinities ((Denley et al., 2004) and chapter 4 (section 4.4.1 and 4.4.2).
Figure 8.3 Competition binding curves of Eu-Insulin binding to immunopurified human IR-A or IR-B. Immunocaptured IR-As or IR-Bs were incubated with Eu-Insulin in the presence or absence of increasing concentrations of insulin, IGF-I, IGF-II or Val44Met IGF-I as described in section 8.3.3. The graphs shown are a representative of three experiments. A, competition for binding to IR-A; B, competition for binding to the IR-B. Results are expressed as a percentage of Eu-Insulin bound in the absence of competing ligand and the data points are means ± S.E.M. of triplicate samples. Errors are shown when greater than the size of the symbols. The ligands are as follows in A and B, Insulin (●); IGF-II (▲); IGF-I (△); Val44Met IGF-I (■).
Table 8.2 Inhibition of europium-labelled insulin for binding to the IR-A and IR-B by insulin, IGF-I, IGF-II and Val44Met IGF-I. The IC$_{50}$ relative to that of IGF-II binding to the IR-A is also shown. Values are the means and ± S.E.M. from three independent experiments. (N.D. = not determined)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>IR-A IC$_{50}$ (nM)</th>
<th>IC$_{50}$ Rel. IGF-I</th>
<th>IR-B IC$_{50}$ (nM)</th>
<th>IC$_{50}$ Rel. IGF-I</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>120.4 ± 34.1</td>
<td>1</td>
<td>366 ± 15</td>
<td>1</td>
</tr>
<tr>
<td>Val44Met IGF-I</td>
<td>&gt; 1000</td>
<td>N.D.</td>
<td>&gt; 1000</td>
<td>N.D.</td>
</tr>
<tr>
<td>Insulin</td>
<td>2.8 ± 0.3</td>
<td>0.02</td>
<td>1.4 ± 0.1</td>
<td>0.004</td>
</tr>
<tr>
<td>IGF-II</td>
<td>18.2 ± 2.4</td>
<td>0.15</td>
<td>68 ± 11</td>
<td>0.19</td>
</tr>
</tbody>
</table>

In summary, IGF-1R binding of Val44Met IGF-I is 90-fold lower than IGF-I and activation is correspondingly lower. Insulin receptor binding and therefore activation is disrupted very significantly by substitution of valine for methionine at residue 44.

8.4.4 Receptor signalling and biological activity in fibroblasts

To examine the effect of the Val44Met mutation on the ability to activate signal transduction in cells with a more physiological number of IGF-1R, activation of the Erk1/2 and Akt/PKB pathways was analysed in cultures of dermal fibroblasts. All assays were performed by Dr. Hermiene van Duyvenvoorde and Dr. Marcel Karperien (Department of Pediatrics, Leiden University Medical Center, Leiden, The Netherlands) and will therefore be only briefly discussed. Val44Met IGF-I was about 100-fold less potent than IGF-I at inducing Erk1/2 phosphorylation. Interestingly, Val44Met IGF-I was 200-fold less potent than IGF-I at inducing Akt/PKB phosphorylation. This suggests the mutation had a greater differential effect on the activation of the Akt/PKB signalling pathways than the Erk1/2 pathway.
The ability of Val44Met IGF-I to stimulate DNA synthesis was measured in fibroblasts of the affected patient and fibroblasts of a matched control subject. These assays were performed by Marie-Jose Walenkamp (Leiden University Medical Centre, The Netherlands). While IGF-I was able to potently stimulate DNA synthesis, as measured by $^3$H-thymidine incorporation, in both sets of fibroblasts, 100-fold more Val44Met IGF-I was required to reach the same level of stimulation as IGF-I.

A more detailed discussion of these collaborators results appears in our manuscript.

**8.4.5 IGFBP binding**

IGFBP binding was assessed using BIAcore analysis with IGFBP-2, IGFBP-3 and IGFBP-6 biosensor surfaces (Figure 8.4). There is no difference in binding affinities between IGF-I and Val44Met IGF-I for any of the surfaces. As expected, IGFBP-2 and IGFBP-3 have similar affinities for IGF-I and Val44Met IGF-I (0.7 nM), whereas IGFBP-6 binds IGF-I and Val44Met IGF-I with a much lower affinity (6.6 nM, see Figure 8.4). As IGFBP binding is not perturbed we can conclude that Val44Met IGF-I is correctly folded. These results are supported by neutral gel filtration of the patient’s serum performed by Dr. Jaap van Doorn (Department of Metabolic and Endocrine Diseases, University Medical Center Utrecht, Utrecht, The Netherlands). Val44Met IGF-I predominantly associates with the 150 kDa complex (comprised of Val44Met IGF-I, IGFBP-3 and the acid labile subunit) as is seen with wild type IGF-I in control serum. Val44Met IGF-I levels are higher in the patient’s serum than IGF-I levels in control serum (Walenkamp et al., 2004).
Figure 8.4 Surface plasmon resonance analysis of V44M-IGF-I binding to IGFBP-2, -3 and -6. Sensorgrams represent binding to IGFBP-2 (A) and IGFBP-3 (B) and IGFBP-6 (C) surfaces at 50 nM IGF-I (black) or Val44Met IGF-I (grey). Kinetic studies with a range of analyte concentrations were determined at a flow rate of 40 µl/min to minimize mass transfer effects, allowing 300 s for association and 900 s for dissociation. Dissociation constants (K_D) were derived using BIAEvaluation 3.2 software and a 1:1 Langmuir binding model.

8.4.6 Structural Analysis of Val44Met IGF-I by NMR

As already stated in the introduction the NMR structural analyses of Val44Met IGF-I were performed by Prof. Ray Norton and Ms. Chunxiao C. Wang (The Walter and Eliza Hall Institute, Parkville, Victoria, Australia). Figures 8.5 and 8.6 and legends were prepared also prepared by Prof. Ray Norton and Ms. Chunxiao C. Wang.

A comparison between the 2D 15N-1H HSQC spectrum of Val44Met IGF-I with those of native IGF-I (Laajoki et al., 1997; Laajoki et al., 1998; Schaffer et al., 2003), allowed a detailed view of the structural variation introduced by the valine at position 44. Chemical shift differences between Val44Met IGF-I and IGF-I were small, except
for Cys6, Ala8, Phe23, Ile43, Asp45, Ser51, Arg56, Leu57 and Tyr60 (Figure 8.5). The residues that are adjacent to the valine mutation, Ile43 and Asp45, exhibited the largest changes compared to their position in wildtype IGF-I. Both aromatic residues Phe23 and Tyr60, implicated in IGF-I binding to the type 1 IGF receptor (Bayne et al., 1990; Hodgson et al., 1996) are affected in Val44Met IGF-I (Figure 8.5). In conclusion, although the structure of Val44Met IGF-I is similar to native IGF-I, chemical shift comparisons suggest that the valine mutation has caused local structural changes around position 44 and in surrounding regions, some of which are involved in binding to the IGF-I receptor. Supporting this conclusion is the analysis of the NOEs from the backbone amide resonances. Almost all the NOEs to Met44 observed were in line with those in the native NMR structure, however there were several new NOEs suggesting that the Met44 side chain is at a different position to the Val44 side chain in the native IGF-I. In addition HN-NH NOEs from Met44 to Ile43 and Asp45 were observed which suggest that the α-helical nature of this region is preserved.
Figure 8.5 Weighted average chemical shift differences between Val44Met IGF-I and native IGF-I (spectra from Prof. Norton’s group), calculated for $^{15}$N and $^1$H resonances using $\Delta \delta_{av} = (\Delta \delta_{NH}^2 + 0.17 \Delta \delta_N^2)^{1/2}$ (Farmer et al., 1996). Residues Gly1, Gly7, Leu10, Glu58 and Cys61 were not assigned. Residues 2, 28, 39, 63 and 66 are proline and Asp12, Lys27, Gly30, Gly32, Ser33, Ser34, Arg37 and Thr41 had zero $\Delta \delta_{av}$ values. The locations of the three helices of native IGF-I are indicated above the plot.
Figure 8.6 Backbone ribbon view of IGF-I with side chains of key residues indicated. Long-[Arg3] IGF-(Laajoki et al., 1998); (PDB accession no. 3LRI) is shown on the left and IGF-I + F1 peptide (Schaffer et al., 2003); (PDB accession no. 1PMX) on the right (with the peptide not shown for clarity); in each case the closest-to-average structure over the family is shown. Side chains are coloured as follows: Met44 in red; Ile43, Asp45 and Arg56, which have the largest chemical shift changes between mutant and native IGF-I, in blue; Cys6, Ala8 and Leu57, which have smaller chemical shift changes between mutant and native IGF-I, in green, and Phe23, Tyr24 and Tyr60, which are implicated in IGF-I binding to the type 1 IGF receptor (Cascieri et al., 1988; Bayne et al., 1990; Sakano et al., 1991; Perdue et al., 1994), in magenta. The upper and lower views of each structure are related by an 80° rotation around the horizontal axis; note that the last five residues in the lower view of IGF-I + F1 peptide, and the first two residues in the upper view, are not shown in order to avoid overlap. The N-terminal extension in long-[Arg3] IGF-I is not shown, so the chain begins at the equivalent of Gly1 of IGF-I.
8.5 DISCUSSION

Reported here is a comprehensive biochemical and structural analysis of Val44Met IGF-I in order to explain the phenotype described of a patient carrying a point mutation in the IGF-I gene. A similar phenotype was previously described resulting from a deletion in the IGF-I gene (Woods et al., 1997). The phenotype is not due to a defective IGF-1R as IGF-I can stimulate the same biological response in fibroblasts derived from the patient or from a normal individual. However, the Val44Met mutation results in a significant reduction (~90-fold) in IGF-1R receptor binding affinity and non-detectable binding to either IR isoform. Poor IGF-1R binding affinity of Val44Met IGF-I results in a corresponding decrease in activation of IGF-1R phosphorylation and downstream signalling molecules, including Erk1/2 and Akt/PKB. Remarkably, Val-Met substitution at position 44 has a greater effect on activation of the Akt/PKB pathway than expected from the reduced receptor binding affinity. It seems feasible that these differences are a direct consequence of the changed kinetics of receptor – ligand interaction, which may have a greater impact on activation of the PKB/Akt-pathway than on the Erk1/2 pathway. The conclusion from these data is that Val44Met IGF-I is partially inactivating. However, at least 100-fold higher concentrations of Val44Met IGF-I are needed to elicit an impaired biological response compared with IGF-I. This makes Val44Met IGF-I effectively inactive in the normal physiological dose-response range for IGF-I action.

Despite a large effect on receptor binding Val44Met IGF-I is still able to bind IGFBP-2, IGFBP-3 and IGFBP-6 with equal affinity to IGF-I. This suggests that the common IGFBP binding site is not disrupted. In support of this conclusion Val44Met IGF-I shows a normal association with the 150kDa complex in serum. Dubaquie and Lowman (Dubaquie and Lowman, 1999) reported a small disruption in IGFBP-1 and IGFBP-3 binding by Val44Ala IGF-I (2.3 and 1.4 fold lower binding than IGF-I
respectively) but did not describe IGF-1R binding. A recent crystal structure of IGF-I in complex with the N-domain of IGFBP-5 shows that Val44 is not included in the N-domain binding site (Zeslawski et al., 2001). Headey et al. (Headey et al., 2004) have reported that binding of IGFBP-6 C-domain to IGF-II affects the two residues either side of Val43, namely Ile42 and Glu44. Although Val43 could not be assessed because of peak overlap it seems that this region of the IGF surface is involved in interaction with the C-domain of IGFBPs. Therefore, the lack of effect of the Val44Met substitution in IGF-I on IGFBP binding may be attributable to the fact that the hydrophobic nature of the surface is preserved. The C-domain of the IGFBPs is apparently less sensitive to the nature of the side chain at position 44 than is the IGF-I receptor.

Interestingly, the results of the NMR analysis of the Val44Met IGF-I structure suggest relatively little disruption of the overall structure. The marked effect on IGF-1R binding could be explained by either local structural disruption around the mutation site and in surrounding areas or by a direct interaction of Val44 with the receptor. Analysis of chemical shift comparisons shows differences in local structure at residues cysteine 6, alanine 8, phenylalanine 23, isoleucine 43, asparate 45, serine 51, arginine 56, leucine 57 and tyrosine 60. Of these residues tyrosine 60 has previously been implicated as important for IGF-1R and IR binding (Bayne et al., 1990; Hodgson et al., 1995). Tyrosine 60 replaced by leucine in IGF-I causes a 20-fold reduction in IGF-1R binding affinity and Tyr60Phe IGF-I has 2.6-fold reduced insulin receptor binding affinity. In addition, Maly and Luthi (Maly and Luthi, 1988) showed that tyrosine 60 was protected from iodination in the presence of the IGF-1R. Phenylalanine 23 has also been identified as important for IGF-1R binding as mutation to glycine results in a 48-fold reduction in receptor binding affinity compared with IGF-I (Hodgson et al., 1996). Whether this mutation is causing a structural
perturbation has not been investigated. The neighbouring residue, tyrosine 24, has been identified in several studies as being important for IGF-1R binding (Cascieri et al., 1988). It has been previously demonstrated that when alanine 8 is mutated to leucine a decrease in IGF-1R binding (~ 6-fold) is observed (Shooter et al., 1996). Only relatively small effects of mutating serine 51 and arginine 56 have been reported (Cascieri et al., 1989; Jansson et al., 1998). Val44 is conserved in all but one (Catfish brain (McRory and Sherwood, 1994)) of the IGF-I sequences reported so far and is also found in the corresponding position in the two structurally related proteins, IGF-II and insulin (Figure 1.2). Interestingly, mutation of valine 43 of IGF-II (which corresponds to valine 44 in IGF-I) to leucine results in a 220-fold lower IGF-1R binding affinity while maintaining IGFBP binding affinities similar to IGF-II (Sakano et al., 1991). This observation confirms the importance of this residue in maintaining IGF-1R binding.

A point mutation in the insulin gene (guanine to thymine at position 1298) resulting in the valine A3 to leucine mutation in the A chain has been termed Insulin Wakayama (Nanjo et al., 1986; Nanjo et al., 1987). Valine A3 in insulin corresponds to valine 44 of IGF-I. Expression of ValA3Leu insulin leads to hyperinsulinemia and in some cases diabetes (Nanjo et al., 1987) resulting from severely defective insulin receptor binding. It has been suggested that valine A3 and isoleucine A2 make direct contact with the IR following a structural change in insulin (Hua et al., 1996). Removal of contact between the beginning of the A chain and the C-terminus of the B chain (involving residue B24) exposes residues isoleucine A2 and valine A3 and thereby allows their interaction with the insulin receptor (Hua et al., 1991; Hua et al., 1996; Xu et al., 2002; Wan et al., 2003; Xu et al., 2004). Substitution of isoleucine A2 with Allo-isoleucine A2 leads to a 50-fold reduction in IR binding affinity while maintaining overall structure (Xu et al., 2002). Direct evidence for interaction with the
CHAPTER 8 – Structural and functional analysis of Val44Met IGF-I

receptor has recently been provided by a cross-linking study using a p-Azido-Phe derivative of ValA3 and suggests an interaction with the insert domain (Xu et al., 2004).

Several substitutions have been made at valine A3 including ValA3Leu insulin, which has only 0.14% of IR binding affinity compared with insulin (Kobayashi et al., 1986; Nanjo et al., 1987). Nakagawa and Tager (Nakagawa and Tager, 1992) reported a similar helical content in both ValA3Leu insulin and native insulin on the basis of CD spectral analysis. Interestingly, NMR analysis of ValA3Leu insulin revealed no significant change in structure (Prof. Michael Weiss, Case Western Reserve Medical School, Cleveland Ohio) unpublished observations) despite the significant effect on IR binding (Kobayashi et al., 1986; Nanjo et al., 1987). Structural analyses of ValA3Ile (Nakagawa and Tager, 1992) and ValA3Thr (Chen and Feng, 1998) by far-UV CD show little disruption to the overall structure, whereas mutation to glycine leads to a complete disruption of the first A-chain α-helix as shown by NMR analysis (Olsen et al., 1998). Furthermore, substitutions at residue isoleucine A2 highlight the importance of the beginning of the A domain helix in IR binding. Substitution of isoleucine A2 with valine reduces the helical content and destabilizes the first A domain helix (Xu et al., 2002). As with ValA3Leu insulin (Nakagawa and Tager, 1992), the data here show that Val44Met IGF-I maintains all helical structures. This is perhaps not surprising as methionine is a residue of reasonable helical propensity (Horovitz et al., 1992; Blaber et al., 1993) and methionine is commonly found in the same position in proteins as valine (Jonson and Petersen, 2001). However, the loop connecting helices 2 and 3 in the native structure (Figure 8.6) is somewhat more flexible in Val44Met IGF-I. Despite this minor structural perturbation, both Val44Met IGF-I and ValA3Leu insulin have severely disrupted receptor binding properties. It seems likely that Val44 in IGF-I plays a similar role in insulin receptor binding to ValA3 in insulin.
In conclusion, reported here is the biochemical and structural analysis of the first naturally occurring mutant of IGF-I. The mutant, V44M IGF-I, exhibits large reductions in IGF-1R and IR binding affinities and correspondingly lower potential to activate signalling events downstream of the IGF1R, while preserving native affinity to several binding proteins. Biological activities of Val44Met IGF-I are only observed when supraphysiological concentrations (at least 100-fold higher) are used. In the normal physiological dose-response range Val44Met substitution is completely inactivating. From this data it can be concluded that the homozygous patient with the Val44Met substitution is effectively null for IGF-I. This fully explains the phenotype of the patient, and is in line with the observed similarities in developmental defects observed in the patient and in one previously described adolescent man with a homozygous IGF-I gene deletion as well as in IGF-I knock out mice (Liu et al., 1993; Woods et al., 1997). The lack of binding to the insulin receptor by Val44Met IGF-I probably plays a minor role in the overall phenotype of the patient as the affinity of IGF-I for either the IR-A or IR-B isoform is relatively low compared to insulin. Structural analyses revealed only minor perturbations in the local structure of residues known to be involved in IGF-1R binding and the overall structure is remarkably well preserved. Finally, the analysis identifies Val44 as a critical residue involved in receptor – ligand interactions and further mutational analysis of this residue could provide valuable insight into the mechanism of IGF-1R binding by IGF-I.
Final Discussion
9.1 DISCUSSION

The structural determinants for the difference in affinity between IGF-I and insulin for their non-cognate receptors has been studied extensively, using various biochemical techniques in the absence of a crystal structure of either ligand with either receptor. In contrast to the large amount of interest in comparing IGF-I with insulin, there has been little study undertaken in investigating the difference in affinity that IGF-I and IGF-II have for almost all proteins with which they both interact. In addition, despite the increasing volume of literature describing the physiological relevance of IGF-II binding to the IR-A, the lack of understanding of how IGF-II binds to the IR-A is a major gap in the understanding of ligand/receptor interaction of the insulin/IGF family. The objective of this thesis has been to investigate the role of the IGF C and D domains in determining the difference in affinity of IGF-I and IGF-II for the IGF-1R, IR isoforms, IGF-2R and IGFBPs. In addition, another aim of this work was to probe the signalling pathways activated by IGF-I and IGF-II through the IR isoforms and define the structural regions of the IGFs that account for the differential ability of IGF-I and IGF-II to induce various signalling pathways. In keeping with the broad structure and function analysis of the IGFs, this thesis also aimed at identifying the structural and biochemical basis for the growth and development characteristics of a subject with the first ever documented naturally occurring mutation of the human IGF-I gene (Val144Met IGF-I).

9.2 SUMMARY OF FINDINGS

As discussed in the Introduction there are 26 amino acid differences between IGF-I and IGF-II with the greatest concentration in the C and D domains. There is also a difference in the number amino acids in the C and D domains, resulting in the IGF-II C and D domains being 4 and 2 amino acids shorter than the IGF-I C and D domains
respectively. With several previous reports suggesting that the IGF-I C domain was important in IGF-1R binding, exchanging the C and D domains between IGF-I and IGF-II was undertaken to probe the receptor binding specificities of the IGFs.

Chapter 2 details the PCR-based construction of the synthetic coding sequences for IGF-I, IGF-II and all IGF chimeras, where the C and D domains have been swapped between IGF-I and IGF-II singly or together. Construction of the coding sequences using totally synthetic oligonucleotides also allowed optimisation of the codon usage for expression in E. coli. The successful fermentation and purification of the first whole domain chimeras of IGF-I and IGF-II is reported in chapter 3. This then allowed analysis of the purified chimeras for binding to the IR isoforms (chapter 4). The results of these studies revealed that the C and D domains of IGF-II were the principal determinants of this ligand's higher affinity binding to the IR-A and IR-B relative to IGF-I. These domains allowed IGF-II to bind the IR-A with an affinity approaching that of the receptor's native ligand, insulin. Interestingly it was the same domains in IGF-I that acted in exactly the opposite way, conferring the lower affinity binding of IGF-I relative to IGF-II to the IR-A and IR-B.

Induction of IR isoform phosphorylation was also studied and reported in chapter 4. Incorporating the IGF-II C and D domains into IGF-I resulted in a slightly more potent ability to induce IR-A and IR-B autophosphorylation relative to IGF-II. Conversely replacing the C and D domains of IGF-II with those of IGF-I generated a protein with a slightly lower potency at inducing autophosphorylation compared to IGF-I.

Little is known about the signalling pathways initiated by either IGF-II or IGF-I binding to the IR-A or IR-B and hence was the focus of studies reported in chapter 5. The ability of IGF-II and IGF-I to induce autophosphorylation of Y1158, Y1162 and Y1163 in the catalytic loop and Y960 in the juxtamembrane domain was consistent
with their relative binding affinities. In addition, the ability of the IGFs and their chimeras to recruit and induce phosphorylation of IRS-1 and induce phosphorylation of Akt/PKB was also proportional to their binding affinity. Their ability to recruit and activate IRS-2, however, was disproportionate to their ability to bind and activate the receptor itself.

The IGF-1R binding specificity of the IGFs and their chimeras was investigated in chapter 6. The difference in affinity of IGF-I and IGF-II for the IGF-1R was in a large part due to the C domains. Unlike the significant role of the IGF-I and IGF-II D domain in regulating IGF binding specificity to the IR-A and IR-B, this region appeared to play only a small role in determining the IGF binding specificity to the IGF-1R. The C domains also regulated to a large extent the difference in the ability of IGF-I and IGF-II to stimulate autophosphorylation of the IGF-1R.

Out of all the proteins with which IGF-I and IGF-II interact the IGF-2R exhibits the largest preference for IGF-II over IGF-I, to the extent that various reports suggest that IGF-I binds poorly or not at all to the IGF-2R (Sakano et al., 1991). The real-time interaction of IGF-I, IGF-II and all IGF chimeras with a fragment of the IGF-2R was probed using surface plasmon resonance technology (chapter 7). The results presented show that the IGF-II C domain contains certain residues that are required for native binding to the IGF-2R. In addition, the IGF-I C domain contains residues that are inhibitory to high affinity binding. These results suggest that the C domain plays a very small role in regulating the IGF-2R binding specificity of the IGFs and that residues within the B and A domains are likely to contain most of the elements that allow IGF-II to bind the IGF-2R and not allow IGF-I to bind the IGF-2R.

The interaction of IGF-I, IGF-II and all IGF chimeras to IGFBP-1, -2, -3 and -6 was also investigated in chapter 7. Both the C and D domains play a negligible role in
determining the IGFBP-1, -2 and -3 binding specificities of the IGFs. The IGF C domains may small role in determining the IGFBP-6 binding specificity of the IGFs.

The structural and biochemical characterisation of the first ever reported mutation in an IGF-I gene is detailed in chapter 8. Mutation of valine to methionine at position 44 causes a 90-fold reduction in IGF-1R binding, totally ablates binding to either IR isoform but does not affect binding to either IGFBP-2, -3 or -6. The NMR structural analyses by Prof. Ray Norton’s group (WEHI, Melbourne), revealed only minor side chain variations compared to the structure of native IGF-I, suggesting that most of the biochemical changes observed are directly due to the different properties between the valine and methionine side chains.

9.3 EVOLUTION OF THE IGFs

A study examining the tolerance of a protein to random amino acid change, mimicking mutation during evolution, found that mutations were more easily tolerated and hence retained in unstructured loops than in structured elements like α helices and β sheets (Guo et al., 2004). The inherent flexibility of the C and D domains of both IGF-I and IGF-II has allowed a less stringent selection for specific amino acids. In Figure 9.1 and 9.2 the number of unique amino acids that occur at each position in IGF-I and IGF-II across all currently sequenced species have been graphed. There are more positions in the C and D domains that have allowed variation in the type of amino acid that can occur compared to the B and A domains e.g. 12 of 29 positions in the B domain and 11 of 20 positions in the A domain are not totally conserved across IGF-I of all currently sequenced species, whereas 9 of 12 positions in the C domain and all 8 positions in the D are not totally conserved across IGF-I of all sequenced species. Similar trends are seen across the four domains of IGF-II (Figure 9.2). In
Cross species amino acid variation in IGF-I sequences

Amino acids sequence of human IGF-I

Figure 9.1 Number of unique amino acids found the corresponding position to that in the human IGF-I sequence. Using the sequence alignment in Figure 1.2, the number of different amino acids found at each corresponding position in the human IGF-I sequence was determined. Bar at top of graph denoted domain boundaries.
Cross species amino acid variation in IGF-II sequences

Amino acid sequence of human IGF-II

**Figure 9.2** Number of unique amino acids found the corresponding position to that in the human IGF-II sequence. Using the sequence alignment in Figure 1.2, the number of different amino acids found at each corresponding position in the human IGF-II sequence was determined. Bar at top of graph denoted domain boundaries.
addition the number of different amino acids tolerated is greater at certain positions in the C and D domains compared to the B and A domains e.g. at the equivalent position of serine 35 in the C domain of human IGF-I, five other amino acids are found in other species, whereas in the A domain of IGF-I not more than two other amino acid types are tolerated at any position.

9.4 DIVERGENCE OF THE IGF-I AND IGF-II C AND D DOMAINS

The sequencing of hagfish prepro-IGF (hagfish evolved before the duplication of IGF) showed that 16 IGF amino acids have been totally conserved throughout vertebrate evolution suggesting that these are critical for maintaining tertiary structure and biological potency. Interestingly, homology between hagfish IGF and human IGF-I and IGF-II is low within the C and D domains suggesting a high level of molecular evolution in these regions (see section 9.3) (Nagamatsu et al., 1991). Hagfish IGF is more closely related to human IGF-I than human IGF-II suggesting that IGF-II has undergone a more rapid and divergent evolution than IGF-I (Nagamatsu et al., 1991) however relative to insulin the evolution of the IGFs has occurred slowly during vertebrate evolution (Duguay et al., 1995). Protein-protein or "functional" interactions put selective pressure on sequence divergence as indicated by residues in ligand binding or active sites being more highly conserved than residues not involved in ligand binding or active sites (Teichmann, 2002). Although residues within the C domain of IGF-I and IGF-II are not essential for structural maintenance, functional importance has not conserved any other residues except Ser 35, Arg 36, Arg 37 of IGF-I and Ser 36, Arg 37, Arg 38 of IGF-II. The results presented in this thesis suggest that the divergent evolution of the IGF-I and IGF-II C and D domains has been important in determining the relative biological actions of the IGFs.
Interestingly, this is not the case for IGFBP and IGF2R binding specificities. The determinants for the different binding affinities of IGF-I and IGF-II for IGFBP-2, 3 and 6 and the IGF-2R must reside in the B and A domains as has been suggested by many mutagenesis studies.

9.5 WHICH C AND D DOMAIN RESIDUES CONFER SPECIFICITY AND WHY?

Which residues in the IGF-I and IGF-II C and D domains regulate binding specificity are currently unknown. Future work mutating or deleting specific residues will determine whether size or charge is influencing the IR interaction (as discussed in chapter 4 and 6). While the exact nature of the residues determining specificity are unknown, the specificity of protein-protein interactions can be regulated by as little as one amino acid. In a series of elegant studies Tony Pawson's group and collaborators have revealed that the specificity of src homology (SH) 2 domains, which bind phosphotyrosine containing peptides, can be switched by mutation of one amino acid (Marengere et al., 1994; Songyang et al., 1995). A single mutation of threonine to tryptophan changes the SH2 domain phospho-peptide binding specificity from Src to that of Grb2 (Marengere et al., 1994; Kimber et al., 2000). Whether only one amino acid in both the C and D domains of IGF-I and IGF-II regulate their IR binding specificity is unknown. If this was the case, during evolution a single mutation at the specificity determining amino acid could have potential disastrous consequences for an organism. Hence it is likely that a single site determining specificity could be highly selected against. Therefore specificity is more likely to be driven across a number of residues that would instead provide a more robust evolutionarily stable framework. Given the high degree of sequence variability in the C and D domains comparing
IGF-I and IGF-II it is more likely that several residues determine the IR binding specificity of the IGFs.

It is not currently known whether size of the C and D domains of IGF-I and IGF-II or specific amino acids regulate their IR and IGF-1R binding specificity. The two extra positive charges in the IGF-II C domain, or lack of these in the IGF-I C domain may influence the IR and IGF-1R binding affinity. Both electrostatic repulsion and steric hinderance have been shown to regulate the specificity of other protein-protein interactions e.g. phospholipase Cγ1 and SHP-2 SH2 domain binding (Kay et al., 1998; Kessels et al., 2002). Indeed, electrostatics have been shown to be important in insulin binding to the IR (Rafaeloff et al., 1992).

As both the C and D domains of IGF-I and IGF-II are flexible, binding of these regions to a receptor, causing a disorder to order transition would be expected to incur an entropy penalty. While several examples of protein domains becoming more flexible upon binding have been reported (Forman-Kay, 1999; Zidek et al., 1999), this is not the most common observation e.g. (Kriwacki et al., 1996). Whether there is a difference in the conformational flexibility between the IGF-I and IGF-II C domains or D domains and consequently a difference in the entropy cost in receptor binding, leading to a difference in affinity is unknown. On the contrary, the more flexible C and D domains could allow higher affinity binding of the bulky IGFs to the IR, whose cognate ligand, insulin is much smaller. The movement and flexibility of a domain could reduce the penalties to the binding affinity in a situation of steric clash and it may also allow a protein to bind to a larger array of structurally divergent binding sites (Kay et al., 1998). In addition, conformational flexibility may reduce the energetic cost associated with an induced-fit binding event (Frankel, 1992), as may be the case for IGF binding to the IR or IGF-1R. An induced fit has been suggested to occur when insulin binds the IR (Hua et al., 1991; Weiss et al., 2000; Weiss et al., 2002).
Recently the in vitro reconstitution of an enzyme from separate fragments opened the way for potential specific isotopic labeling of segments of proteins for NMR analysis (Southworth et al., 1998). This technique has been applied to specific labeling of a maltose binding protein (MBP), which is 370 amino acids, traditionally too large for structure determination by NMR (Otomo et al., 1999). A major advance was the $^{15}$N labeling of a central segment (Gly$^{101}$-Ser$^{238}$) of MBP and confirmation that the signals resulting from the specifically labeled segment was at the same position as for the uniformly labeled protein (Otomo et al., 1999). This could be applied to specific labeling of the C domain of IGF-I and IGF-II. While IGF-I and IGF-II are small enough for complete labeling, the intein approach to specific segment labeling would aid in the assignment process as less than a quarter of the protein would be labeled and this would allow easier assignment of residues. NMR analysis of chemical shifts of a segmentally labeled IGF-I and IGF-II C domain in the absence and presence of either IGFBPs, IGF-2R fragments or IGF-1R or IR fragments, would provide evidence for the interaction of C domain residues with an interacting protein. It could also shed light on the relative flexibilities of the IGF-I and IGF-II C and D domains.

Interpreting the process of co-evolution of interacting proteins has been exploited to try and determine sites of interaction (Pazos et al., 1997; Goh et al., 2000; Ramani and Marcotte, 2003). This process has been successful in determining the specific binding of chemokines for their specific receptors (Goh et al., 2000; Ramani and Marcotte, 2003). Hence, the various co-evolution programs could be used to determine sites of insulin/IGF ligand/receptor interaction.
The work described here in this thesis has raised several fundamental questions about IGF/IR interactions that could be investigated in the future:

- What are the specific factors, size or charge, of the IGF C domains that regulate IR isoform binding specificity?

Mutants of IGF-I and IGF-II are currently being made to provide an answer to this question. Deletion of amino acids in IGF-I C domain to reduce overall size and conversely introduction of amino acids into the IGF-II C domain, will reveal whether domain size is regulating IGF-1R and IR binding specificity. In addition, to determine whether charge interactions are regulating binding specificity the extra charged residues in the IGF-II C domain will be substituted with either neutral or negatively charged amino acids.

- Do the C or D domains of the IGFs contact the IR and IGF-1R upon binding? If so what region of the receptor, either IR or IGF-1R, is involved?

Our laboratory has forged a collaboration with Dr. Jonathon Whittaker (Case Western Reserve School of Medicine, Cleveland, Ohio). We have been provided with receptor mutants created in his lab, which can be used in complementary binding studies with the chimeras reported here in an attempt to locate possible sites of receptor-ligand contact.
• How does IGF-I induce potent phosphorylation of IRS-2 via the IR-A and IR-B? What is the biological relevance of this event?

As stated in the discussion of chapter 5, a time course of IGF-I induced phosphorylation of IRS-2 has revealed that the surprising phosphorylation level of IRS-2 after a 5 minute stimulation with IGF-I was not a result of unusual kinetics of activation (Charles T. Roberts Jr, personal communication). Our laboratory is in the process of receiving cells from an IR/IRS-2 knockout mouse which can be used to ectopically express either IR isoform and IRS-2. A direct comparison between these cells and cells expressing either IR isoform (hence lacking IRS-2) would reveal the biological significance of IGF-I activation of IRS-2. The molecular mechanism by which IGF-I induced a higher than expected level of IRS-2 phosphorylation would be difficult to study. However, creating deletion mutants of IRS-2 and expressing these constructs in IRS-2 deficient cells would reveal what part of IRS-2 was involved in the recruitment to the IR and possibly what regions of IRS-2 are phosphorylated by the IR kinase.

9.7 DESIGNING INHIBITORS OF IGF ACTION RATHER THAN OF IGF-1R ACTION

As shown in chapter 5 and in number of previous publications, IGF-II can potently signal through the IR-A. In doing so, IGF-II can induce cancer cell proliferation, migration and protection from various apoptotic insults. As discussed in section 1.3.2, due to the action of the IGF-1R, it has been explored as a potential cancer therapeutic target (reviewed in (Bahr and Groner, 2004)). However, an antibody, a tyrosine kinase inhibitor or an antisense oligonucleotide directed against the IGF-1R may not prevent IGF-II action via the IR-A. In this way a cancerous cell expressing the IR-A may have a selective growth advantage in the presence of IGF-1R
inhibitors and continue the cancer program of the mutant cell. A study examining the role of the two receptors in breast cancer showed that despite both the IGF-IR and IR-A being present on the surface of the cell, the mitogenic action of IGF-II was primarily mediated by the IR-A, hence an inhibitor to the IGF-IR would not be efficacious (Sciacca et al., 1999). In addition there is mounting evidence that insulin may also promote tumour cell growth via the IR isoforms (reviewed in (Boyd, 2003)). There is no obvious answer to this conundrum as systemic inhibition of the IR function may induce insulin resistance and alter the metabolic state of the patient. In the generation of an inhibitor of the IGF-1R kinase the researchers were careful not to create a molecule that could inhibit the IR kinase as to prevent potential disruption of glucose/insulin metabolism (Garcia-Echeverria et al., 2004). Despite this perceived problem, local administration of an inhibitor that targets both the IGF-1R and IR maybe the most efficacious therapy. Studies that examine the potential therapeutic value of IGF-1R inhibition should be conducted with wildtype cells that have a native receptor expression profile. Indeed, non-selective IGF-1R inhibitors, which also target the IR, should not be discarded as potential treatments for cancer, as inhibition of the IR may be as important as inhibition of the IGF-1R.

9.8 CONCLUSION

The major aim of this thesis was to probe the receptor/ binding protein binding specificity of the IGFs and in particular to determine the structural basis of why IGF-I and IGF-II have different affinities for all proteins that they interact with. The studies presented here were successful in defining the relative roles of the IGF-I and IGF-II C and D domains in binding to the IGF-1R, IR isoforms, IGFBPs and IGF-2R. In addition, the IR signaling specificity of the IGFs was also defined. These studies have
contributed to a better understanding of ligand/receptor interactions of the insulin/IGF system.
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