CHARACTERIZATION AND PURIFICATION OF INSULIN-LIKE GROWTH FACTOR-BINDING PROTEINS OF HUMAN FIBROBLASTS

by

Briony Evelyn Forbes, B.Sc. (Hons)

A thesis submitted to the
University of Adelaide, South Australia
for the degree of
Doctor of Philosophy

August, 1991

Department of Biochemistry
University of Adelaide,
South Australia.

TABLE OF CONTENTS

Summary i
Statement iii
Acknowledgments iv
Publications v
Abbreviations vi

CHAPTER ONE INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION AND LITERATURE REVIEW 1
- 1.1.1 Insulin-like growth factors (IGFs) 1
- 1.1.2 Primary Structure of IGFs 1
- 1.1.3 Similarities Between IGFs and Insulin 2
- 1.1.4 Serum Levels of IGFs and Factors Affecting IGF Levels 3
- 1.1.5 IGF-I and IGF-II cDNAs and Genes 4
- 1.1.6 Regulation of IGF Expression 5
- 1.1.7 IGF Cellular Receptors 7
- 1.1.8 Biological Effects of IGFs 8

1.2 IGF BINDING PROTEINS (IGFBPs) 10
- 1.2.1 Identification of IGF Carrier Proteins 10
- 1.2.2 Classification of IGFBP 10
- 1.2.3 Purification and Characterization of IGFBPs 11
- 1.2.4 IGFBP-1 12
- 1.2.5 Factors Affecting Serum Levels of IGFBP-1 13
- 1.2.6 IGFBP-1 and Development 14
- 1.2.7 IGFBP-2 15
- 1.2.8 Factors Affecting Serum IGFBP-2 Levels 16
- 1.2.9 IGFBP-2 and Development 17
- 1.2.10 The 150 kDa IGFBP 18
- 1.2.11 IGFBP-3 20
- 1.2.12 Factors Affecting Serum IGFBP-3 Levels 20
- 1.2.13 IGFBP-3 and Development 21
- 1.2.14 IGFBP-4 22
- 1.2.15 Other Binding Proteins 23
- 1.2.16 Actions of IGFBPs 24
- 1.2.17 The IGFBP - IGF Interaction 25

1.3 BACKGROUND TO THESIS 27

CHAPTER TWO MATERIALS AND METHODS

2.1 MATERIALS 29
- 2.1.1 General Chemicals and Reagents 29
- 2.1.2 Antibodies and Protein Standards 29
- 2.1.3 Peptides and Hormones 30
- 2.1.4 Cell Culture Materials 30
- 2.1.5 Radiolabelled Peptides and Nucleotides 30
- 2.1.6 Cell Lines 31
- 2.1.7 Enzymes and Kits for Molecular Biology 31
- 2.1.8 Oligonucleotides 31

2.2 METHODS 32
- 2.2.1 Charcoal Binding Assay 32
- 2.2.2 Polyethylene Glycol (PEG) IGFBP Assay 33
- 2.2.3 Radioreceptor Assay 33
- 2.2.4 IGF-I Radioimmunoassay (RIA) 33
- 2.2.5 IGF-II radioreceptor assay (RRA) 34
- 2.2.6 Protein Synthesis Determination 34
- 2.2.7 Collection and Concentration of Conditioned Media 35
- 2.2.8 Stimulation of cells with Growth Factors and Hormones 35

2.2.9 SDS-Polyacrylamide Gel Electrophoresis 35
2.2.10 Silver Staining SDS-Polyacrylamide Gels 35
2.2.11 Western Ligand Blots and Immunoblots 36
2.2.12 Affinity Chromatography 36
2.3 PURIFICATION of He[39]L BINDING PROTEINS 37
2.3.1 Collection of Medium 37
2.3.2 S-Sepharose Chromatography 37
2.3.3 IGF-I Affinity Chromatography 37
2.3.4 High Performance Liquid Chromatography (HPLC) 38
2.3.5 Peptide Sequencing 38
2.4 METHODS FOR MOLECULAR BIOLOGY 38
2.4.1 Polymerase Chain Reaction 38
2.4.2 Northern Analysis 39
2.4.3 cDNA Library Screening 40
2.4.4 Preparation of Lambda gt11 DNA 40
2.4.5 Southern Blotting 41
2.4.6 Preparation of DNA for Subcloning 41
2.4.7 Ligations 41
2.4.8 Transformation of Bacteria 41
2.4.9 Preparation of Double Stranded Plasmid DNA 42
2.4.10 Preparation of Single Stranded Plasmid DNA 42
2.4.11 DNA Sequencing 42
2.5 COMPUTER PROGRAMMES 43

CHAPTER THREE CLASSIFICATION OF IGFBPs ACCORDING TO IGF-BINDING SPECIFICITIES

3.1 INTRODUCTION 44
3.2 RESULTS AND DISCUSSION 47
3.2.1 Competition binding assays with IGF-I and IGF-II as competing ligands 47
3.2.2 Competition binding assays with des-(1-3)-IGF-I as the competing ligand 50

CHAPTER FOUR CHARACTERIZATION OF IGFBPs PRODUCED BY HUMAN FIBROBLASTS

4.1 INTRODUCTION 54
4.2 RESULTS AND DISCUSSION 55
4.2.1 Competition binding assays with fibroblast conditioned media 55
4.2.2 Western ligand blotting of fibroblast conditioned medium 57
4.2.3 IGF production by He[39]L cells 60
4.2.4 He[39]L biological response to IGF 61
4.2.5 Stimulation of He[39]L binding protein production 61
4.2.6 Stimulation of SF 1972 binding protein production 63
4.2.7 Stimulation of Binding Protein Production by Growth Factors and Hormones 65

CHAPTER FIVE PURIFICATION AND CHARACTERIZATION OF He[39]L IGFBPs

5.1 INTRODUCTION 68
5.2 RESULTS 69
5.2.1 Purification of the 32 kDa He[39]L binding protein 69
5.2.2 Characterization of the 32 kDa He[39]L binding protein 70
5.2.3 Purification of 17-19 kDa binding protein 72
5.3 DISCUSSION 73

CHAPTER 6 CLONING OF THE 32 kDa He[39]L IGFBP

6.1 INTRODUCTION 80
6.2 RESULTS AND DISCUSSION 84
6.2.1 Original Polymerase Chain Reaction (PCR) Approach 84
6.2.2 PCR of bIGFBP-2 sequences 89
6.2.3 The second He[39]L PCR reaction 90
6.2.4 Oligonucleotide Screening of the IMR 90 cDNA library 95
6.4 CONCLUSION 97

CHAPTER 7 GENERAL DISCUSSION 99

BIBLIOGRAPHY 105

Summary

Insulin-like growth factors (IGF) are growth promoting peptides which act via cell membrane receptors (Type 1 and Type 2 receptors). The two forms of IGF, IGF-I and IGF-II, are found in the circulation associated with carrier proteins referred to as the insulin-like growth factor binding proteins (IGFBP). IGFBP are found in two molecular weight groups, namely the large (40-55 kDa) and the small (24-34 kDa) molecular weight binding proteins. The large molecular weight IGFBP (IGFBP-3) in association with an acid labile subunit forms the main carrier of IGFs in the circulation. The role of the smaller IGFBPs is not clear at this stage but they may act in the enhancement or inhibition of IGF action.

Four binding proteins available at the time of this study (IGFBP 1, 2, 3 and the 32 kDa He[39]L IGFBP described below) were used in the classification of IGFBP into three distinct groups according to their relative IGF-binding affinities. The three binding patterns were determined using competitive binding assays using purified IGFBPs and IGFs, including IGF-I, IGF-II and a truncated form of IGF-I (des-(1-3)-IGF-I). Earlier studies in the group determined that the IGF-I variant has a 5-10 fold enhanced biological activity over the full length IGF-I (Francis *et. al.*, 1988b). Classification of the binding proteins into groups on their ability to bind the variant lead to a speculation of the possible roles of the various binding proteins in the modulation of IGF action.

Further studies outlined in this thesis involved the characterization of binding proteins produced by a human embryonic fibroblast cell line (He[39]L). Techniques such as Western ligand blotting, immunoblotting and charcoal binding assays were used in the comparison of IGFBP produced by this cell line with IGFBPs of other human fibroblasts.

He[39]L cells produced relatively low amounts of binding protein when compared with cell lines used previously as a source of IGFBP purification (Hep G2 human hepatoma cells, MDBK bovine kidney cells). A series of stimulation experiments revealed that He[39]L binding protein production could be enhanced by co-incubation with IGF but not other factors or hormones tested. This suggests a mechanism by which IGF controls the levels of its own carrier.

Serum-free conditioned medium of He[39]L cells was identified as a source for the purification of novel binding proteins as it presented an unusual IGF-binding pattern. Two novel binding proteins were isolated and purified using cation exchange chromatography, affinity chromatography and reverse phase high performance liquid chromatography. A 25 amino acid N-terminal sequence was obtained for a 32 kDa He[39]L binding protein using the technique of electroblotting and the sequence differed from previously sequenced IGFBPs. Analysis of the protein revealed a small molecular weight (32 kDa) binding protein

with N-linked glycosylation. The He[39]L binding protein was shown to be immunologically unrelated to the bovine small molecular weight IGFBP-2.

The 32 kDa He[39]L binding protein did not bind des-(1-3)-IGF-I, and IGF-II competed for the binding sites on this protein better than IGF-I. This binding pattern corresponds to that of bovine IGFBP-2 which belongs to one of the three IGFBP groups described in this thesis.

A smaller 17-19 kDa binding protein was purified from He[39]L conditioned medium and had an N-terminal sequence of 13 residues, also differing from previously sequenced IGFBPs. This binding protein was not further characterized.

Having isolated the 32 kDa He[39]L binding protein with an N-terminal sequence quite different to other IGFBP an attempt was made to isolate a cDNA clone encoding that binding protein. Two approaches were taken. The first involved screening a human embryonic lung fibroblast library made from the IMR 90 cell line. These cells secrete a binding protein of the same size as the He[39]L binding protein. The library was probed with a degenerate oligonucleotide made to the predicted DNA sequence corresponding to amino acids 13-22. Three cDNA clones were isolated, all of which did not encode binding proteins.

The second parallel approach involved the use of the polymerase chain reaction (PCR) with the intention of amplifying He[39]L binding protein cDNA made from RNA of those cells. In a control reaction bovine IGFBP-2 sequences were amplified using the clone isolated in our laboratory as a template. However, even with the system optimized for the amplification of binding protein sequences no specific amplification of He[39]L cDNA was achieved. Further control experiments suggested a possible RNA secondary structure problem in the synthesis of cDNA used as a template in the He[39]L PCR.

The results presented in this thesis will be useful in the classification of novel binding proteins in the future. Further information is provided about the ever increasing numbers of IGFBPs with the purification of two novel IGFBPs. Isolation of novel IGF binding proteins and their characterization will ultimately be vital in elucidating the control mechanisms regulating the action of IGFs.

Statement

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

Briony E. Forbes

Acknowledgments

I wish to thank Professors W. H. Elliot and G. E. Rogers for permission to undertake this research in the Department of Biochemistry, University of Adelaide, South Australia. I am extremely grateful to Dr. John Wallace for his continual encouragement, criticism and total support. I also greatly appreciate the supervision and honest direction given by Dr. John Ballard. I acknowledge the financial support of the University of Adelaide Postgraduate Research Award.

I would like to thank Dr. A. V. Sivaprasad and Ian Dodd for assistance with computing and Dr. Rick Tearle for criticism and encouragement through writing. Both Geoff Francis and Dr. Paul Walton are to be thanked for many useful discussions. Many thanks also go to Dr. Helena Richardson, Dr. Jack Ohmdahl, Tim Cox and Paul Morretti for suffering PCR with me.

I am very grateful to Dr. Jane Stanley and Dr. Henry Forbes who generously provided me with the necessary equipment for writing. Barbara Magee and Paddy also had to forgo Crystal for several weeks and I greatly appreciate that. Many thanks to Robyn Thomson for proof-reading. I also acknowledge the assistance given by Marina Ross, Denise Turner and Shuichi Saki.

I cannot thank Grant Booker enough for his apparently endless patience and helpfulness, particularly in the last few months. I would also like to thank the other past and present laboratory members including Fil Lim, Christopher Bagley, David Mottershead, Michelle Walker, Helen Healey, Sharron Gargosky, Nick Kallincos, Neil Brewster, Dale Val and Jill Simes for being a great crowd to work with. Laszlo Szabo, Zee Upton and Sally Aplin need a particular mention for providing a refreshing angle to laboratory life which always kept me going.

Melissa Brown and Jane Stanley have been two friends who have been through many years of study and play with me and I truly appreciate their friendships. I also give many thanks to my two new found sisters, Natalie Neville and Cathy Sage, and to Louise O'Keefe, Phil Govey and the extended families who have lived through this PhD with me .

Particular appreciation goes to Rob King who has provided me with an endless source of strength throughout my PhD and I am truly grateful for all the support and encouragement of my parents and family.

Publications arising from work presented in this PhD thesis

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

Forbes, B., Szabo, L., Baxter, R. C., Wallace, J. C. and Ballard, F. J. (1988) A comparative study of the binding proteins of the human 53k, 28k and the MDBK-cell binding proteins using IGF-I, IGF-I variants and IGF-II. Abstract, IGF Symposium, Adelaide.

Forbes, B., Ballard, F. J. and Wallace, J. C. (1989) Characteristics of a small molecular weight binding protein isolated from a human lung fibroblast cell line (He[39]L). Abstract, 32nd Annual Meeting of the Endocrine Society of Australia

Forbes, B., Ballard, F. J. and Wallace, J. C. (1989) Characterization of an insulin-like growth (IGF) factor binding protein from medium conditioned by human lung fibroblasts (He[39]L). Abstract, 28th National Scientific Conference for the Australian Society for Medical Research.

Forbes, B., Ballard, F. J. and Wallace, J. C. (1990) *J. Endocrinol.* **126**, 497-506, An insulin-like growth factor-binding protein purified from medium conditioned by a human lung fibroblast cell line (He[39]L) has a novel N-terminal sequence.

Abbreviations

In addition to those accepted for the use in the Journal of Biological Chemistry, the following abbreviations are used in this thesis:

ALAS	δ-aminolevulinate sythetase
ALS	acid labile subunit
BSA	bovine serum albumin
cpm	counts per minute
CSF	cerebrospinal fluid
C-terminus	carboxy terminus
EGF	epidermal growth factor
FCS	fetal calf serum
FGF	fibroblast growth factor
IGF-I	insulin-like growth factor I
IGF-II	insulin-like growth factor II
IGFBP	insulin-like growth factor binding protein
bIGFBP	bovine IGFBP
hIGFBP	human IGFBP
mIGFBP	mouse IGFBP
pIGFBP	porcine IGFBP
rIGFBP	rat IGFBP
GH	growth hormone
MSA	multiplication-stimulating activity
Mr	relative molecular mass
NSILA	non-suppressible insulin-like activity
N-terminus	amino terminus
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PP12	placental protein 12
PTH-	phenylthio-hydantoin-
PVDF	polyvinylidene difluoride
RIA	radioimmunoassay
RRA	radioreceptor assay
rpHPLC	reverse phase high performance liquid chromatography
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION AND LITERATURE REVIEW

1.1.1 Insulin-like growth factors (IGFs)

Insulin-like growth factor binding proteins (IGFBPs) are directly involved in modulating the actions of insulin-like growth factors (IGFs) and for this reason a brief introduction into the IGFs will follow. The review of both IGFs and IGFBPs will encompass the literature available until the end of December, 1990. The most recent information relating to my PhD work will be included in the appropriate chapters.

It was about ten years after the identification of IGF activity that binding proteins were first detected. In that time the action of IGFs was extensively defined: firstly as sulphation activity (Salmon and Daughaday, 1957) stimulating sulphate incorporation by cartilage; then as non-suppressible insulin-like activity (NSILA) where the identified factor acted like insulin in adipose tissue, but this activity was not neutralized by an anti-insulin antibody (Froesch *et. al.*, 1963); and finally as multiplication-stimulating activity (MSA), a factor produced by rat liver cells which could promote growth of cells dependent normally on serum factors in culture medium (Dulak and Temin, 1973). Following purification and characterization, these factors were given the common name of IGF in 1987 (Daughaday *et. al.* 1987), also avoiding confusion with the term "somatomedin" designated to the various defined activities (Daughaday *et. al.*, 1972).

1.1.2 Primary Structure of IGFs

Two forms of IGF have been purified from many sources including mammalian and non-mammalian species. IGF-I (70 amino acids) and IGF-II (67 amino acids) were first isolated from human plasma (Rinderkneckt and Humbel, 1976a; Rinderknecht and Humbel, 1976b; Rinderknecht and Humbel, 1978a; Rinderknecht and Humbel, 1978b). The mammalian forms of IGF-I differ in only a few residues, human, bovine, and porcine IGF-I being identical while ovine, rat and mouse differ from these by only 1, 3 and 4 residues, respectively. The IGF-II molecules from the different mammalian species show a similar degree of species diversity (see Figure 1.1 and reviewed by Humbel, 1990).

Greater differences are seen between avian and mammalian IGFs than between the mammalian IGFs. Chicken IGF-I differs from human IGF-I by 8 residues (Roberts *et. al.*, 1987; Dawe *et. al.*, 1988; Kajimoto and Rotwein, 1989; Ballard *et. al.*, 1990a) and there are significant differences between human and chicken IGF-II in regions of the molecule believed to play a role in receptor binding and binding to IGFBPs (Kallincos *et. al.*, 1990). The sequences of salmon and Xenopus IGF-I have a similar degree of divergence from human IGF-I as chicken IGF-I, with 10 and 13 different amino acids respectively (Cao *et. al.*, 1989; Kajimoto and Rotwein, 1990).

More interestingly from the evolutionary viewpoint, the sequence of Atlantic hagfish IGF represents an intermediate between IGF-I and IGF-II (see Figure 1.1), particularly in the A and B domains (Nagamatsu *et. al.*, 1991). Agnatha to which the Atlantic hagfish belongs, is a class of chordates that diverged from the main line of vertebrates 550 million years ago. Therefore, the IGFs represent a family of slowly diverging, structurally and functionally - related peptides, and are probably present in all vertebrates.

1.1.3 Similarities Between IGFs and Insulin

Not only do the different IGFs show similarity to each other but they are related to insulin on both the amino acid and structural levels. IGF-I and IGF-II are 62% similar (Rinderknecht and Humbel, 1978b). Both have a similar domain structure to insulin with IGF-I sharing 25/51 amino acid residues with insulin, the main differences being on the surface of the IGF molecule. Two differences in structure between IGFs and insulin are the presence of a connecting C domain, normally cleaved from the proinsulin on secretion, and a carboxy-terminal extension, or D domain, in IGFs not found in proinsulin.

The similarity in structure of IGF to proinsulin suggests a common ancestral gene, with proinsulin probably the more recent product of evolution as it is proteolytically cleaved to form the dimeric molecule (Rinderknecht and Humbel, 1978b). A model of the tertiary structure of the IGFs has been proposed on the basis of the known structure of insulin (Blundell, Bedarkar and Humbel, 1983).

The degree of conservation of the members of the family of peptides including both IGFs and insulin suggests a elementary role of the peptides in the function of all vertebrates.

Figure 1.1: Sequence comparison of insulin and insulin-like growth factor (IGF)-related proteins

Those residues conserved in IGF-I or IGF-II are boxed and those residues also conserved in the insulin sequence are highlighted in black. The references from which these sequences are obtained are as follows:

IGF-I

Man	Rinderknecht and Humbel, 1978a
Cow	Honegger and Humbel, 1986
Cow [des (1-3)]	Francis *et. al.*, 1986
Pig	Francis *et. al.*, 1989b
Sheep	Francis *et. al.*, 1989a
Guinea Pig	Bell *et. al.*, 1990
Rat	Tamura *et. al.*, 1989
Mouse	Bell *et. al.*, 1986
Domestic fowl	Kajimoto and Rotwein, 1989
Xenopus	Kajimoto and Rotwein, 1990
Salmon	Cao *et. al.*, 1990
Hagfish	Nagamatsu *et. al.*, 1991

IGF-II

Man	Rinderknecht and Humbel, 1978b
Pig	Francis *et. al.*, 1989b
Cow	Honegger and Humbel, 1986
Sheep	Francis *et. al.*, 1989a
Rat 1	Dull *et. al.*, 1984
Rat 2	Marquardt *et. al.*, 1981
Mouse	Stempien *et. al.*, 1986
Domestic fowl	Kallincos *et. al.*, 1990

```
                       1        10        20        30        40        50        60        70
IGF-I  Man             GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPAKSA
       Cow             GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPAKSA
       Cow [des(1-3)]  ---TLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPAKSA
       Pig             GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPAKSA
       Sheep           GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKAAKSA
       Guinea Pig      GPETLCGAELVDALQFVCGDRGFYFNKPTGYGSSSRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPAKSA
       Rat             GPETLCGAELVDALQFVCGPRGFYFNKPTGYGSSIRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPTKSA
       Mouse           GPETLCGAELVDALQFVCGPRGFYFNKPTGYGSSIRRAPQT-GIVDECCFRSCDLRRLEMYCAPLKPTKAA
       Domestic fowl   GPETLCGAELVDALQFVCGDRGFYFSKPTGYGSSSRRLHHK-GIVDECCFQSCDLRRLEMYCAPIKPPKSA
       Xenopus         GPETLCGAELVDTLQFVCGDRGFYFSKPTGYGSNNRRSHHR-GIVDECCFQSCDFRRLEMYCAPAKPAKSA
       Salmon          GPETLCGAELVDTLQFVCGERGFYFSKPTGYGPSSRRSHNA-GIVDECCFQSCELRRLEMYCAPVKSGKAA
Hagfish IGF            LSETLCGSELVDTLQFVCDDRGFFFVPQVPPRAGAHAASAAAKGIVEECCFKGCSLALLEMYCAA--PSKAERDVA
IGF-II Man          AYRPSETLCGGELVDTLQFVCGDRGFYFSRPA--SRVSRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Pig          AYRPSETLCGGELVDTLQFVCGDRGFYFSRPA--SRVNRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Cow          AYRPSETLCGGELVDTLQFVCGDRGFYFSRPS--SRINRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Sheep        AYRPSETLCGGELVDTLQFVCGDRGFYFSRPS--SRINRRSR---GIVEECCFRSCDLALLETYCAA--PAKSE
       Rat 1        AYRPSETLCGGELVDTLQFVCSDRGFYFSRPS--SRANRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Rat 2        AYRPSETLCGGELVDTLQFVCSDRGFYFSRPG--SRANRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Mouse        AYGPGETLCGGELVDTLQFVCSDRGFYFSRPS--SRANRRSR---GIVEECCFRSCDLALLETYCAT--PAKSE
       Domestic fowl-YGTAETLCGGELVDTLQFVCGDRGFYFSRPV--GRNNRRIN---GIVEECCFRSCDLALLETYCAT--PAKSE
                    1        10        20        30        40        50        60           67

                        B1       B10       B20       B30         A1       A10       A20
Insulin Man             FVNQHLCGSHLVEALYLVCGERGFFYTPKA           GIVEQCCTSICSLYQLENYCN
```

The importance of IGFs as well as insulin in growth and development is emphasized by the fact that IGF-II is detected in preimplantation mouse embryos and IGF-I is detected in unfertilized chicken eggs. Both forms are also detected in the early stages (first days) of embryogenesis of those species (Heyner *et. al.*, 1989; De Pablo *et. al.*, 1990).

1.1.4 Serum Levels of IGFs and Factors Affecting IGF Levels

The normal serum level of IGF-I in adult humans is approximately 200 ng/ml and IGF-II is about 600 ng/ml (Zapf *et. al.* 1981). Some difficulty in interpretation of radioimmunoassays developed for the estimation of IGF levels has arisen due to the interference of binding proteins. The assays using separation by acid gel chromatography are the most appropriate as this method allows elimination of all binding proteins in the sample to be measured (Mesiano *et. al.*, 1988).

The serum levels of IGF-I and IGF-II in humans vary during prenatal to postnatal development and during puberty to adult maturity. Initially, IGF-I levels do not appear to vary in the first 9-19 weeks of gestation of the human fetus. During that period the levels in the lung, kidney and intestine are higher than the liver suggesting that the liver may not be the major source of circulating IGF-I as is believed for the adult (D'Ercole *et. al.*, 1986). The cord blood and newborn serum levels are much lower than adult levels but there is a surge in the first 10-15 days after birth (Lineham *et. al.*, 1986). Thereafter serum IGF-I levels continue to increase reaching a peak during puberty (Zapf *et. al.* 1981). After puberty the levels drop and remain constant till old age (>65 years) when they are again reduced (Donahue *et.al.*, 1990).

Human IGF-II levels are low in the term infant (282 ng/ml, Bennet *et. al.*, 1983) but increase after birth and reach a steady level after one year (Rosenfeld *et. al.*, 1986). The level of IGF-II in the developing fetus correlates with fetal gestational age (Bennet *et. al.*, 1983). This is in contrast to development in the rat where IGF-II levels are much higher in fetal than adult serum (Daughaday *et. al.*, 1982a). Also, IGF-II levels are higher than IGF-I in the developing fetus and IGF-II is believed to play a prominent role in organogenesis and the development of neural tissue and vasculature as well as in muscle and cartilage (Bondy *et. al.*, 1990). The significance of the differences in IGF levels between the human and rat is not as yet understood. However, the situation in the rat is also observed in fetal lamb serum (Gluckman and Butler, 1983).

Originally it was believed that all IGFs were regulated by growth hormone (GH), hence the term somatomedin (Daughaday *et. al.*, 1972). The somatomedin hypothesis was proposed after the observation that sulphate incorporation into cartilage of hypophysectomized rats could be stimulated by the administration of GH. IGF-I levels in human and rat sera are affected by GH. GH-deficiency can lead to lower IGF-I levels and correspondingly smaller stature in humans (Rosenfeld *et. al.*, 1986), whereas overexpression of GH results in acromegaly, associated with high IGF-I levels (Clemmons *et. al.*, 1979).

Contrary to the somatomedin hypothesis, it has been shown that IGF-II is only weakly GH regulated, if at all (Baxter, 1986). Serum IGF-II levels remain unchanged in acromegaly but are reduced in cases of GH deficiency and also in hypophysectomized rats; hence the reference to weak GH regulation (Zapf *et. al.*, 1981; D'Ercole *et. al*, 1984).

1.1.5 IGF-I and IGF-II cDNAs and Genes

Several cDNA clones have been isolated for both human and rat IGF-I and IGF-II and the genes encoding these peptides have also been identified (reviewed by Sussenbach, 1989). IGF-I cDNA clones have also been isolated from several other species (Bell *et. al.*, 1986; Travakkol, *et. al.*, 1988; Kajimoto and Rotwein, 1989). Jansen *et. al.* (1983) were the first to isolate an IGF cDNA clone for human IGF-I. They revealed that the IGF-I messenger RNA (mRNA) gives rise to a precursor peptide which is proteolytically cleaved to form the native peptide. In fact two different mRNAs are formed from the IGF-I gene and these arise by alternative splicing of the 3' untranslated region (Rotwein *et. al.*, 1986). The prepro mRNAs are referred to as IGF-Ia and IGF-Ib. In rat and man these two mRNAs arise by different means (Roberts *et. al.*, 1987). Indeed, there are many mRNA lengths present when tissues from both species are probed by Northern analysis. The different mRNAs arise due to alternative splicing from the primary transcript of exons 5 and 6 as well as multiple polyadenylation sites at the 3' end of exon 5 (Sussenbach, 1989). Further heterogeneity of rat IGF-I mRNA also arises due to alternative splicing of the 5' untranslated region.

Human IGF-I and IGF-II are encoded by single genes on chromosome 12 and 11 respectively (Brissenden, Ullrich and Francke, 1984; Tricoli *et. al.*, 1984). Three mRNA species arise from the human IGF-II gene and theses are a result of the presence of four promotors and two polyadenylation sites.

promoters. The rat IGF-II gene also has the same three promoters but multiple mRNAs are generated from this gene. This is most likely due to variable RNA processing or polyadenylation sites (Daughaday and Rotwein, 1989).

A number of variant IGF-I and IGF-II peptides have been purified from human serum, cerebrospinal fluid (CSF) and the brain. Most of these are believed to be a result of incorrect processing of the propeptides into the native IGF. A variant of IGF-II lacks the amino terminal alanine and represents 25% of the total serum IGF-II (Rinderknecht and Humbel, 1978b). Alternative processing is the likely origin of a peptide representing the carboxy terminal extension of the propeptide of IGF-II. The first amino acid of the variant lies two residues downstream of a potential prohormone processing site (Hykla *et. al.*, 1985). Two other variants of human IGF-II have insertions at positions 29 and 33, the latter having also a carboxy terminal 21 amino acid extension which is derived from the propeptide E domain (Hampton *et. al.*, 1989; Zumstein *et. al.*, 1985). Six other variants of IGF-I and IGF-II separated on the basis of isoelectric point have been isolated from human plasma (Blum *et. al.*, 1986). CSF contains mature and "big" IGF-II (approximately 7 kDa and 9 kDa respectively; Haselbacher and Humbel, 1982). The biological significance of these variants is unknown but all are biologically active in vitro.

A truncated variant of IGF-I has attracted substantial attention as it more biologically active than IGF-I. It has been purified from bovine colostrum (Francis *et. al.*, 1986), human brain (Carlsson-Skwirut *et. al.*, 1986; Sara *et. al.*, 1986), porcine uterus (Ogasawara *et. al.*, 1989) and human platelets (Karey *et. al.*, 1989). The truncated IGF, des-(1-3)-IGF-I, has the first three amino acids deleted. In protein synthesis and degradation assays des-(1-3)-IGF-I has an approximately ten fold greater potency than IGF-I. This phenomenon is due to a lack of interaction with certain IGFBPs (Szabo *et. al.*, 1988; Bagley *et. al.*, 1989) and will be discussed later.

1.1.6 Regulation of IGF Expression

Since the isolation of both IGF-I and IGF-II cDNAs from various species and subsequent isolation of the genes encoding those peptides a great deal of information on the expression of IGFs has been acquired (Sussenbach, 1989). It has been revealed that developmental and tissue specific factors regulate IGF expression in the fetus and adult. In the human fetus IGF-I is present in all tissues in the form of different sized transcripts (Han

et. al., 1988). The transcripts are expressed preferentially in specific tissues and in the rat have been shown to represent mRNAs with different 3'and 5' untranslated regions (Hoyt *et. al.*, 1988). The longer 3' untranslated regions may confer stability to the larger mRNAs and in some way be used in tissue specific expression (Hepler *et. al.*, 1990).

IGF-II is also expressed in a tissue specific manner, being present in all human fetal tissues except the cerebral cortex and hypothalamus. Messenger RNAs with different 5' untranslated regions arising from three promoters are preferentially expressed in various tissues (Sussenbach, 1989).In general IGF-II is at higher levels than IGF-I (Han *et. al.*,1988). In the adult rat IGF-II mRNA is expressed in the brain, uterus, kidney and heart and barely detectable in the liver whereas IGF-I mRNA is present in all tissues (Murphy *et. al.*, 1987a).

Developmental control is also affected with the onset of puberty when there is an increase in IGF-I levels as mentioned above. This increase appears to be earlier in girls than boys and the earlier growth and maturation of girls may be partly a reflection of the IGF-I levels. Therefore gender and sex hormones also seem to be factors affecting IGF expression (Zapf *et. al.*, 1981).

As mentioned, regulation of IGF expression by hormonal control is not only observed in response to GH but also with a number of other hormones and growth factors. In particular, IGF-I levels are locally increased in the uterus of immature rats in response to administration of estrogen (Murphy *et. al.*, 1987b) and may be important in the growth and development of the uterus. In fact, pregnancy in humans results in increased maternal IGF-I serum levels (Bala *et. al.*, 1981; Hall *et. al.*, 1984). Placental lactogen can also stimulate the production of IGF-I and IGF-II *in vitro* (Adams *et. al.*, 1983). In addition, fibroblast growth factor , angiotensin II and acute corticotropin (ACTH) stimulate the secretion of IGF-I by bovine adrenal cells (Penhoat, *et. al.*, 1989).

Another factor affecting the expression of IGFs is the nutritional status of the individual. Studies using nutritionally deprived rats have measured both decreased serum IGF-I levels and decreased expression of IGF-I mRNA transcripts in various tissues (Emler and Schalch, 1987; Maiter *et. al.*, 1989; Bornfeldt *et. al.*, 1989). Similar reduction in serum levels of IGFs was observed in fasting humans (Clemmons *et. al.*, 1981a; Merimee *et. al.*, 1982). In the liver there is selective reduction in expression of particular IGF-II mRNAs,

again demonstrating the control of IGF expression at the level of mRNA as observed in tissue specific control of IGF expression (Straus *et. al.*, 1988).

1.1.7 IGF Cellular Receptors

There are two membrane bound IGF receptors. Both, or usually at least one, are expressed on the surface of most cells. The type 1 IGF receptor is a heterotetramer consisting of two α (135 kDa) and two β (95 kDa) subunits linked by disulphide bonds (Rechler and Nissley, 1985). The β subunits of the type 1 receptor represent the intracellular domain. Each α and β subunit arises from a single propeptide which is postranslationally cleaved to form the dimeric structure (Ullrich *et. al.*, 1986). A cDNA clone has been isolated encoding the type 1 receptor and the sequence reveals a significant homology to the insulin receptor (Ullrich *et. al.*, 1986). The insulin receptor is also a heterotetramer. Both receptors have intracellular tyrosine kinase sites (Jacobs *et. al.*, 1983) and in fact the domains in each receptor containing these sites show 84% similarity.

The type 2 IGF receptor is a single peptide containing no intermolecular disulphide bonds. It has a short intracellular domain and an extracellular domain consisting of 15 cysteine rich repeats (Morgan *et. al.*, 1987). The cDNA sequence encoding the type 2 receptor revealed sufficient similarity with the bovine cation-independent mannose 6-phosphate receptor that they are now considered identical proteins (Lobel *et. al.*, 1988). Hence the receptor can bind not only IGF-II but also proteins containing mannose 6-phosphate moieties. A soluble form of the type 2 receptor extracellular domain is found in blood, but its role in IGF binding is not understood.

The three receptors are encoded by single genes located on separate chromosomes. The insulin receptor is on the short arm of chromosome 19 (Yang-Feng *et. al.*, 1985), the type 1 receptor is on the long arm of chromosome 15 (Ullrich *et. al.*, 1986) and the type 2 receptor is located on the long arm of chromosome 6 (Laureys *et. al.*, 1988).

There is a very limited structural similarity between the type 2 receptor and the type 1 and insulin receptors (Morgan *et. al.*, 1987). This is reflected in the relative abilities of the receptors to bind the various peptides. With the advent of production of both IGF-I and IGF-II by recombinant technology techniques pure sources were available to test the relative binding by the two receptors. The type 1 receptor not only binds IGF-I but also binds

IGF-II to only a slightly lesser extent and binds insulin weakly. The type 2 receptor on the other hand has greatest affinity for IGF-II and binds IGF-I at a very much lower affinity but does not bind insulin (Roth, 1988). Previously, using preparations of IGF-I containing trace contaminations of IGF-II, a less marked difference was noted in type 2 receptor binding studies (Rechler and Nissley, 1985). Although the type 1 and type 2 receptors are so different in their primary structure, obviously they must share some conformational similarity in their IGF binding domains.

There is considerable evidence demonstrating that IGF-II elicits most of its insulin-like activities through the type 1 receptor rather than the type 2 receptor (Mottola and Czech, 1984; Ewton *et. al.*, 1987). Possibly the mannose-6-phosphate/type 2 receptor acts to recirculate IGF-II and type 2 receptors (Czech, 1989).

A great deal of effort has been concentrated on the analysis of the receptor binding of IGFs. Systematic site-directed mutagenesis of IGF-I followed by receptor binding studies has been the approach to determine the residues on the peptides involved in the interaction with receptors. The structures of IGF-I, IGF-II and insulin are represented in Figure 1.2. The different domains and some specific residues which interact with receptors are highlighted. Residue 24 of IGF-I and the C domain are important for IGF-I binding to type 1 receptors (see Figure 1.2; Cascieri *et. al.*, 1988a; Bayne *et. al.*, 1989). Changing the amino acids of the B and A domains to residues found in insulin in the corresponding positions significantly reduces the IGF-I binding affinity to type 2 receptors (see Figure 1.2; Bayne *et. al.*, 1988). These experiments demonstrate that there are two different receptor binding sites on IGF-I for the different IGF receptors. Also, each receptor recognises a different structural determinant on the various IGF peptides. In fact, the IGF determinants recognised by IGFBPs are also unique and will be discussed in more detail in section 1.2.17.

1.1.8 Biological Effects of IGFs

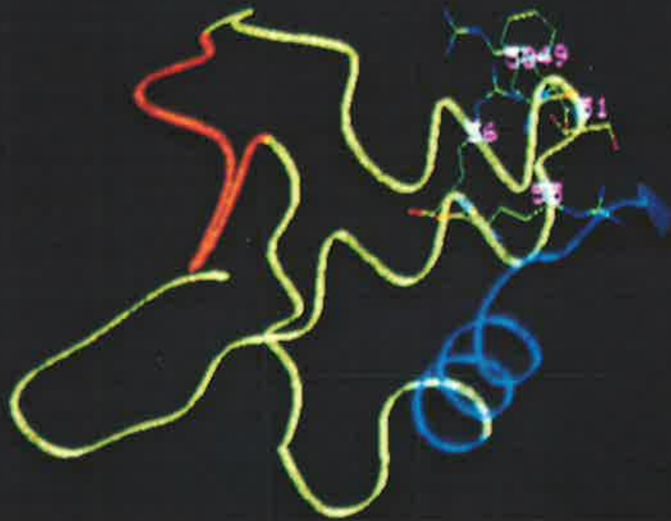
IGFs are found in serum and other body fluids including CSF (Haselbacher and Humbel *et. al.*, 1982), amniotic fluid (Merimee *et. al.*, 1984) and saliva (Costigan *et. al.*, 1988). They are also secreted by a range of different cell lines including human fibroblasts (Clemmons and Shaw, 1986), rat liver cells (Marquardt *et. al.*, 1981), fetal rat calvariae (Canalis *et. al.*, 1988), Sertoli cells (Smith *et. al.*, 1987) and adrenal cells (Penhoat *et. al.*,

Figure 1.2: Insulin-like growth factor (IGF) receptor binding domains in the IGF-I, IGF-II and insulin structures

Three dimensional models of IGF-I, IGF-II and insulin were generated using the Insight II program (Biosym Technologies Inc., San Diego, CA, USA, 1990) with coordinates obtained from the Protein databank, Brookhaven National Laboratory, Upton, NY, U.S.A. IGF coordinates were determined by comparison with the insulin structure and predictions made by Blundell *et. al.*, (1983). The models highlight the similarity in tertiary structure shared between the three proteins. The domains are shown in different colours as follows:

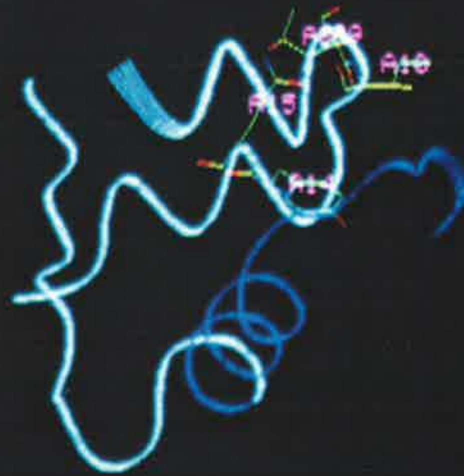
A domain, IGF-I,	green
A domain, IGF-II,	yellow
A domain, Insulin,	pale blue
B domain,	blue
C domain,	red

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



IGFI

IGFII



INSULIN

1989). Also, IGF mRNA is found in most tissues of the body as described above. These observations support the theory that IGFs not only function in an endocrine fashion being secreted by the liver and distributed to target tissues via the serum, but also may exert their effects in a paracrine and/or autocrine manner when produced locally.

In vivo studies provide evidence to support the somatomedin hypothesis and an endocrine action of IGF in that IGF can replace some of the functions of GH in hypophysectomized rats. With the lack of GH these rats are growth retarded. Subcutaneous infusion of IGF-I (103 µg/100 g rats/day) into hypophysectomized rats results in an increase in body weight (Schoenle *et. al.*, 1985). Similar results were seen with normal neonatal rats. Their growth rate and certain organ sizes were greater than litter mates not receiving IGF-I and an accelerated eye opening associated with a faster epithelial cell differentiation was observed. An effect on differentiation of erythroid progenitor cells was noted in that model and also reported by Kurtz *et. al.*, (1988), who infused 120 µg IGF-I/day into hypophysectomized rats and measured an increase in ^{59}Fe incorporation into erythrocytes and an increase in the number of reticulocytes.

Infusion of 20 µg IGF-I/kg body weight/hour was performed on normal adult humans (Guler *et. al.*, 1989). The result of the infusion over six days was to reduce circulating GH and IGF-II levels presumably by negative feedback regulation of GH secretion and displacement of IGF-II from IGFBPs. The lack of effect on nitrogen balance in the normal subjects may indicate the need for damage or body stress to see a positive effect induced by IGF-I.

Many studies have looked at the effects of IGFs *in vitro* using cultured cells and tissue explants. The earlier studies with tissue explants demonstrated the NSILA and sulphation activities as described earlier (Salmon and Daughaday, 1957; Froesch *et. al.*, 1963), whereas the cell culture experiments revealed more specifically the abilities to act as mitogens and to stimulate differentiation. IGF stimulates growth of chicken embryo fibroblasts measured by an increase in thymidine incorporation (Moses *et. al.*, 1978). IGF-I and IGF-II promote the differentiation of chicken myoblasts to mature muscle cells in primary culture as measured by an increase in acetylcholinesterase activity (Schmid *et. al.*, 1983). Similar experiments using alkaline phosphatase as a marker of differentiation show stimulation of differentiation by IGF-I and IGF-II of cultured osteoblasts resulting in collagen synthesis (Schmid *et. al.*, 1984). IGF can also stimulate differentiation of

oligodendrocytes (McMorris *et. al.*, 1986), erythroid cells (Kurtz *et. al.*, 1985) and ovarian cells (Veldhuis and Demers, 1985), and so appears to have a broad spectrum of target tissues in which it can elicit its effects.

1.2 IGF BINDING PROTEINS (IGFBPs)

1.2.1 Identification of IGF Carrier Proteins

For a long time it has been recognized that IGF is found in serum in a large molecular weight form. Under neutral gel filtration conditions the insulin-like activity was identified in a 70-150 kDa complex (Bürgi *et. al.*, 1966; Jakob *et. al.*, 1968), which after acidification appeared as a 6-10 kDa active species. Zapf *et. al.* (1975a) demonstrated the existence of a specific carrier protein in serum. The carrier protein could compete for somatomedin binding sites in a charcoal binding assay in which iodinated somatomedin was separated from somatomedin-carrier complexes by adsorption of the unbound radiolabel to activated charcoal.

The identification of IGF carrier proteins by Zapf *et. al.* (1975a) was confirmed by others (Hintz and Liu, 1976). Binding proteins in serum were also identified by Kaufmann *et. al.* (1977) by following the clearance rate of radiolabelled IGF with neutral gel chromatography. In the first 20 minutes after injection into normal rats the IGF was shown to be associated with smaller molecular weight fractions (approximately 50 kDa). Thereafter the IGF was associated with the larger molecular weight fractions (up to 200 kDa) for up to three hours. The presence of binding proteins provided an explanation for such high levels of IGF not leading to hypoglycemia. In fact, the IGF in human serum is found totally in association with binding proteins eliminating any possibility of hypoglycemia (Daughaday *et. al.*, 1982b; Zapf *et. al.*, 1975a; Martin and Baxter, 1985). Both gel filtration and ultracentrifugation of serum show that IGF is associated predominantly with the larger 70-150 kDa binding protein rather than the 30-50 kDa IGF-binding species (Hintz and Liu, 1976; Daughaday *et. al.*, 1982b).

1.2.2 Classification of IGFBP

Since the identification of IGF carrier proteins in serum a number of binding proteins have been purified and extensively characterized. In 1989 a general concensus for the

nomenclature of binding proteins was agreed upon. Following the isolation of a cDNA clone for a novel binding protein it is named as IGFBP-1, IGFBP-2 etc. according to the order of published sequence (Ballard *et. al.*, 1990b). Up to late 1990 four distinct binding proteins have been sequenced at the cDNA level. Others have been purified and partial amino acids sequences have been reported. Table 1.1 lists the references for the purification of IGFBPs for which the cDNA sequences have been reported. The accepted name of each binding protein is given as well as any previous names. Table 1.2 outlines the references to the sequences of binding protein cDNA clones.

Prior to the cloning of any binding proteins, they were classified in a number of ways. Initially binding proteins were classed according to size. Hossenlopp *et. al.*, (1986) developed the Western ligand blot technique which lead to the discovery that there are not only two binding protein species in serum as identified by gel chromatography but there are the high molecular weight and several low molecular weight binding proteins (< 40 kDa). This observation was confirmed by others using crosslinking techniques (D'Ercole and Wilkins, 1984; Wilkins and D'Ercole, 1985; Ooi and Herington, 1986). It should be noted that identification of binding proteins having the same molecular weight does not infer molecular identity.

Binding proteins found in the high molecular weight range after neutral gel chromatography are GH-dependent and are distinguished from low molecular weight binding proteins, therefore, not only on the basis of size but by the nature of the control of their expression (Cohen and Nissley, 1976; Moses *et. al.*, 1976). This will be discussed in more detail in reference to particular binding proteins.

Further classification of binding proteins on the basis of their relative IGF-binding affinities is a topic for discussion in this thesis and has revealed at least three classes of binding proteins (Forbes *et. al.*, 1988).

1.2.3 Purification and Characterization of IGFBPs

Of the four binding proteins characterized at the cDNA, level IGFBP-1, IGFBP-2 and IGFBP-3 are by far the most extensively researched. Both amino acid and cDNA sequences of the different binding proteins have revealed a remarkable conservation of the relative positions of 18 cysteines. The cysteines are obviously playing an important role in

NAME	PREVIOUS NAME	SOURCE	Mr	REFERENCE
hIGFBP-1	AFBP	human amniotic fluid	35-40 kDa	Drop *et. al.*, 1984a
			32 kDa	Povoa *et. al.*, 1984a
				Clemmons *et. al.*, 1986
				Busby *et. al.*, 1988
			31 kDa	Busby *et. al.*, 1989
	HEP G2 BP	human hepatoma cell (HEP G2) conditioned medium	32 kDa	Povoa *et. al.*, 1985
	Placental protein 12 (PP12)	human decidua	34 kDa	Koistinen *et. al.*, 1986
rIGFBP-2	BRL 3A BP	rat liver cell (BRL 3A) conditioned medium	36 kDa	Mottola *et. al.*, 1986
				Lyons and Smith, 1986
				Romanus *et. al.*, 1987
		rat serum	33 kDa	Zapf *et. al.*, 1988
bIGFBP-2	MDBK BP	bovine kidney cell (MBDK) conditioned medium	32 kDa	Szabo *et. al.*, 1988
hIGFBP-3	BP53	human serum	53 kDa, 47 kDa	Baxter and Martin, 1987
			40 kDa	Martin and Baxter, 1986
			45 kDa, 42 kDa, 31 kDa	Grant *et.al.*, 1987
			45 kDa	Zapf et. al., 1990a
	IDF45	ovarian follicular fluid	79 kDa	Blat *et.al.*, 1989a
rIGFBP-3	BP53	rat serum	48 kDa, 44 kDa	Baxter and Martin, 1987
			45 kDa, 42kDa	Zapf *et. al.*, 1988
			45 kDa, 37 kDa, 22-28 kDa	Shimonaka *et. al.*, 1989
pIGFBP-3		porcine serum	45 kDa, 41 kDa	Walton *et. al.*, 1989
hIGFBP-4		human bone cell conditioned medium	24 kDa	Mohan *et. al.*, 1989
		human seminal plasma	25 kDa	Rosenfeld *et. al.*, 1990
rIGFBP4		rat serum	32 kDa	Shimonaka *et.al.*, 1989

Table 1.1: Purification of IGFBPs. The purification of IGFBP 1, 2, 3 and 4 has been performed by the authors described in this table. The names formerly given to the respective IGFBPs is shown. The source and molecular weight measured in each purification are also listed.

IGFBP TYPE	SOURCE	REFERENCE
hIGFBP-1	Human decidua cDNA library	Brewer *et. al.*, 1988
	Human placenta cDNA library	Brinkman *et. al.*, 1988a
	Human decidual cDNA library	Julkunen *et. al.*, 1988
	HEP G2 cDNA library	Luthman *et. al.*, 1989
	HEP G2 cDNA library	Lee *et. al.*, 1988
	Human genomic library	Brinkman *et. al.*, 1988a
	Human leukocyte genomic library	Cubbage *et. al.*, 1989
rIGFBP-1	Rat decidual cDNA library	Murphy *et. al.*, 1990
hIGFBP-2	Fetal liver cDNA library	Binkert *et. al.*, 1989
	HEP G2 cDNA library	Zapf *et. al.*, 1990a
bIGFBP-2	MDBK cDNA library	Upton *et. al.*, 1990
rIGFBP-2	BRL 3A cDNA library	Brown *et. al.*, 1989
	Adult rat liver cDNA library	Margot *et. al.*, 1989
	Rat genomic library	Brown and Rechler, 1990
hIGFBP-3	Human liver cDNA library	Spratt *et. al.*, 1990
	Human liver cDNA library	Wood *et. al.*, 1988
	Human genomic library	Cubbage *et. al.*, 1990
rIGFBP-3	Rat liver cDNA library	Albiston and Herington, 1990
pIGFBP-3	Porcine ovarian cDNA library	Shimasaki *et. al.*, 1990a
hIGFBP-4	Osteocarcinoma cell cDNA library	LaTour *et. al.*, 1990
rIGFBP-4	Rat liver cDNA library	Shimasaki *et. al.*, 1990a

Table 1.2: Cloning of IGFBPs. IGFBP 1, 2, 3 and 4 have been cloned from cDNA libraries. The source of library used by each researcher is listed. The genes for IGFBP 1, 2 and 3 have also been isolated.

the maintenance of the tertiary structure of binding proteins leading to the structural conformation involved in IGF binding. There are 12 cysteines in the amino terminal domain of binding proteins and 6 in the carboxy terminal domain. Both ends of the proteins contain the areas of greatest similarity between the different binding proteins with a nonconserved region connecting them (see Figure 1.3).

1.2.4 IGFBP-1

The first binding protein to be purified was the small molecular weight binding protein now termed human IGFBP-1 (hIGFBP-1). Formerly IGFBP-1 was called amniotic fluid binding protein referring to the original source (see Table 1.1; Drop *et. al.*, 1984a; Povoa *et. al.*, 1984a). It has also been called BP 28 (28 kDa) after isolation from conditioned medium of human hepatoma cells (HEP G2; Povoa *et. al.*, 1985) and placental protein 12 (PP12) when purified from human decidua (Koistinen *et. al.*, 1986). hIGFBP-1 has a molecular weight of just over 25 kDa as deduced from the cDNA sequence. Determination of size by SDS polyacrylamide gel electrophoresis (SDS-PAGE) led to reports of 28 kDa (nonreduced, Baxter *et. al.*, 1987) and 32-35 kDa (reduced, Drop *et. al.*, 1984a; Povoa *et. al.*, 1984a; Rutanen *et. al.*, 1988). The binding protein does not appear to be N-glycosylated but has 5 potential O-glycosylation sites as deduced from the cDNA sequence (Brinkman *et. al.*, 1988a).

Both human and rat cDNA sequences are now available (see Table 1.2 for references). There is approximately 66% similarity between the IGFBP-1 of the two species with most similarity in the amino and carboxy terminal domains of the protein (Murphy *et. al.*, 1990). The gene for IGFBP-1 has been isolated and spans over 5 kb with four exons (Brinkman *et. al.*, 1988b; Cubbage *et. al.*, 1989). The gene is localized on chromosome 7 (Brinkman *et. al.*, 1988b). It gives rise to a single mRNA of approximately 1.4-1.5 kb. Rat IGFBP-1 (rIGFBP-1) is thirteen amino acids longer than its human counterpart due to amino acid substitutions in two sites of the sequence. IGFBP-1 sequences contain an Arg-Gly-Asp (RGD) motif near the carboxy-terminus. The motif is found in several matrix proteins and is involved in recognition by specific adhesion receptors (Ruoslahti and Pierschbacher, 1986), although such a role has not been determined for binding proteins.

```
IGFBP-1          AP--WQCAPCSAEKLALCPPVSA---------------SCSEVTASAGCGCCPMCALPLGAACG
IGFBP-2             EVLFRCPPCTPERLAACGPPPVAPPAAVAAVAGGARMPCAELVREPGCGCCSVCARLEGEACG
IGFBP-3  GASSGGLGPVVRCEPCDARALAQCAPPPA---------------VCAELVREPGCGCCLTCALSEGQPCG
IGFBP-4             DEAIHCPPCSEEKLARCRPP----------------VGCEELVREPGCGCCATCALGLGMPCG

IGFBP-1  VATARCARGLSCRALPGEQQPLHALTRGQGACVQES-----------DASAPHAAEAGSPESPESTEITE
IGFBP-2  VYTPRCGQGLRCYPHPGSELPLQALVMGEGTCEKRR-----------DAEYGASPEQVADNGDDHSE---
IGFBP-3  IYTERCGSGLRCQPSPDEARPLQALLDGRGLCVNAS-----------AVSRLAAYLPAPPAP-------L
IGFBP-4  VYTPRCGSGLRCYPPAGVEKPLHTLMHGQGVCMELAEIEAIQESLQPSDKDEGDHPNNSFSPCSAHDRRC

IGFBP-1  EELLDNFHLMAPSEEDHS----ILWDAIST------YDGSKALHVTNIKKWKE-----------------
IGFBP-2  -------GGLVENHVD---STMNMLGGGGSAGRKPLKSGMKELAVFREKVT-EQHRQMGKGGKHHLGLEE
IGFBP-3  -------GNASESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIIIKKGHAKDSQRYKVDYESQSTDT
IGFBP-4  ------LQKHFAKIRDRSTSGGKMKVNGAPREDARPVPQG------------------------------

IGFBP-1  --------------PCRIELYRVVESLAKAQETSGEEI----SKFYLPNCNKNGFYHSRQCETSMDGEAG
IGFBP-2  PKKLRPPPART---PCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRG
IGFBP-3  QNFSSESKRETEYGPCRREMEDTLNHLKFLNVLSPRGV-------HIPNCDKKGFYKKKQCRPSKGRKRG
IGFBP-4  --------------SCQSELHRALERLAASQ--S-RTH-EDLYIIPIPNCDRNGNFHPKQCHPALDGQRG

IGFBP-1  LCWCVYPWNGKRIPGSPEIRGDPNCQMYFNVQN
IGFBP-2  ECWCVNPNTGKLIQGAPTIRGDPECHLFYNEQQEACGVHTQRMQ
IGFBP-3  FCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK
IGFBP-4  KCWCVDRKTGVKLPGGLEPKGELDCHQLADSFRE
```

Figure 1.3: Sequence comparison of four human insulin-like growth factor binding proteins (IGFBPs). The amino acid sequences of human IGFBP 1, 2, 3 and 4 were deduced from cDNA sequences. Eighteen cysteines are conserved throughout. Human IGFBP-2 has two extra cysteines in the central nonconserved region and possibly another at the C-terminal end (Binkert *et. al.*, 1989). For references reporting the sequences of these IGFBPs refer to Table 1.2.

1.2.5 Factors Affecting Serum Levels of IGFBP-1

Soon after its purification a radioimmunoassay (RIA) was developed for hIGFBP-1 and levels were measured in serum (Drop *et. al.*, 1984b; Povoa *et. al.*, 1984b). Adult serum has lower levels of hIGFBP-1 than amniotic fluid (9 ng/ml and 37 ng/ml respectively; Busby *et. al.*, 1988b; Baxter *et. al.*, 1987). The major source of serum IGFBP is believed to be the the liver. hIGFBP-1 is found in other body fluids including lymph (Binoux and Hossenlopp, 1988) and milk (Suikkari, 1989) but is not present in cerebrospinal and seminal fluids (Rosenfeld *et. al.*, 1989; Rosenfeld *et. al.*, 1990).

As a member of the small molecular weight binding protein complex identified by neutral gel chromatography, hIGFBP-1 is classed as GH-independent (Cohen and Nissley, 1976; White *et. al.*, 1981). However, this term appears to be not strictly agreed upon as, using a RIA, serum hIGFBP-1 levels in acromegaly are reduced whereas in GH-deficient patients hIGFBP-1 is found at twice the concentration of normal adults (Hardouin *et. al.*, 1989; Povoa *et. al.*, 1984b). Also, using rIGFBP-1 cDNA as a probe, Seneviratne *et. al.* (1990) showed that hepatic rIGFBP-1 mRNA levels are increased following hypophysectomy but reduced by subsequent GH treatment. However, Baxter and Cowell (1987) showed by RIA that serum hIGFBP-1 levels remained the same in normal and GH-deficient subjects and Ooi *et. al.* (1990) saw very small increases if any in hypophysectomized rat liver IGFBP-1 mRNA. These discrepancies remain unexplained.

Baxter and Cowell (1987) also showed that hIGFBP-1 levels fluctuate throughout a 24 hour period. The diurnal rhythm is actually influenced by nutritional status, that is the intake of food leads directly to a decrease in IGFBP-1 levels (Cotterill *et. al.*, 1988; Busby *et. al.*, 1988b). Prolonged fasting leads to elevated hIGFBP-1 concentrations in serum which can be restored to normal upon resumption of food intake (Busby *et. al.*, 1988b). This may reflect the effect of insulin which when increased in concentration, for example after feeding, leads to a decrease in serum hIGFBP-1 (Suikkari *et. al.*, 1988; Unterman *et. al.*, 1990).

Although hIGFBP-1 levels are increased in insulin-dependent diabetes (decreased insulin levels), there is no correlation with IGF-I levels, which are decreased, remain normal or are increased according to different reports (Suikkari *et. al.*, 1988). The effect of insulin on IGFBP-1 levels has been confirmed by in vitro studies using human hepatoma cells

(HEP G2). Increasing concentrations of insulin in the culture medium leads to an decrease in IGFBP-1 production by those cells (Cotterill *et. al.*, 1989; Conover and Lee, 1990). 50% reduction of IGFBP-1 secretion by cultured human fetal explants resulted after exposure to insulin (Lewitt and Baxter, 1989).

1.2.6 IGFBP-1 and Development

Developmental factors affect the circulating levels of IGFBP-1. Using the specific RIA described above, the serum hIGFBP-1 levels were shown to decrease with age (Drop *et. al.*, 1984b). Western ligand blotting was used to confirm this observation. Unfortunately this technique fails to identify exact types of IGFBP but the changes can be described for each IGFBP size. All binding proteins except a 34 kDa species were shown to be reduced in elderly women (Donahue *et. al.*, 1990). Looking at rat liver binding proteins by Northern analysis rIGFBP-1 mRNA levels also decreased with maturation (Ooi *et. al.*, 1990).

There is a switch in the distribution of serum IGF from predominantly small molecular weight binding proteins to high molecular weight IGFBP during the late stages of human fetal development (D'Ercole *et. al.*, 1980). A similar transfer occurs in other species including sheep and rats at or near the time of birth (White *et. al.*, 1982; Butler and Gluckman, 1986). The switch may indicate the induction of GH-dependent growth and hence the appearance of high molecular weight IGF-binding protein complexes.

IGFBP-1 appears to play an important role in fetal development. Even in the differentiation stage of formation of the placenta (decidualization) hIGFBP-1 levels are elevated in the deciduoma (Croze *et. al.*, 1990). The deciduoma provides the neighouring placenta with immunoreactive hIGFBP-1 not produced by this tissue (Rutanen *et. al.*, 1985). The concentration of hIGFBP-1 in amniotic fluid is an indicator of fetal maturity as it declines with time (Baxter *et. al.*, 1987).

Maternal serum binding protein concentrations change during pregnancy. In general, prenatal and one day postpartum hIGFBP-1 levels are greatly elevated over those found in the non-pregnant adult (Ooi *et. al.*, 1990). Western ligand blotting reveals that IGFBP-1 levels in pregnant rat and human sera are increased whereas other binding proteins are decreased, especially in the later stages of pregnancy (Gargosky *et. al.*, 1990a; Guidice *et. al.*, 1990). This is believed to be a result of pregnancy specific serum proteases which

degrade or inactivate the large molecular weight binding proteins (Davenport *et. al.*, 1990; Guidice *et. al.*, 1990; Hossenlopp *et. al.*, 1990).

In fetal tissues immunostaining with anti-IGFBP-1 antibody shows the presence of the binding protein in all tissues except cerebral cortex, spleen and thyroid and is mainly in cells undergoing differentiation (Hill *et. al.*, 1989). In contrast, other studies with adult tissues have detected hIGFBP-1 mRNA only in liver, secretory endometrium and pregnancy decidua but not in kidney, adrenal and proliferative endometrium (Julkanen *et. al.*, 1988; Waites *et. al.*, 1990). This suggests that the liver may be the primary source of non-pregnant adult hIGFBP-1. In the adult rat, rIGFBP-1 is not only present in liver and deciduoma but also in kidney and to a lesser extent in brain and uterus from pseudopregnant rats (Murphy *et. al.*, 1990). This may indicate some species specific variation in the expression of IGFBP-1.

Binding proteins are present in the sera of the mammalian species studied so far. Both high and low molecular weight IGF binding proteins have been identified by gel filtration in the serum of sheep (Butler and Gluckman, 1986; Hodgkinson *et. al.*, 1989) and cows (Hossner *et. al.*, 1988) and by cross-linking to radiolabelled IGF in pigs (McCusker *et. al.*, 1988). However, IGFBP-1 has not yet been purified from these sera. Both large and small molecular weight binding proteins are present in chicken serum (Armstrong *et. al.*, 1989; Francis *et. al.*, 1990).

1.2.7 IGFBP-2

IGFBP-2 was originally purified from serum-free medium conditioned by rat liver cells (BRL 3A, Mottola *et. al.*, 1986) and bovine kidney cells (MDBK, Szabo *et. al.*, 1988). It has a molecular weight of 40 kDa under reducing conditions and 34 kDa under non-reducing conditions (Szabo *et. al.*, 1988; Mottola *et. al.*, 1986). Although an antibody for rIGFBP-2 fails to detect the protein in adult rat serum, rIGFBP-2 has been purified from this source (Zapf *et. al.*, 1988). The antibody detects significant levels of IGFBP-2 in fetal rat serum and the binding protein has therefore been implicated in fetal development (Romanus *et. al.*, 1986). However, the antibody raised against bIGFBP-2 detects IGFBP-2 in both fetal and adult bovine serum. Perhaps there is some difference in serum IGFBP-2 levels between species. Interestingly, this IGFBP-2 antibody shows crossreactivity with other species, unlike the anti-human IGFBP-1 antibody. It detects IGFBP-2 in fetal and adult

sheep sera and cross reacts with adult chicken serum but not adult human serum (Upton *et. al.*, 1990).

Not only the rat (Brown *et. al.*, 1989; Margot *et. al.*, 1989) but also bovine (Upton *et. al.*, 1990) and human IGFBP-2 (Binkert *et. al.*, 1989; Zapf *et. al.*, 1990a) cDNA clones have been isolated (see Table 1.2). The deduced molecular weight of each IGFBP-2 is approximately 30 kDa which is significantly lower than is visualized by SDS-PAGE. However, all IGFBP-2 appear not to be glycosylated, so this can not account for the differences in observed molecular weights. The 18 cysteines are conserved in each species as they are for IGFBP-1. The rat sequence has four in-frame deletions in comparison with the human and bovine IGFBP-2 and there is a 82% and 88% similarity when comparing bovine IGFBP-2 with rat and human, respectively (Binkert *et. al.*, 1989; Upton *et. al.*, 1990). The single messenger RNA encoding IGFBP-2 is approximately 1.5-2 kb. Like IGFBP-1, IGFBP-2 sequences also have RGD motifs.

1.2.8 Factors Affecting Serum IGFBP-2 Levels

The regulation of IGFBP-2 expression by GH follows much the same pattern as for IGFBP-1. Although IGFBP-2 is classed as a small molecular weight binding protein, it too is influenced by the circulating levels of GH. In acromegaly hIGFBP-2 serum levels are reduced, noted in Western ligand blots as a decline in the 34-36 kDa species (Hardouin *et. al.*, 1987). Hypopituitary sera have increased concentrations of hIGFBP-2 as assessed by the same technique (Hossenlopp *et. al.*, 1986; Hardouin *et. al.*, 1987; Binoux and Hossenlopp, 1988). Similarly, in hypophysectomized rats (GH-deficient) IGFBP-2 hepatic mRNA levels are increased by 10-20 fold, and this is reflected in serum levels (Orlowski *et. al.*, 1990a). Administration of GH alone barely reverses the IGFBP-2 levels but GH given in conjunction with T_4, cortisone acetate and testosterone reduces IGFBP-2 to 50% of the hypophysectomized rat serum levels (Ooi *et. al.*, 1990). Obviously the regulation of IGFBP-2 serum levels is not simply dependent on GH.

Fasting leads to elevated IGFBP-2 levels in rats but has not been studied in humans (Ooi *et. al.*, 1990; Orlowski *et. al.*, 1990a). More specifically, rIGFBP-2 mRNA in the liver of fasted rats is significantly increased but the brain remains unaffected (Straus and Takemoto, 1990), indicating a distinct mechanism for maintaining IGF levels in the brain.

As for IGFBP-1, the reversal of the malnourished state leads to a reversal of elevated IGFBP-2 levels.

The other major factor influencing IGFBP-2 expression is the level of serum insulin. Thus Diabetes mellitus leads to an increase in IGFBP-2. It appears that chronic infusion of insulin reverses the IGFBP-2 increase (Böni-schnetzler *et. al.*, 1990) but short term administration does not (Ooi *et. al.*, 1990; Unterman *et. al.*, 1990). It may be that insulin infusion is leading to a restoration of GH levels which are reduced in diabetes and that the GH may in turn ultimately reverse the IGFBP-2 levels.

1.2.9 IGFBP-2 and Development

IGFBP-2 levels vary throughout pregnancy. Human maternal serum levels gradually decrease as the pregnancy progresses (Guidice *et. al.*, 1990). A similar pattern is seen for pregnant rats with the concentrations returning to normal as soon as 2 days post partum (Gargosky *et. al.*, 1990b). Interestingly, blood IGF-I levels follow much the same pattern as rIGFBP-2 during and after pregnancy, suggesting some coordination in their expression during pregnancy.

In the developing rat fetus, the serum levels of IGFBP-2 are generally higher than in an adult. Then several days postpartum serum levels begin to fall as do the rat serum IGF-II levels (Donovan *et. al.*, 1989; Orlowski *et. al.*, 1990a). At term gestation rIGFBP-2 mRNA is most abundant in the liver and is present at lower levels in most other tissues (Brown *et. al.*, 1989). Of note is the large difference in concentrations of rIGFBP-1 mRNA versus rIGFBP-2 mRNA in fetal kidney (8 fold more rIGFBP-2) and fetal brain (25 fold more rIGFBP-2) perhaps indicating specific developmental roles for IGFBP-2 in these tissues (Ooi *et. al.*, 1990).

Looking at specific cellular localization of rat IGFBP-2 and IGF-II in the developing fetus, Wood *et. al.* (1990) observed that very rarely are both expressed in the same cell types. Rat IGFBP-2 is generally found in cells of ectoderm or endoderm origin whereas IGF-II is found mainly in mesodermal cells. The reasons for this distribution pattern are unknown, but it is possible the binding proteins are having a role as carrier proteins in directing IGF to specific tissues.

In the adult rat the pattern of IGFBP-2 mRNA abundance is somewhat different to the fetus. There are significant amounts of rIGFBP-2 mRNA in the kidney, brain and testes, very little in the liver and rIGFBP-2 mRNA is undetectable in the heart and muscle (Margot *et. al.* 1989; Binkert *et. al.*, 1989). Human IGFBP-2 mRNA is also found in adult human liver (Zapf *et. al.*, 1990a).

Little is known about the actual concentrations of hIGFBP-2 during human development as the specific probes for this protein have only recently become available. However, Donahue *et. al.* (1990) have studied the level of IGFBP in serum of elderly women by Western ligand blotting and noted an increase in a 34 kDa IGFBP. It is possible this represents hIGFBP-2 as IGFBP-1 has been specifically been shown to decrease with age. However, this must be confirmed by specific probing.

1.2.10 The 150 kDa IGFBP

For a long time it has been recognized that the large molecular weight binding protein species identified by neutral gel chromatography of serum is a multi subunit complex (Hintz and Liu, 1976). Much speculation has arisen over the subunit nature of the 150 kDa complex with two models arising from different sources of evidence.

Several low molecular weight binding proteins having some characteristics similar to the 150 kDa complex such as glycosylation and GH-dependence were identified by cross-linking in high molecular weight preparations. Using this evidence, Wilkins and D'Ercole (1985) proposed a hexamer structure of 24 kDa subunits, which would infer a 150 kDa complex with each subunit having an IGF binding site. However, Martin and Baxter (1986) determined that the large molecular weight binding protein has only one binding site, suggesting the need for an alternative subunit model.

The second model for the 150 kDa complex structure has been developed following the identification of an acid labile and an acid stable subunit (Furlanetto, 1980). The 150 kDa complex can be dissociated under acid conditions to give a 50-60 kDa IGF-binding species identified by gel chromatography, which represents the acid stable subunit of the complex now called IGFBP-3. The subunit has a single binding site for IGF and does not require the presence of the acid labile subunit (ALS) for IGF binding. The ALS on the other hand does not bind IGF itself and can only associate with IGFBP-3 when IGFBP-3 is bound to IGF

(Baxter, 1988). Therefore, this model accounts for a multisubunit structure containing a single IGF binding site.

The smaller cross-linked IGFBPs identified by Wilkins and D'Ercole (1985) and also by Ooi and Herington (1986) were possibly glycosylation variants of IGFBP-3 or breakdown products due to storage as experienced by others (Martin and Baxter, 1986; Baxter and Martin, 1989). Indeed Shimonaka *et. al.* (1989) and Zapf *et. al.* (1990a) have isolated IGFBP-3 truncated at the carboxy-terminus. Another smaller species (16-18 kDa) which cross reacts with antiserum directed against hIGFBP-3 (Baxter and Martin, 1986) has been identified by Herington and Kuffer, (1981). It is also present in amniotic fluid (Ooi and Herington, 1990). The significance of this species and other truncated binding proteins in both serum and amniotic fluid is unknown.

An assay for the ALS has been developed and used to follow its purification from human plasma (Baxter, 1988; Baxter *et. al.*, 1989). The assay relies on the identification of a shift in mobility using G200 gel chromatogragphy of cross-linked, radiolabelled hIGFBP-3 - IGF on association with the ALS. The ALS is separated from the 53 kDa hIGFBP-3 under alkali conditions and remains stable under neutral conditions. It appears as a doublet of 84 and 86 kDa on reducing SDS-PAGE, the two sizes being due to different glycosylation forms. In serum the ALS appears in molar excess over IGF, whereas hIGFBP-3 is in equimolar concentrations (Baxter, 1990). This may be in order to favour the formation of the large molecular weight complex over the IGFBP-3 - IGF complex in the circulation, as IGF is almost exclusively associated with the 150 kDa complex.

Low concentrations of IGFBP-3 have been detected in human lymph. This suggests that the IGF is specifically dissociated from the large 150 kDa binding protein - IGF complex in serum and is released for its movement across the capillary barrier (Binoux and Hossenlopp, 1988). Once in the lymph the IGF can bind either to IGFBP-3 or the smaller molecular weight binding proteins. Clemmons *et. al.* (1983) reported that heparin facilitates in the breakdown of the 150 kDa-binding protein complex and Chatelain *et. al.* (1983) showed the susceptibility of the complex to serum proteases. A role of proteases in pregnancy sera has also been proposed (Guidice *et. al.*, 1990; Davenport *et. al.*,1990; Hossenlopp *et. al.*, 1990). These may be mechanisms by which IGF is released from the binding protein in a form which is readily accessible to cellular receptors and which can pass from the blood stream into the extracellular space to target cells.

1.2.11 IGFBP-3

Human IGFBP-3 has been purified from Cohn fraction IV of plasma (Martin and Baxter, 1986; Grant *et. al.*, 1987) and it appears as a doublet of 47 and 53 kDa after SDS-PAGE under nonreducing conditions. The two species are due to different glycosylation forms (Martin and Baxter, 1986). Human IGFBP-3 is immunologically unrelated to hIGFBP-1 (Baxter *et. al.*, 1986).

Porcine, rat and mouse IGFBP-3 have also been purified (Baxter and Martin, 1987; Zapf *et. al.*, 1988; Walton *et. al.*, 1989; Blat *et. al.*, 1989a). Their molecular weights are similar to hIGFBP-3 although rat serum IGFBP-3 can appear as a triplet of 43, 41 and 39 kDa on ligand blots (Hossenlopp *et. al.*, 1987). Human, porcine and rat IGFBP-3 cDNA sequences are available (see Table 1.1, Wood *et. al.*, 1988; Shimasaki *et. al.*, 1989; Spratt *et. al.*, 1990; Shimasaki *et. al.*, 1990a; Albiston and Herington, 1990) and, as seen with IGFBP-1 and IGFBP-2, there is considerable conservation of the sequences between species at both ends of the molecule. Rat IGFBP-3 is one amino acid smaller than human IGFBP-3 and porcine IGFBP-3 is one larger. Again the 18 cysteines are conserved.

The molecular weight of hIGFBP-3 predicted from the cDNA sequence is 28.7 kDa. The discrepancy between this and the size indicated by SDS-PAGE is due to glycosylation. Both porcine and human IGFBP-3 have three potential N-linked and two potential O-linked glycosylation sites, whereas rat has an extra N-linked glycosylation site (Wood *et. al.*, 1988; Shimasaki *et. al.*, 1989; Shimasaki *et. al.*, 1990a). Unlike the smaller binding, proteins IGFBP-3 does not have an RGD sequence.

Recently the gene for hIGFBP-3 has been isolated. It spans 8.9 kb and has four exons corresponding to coding sequence and a fifth exon containing 3' untranslated sequence (Cubbage *et. al.*, 1990). The mRNA derived from the hIGFBP-3 gene is a single 2.5kb species.

1.2.12 Factors Affecting Serum IGFBP-3 Levels

As mentioned earlier, large molecular weight binding proteins are GH-dependent. In fact both the acid stable IGFBP-3 and the ALS are GH-dependent (Binoux *et. al.*, 1984; Furlanetto, 1980; Baxter and Martin, 1986). Circulating hIGFBP-3 levels are significantly

decreased in patients with GH-deficiency and increased 2-3 fold in acromegaly (Hardouin *et. al.*, 1989; Hossenlopp *et. al.*, 1986; Hardouin *et. al.*, 1987; Baxter and Martin, 1986). Hypophysectomized rats also have decreased serum IGFBP-3 concentrations and GH infusion reverses this effect (Clemmons *et. al.*, 1989). Hypophysectomized pigs show the same pattern of responses (Walton and Etherton, 1989).

IGF-I infusion into healthy humans leads to an increase in IGFBP-3 expression as seen by Western ligand blotting. The effect is enhanced with concomittant infusion of GH and IGF-I (Zapf *et. al.*, 1990b). Similar experiments using hypophysectomized and diabetic rats detect an increase of IGFBP-3 on IGF-I infusion (Zapf *et. al.*, 1989). Diabetic rats, which unlike hypophysectomized rats are able to produce GH, form the 150 kDa complex following IGF-I infusion. On the other hand IGF-I only induces IGFBP-3 in hypophysectomized rats suggesting that IGF-I alone can induce IGFBP-3 but GH is important in inducing ALS.

Unlike IGFBP-1, there is no diurnal rhythm associated with IGFBP-3 levels which remain constant at approximately 6 μg/ml (Baxter and Martin, 1989). Nutritional deprivation does, however, influence serum IGFBP-3 levels. Protein-deprived rats have one third of the normal adult IGFBP-3 levels. GH or IGF-I infusion partially adjust for protein deprivation suggesting that neither are solely responsible for IGFBP-3 levels.

Other factors affecting IGFBP-3 levels are diseased states such as diabetes mellitus which leads to reduced IGFBP-3 and chronic renal failure which has the opposite effect (Goldberg *et. al.*, 1982; Baxter and Martin, 1986).

1.2.13 IGFBP-3 and Development

IGFBP-3 levels follow the same pattern as IGF-I with aging, increasing from early childhood to puberty and then gradually decreasing with age (Baxter and Martin, 1986). A similar situation applies for ALS (Baxter, 1989). By Western ligand blotting a gradual decrease of IGFBP-3 in aging women was also identified (Donahue *et. al.*, 1990).

As mentioned previously, pregnancy leads to dramatic effects on maternal serum IGFBP-3 levels with a specific protease apparently degrading IGFBP-3 (Guidice *et. al.*, 1990; Gargosky *et. al.*, 1990a; Davenport *et. al.*, 1990). An antiserum directed to

hIGFBP`3 recognizes a 30 kDa protein in pregnancy serum while the 47 and 53 kDa IGFBP-3 doublet are essentially absent (Hossenlopp *et. al.*, 1990). It is possible the 30 kDa IGFBP is the same as the truncated IGFBP-3 purified by Zapf *et. al.* (1990a). Two smaller IGFBP (21.5 and 21 kDa) appear at the same time as the 30 kDa immunoreactive species and all may be products of the enzymatic degradation. Interestingly, Baxter and Martin (1986) measured mildly elevated maternal serum levels of hIGFBP-3 in the third term of pregnancy, suggesting the presence of immunoreactive IGFBP-3 in an altered form not detectable by Western ligand blotting.

IGFBP-3 is not degraded in human amniotic fluid during pregnancy. Human amniotic fluid contains IGFBP-3 but at much lower levels than IGFBP-1 (Baxter *et. al.*, 1987). Amniotic fluid IGFBP-3 levels increase between day 50 and 80 of porcine pregnancy as do allantoic fluid levels (Walton and Etherton, 1989). Similar levels are measured in porcine colostrum and follicular fluid and lower concentrations are in milk. Interestingly, ALS is not detected by a specific antibody in human amniotic fluid, CSF and seminal plasma (Baxter, 1989)

Fetal tissue IGFBP-3 levels have not been specifically studied, although it is generally believed that large molecular weight binding protein levels are low in the developing fetus (D'Ercole *et. al.*, 1980). With cDNA probes, IGFBP-3 mRNA has been identified in many tissues of the adult rat including liver, kidney, spleen, heart, lung, stomach, ovary and testes but not in brain cortex and hypothalamus (Shimasaki *et. al.*, 1989). In the adult pig IGFBP-3 mRNA has been localized in ovary, testes, brain and liver (Shimasaki *et. al.*, 1990a).

1.2.14 IGFBP-4

A fourth binding protein has been recently characterized. Its presence in serum and conditioned medium of various cell lines as a 24 kDa IGFBP has, however, been noted for some time (Hossenlopp *et. al.*, 1987; Ocrant *et. al.*, 1989; Conover *et. al.*, 1989; Thrailkill *et. al.*, 1990). It is also present in seminal plasma (Rosenfeld *et. al.*, 1990). The binding protein has been purified by three groups; Shimonaka *et. al.*(1989) have purified rat serum IGFBP-4, Mohan *et. al.* (1989) isolated IGFBP-4 from conditioned medium of human osteocarcinoma cells and Walton *et. al.* (1990) has purified porcine serum IGFBP-4.

Little is known about this binding protein so far and identification has been mainly by Western ligand blotting. Although the 24 kDa IGFBP is obviously a small molecular weight binding protein, it appears to respond to GH in a similar manner to IGFBP-3 (Hardouin *et. al.*, 1987). For example, in acromegalics there is an increase in IGFBP-4 expression. IGFBP-4 has a possible role in fetal development as proposed for other small molecular weight binding proteins. In fetal rats the predominant binding proteins are 30 and 24 kDa (Hossenlopp *et. al.*, 1987). Curiously the IGFBP-4 levels in pregnant serum are reduced at the same time as IGFBP-3 is degraded. With increasing age the levels of the 24 kDa binding protein decrease as seen for IGFBP-1 and IGFBP-3 (Donahue *et. al.*, 1990). It appears that the 24 kDa binding protein has some characteristics common to both large and small binding proteins. Until specific probes are used it is not possible to speculate on its role in modulation of IGF effects.

The cDNA clone for IGFBP-4 has been isolated from rat liver and human placenta, liver and ovary cDNA libraries (Shimasaki *et. al.*, 1990b). IGFBP-4 is quite unusual in that it has two extra cysteine residues apart from the 18 conserved cysteines present in all binding proteins so far sequenced. The human IGFBP-4 sequence is 4 residues longer than rat IGFBP-4 and the two sequences differ by 8%. Again most homology lies at either end of the sequences. The IGFBP-4 sequence contains a single potential N-glycosylation site. The mRNA encoded by the IGFBP-4 gene is a single 2.6 kb species. It has been localized in several rat tissues including liver, adrenal, testis, spleen, heart, lung, kidney, stomach, hypothalamus and brain cortex. It is in greatest abundance in the liver.

1.2.15 Other Binding Proteins

Yet another binding protein has been isolated from fibroblasts (Forbes *et. al.*, 1990; Martin *et. al.*, 1990) and cerebrospinal fluid (Roghani *et. al.*, 1989). It is a 32 kDa, glycosylated binding protein with a similar IGF-binding pattern to one of the three classes of binding proteins defined on the basis of IGF binding affinity (see Chapters 3 and 5). Little is known about the expression of this binding protein but it has been recently identified in human serum (Zapf *et. al.*, 1990a).

1.2.16 Actions of IGFBPs

The specific actions of IGFBPs are not yet understood. *In vivo* it is generally accepted that IGFBPs act to increase the half lives of IGF-I and IGF-II (Kaufmann *et. al.*, 1977; Cohen and Nissley, 1976). This phenomenon has also been demonstrated in lambs (Francis *et. al.*, 1988a). Thus upon reduction of ^{125}I IGF-I and its infusion into lambs, its half life was greatly reduced in comparison with that of native IGF-I. This was due to the incorrect refolding of a proportion of the reduced IGF-I which resulted in the production of peptides unable to bind to IGFBPs. The unfolded peptides were subsequently more rapidly degraded. Since IGFBPs increase IGF serum half life it has been suggested that they act as serum carrier proteins assisting in the distribution of IGFBPs throughout the body.

Two lines of evidence point towards distinct and opposite actions of IGFBPs. They can inhibit or potentiate IGF action. Support for these concepts comes from many *in vitro* experiments. Meuli *et. al.* (1978) used perfused rat hearts to demonstrate that binding of iodinated IGF to receptors is reduced by its preincubation with partially purified serum binding proteins. Glucose incorporation into fatty acids and sulphate into chondrocytes is inhibited in adipose tissue by partially purified amniotic fluid binding protein (Drop *et. al.*, 1979). These are examples of the inhibition of IGF action by IGFBPs.

Several groups have demonstrated an inhibition of IGF receptor binding by IGFBPs with various cells (Knauer and Smith, 1980; Rutanen *et. al.*, 1988; Gopinath *et. al.*, 1989). Some interesting clues to the action of binding proteins have appeared with the use of cell lines which do not produce IGFBPs. In this way the action of added binding protein is not complicated by the presence of endogenous binding proteins. Using human choriocarcinoma cells (which do not produce binding proteins) the addition of IGFBP-1 along with IGF-I lead to an inhibition of IGF-I - stimulated aminoisobutyric acid uptake (Ritvos *et. al.*, 1988). This was via an inhibition of IGF-receptor binding. A similar line of experiments was performed by Ross *et. al.* (1989) who showed a decrease in both DNA synthesis and protein accumulation on addition of IGFBP-1 or IGFBP-2 to chicken embryo fibroblast cultures containing IGF.

Quite the opposite effect has been reported, initially by Clemmons *et. al.* (1986) and then by his colleagues (Elgin *et. al.*, 1987). Incubation of human fibroblasts with IGF-I and IGFBP-1 in the presence of platelet-poor plasma leads to an enhanced response compared

with IGF-I alone. However without platelet-poor plasma the same preparation of IGFBP-1 did not enhance the response to IGF-I (Ross *et. al.*, 1989). Evidently an unknown plasma associated factor can act to enhance the response to IGF (Clemmons and Gardiner, 1990).

Enhancement of IGF action can be achieved with another binding protein. Although coincubation of IGF with IGFBP-3 with human skin fibroblasts results in an inhibition of the action of IGFBP-3, preincubation of those cells with IGFBP-3 for 8-24 hours enhanced the subsequent effect of IGF-I above control values with no preincubation (De Mellow and Baxter, 1988). It is tempting to suggest the existence of a cellular receptor for IGFBPs which allows the association of binding proteins with the cell membrane in a way that might assist the presentation of IGF to cellular receptors. However, there is no evidence for such a mechanism. Cross-linking to cellular membranes by several groups has identified an IGF-binding species of the same size as IGFBPs. Whether this is a membrane bound form of IGFBPs or a fragment of an IGF receptor has not been reported. If IGFBPs are associated specifically with the cell membrane, it will be interesting to establish if they have a role in IGF signalling or whether this represents part of the IGFBP secretion pathway.

1.2.17 The IGFBP - IGF Interaction

Very little is known about the IGF binding site of IGFBPs. In some way the IGFBP amino acid sequence homologies (see Figure 1.3) result in a common tertiary IGFBP structure with all binding proteins having a similar IGF binding site. Most clues about the nature of the binding site have been deduced from the isolation of fragmented binding proteins. A 21 kDa IGFBP-1 has been isolated which is amino-terminally truncated. A similarly sized IGFBP-2 lacking some of the carboxy-terminus has also been reported (Huhtala *et. al.*, 1986; Wang *et. al.*, 1988). Both can bind IGF and therefore, the binding site must still be maintained by the remaining central region of the peptide (the nonconserved region between IGFBPs) in the truncated isolates.

In addition, a carboxy terminal deletion of IGFBP-3 still maintains the binding capacity of the binding protein. The 30 kDa fragment is one of the several smaller fragments of IGFBP-3 identified by Western ligand blotting, i. e. still able to bind IGF (Zapf *et. al.*, 1990a). Ooi *et. al.* (1989) have partially sequenced a 17 kDa binding protein which shows extensive homology to the amino terminus of IGFBP-3 and some similarity to the carboxy end. This binding protein may arise by breakdown of IGFBP-3 but the total sequence has

not yet been related to IGFBP-3. Its sequence may also provide information on the IGF binding site.

Other evidence strongly suggests that the maintenance of the tertiary structure of IGFBPs by the common cysteines is important in retaining the IGF binding site. The fact that the 18 cysteines are conserved throughout all IGFBP sequences supports this concept. An expression vector containing IGFBP-1 cDNA but lacking the sequence encoding the last 24 amino acids produces a truncated form of IGFBP-1 which is unable to bind IGF. Presumably the removal of the carboxy-terminal cysteine disrupts the tertiary structure of the binding protein and the IGF binding site in turn (Brinkman *et. al.*, 1989). One report suggests the replacement of cysteines at positions 16 and 35 with serine also disrupts IGF binding (Powell *et. al.*, 1989).

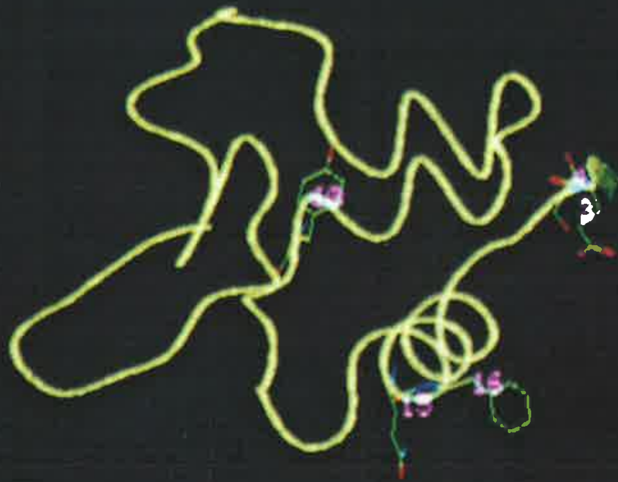
More information is available on the IGFBP binding site on the IGF peptide. The variant of IGF-I, des-(1-3)-IGF-1, has been used to determine an area of the IGF-I sequence important in binding to IGFBPs. Ross *et. al.* (1989) observed that IGFBP-2 failed to inhibit the action of the biologically more active IGF, des-(1-3)-IGF-1, although it does inhibit IGF-I stimulated protein synthesis. The loss of the first three amino acids of IGF is vital in IGF-I binding by IGFBP-1 and IGFBP-2 but not IGFBP-3 (Forbes *et. al.*, 1989). The subsequent lack of binding to IGFBP-1 and IGFBP-2 means that more of the truncated IGF is available to bind to cellular receptors resulting in the enhanced biological activity (Bagley *et. al.*, 1989).

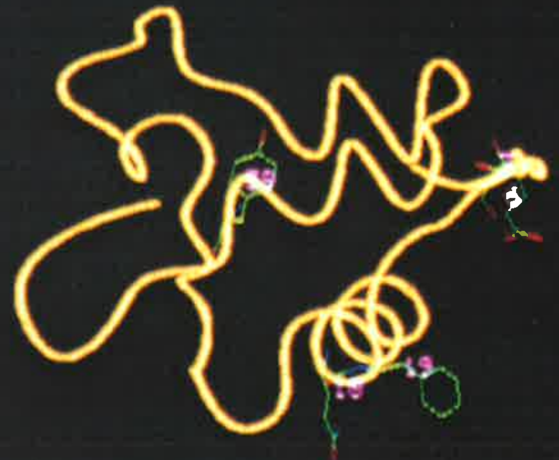
Synthetic peptides of the B domain of both IGF-I and IGF-II do not bind bIGFBP-2 showing that the amino terminal end of IGF is important but is not sufficient for IGFBP binding (Szabo *et. al.*, 1988). DeVroede *et. al.* (1985) also demonstrated the importance of the IGF B domain.

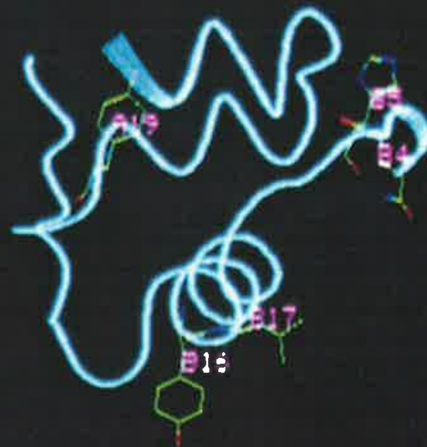
Residues important in the IGF-IGFBP interaction are highlighted on models of the structures of IGF-I, IGF-II and insulin shown in Figure 1.4. Site-directed mutagenesis has been used to alter specific residues in IGF-I to elucidate the IGFBP binding site of IGF. Modifications at positions 3, 15 and 16 of IGF-I to residues found in insulin and at position 4 (Thr^4 to Ala^4) reduces the binding to serum binding proteins 600 fold (Cascieri *et. al.*, 1988b). Substitution of the B domain with insulin B domain also reduces binding by 1000

Figure 1.4: Domains of Insulin-like growth factor-I (IGF-I) which interact with IGF-protein bindings and corresponding domains in the IGF-II and insulin structures

Three dimensional models of IGF-I, IGF-II and insulin were generated using the Insight II program (Biosym Technologies Inc., San Diego, CA, USA, 1990) with coordinates obtained from the Protein databank, Brookhaven National Laboratory, Upton, NY, U.S.A. IGF coordinates were determined by comparison with the insulin structure and predictions made by Blundell *et. al.*, (1983). The models highlight the similarity in tertiary structure shared between the three proteins. Residues Glu^{3}, Thr^{4}, Gln^{15}, Phe^{16}, and Tyr^{60} IGF-I are highlighted as they have been shown by site-directed mutagenesis or chemical modification to be involved in IGF-I binding by binding proteins (Cascieri *et. al.*, 1988b; Moss *et. al.*, 1991). The corresponding residues of IGF-II (Glu^{6}, Thr^{7}, Gln^{18}, Phe^{19}, Tyr^{59}) and insulin (Gln^{B4}, His^{B5}, Tyr^{B16}, Leu ^{B17}, Tyr^{A19}) are also highlighted.


IGF I


IGF II


B17
INSULIN

fold. Mutations in the A, C and D domains have minimal effect on binding to acid stable, serum binding proteins.

Clemmons *et. al.* (1990) revealed some differences in the binding sites of acid stable, serum binding proteins (most probably representing IGFBP-3) and the purified IGFBP-1. The substitutions at positions 3, 4, 15 and 16 of IGF-I still reduce IGFBP-1 binding, but 10 fold less than for acid stable, serum binding proteins. Mutation of the whole B domain has less effect on IGFBP-1 binding (200 fold reduction in IGF-I binding) and substitutions in positions 49-56 of the A chain alter IGFBP-1 binding. Therefore a distinction can be made between IGFBP-1, IGFBP-2 and serum binding proteins with respect the residues of IGF-I involved in IGFBP - IGF interaction. All positions discussed here are residues exposed on the same surface of IGF-I (see Figure 1.4).

Moss *et. al.* (1991) demonstrated that the association with IGFBP blocked the iodination of Tyr 60 of IGF-I and the corresponding residue (Tyr 59) of IGF-II. Both tyrosines are found within the structures of IGF (see Figure 1.4) and apparently the presence of IGFBP excludes the iodinating agent (Chloramine T). It appears that this region is also important in IGFBP-2 binding to both IGF-I and IGF-II.

Isolation of new binding proteins and elucidation of their amino acid sequence will assist in determining the structural determinants involved in the IGF-binding protein interactions and the differences which lead to the various affinities for IGF-I, IGF-II and des-(1-3)-IGF-1. Analysis of the IGFBPs will aid in prediction of the nature of these sites.

1.3 BACKGROUND TO THESIS

The knowledge of the IGF binding proteins is expanding extremely rapidly. Many researchers have turned to examining the factors influencing the expression of IGF binding proteins in the last two to three years and since the isolation of molecular and immunological probes for binding proteins. This information will eventually provide us with the key to the mechanisms of the control of IGF action.

At the commencement of this thesis, however, relatively little was known about the IGF binding proteins. Several binding proteins had been purified but there was some confusion as to the relationship of the different binding protein isolates to each other.

Limited N-terminal amino acid sequence of the human amniotic fluid binding protein, the MDBK binding protein isolated from bovine kidney cells in our laboratory, a binding protein isolated from rat liver cells (BRL 3A) and the large serum binding protein suggested these proteins were in fact three distinct binding proteins, with the BRL 3A binding protein being related to the MDBK binding protein. However, the sequence data available were insufficient to definitely distinguish the individual binding proteins, particularly as a significant degree of homology existed between each sequence. Adding to the confusion was the variation in size between similar binding proteins isolated by different groups. As a result there was a great need for classification of the different binding proteins.

Furthermore, no more than three distinct binding proteins had been isolated at the commencement of this work. However, western ligand blotting had been used to identify five binding proteins in human serum (Hossenlopp *et. al.*, 1987; Hardouin *et. al.*, 1987). Therefore, at least two and possibly more binding proteins had not yet been purified. Apparently the control of IGF action is mediated by all of these different binding species.

Preliminary competition binding assays performed by coworkers in our laboratory indicated that the human lung fibroblast (He[39]L) cell line had an unusual IGF-binding pattern when compared with cell lines which secreted known binding proteins. On the basis of this observation the He[39]L cell line appeared to be producing novel binding proteins.

With this background I formulated three overall aims of my PhD. The initial aim was to address the problems of identification of one binding protein from another. The availability of the truncated IGF-I (des-(1-3)-IGF-1) in our laboratory provided me with a unique opportunity to do this using competitive binding assays. The second aim was to analyze the binding of different binding proteins to des-(1-3)-IGF-1 and to thereby aid in the explanation for the enhanced biological activity of this truncated form of IGF-I. The final aim was to isolate and characterize novel binding proteins to ultimately understand the different actions of all the binding proteins in the regulation of the effects of IGFs.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 General Chemicals and Reagents

Activated charcoal, acrylamide, $AgNO_3$, agarose (type 1), ampicillin, Bacitracin, RIA grade bovine serum albumin (BSA), chloramphenicol, Coomassie Brilliant Blue (R_{250}), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), deoxynucleotides, ethylenediaminetetraacetic acid (EDTA), ethidium bromide, N-2-hydroxyethyl-piperanzine-N'-2-ethanesulfonic acid (Hepes), $MgCl_2$, 2-mercaptoethanol, N,N"-methylene-bis-acrylamide, N-acetyl-glucosamine, nitro blue tetrazolium (NBT), Nonidet P-40, phenylmethylsulphonyl fluoride (PMSF), polyethylene glycol (PEG) 6000, polyoxethylenesorbitan monolaurate (Tween 20), potassium ferricyanide, sodium dodecyl sulphate (SDS), sodium thiosulfate and tetracycline were purchased from Sigma Chemical Co., St. Louis, MO.,USA.

Wheatgerm agglutinin-Sepharose 6MB and concanavalin A-Sepharose were purchased from Pharmacia, Uppsala, Sweden. Methyl-α-D-mannopyranoside for affinity chromatography was from Calbiochem, San Diego, CA, USA. Avidin-alkaline phosphatase conjugate used in Western immunoblots was from Bresatec Pty. Ltd., Adelaide, South Australia.

Solvents for reverse phase high performance liquid chromatography (rpHPLC) and fast-phase liquid chromatography were from BDH Australia, Port Fairy, Victoria, Australia. Trifluoroacetic acid (TFA) was from Applied Biosystems, Foster City, CA, USA.

Nitrocellulose and Nytran membranes were from Schleicher and Schuell whereas polyvinylidenedifluoride (PVDF) was supplied by Millipore, Bedford MA., USA. Colony/Plaque Screen filters (NEN Research products, Boston, MA., USA.) were used for cDNA library screening.

2.1.2 Antibodies and Protein Standards

Goat Anti-rabbit IgG coupled to biotin and molecular weight markers (6H) were purchased from Sigma Chemical Co., St. Louis, MO.,USA. A polyclonal antibody was raised against IGFBP-2 in our laboratory by L. Szabo. Polyclonal antibodies raised against

IGFBP-1 were kind gifts of Drs. R. C. Baxter (Camperdown, NSW; Antibody A2) and P. D. K. Lee (Texas Children's Hospital, Houston, Tx, USA.). Goat anti rabbit gamma globulin and rabbit IgG were from Silenus Laboratories, Hawthorn, Australia. ^{14}C-Labelled rainbow markers for ligand blots were obtained from Amersham, North Ryde, NSW., Australia.

2.1.3 Peptides and Hormones

IGFs were obtained from different sources for various experiments and will be quoted in figure legends where appropriate. hIGFBP-3 and hIGFBP-1 were provided for competition binding studies in Chapter 4 by Dr. R. C. Baxter. Human IGFBP-1 used as a standard for ligand blotting and in lectin affinity experiments was a gift from Dr. P. D. K. Lee. Bovine IGFBP-2 was purified by L. Szabo in our laboratory as described in Szabo *et. al.* (1988).

Platelet-derived growth factor (PDGF), epidermal growth factor (EGF) and bovine fibroblast growth factor (bFGF) for stimulation of cultured cells were provided by Chiron Corporation, Emeryville, California. Dexamethasone and estradiol were from Sigma Chemical Co., St. Louis, MO.,USA. Insulin (Actrapid) was from Commonwealth Serum Laboratories, Melbourne, Australia.

2.1.4 Cell Culture Materials

Plasticware for routine cell assays was from Nunc, Kamstrup, Roskilde, Demark, as was a cell factory for large scale collection of conditioned medium. All cells were grown in Dulbecco's modified Eagle's minimal essential medium (DME; Gibco, Glen Waverley, Victoria, Australia) containing 10% (v/v) fetal calf serum (FCS; Flow Laboratories, North Ryde, NSW., Australia) and 100 mg/ml streptomycin and 60 mg/ml penicillin (both from Glaxo, Boronia, Victoria, Australia) unless stated otherwise. Hepes buffer was made with 0.1M Hepes, 5mM KCl, 1.2mM $MgS0_4.7H_20$, 8mM glucose and adjusted to pH 7.6.

2.1.5 Radiolabelled Peptides and Nucleotides

IGFs were iodinated by M. Conlon (Child Health Research Institute, Adelaide Children's Hospital, Adelaide) to a specific activity of 80-150 Ci/g using the Chloramine T method as described by Ballard *et. al.* (1987). L-[4,5-^{3}H] leucine (40-60 Ci/mmol) was

from New England Nuclear Research Products, Boston, MA. USA. [α-^{32}P] dATP, [α-^{35}S] dATP and [γ-^{32}P] dATP were purchased from Bresatec Pty. Ltd., Adelaide, South Australia.

2.1.6 Cell Lines

He[39]L cells	Commonwealth Serum Laboratories, Parkville, Victoria, Australia.
Hep G2 cells	American Type Tissue Culture (ATCC HB 8065), Rockville, MD, USA.
L6 myoblasts	Dr. J. M. Gunn, Texas A&M University, College Station TX., USA.
IMR 90 cells	Adelaide Children's Hospital, South Australia.
MDBK cells	Flow Laboratories, North Ryde, NSW., Australia.
Ag2804	Institute of Medical Research, Camden, NJ, USA.
SF1972	Adelaide Children's Hospital, South Australia.
WI38 cells	Flow Laboratories, North Ryde, NSW., Australia.

2.1.7 Enzymes and Kits for Molecular Biology

T4 DNA ligase [EC 6.5.1.1], E. coli DNA polymerase I (Klenow fragment), *Taq* polymerase, *Taq* polymerase buffer and oligonucleotide labelling-, kinasing- and dideoxynucleotide sequencing kits were supplied by Bresatec Pty. Ltd., Adelaide, South Australia. Calf intestinal phosphatase [EC 3.1.3.1] was from Boehringer Mannhein Australia, Sydney, Australia. Restriction endonucleases were mainly from New England Biolabs Inc., MA., USA. and Pharmacia LKB Biotechnology, Uppsala, Sweden. RNAsin was also from Pharmacia LKB Biotechnology. Murine moloney leukemia virus reverse transcriptase was from Bethesda Research Laboratories, Life Technologies Inc., Gaithersburg, MD, USA.

2.1.8 Oligonucleotides

Oligonucleotides were synthesized by Dr. R. Warren in the Department of Biochemistry, University of Adelaide, South Australia.

MDBK 24-mer (P24)	5'- GAG GTG CTG TTC CGC TGC CCG CCC -3' Directed against sequence encoding the first 8 amino acids of bIGFBP-2

He[39]L 23-mer (P23)	5'- GGA(G,C,T) GGA(G,C,T) TGC(T) GTA(G,C,T) GAA(G) GAA(G) GAA(G) GA -3' Directed against sequence encoding amino acids 15-22 of the He[39]L IGFBP. Degeneracies are shown in brackets.
He[39]L 29-mer (P29)	5'- TGC(T) CCI GGI GGI TGC(T) GTI GAA(G) GAA(G) GAA(G) GA- 3' Directed against sequence encoding amino acids 13-22 of the He[39]L IGFBP. Degeneracies are shown in brackets and inosine residues are denoted as I.
IGFBP 30-mer (P30)	5'- GAG GAA TTC G(T)CC A(C)TG(T) CTT GTC(T) A(G)CA GTT GGG -3' Directed against a sequence common to IGFBPs 1,2 and 3 (encoding the amino acids PNCDKHGLY in bIGFBP-2) at the C-terminal end of the IGFBP sequences. An EcoR I restriction endonuclease site is at the 5'end.
IGFBP 15-mer (P15)	5'- GCA A(G)CA A(G)CC GCA GCC -3' Directed against a sequence common to IGFBPs 1,2 and 3 (encoding the amino acids GCGCC) at the N-terminal end of the IGFBP sequences.

2.2 METHODS

2.2.1 Charcoal Binding Assay

The charcoal binding assay for the measurement of binding protein binding to IGF was performed as described in Szabo *et. al.* (1988). Briefly, samples were assayed by incubating with ^{125}I-IGF-I or ^{125}I IGF-II (4000 dpm/tube) for 1 hour at room temperature in assay buffer (10mM sodium phosphate pH 7.4, 150mM NaCl, 2 mg/ml BSA). Unbound tracer was adsorbed by activated charcoal (5mg/ml) added as a slurry in the same buffer for 30 minutes on ice. IGF-binding protein complexes were separated from unbound IGF by centrifugation at 10,000g for 10 minutes. The amount of IGFBP present was determined by counting the radioactivity in the supernatant.

2.2.2 Polyethylene Glycol (PEG) IGFBP Assay

IGFBP-1 was detected using a PEG separation method rather than the charcoal binding assay as described by Baxter *et. al.* (1987). Following incubation of IGFBP-1 with ^{125}I-IGF-I or ^{125}I-IGF-II in assay buffer (10mM sodium phosphate pH 7.4, 150mM NaCl, 2 mg/ml BSA), binding protein - IGF complexes were separated from unbound, iodinated peptide using a specific polyclonal antibody for IGFBP-1 (Antibody A2, 1/3500, 4°C, overnight). A second antibody, goat anti rabbit gamma globulin (1/164), and carrier rabbit IgG (1/2050) were added for 30 minutes at 4°C and then precipitated with 3.9% (w/v) PEG, 0.1M NaCl by centrifugation at 4000 rpm for 25 minutes at 4°C. Pellets were counted in a gamma counter.

2.2.3 Radioreceptor Assay

The radioreceptor assay measures the binding of IGF to its cell surface receptors. Addition of binding protein interferes with this interaction and thus the assay can be used in the detection of binding proteins. The method for the radioreceptor is described by Ballard *et. al.* (1986). Briefly, cells were grown to confluence in 24 place multiwell trays and washed in Hepes/0.25% (w/v) BSA (2 hours, 4°C) before adding ^{125}I IGF-I or ^{125}I IGF-II (10,000 cpm in the same buffer) and competing ligand. Cells were incubated overnight at 4°C and subsequently washed in Hanks balanced salts (Flow Laboratories, 2x1ml, 1x2.5ml). Following solubilization with 0.1% (v/v) Triton/0.5M NaOH surface bound tracer was measured in a gamma counter.

2.2.4 IGF-I Radioimmunoassay (RIA)

Prior to measurement of IGFs in conditioned medium binding proteins were separated from IGF by acidification to pH 2.8 with glacial acetic acid and separation on a Waters Protein Pak 125 column (Waters/Millipore, Lane Cove, NSW, Australia) in mobile phase (0.2M acetic acid, 0.05 M triethylamine and 0.5% (v/v) Tween 20). Fractions were collected at 0.25 minute intervals (250µl) and 50µl were assayed in triplicate for the presence of IGFs.

The measurement of IGF-I content in He[39]L conditioned medium was determined using the radioimmunoassay described by Owens *et. al.* (1990). Fractions from the Protein

Pak column or standards in mobile phase were assayed by the addition of 30μl TrisCl pH 7.5 (0.4M), 200μl RIA buffer (30mM NaH_2PO_4, 0.2% (w/v) protamine sulphate, 10mM disodium EDTA, 0.2% (w/v) NaN_3 and 0.05% (v/v) Tween 20 at pH 7.5), 50μl ^{125}I IGF-I (20,000 cpm) and 50μl anti-IGF-I antibody in RIA buffer (1/40,000). After 18 hours at 4°C 50μl goat anti-rabbit serum (1/20) and 50μl rabbit serum (1/250) were added to each sample for 1 hour at 4°C. Cold PEG 6000 (1ml, 6% w/v) in 150 mM sodium chloride was added and the tubes were centrifuged at 4000 rpm for 30 minutes at 4°C. The supernatants were aspirated and the pellet counted in a gamma counter. The limit of detection of the IGF-I RIA was 11 pg.

2.2.5 IGF-II radioreceptor assay (RRA)

The levels of IGF-II in conditioned medium were measured by binding of IGF to sheep placental membranes as outlined by Read *et. al.* (1986). Following Protein Pak separation 50μl of fractions or standards in mobile phase were assayed by the addition of 30μl TrisCl pH 7.5 (0.4M), RRA buffer (10mM TrisCl, 0.5% (w/v) BSA and 10mM $CaCl_2$ at pH 7.4), 50μl ^{125}I IGF-II (20,000 cpm) and 100μl ovine placental membranes (final concentration of 0.2mg protein/ml) in RRA buffer. This amount of membranes was sufficient to bind 30-40% of ^{125}I IGF-II in the abscence of competing ligand. Samples were incubated overnight at 4°C. Cold 10mM TrisCl, 0.5% (w/v) BSA and 10mM $CaCl_2$ at pH 7.4 (1ml/tube) was added and tubes were centrifuged at 4000 rpm at 4°C. Supernatants were aspirated and the pellets were counted in a gamma counter. The limit of detection of IGF-II by RRA was 30pg.

2.2.6 Protein Synthesis Determination

The method of the protein synthesis assay has been described previously by Francis *et. al.* (1986). It involved the incorporation of [^{3}H] leucine (0.5mM in PBS/0.1% (w/v) BSA) into total cell protein in the presence of stimulating peptide during an 18 hour incubation of confluent cell monolayers in 24-place multiwell trays (37°C, 5% (v/v) CO_2). The cells were washed at 0°C twice with Hank's salts, twice with 5% (v/v) trichloroacetic acid over 10 minutes and once with glass distilled water before solubilizing in 0.1% (v/v) Triton/0.5M NaOH. Activity is expressed as a percentage of the total counts incorporated into control wells with no added growth factor (=100%).

2.2.7 Collection and Concentration of Conditioned Media

Cells were grown to confluence in DME containing 10% (v/v) FCS at 37°C in 5% (v/v) CO_2. They were washed for 2 hours in DME without serum and subsequently incubated for 12 to 48 hours before collection. The medium was centrifuged at 500g for 15 minutes before storage at -20°C. Serum-free conditioned media were concentrated 5-10 fold using Centricon P10 microconcentraters (Amicon, Denvers, MA., USA.).

2.2.8 Stimulation of cells with Growth Factors and Hormones

A similar protocol to the routine collection of conditioned media (section 2.2.6) was used in stimulation experiments. After 2 hours wash in serum-free DME, peptides were added to fresh serum-free culture medium. Cells were cultured for 48 hours at 37°C in 5% (v/v) CO_2 before collection of medium. This was subsequently centrifuged briefly to remove any cell debris and concentrated as described above (section 2.2.6)

2.2.9 SDS-Polyacrylamide Gel Electrophoresis

Proteins were separated by electrophoresis using the system described by Laemmli (1970). Gels for the separation of conditioned medium and serum samples were generally 13cm x 13cm x 0.15mm and were electrophoresed at 30mA for several hours. Thinner gels were used for purified peptides. The percentage acrylamide used for each gel is indicated in figure legends and was made from a stock solution of 40% acrylamide/0.32% bis. Molecular weight markers (Sigma SDS-6H) consisted of myosin (Mr 205,000) β galactosidase (Mr 116,000), phosphorylase b (Mr 97,000), bovine serum albumin (66,300), ovalbumin (Mr 45,000) and carbonic anhydrase (29,000). Lysozyme (14,000) was also used as a marker in some instances.

2.2.10 Silver Staining SDS-Polyacrylamide Gels

Between 50-200 ng protein were loaded onto 0.5 mm SDS-polyacrylamide gels for silver staining. Proteins were fixed for at least 3 hours in 30% (v/v) ethanol and 10% (v/v) acetic acid. The method for silver staining followed that of Heueshoven and Dernick (1985). After fixation gels were washed in glass distilled H_2O to remove excess fixer and treated with a solution of 1.5% (w/v) potassium ferricyanide, 3% (w/v) sodium thiosulphate and 0.5% (w/v) sodium carbonate for 30 seconds. Gels were washed quickly under a

monodistilled H_2O tap, then in glass distilled H_2O for 15 minutes and were subsequently stained with 0.1% (w/v) $AgNO_3$ for 30 minutes. Staining was developed with 2.5% (w/v) Na_2CO_3 and 0.5% (v/v) formaldehyde, changing the developer several times until proteins became evident. The reaction was stopped with 1% (v/v) acetic acid.

2.2.11 Western Ligand Blots and Immunoblots

Proteins were separated by 12.5% (w/v) SDS-polyacrylamide electrophoresis under non-reducing conditions as described by Laemmli (1970). They were transferred to nitrocellulose at 300mA for 3 hours using a Hoeffer Transphor TE24 apparatus (Hoeffer Scientific, San Francisco, CA, USA.). Ligand blots were performed as outlined by Hossenlopp *et. al.* (1986). Briefly, nitrocellulose was washed with 0.15 M NaCl, 0.1 M Tris-HCl pH 7.4, 0.05 mg/ml sodium azide containing 3% (v/v) Nonidet P40 for 10 minutes followed by a 2 hour wash in the same buffer containing 1% (v/v) RIA grade BSA and a 10 minute wash in buffer containing 1% (v/v) Tween 20. The filters were incubated with 1 x 10^6 cpm ^{125}I-IGF-II overnight at 4°C in buffer containing 1% (v/v) BSA and 1% (v/v) Tween 20. Following 2 x 45 minute washes in buffer to remove unbound tracer, filters were exposed to X-ray film (A8323, Konica, Tokyo, Japan) for 2-4 days at -80°C with 2 intensifying screens.

Immunoblots were blocked in phosphate buffered saline (PBS) with 2% (v/v) BSA at least 3 hours, washed 3 times in PBS containing 1% (v/v) Tween 20 and incubated with polyclonal antibody (1/100) overnight at 4°C in the same buffer. After a similar washing procedure the filters were incubated with a second antibody-biotin conjugate (1/1000 dilution; 2-3 hours), washed again and incubated with avidin-alkaline phosphatase (1/1000 dilution; 1 hour). Following 3x20 minute washes in Tris-HCl buffer pH 9.5 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5mM $MgCl_2$) the reaction was developed after several minutes incubation with NBT (0.3mg/ml) and BCIP (0.2mg/ml) in the same buffer, as described by Bers and Garfin (1985). The reaction was stopped with 1 mM EDTA.

2.2.12 Affinity Chromatography

Lectin affinity columns were used to identify glycosylated binding proteins. IGFBPs were applied to a wheatgerm agglutinin-Sepharose 6MB (Pharmacia) column (0.75 x 1.5 cm) and a concanavalin A-Sepharose (Pharmacia) column (0.75 x 1.5 cm) in

0.05 M Na_2PO_4 plus 0.5 M NaCl pH 6.5 containing 0.25% (w/v) BSA. A 3 ml wash (0.2 ml/minute) in the same buffer preceded elution. Glycosylated proteins binding to the wheatgerm agglutinin were eluted in a stepwise fashion with 0.5 M N-acetyl-glucosamine, whereas elution of the concanavalin A column was with 0.5 M methyl-α- mannopyranoside. Both elutions were at a flow rate of 0.2 ml/minute. IGFBPs were detected by either charcoal binding assay (IGFBP-2 and IGFBP-3) or PEG precipitation assay (IGFBP-1) as described above.

2.3 PURIFICATION of He[39]L BINDING PROTEINS

2.3.1 Collection of Medium

He[39]L cells were grown to confluence in a cell factory in DME containing 10% (v/v) FCS. The cells were washed twice in serum-free medium and incubated 3 days before collection. Cells were maintained for 4 weeks with intermittent periods of growth in DME plus 10% (w/v) FCS. The conditioned medium was stored at -15°C.

2.3.2 S-Sepharose Chromatography

A single purification involved acidification of 10-20 litres of medium with glacial acetic acid to a final concentration of 50 mM. The pH was adjusted to 3.0 with 6 M HCl. Following filtration through Whatman No. 1 paper the medium was pumped onto a S-Sepharose Fast Flow cation exchange column (3.2 x 25 cm; Pharmacia, Uppsala, Sweden) at 10 ml/minute at 4°C. The column was washed with 50 mM acetic acid (600 ml = 2 column volumes) and 50 mM ammonium acetate (300 ml) containing 0.5 mM Bacitracin, 5 mM EDTA, 0.5 mM PMSF. Fractions were eluted at 5 ml/minute and collected at 2.8 minute intervals using a 1.2 l gradient from 50 mM ammonium acetate pH 6.0 to 1.0 M ammonium acetate pH 7.5 in the presence of the same protease inhibitors. Throughout the purification procedure IGFBP activity was detected with the charcoal binding assay described in section 2.2.1 and by interference in IGF-II radioreceptor assays described in section 2.2.3.

2.3.3 IGF-I Affinity Chromatography

IGFBP active fractions from S-Sepharose purification were pumped at 4°C with a flow rate of 1 ml/minute onto an IGF-I affinity column equilibrated in 50 mM Tris-HCl,

150 mM NaCl pH 7.0. The affinity column was provided by Dr. P. E. Walton, CSIRO Division of Human Nutrition, Adelaide, South Australia and was prepared by attaching 200 µg IGF-I to Affi-Gel 10 (Biorad, North Ryde, Australia). Specifically bound IGFBP was eluted with 0.5 M acetic acid at 0.5 ml/min.

2.3.4 High Performance Liquid Chromatography (HPLC)

The final purification step involved adsorption of IGFBP pooled fractions to a reverse-phase HPLC aquapore butyl cartridge (4.6 mm x 3.0 cm; Applied Biosystems, Santa Clara, CA. USA.) in 0.1% (v/v) trifluoroacetic acid. A 25-45% (v/v) acetonitrile gradient over 40 minute was used to elute the IGFBP. Chromatography was performed at low flow rates using equipment purchased from Waters^{TM} (Millipore Corporation) and eluted material was monitored using a flow-through ultraviolet detector (Waters, model 490). Data and pump management were controlled by Waters Expert^{TM} Chromatography software on a Digital Electronic Corporation P350 personal computer. Following rpHPLC purification He[39]L binding protein was stored lyophilized at -80°C.

2.3.5 Peptide Sequencing

Samples for peptide sequencing were prepared using the electroblotting method of Matsudaira (1987). He[39]L binding protein was transferred to polyvinylidenedifluoride for 15 minutes using 10 mM 3-[cyclohexylamino]-1-propanesulphonic acid plus 10% (v/v) methanol, stained for 10 minutes with Coomassie Brilliant Blue (R_{250}) in 50% (v/v) methanol and 5% (v/v) acetic acid and destained for 10 minutes in 50% (v/v) methanol and 10% (v/v) acetic acid. Protein bands were cut out and stored at -20°C or immediately applied directly submitted for sequencing with an Applied Biosystems 470 A Sequenator. The peptides were subjected to Edman degradation as described by Hunkapiller *et. al.* (1983). Peptide sequencing was kindly performed by Ms. Denise Turner, Department of Biochemistry, University of Adelaide, South Australia.

2.4 METHODS FOR MOLECULAR BIOLOGY

DNA was cloned, isolated and analysed using standard procedures described by Sambrook *et. al.* (1989).

2.4.1 Polymerase Chain Reaction

The method for polymerase chain reactions (PCR) was described previously by Saiki *et. al.* (1985) and Mullis and Faloona (1987). Reactions involved the addition of 2mM deoxynuleotides, 1x *Taq* polymerase buffer (67mM Tris HCl pH 8.8, 16.6 mM ammonium sulphate, 0.45% Triton X-100, 200µg/ml gelatine), 2mM $MgCl_2$, 2 primers (100ng of 30-mers or 1µg of degenerate 23-mer) and 0.5 units of *Taq* polymerase to template DNA. Polymerase chain reactions (PCR) were performed with a Perkin Elmer Cetus Thermal Cycler (Norwalk, CT, USA). Following a cycle of 94°C for 5 minutes, 55°C for 3 minutes, 72°C for 3 minutes PCR products were amplified for 30 cycles (94°C for 45 seconds, 55°C for 3 minutes and 72°C for 3 minutes) unless stated otherwise.

The cDNA clone encoding bIGFBP-2 (10ng/reaction; Upton *et. al.*, 1990) was used as template DNA. cDNA was also synthesized from total cellular RNA (isolated as described in Sambrook *et. al.*, 1989) and used as template for PCRs. cDNA synthesis involved heating RNA (2µg) in the presence of 0.5 units RNasin at 65°C for 5 minutes. Deoxynucleotides (2mM), BRL-reverse transcriptase buffer (1x = 250mM Tris-HCl pH8.3, 375mM KCl, 50mM DTT, 15mM $MgCl_2$), RNAsin (0.5 units) and BRL-reverse transcriptase (200 units) were added to a 20 µl reaction for 40 minutes at 37°C. Newly synthesized cDNA was added directly to PCR reactions.

2.4.2 Northern Analysis

RNA extraction from tissue culture cells was performed by a guanidium/caesium chloride method described in Sambrook *et. al.* (1989). Northern analysis (Upton *et. al.*, 1990) involved separation of RNA for 4 hours at 90mA through a 1% (w/v) agarose gel prepared in 6.5% (v/v) formaldehyde and transfer to Nytran membrane. Hybridization with a ^{32}P nick translated probe was at 42°C in 50% (v/v) formamide, 5x Denhardt's solution (100x Denhardt's is 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA and 1% (v/v) ficoll), 0.1mg/ml denatured salmon sperm, 0.1% (w/v) SDS and 5x SSPE (20X SSPE is 3M NaCl, 0.2M $Na_2PO_4.H_2O$, 0.02M EDTA). Blots were washed at a final temperature of 50°C in 0.1% (v/v) SSPE /0.1% (w/v) SDS and exposed to X-ray film (Konica) at -80°C with 2 intensifying screens. Radioactively labelled marker RNA was also included on the Northern

blot (0.24kb-9.5kb RNA ladder, Bethesda, Research Laboratories, Gaithersburg, MD, USA.).

2.4.3 cDNA Library Screening

A human lung fibroblast cell line (IMR 90) lambda gt11 cDNA library was a gift from Dr. P. Morris, Adelaide Children's Hospital, Adelaide, South Australia and was originally purchased from Clontech Laboratories Inc., Palo Alto, CA., USA. (catalogue No. HL1011). A single copy of the original library stock contained $2x10^6$ phage. The library had been amplified once by Dr. P. Morris.

Phage were incubated with Y1090 cells in L-broth (LB; 10g/l bacto-tryptone, 5g/l bacto-yeast extract, 10g/l NaCl) for 20 minutes at 37°C They were mixed with 0.7% soft agar in LB + 0.2% maltose/10mM $MgSO_4$ and poured as an overlay onto agar plates made with the same medium. Plates were incubated for 7 hours at 37°C. Phage were lifted onto duplicate Plaquescreen filters and crosslinked using a fixed dosage of ultraviolet light (120,000μJoules).

A total of 5 x 10^5 plaques (approximately 1/4 of the library prior to amplification) were screened with two oligonucleotides on separate occasions (P29 and P30; see Chapter 6). Oligonucleotides were radiolabelled using ^{32}P γ dATP by kinasing (Sambrook *et.al.*, 1989). Filters were hybridized at 42°C in 5 x Denhardt's solution, 0.1% (v/v) SDS, 1.0 M NaCl, 0.05 M Tris-HCl pH 7.5 and 100 μg/ml denatured salmon sperm DNA. Nonspecific hybridization was removed by 2 x 30 min washes with 5 x SSC (1 x =150 mM NaCl, 15 mM sodium citrate pH 7.0), 0.1% (v/v) SDS at 42°C. For some oligonucleotides filters were washed further at 50°C for 30 minutes. Filters were exposed to X-ray film (Konica) for 2 to 3 days at -80°C with 2 intensifying screens.

2.4.4 Preparation of Lambda gt11 DNA

Cultures of Y1090 bacteria were grown to $O.D._{600}$ 0.5 in 20ml LB+0.2% (w/v) maltose, 10mM $MgCl_2$. Lambda phage picked as plaques from library screening were eluted into 1ml phage storage buffer (0.01M TrisCl pH 7.4, 0.1M NaCl, 0.01M $MgCl_2$, 0.05% (w/v) gelatin) overnight at 4°C and 200μl were added to the Y1090 cultures. After 4 hours at 37°C lysis of bacteria had occured and a few drops of chloroform were added. The

preparations were RNase (2.5μg/ml) and DNase (2.5μg/ml) treated for 1 hour at 37°C and pelleted at 12,000g, 4°C. Pellets were resuspended in 5.7ml NaCl/3.0ml PEG 6000 (50% w/v) for 2 hours at 4°C. Lambda DNA was pelleted at 10,000 rpm for 20 minutes at 4°C before resuspending in 500μl PSB and proteinase K treatment (1.6mM EDTA, 0.16% (w/v) SDS and 4μg/ml proteinase K at 65°C for 30 minutes). DNA was phenol extracted 3 times and ethanol precipitated.

2.4.5 Southern Blotting

Southern blotting of lambda gt11 cDNA was performed as described in Sambrook *et. al.* (1989). Briefly, DNA was separated on a 1% agarose/TBE (90mM TrisCl pH 8.3, 40mM borate, 2.5mM EDTA) gel at 30mA. DNA was transferred to nylon membranes by diffusion in 10 x SSC and cross-linked to the membrane by exposure to ultraviolet irradiation (120,000μJoules). Hybridization conditions were identical to those used in library screening (section 2.4.3).

2.4.6 Preparation of DNA for Subcloning

Double stranded fragment and vector DNA was prepared for subcloning by restriction endonuclease digestion. DNA fragments were separated on 1% (w/v) agarose/TBE gels and eluted either by centrifugation at 5000g over siliconized glass wool or electroelution into TE (25mM TrisCl pH8.0, 10mM EDTA) in dialysis tubing. DNA was precipitated in ethanol after phenol extraction.

2.4.7 Ligations

Vector and fragment DNA were ligated at a molar ratio of 1:3 in ligase buffer (66mM TrisCl pH 7.6, 6.6 mM $MgCl_2$, 66μM ATP) containing T4 DNA ligase (1 unit per ligation) overnight at 14°C.

2.4.8 Transformation of Bacteria

Cultures of bacteria were grown up overnight at 37°C in LB containing antibiotics when appropriate. A subculture of these bacteria were grown to an $O.D._{600}$ of 0.6, placed on ice for one hour and then pelleted. Cells were resuspended in 50mM $CaCl_2$, 50mM $MgCl_2$ and left on ice for 30 minutes. After repelleting the cells were resuspended in

80 mM $CaCl_2$. Ligated DNA (1 to 10μg) was added to competent cells at 37°C for 30 minutes. Cells were heat shocked at 42°C for 90 seconds, on ice for 5 minutes, at room temperature 5 minutes and were finally spread over agar plates (made from LB containing appropriate antibiotics). Plates were incubated overnight at 37°C.

2.4.9 Preparation of Double Stranded Plasmid DNA

Overnight 2ml cultures of bacteria transformed with plasmid DNA were pelleted at 6000g for 1 minute. Cells were resuspended in 1 volume 25mM TrisCl pH8.0, 10 mM EDTA, 50 mM glucose and 2 volumes of 0.2M HaOH/0.1% SDS and incubated on ice for 5 minutes. Sodium acetate (1.5 volumes of 3M solution pH 5.2) was added for 5 minutes on ice and the mixture was centrifuged for 30 minutes at 4°C. DNA in the supernatant was phenol extracted and ethanol precipitated.

2.4.10 Preparation of Single Stranded Plasmid DNA

Cultures of transformed XL1-B cells in 2YT (16g/l bacto-tryptone, 10g/l bacto-yeast extract, 5g/l NaCl) containing tetracycline (10μg/ml) were grown to an $O.D._{600}$ of 0.5 before inoculating with the helper phage M13K07. Cultures were grown for 2 hours at 37°C and for a further 18 hours at 37°C in the presence of kanamycin (70μg/ml). Phage particles were precipitated from culture supernatants in the presence of 700mM NaCl/5.5% PEG 6000 at 12,000g for 10 minutes. The pellet was resuspended in TE, extracted with phenol and single stranded DNA was precipitated with ethanol.

2.4.11 DNA Sequencing

DNA was sequenced either by the single stranded sequencing method of Sanger *et. al.* (1977) or double stranded DNA sequencing described by Chen and Seeburg (1985). Both methods involved the synthesis of DNA with either α-[^{35}S]-dATP or α-[^{32}P]-dATP using the Sequenase sequencing kit (United States Biochemical Corporation, Clevland, Ohio, U.S.A.) or the Klenow sequencing kit from Bresatec Pty. Ltd., Adelaide, South Australia.

Reactions were separated at 1600mA on 6% polyacrylamide gels (acrylamide:bis, 19:1) containing 8M urea in TBE buffer. Gels were dried following washing with

12% acetic acid and 20% ethanol to remove the urea and exposed to X-ray film (Konica) at room temperature.

2.5 COMPUTER PROGRAMMES

Homology searches were made by screening the GENBANK (release 67.0, 1991), EMBL (release 20, 1989), National Biomedical Research Foundation (NBRF) protein identification research (release 23, 1989) and nucleic acid (release 36, 1990) databases. The collection of programmes from the Genetics computing Group of the University of Wisconsin (Devereux *et. al.*, 1984) were used for database searches, in particular with the WORDSEARCH and FASTA programmes which identify sequences similar to query sequences in the databases using the Wilbur and Lipman (1983) and the Pearson and Lipman (1988) style searches, respectively. Localized protein sequence similarities were also identified using SEQHP (Dayhoff *et. al.*, 1978; Goad and Kanehisa, 1982) from the ANALYSEQ suite of programmes (Staden, 1982).

Chromatographs were produced from printed output using a digitiser (Houston Instruments) and AutoCAD (Autodesk Inc.). Word processing and typesetting were performed on an IBM compatible personal computer and on a Mactinosh SE personal computer using Word versions 5.00 and 4.00 A respectively (Microsoft Corporation). Diagrams, graphs and tables were produced using MacDraw II (Claris), Cricket Graph (Claris) and Excel (Microsoft Corporation). Protein models shown in Chapter 1 were constructed with the Insight II programme (Biosym Technologies Inc., San Diego, CA, USA., 1990).

CHAPTER 3

CLASSIFICATION OF IGFBPs ACCORDING TO IGF-BINDING SPECIFICITIES

3.1 INTRODUCTION

The IGFs are members of a family of proteins generally termed growth factors, which act to stimulate growth throughout all stages of development and regulate growth and differentiation in regenerating tissues (Heldin and Westermark, 1989). IGFs are unusual in that their action appears to be modulated by a family of binding proteins. In contrast, carrier proteins or binding proteins do not apparently play a role in the action of well characterized growth factors such as platelet-derived growth factor, epidermal growth factor, fibroblast growth factors, nerve growth factor, transforming growth factors α and β and the interleukins or colony-stimulating factors.

IGFs are, however, not unique peptides in their association with binding proteins. Some hormones exist in the circulation bound to carrier proteins. Indeed growth hormone (GH), which regulates the production of IGFs, has a specific carrier protein. However, the carrier/binding protein regulation of IGFs is extremely complex in comparison with the actions of the GH carrier and other hormonal carrier proteins (thyroid hormone, testosterone and corticosteroid carrier proteins, for example). There are up to six IGF-binding species in adult human serum as identified by Western ligand blotting. At the commencement of this study we were aware of the numbers of binding proteins in serum but little was known about the IGF-binding capabilities of the individual binding proteins.

In the earliest studies identifying the existence of IGF-binding proteins a method was developed for the separation of radiolabelled IGF complexed to IGFBP from unbound IGF. The method involved the adsorption of unbound IGF to activated charcoal (Zapf *et. al.*, 1975a). After modification of the original method the competitive binding assays were developed, using increasing amounts of IGF to compete with iodinated peptide for binding to IGFBPs (Zapf *et. al.*, 1977; Schalch *et. al.*, 1978). Both Zapf *et. al.* (1978) and Rechler *et. al.* (1980) demonstrated the relative binding of serum binding proteins to IGF-I and IGF-II in humans and rats, respectively. In both cases serum, representing a pool of different binding proteins, bound IGF-II with greater potency than IGF-I.

Binoux *et. al.* (1982) were the first to demonstrate that serum contained a mixture of IGF-binding proteins which could be identified not only by their size but also their relative

IGF-binding. The serum of adult humans was separated on Sephadex G200 into two pools containing the large and small molecular weight binding proteins. Each pool was subjected to competitive binding assays using purified IGF-I or IGF-II as radioligands and competing ligands. Two distinct binding patterns were revealed. In addition, a comparison of normal human adult serum with fetal human serum demonstrated two binding patterns resulting from the different binding proteins in the two sera. In contrast to adult serum, fetal serum contains predominantly the smaller molecular weight binding proteins (D'Ercole *et. al.*, 1980).

The competitive binding assay has been used in the analysis of binding proteins in other body fluids and culture medium of cell lines or organ cultures in an attempt to analyze the relative binding of these mixtures of binding proteins. For example, the binding pattern derived from cerebrospinal fluid (CSF) is very similar to that of the small molecular weight serum binding proteins (Binoux *et. al.*, 1982; Hossenlopp *et. al.*, 1986) but the serum-free culture medium of bovine vascular endothelial cells binds IGF-I to a greater extent than IGF-II (Bar *et. al.*, 1987). These relative binding patterns reflect the combined binding abilities of all binding components of the conditioned medium, body fluid or serum rather than the individual binding protein input.

As different isolates of purified binding proteins were reported there was much need for clarification of their nomenclature and classification. The purified binding proteins were distinguished purely on the basis of size as some confusion arose from the comparison of N-terminal sequences of limited length. Hossenlopp *et. al.* (1986) had attempted to adapt the Western ligand blot technique to measure the IGF-binding of individual IGFBPs in serum but without success.

It was not until purified IGFBPs were available that the marked differences in IGF-binding between various binding proteins became evident. However, discrepancies arose between various research groups. For example, rat IGF-II (MSA) was 2.5% as potent as IGF-I in displacing iodinated IGF from the placental protein 12 (human IGFBP-1, hIGFBP-1) purified by Ritvos *et. al.* (1988). Using the same protein Baxter *et. al.* (1987) reported that IGF-II was 60% as potent as IGF-I in competitive binding assays. The differences may have been due to the different preparations of IGFs and binding proteins

used in the assays. Also Baxter *et. al.* (1987) noted that, unlike IGFBP-2 and IGFBP-3, the IGF-IGFBP-1 complex is partially absorbed by charcoal and therefore the charcoal binding assay is not appropriate for this IGFBP. Instead they used an antibody/polyethylene glycol separation method. Despite the variation of IGF-binding reports, the preliminary information suggested significant differences between the IGFBP isolated at the time.

The aim of this study was to identify and compare the IGF-binding patterns of three binding proteins available at the time (human amniotic fluid binding protein now called hIGFBP-1, bovine kidney cell (MDBK) binding protein or bIGFBP-2 and the human serum binding protein, hIGFBP-3). The assays were performed under standardized conditions to clarify reports of different binding patterns of various isolates of the same protein, as mentioned above. Using this information I aimed thereby to assist in the identification of one binding protein from another.

A unique opportunity was also available at the time of this study to test the ability of the three IGFBPs to bind des-(1-3)-IGF-1. Our group had purified a truncated variant of IGF-I from bovine colostrum (Francis *et. al.*, 1986) and it has also been isolated from adult and fetal human brain, porcine uterine tissue and platelets (Carlsson-Skwirut *et. al.*, 1986; Sara *et. al.*, 1986; Ogasawara *et. al.*, 1989; Karey *et. al.*, 1989). The variant showed an enhanced biological activity over the full length IGF-I (Francis *et. al.*, 1988b; Sara *et. al.*, 1986) and an explanation was sought for this phenomenon. As des-(1-3)-IGF-1 exhibited a nearly identical receptor binding to IGF-I (Ballard *et. al.*, 1987) a solution was sought in the binding of des-(1-3)-IGF-1 to binding proteins. As a result the enhanced biological activity of the IGF-I variant has been explained. Classification of binding proteins on this basis has allowed not only the distinction of one type of binding protein from another but also speculation on the functions of individual binding proteins.

3.2 RESULTS AND DISCUSSION

3.2.1 Competition binding assays with IGF-I and IGF-II as competing ligands

Competition binding assays performed in this chapter were based on the method for charcoal separation of unbound IGF from IGF-binding protein complexes as described in Szabo *et. al.* (1988) and see section 2.2.1. Assays were performed in collaboration with L. Szabo. As mentioned above, Baxter *et. al.* (1987) experienced some difficulty in the separation of IGF complexed to hIGFBP-1 from unbound IGF, as they demonstrated a nonspecific adsorption of the complex to charcoal. This phenomenon also arose in our hands and prompted us to use antibody separation with polyethylene glycol precipitation.

In order to standardize the IGFs used as ligand in the competition assays, each peptide was measured by high performance liquid chromatography (HPLC) against a weighed amount of recombinant human IGF-I (rhIGF-I), as weighable amounts of IGF-II and des-(1-3)-IGF-1 were unavailable at the time. I assumed that the absorbances at 215 nm of the same amounts of IGF-I, IGF-II and des-(1-3)-IGF-1 were equivalent.

Initially each binding protein tested in these assays was titrated against IGF-I and IGF-II tracers to determine the amount of binding protein which bound at least 30 % of the tracer (see Figure 3.1). The amounts chosen for the following assays were 2.5 ng hIGFBP-1, 10 ng bIGFBP-2 and 1.0 ng hIGFBP-3 for the IGF-I tracer and 2.5 ng hIGFBP-1, 2.0 ng bIGFBP-2 and 1.0 ng hIGFBP-3 for the IGF-II tracer.

The three binding proteins (hIGFBP-1, bIGFBP-2, hIGFBP-3) were tested for their IGF-binding characteristics using rhIGF-I tracer. Bovine IGF-II (bIGF-II) competed at slightly lower concentrations than rhIGF-I for binding to hIGFBP-1 (Figure 3.2a), bIGFBP-2 (Figure 3.2b) and hIGFBP-3 (Figure 3.2c).

With bIGF-II tracer a marked difference between bIGFBP-2 and the other binding proteins was evident. Bovine IGF-II competed 40 fold better than rhIGF-I for binding to bIGFBP-2 this time (Figure 3.3b). In contrast, competition for hIGFBP-1 (Figure 3.3a) and

hIGFBP-3 (Figure 3.3c) remained similar to the pattern seen for rhIGF-I tracer with a slightly better binding of bIGF-II than rhIGF-I.

To establish that the IGF-binding patterns observed with rhIGF-I and bIGF-II as competing ligands were not a result of a species specific interaction of the ligands with the three binding proteins or a difference in the natural form of IGF-I versus rhIGF-I, the competition assays were repeated using preparations of IGF from various species.

Human IGF-II was assayed in parallel with bIGF-II and the results are shown in Figures 3.2 and 3.3. With all three binding proteins and both the rhIGF-I and bIGF-II tracers there was an identical binding pattern for hIGF-II and bIGF-II. Furthermore, oIGF-I and oIGF-II exhibited the same binding potencies as rhIGF-I and bIGF-II using rhIGF-I and bIGF-II tracers in bIGFBP-2 competition assays (see Figure 3.4).

Another series of assays (Figure 3.5) was performed with hIGF-I and hIGF-II as tracers rather than rhIGF-I and bIGF-II tracers. These assays were performed in collaboration with Dr. R. C. Baxter, Camperdown, NSW, Australia. Using hIGF-I, hIGF-II and bIGF-II as competing ligands in bIGFBP-2 competition assays there was essentially no difference in the binding patterns for bIGFBP-2 (compare Figure 3.5a with 3.2b and Figure 3.5b with 3.3b).

Comparisons of the amino acid sequences of the different forms of IGF-I and IGF-II used in these assays indicates significant homology between species. Ovine and human IGF I have identical sequences. Bovine and human IGF-II differ by only three residues in the C domain. Ovine and bovine IGF-II differ by only one residue in the A domain. Conservation of sequences maintains a structural homology between isolates of IGFs from different species resulting in similar competition for binding to the three IGFBPs used in the assays described above.

My binding patterns confirmed the previous reports of Baxter *et. al.* (1987) and Martin and Baxter (1986) for hIGFBP-1 and hIGFBP-3 respectively. Baxter and Martin (1987) obtained similar competition for binding to rat IGFBP-3 to that shown here for binding to hIGFBP-3. Sequence data now available indicates substantial amino acid

homology of IGFBPs between species. It is likely that isolates of IGFBP 1, 2 and 3 from different species will behave similarly in competition binding assays as a result of maintenance of structural homology. IGFBP-3 may, however, be an exception as it is glycosylated. Alterations in glycosylation may alter the interaction with IGFs. In fact recently a small difference in des-(1-3)-IGF-1 binding has been noted between hIGFBP-3 and ovine IGFBP-3 in competition binding assays (Walton *et. al.*, 1991). Whether this attributable to glycosylation at this stage has not been established, however, there is significant homology between the N-terminal sequences of the IGFBP-3 isolates of the two species. In contrast, relative binding of IGF-I and IGF-II to porcine IGFBP-3, which is also glycosylated, is essentially the same as to hIGFBP-3 (Walton *et. al.*, 1989).

The difference between the binding patterns of bIGFBP-2 with rhIGF-I tracer versus bIGF-II tracer probably results from two factors. Most likely, IGFBP-2 interacts with both IGF-I and IGF-II at a common binding site but the binding of IGF-II incorporates another part of the bIGFBP-2 structure which does not interact with IGF-I. A greater affinity for IGF-II results (Figure 3.2b with IGF-I tracer). The second factor affecting bIGFBP-2 binding results from iodination of IGF-II. A greater difference is observed between the IGF-I and IGF-II competition for binding to IGFBP-2 using IGF-II tracer rather than IGF-I.

The effect of iodination on binding to binding proteins has been recently addressed by Moss *et. al.* (1991). The different interaction of bIGFBP-2 with ovine IGF-II (oIGF-II) compared with ^{127}I oIGF-II was described, using either human IGF-I (hIGF-I) or hIGF-II as tracers. The ^{127}I oIGF-II was 2.5 and 5 fold less potent than noniodinated oIGF-II in each respective competitive binding assay. The result of iodination of IGF-II was, therefore, to decrease binding to bIGFBP-2.

The tyrosine involved in the change in binding upon iodination was determined with further experiments analyzing the distribution of ^{125}I in iodinated IGF-II in the presence or absence of bIGFBP-2. It was concluded that Tyr59 is either directly involved in IGFBP binding or lies in a region of the IGF-II structure enveloped by bIGFBP-2 binding (Moss *et. al.*, 1991).

Although the ^{127}I iodination studies have not been extended to IGF-I, it appears from my competition studies that there is not as dramatic an effect of iodination on IGF-I binding when comparing the competition curves with iodinated IGF-I and IGF-II (Figures 3.2 and 3.3) using IGF-I as the competing ligand for all three binding proteins.

With this information we can conclude that the iodination of IGF-II results in a reduction in binding to bIGFBP-2. However, this effect does not totally account for the increased potency of IGF-II in bIGFBP-2 competition assays. A preference for IGF-II binding by bIGFBP-2 still exists.

3.2.2 Competition binding assays with des-(1-3)-IGF-I as the competing ligand

The second aspect of this study was to determine which binding proteins bind the truncated form of IGF-I, des-(1-3)-IGF-1. Very different binding patterns were found when des-(1-3)-IGF-1 was tested with each binding protein (Figures 3.2 and 3.3). With both rhIGF-I and bIGF-II tracers there was little or no detectable competition of the synthetic des-(1-3)-IGF-1 for bIGFBP-2 (Figures 3.2b and 3.3b). Up to 100 fold higher concentrations of des-(1-3)-IGF-1 than rhIGF-I were needed for binding to be detected to hIGFBP-1 (Figures 3.2a and 3.3a). However, the variant was able to compete for binding to hIGFBP-3 at just two to three fold higher concentrations than hIGF-I (Figures 3.2c and 3.3c).

Upon observation of the striking differences between the three binding proteins with respect to their ability to bind des-(1-3)-IGF-1 I defined three binding protein classes. Firstly, the large molecular weight binding protein, hIGFBP-3 (38-43 kDa), is able to bind des-(1-3)-IGF-1 whereas the smaller binding proteins, hIGFBP-1 and bIGFBP-2, bind des`(1-3)-IGF-1 weakly, if at all. The distinction between the smaller molecular weight binding proteins is based on the preference of bIGFBP-2 to bind IGF-II over IGF-I and is facilitated by the marked affect of iodination of IGF-II on the interaction with bIGFBP-2. That is, bIGFBP-2 can be distinguished by its enhanced binding to IGF-II in the presence of iodinated IGF-II and its inability to bind des-(1-3)-IGF-1.

The three binding classes defined in this study have been used in the classification of a novel binding protein, the He[39]L binding protein (see Chapter 5 and Forbes *et. al.* 1990). It has similar binding characteristics to bIGFBP-2 which suggests that alteration to IGF-II through iodination can also affect the binding to binding proteins than other bIGFBP-2. It also confirms that there are at least three IGF-binding patterns exhibited by IGFBPs and the classification may be useful in the characterization of novel binding proteins.

These results implicated the first three amino acids of IGF-I as being crucial in the binding to the small molecular weight binding proteins. A systematic study of the effect of sequential truncation of IGF-I up to residue 5 established that the removal of Glu at position 3 destroys binding to small binding proteins (Bagley *et. al.*, 1989). Other approaches have also implicated this residue in binding to IGFBPs. Bayne *et. al.* (1988) produced a mutant of IGF-I having the residues Gln and Ala at positions three and four (normally occupied by Glu and Thr). There was a reduction in binding to acid stable serum binding proteins but not to the extent demonstrated in this study. The residues in positions 3 and 4 are highlighted in the Figure 1.4. As the acid stable serum binding proteins most likely represent a mixture of large and small molecular weight binding proteins the reduction in binding must in part reflect the inability of this analogue to bind to the small IGFBPs. As reviewed in section 1.2.17 mutation of the B chain of IGF-I disrupts binding to all IGFBPs (DeVroede *et. al.*, 1985; Cascieri *et.al.*, 1988b). Therefore, the B region is likely to be involved in the binding to all binding proteins with the N-terminal amino acid at position 3 being particularly important in binding to small molecular binding proteins.

The large molecular weight binding proteins are the major carrier proteins of IGF in the circulation and have been shown to increase the half life of IGF-I in clearance studies (Kaufmann *et. al.*, 1977). Similarly, after infusion of rats with des-(1-3)-IGF-1 the small proportion which was bound to free carrier in rat serum remained whereas unbound des-(1-3)-IGF-1 was rapidly cleared (Ballard *et. al.*, 1991a). The des-(1-3)-IGF-1 initially in the bound form was, however, cleared at a faster rate than IGF-I. This is probably a reflection of the fact that des-(1-3)-IGF-1 is bound by IGFBP-3 with slightly less potency than IGF-I indicated by the competition assays above.

In the same clearance studies levels of des-(1-3)-IGF-1, IGF-I and IGF-II were measured in various organs and tissues of the infused rats. Interestingly, des-(1-3)-IGF-1 remained in higher levels than IGF-I and IGF-II in the adrenals, brain and gut organs whereas the opposite pattern was seen in the blood. Receptor levels are unlikely to account for the differences in IGF-I and des-(1-3)-IGF-1 levels in these tissues as both peptides have the same receptor binding characteristics (Ballard *et. al.*, 1987). However, as demonstrated in this chapter, des-(1-3)-IGF-1 binds to binding proteins with less potency than IGF-I (if at all) and is, therefore, more readily available to bind to receptors. Hence the higher levels of des-(1-3)-IGF-1 in those tissues.

The fact that only certain tissues in the des-(1-3)-IGF-1 clearance study had higher levels of des-(1-3)-IGF-1 bound to receptors than IGF-I must be a reflection of the types of binding proteins in those tissues. The enhanced biological activity of des-(1-3)-IGF-1 *in vitro* is due to the inability of the smaller binding proteins to bind des-(1-3)-IGF-1. Ross *et. al.* (1989) have confirmed this with an *in vitro* model based on a cell line which does not produce IGFBPs. On addition of bIGFBP-2 and des-(1-3)-IGF-1 an enhanced response was seen in comparison with the response to IGF-I. However, it has not as yet been established whether the small molecular weight binding proteins predominate in the adrenals, brain and gut of rats.

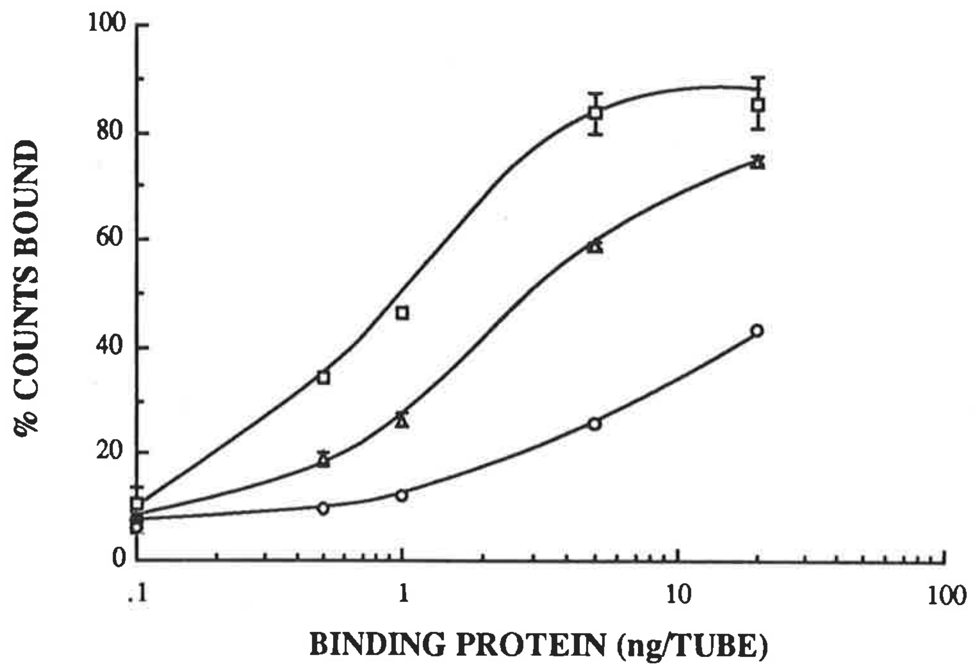
To understand the control mechanisms allowing IGFs to bind to IGF receptors in particular tissues it will be necessary to determine which types of IGFBPs are found in the different tissues. Currently we have two indicators of the types of binding proteins found in different tissues. Firstly, lymph has been analysed to determine which binding proteins are found within tissues in general. Some reports indicate relatively low levels of the large molecular weight carrier in lymph, in particular in human (Binoux and Hossenlopp, 1988) and sheep lymph (Lord *et. al.*, 1991). In contrast, similar levels of IGFBP-3 are present in rat lymph and serum (Gargosky *et. al.*, 1990c). In the human at least, the low levels of IGFBP-3 in lymph suggest that the small molecular weight binding proteins modulate the action of IGFs within the tissues whereas IGFBP-3 and the large binding protein complex are important in IGF circulation.

Secondly, cell lines or organ cultures have been shown to produce a range of differently sized binding species. Analysis of medium conditioned by cells lines may provide useful information about the types of binding proteins produced by different tissues. In the following chapters I will describe the characterization of IGF binding proteins produced by various human fibroblast cell lines, in particular the He[39]L embryonic lung fibroblasts.

Figure 3.1: Determination of binding protein binding capacity

Increasing amounts of binding protein were incubated with either **A** rhIGFI tracer or **B** bIGF-II tracer. Separation of IGF-binding protein complexes from unbound IGF was achieved by charcoal adsorption (section 2.2.1) or in the case of hIGFBP-1 by PEG precipitation of antibody A2-IGFBP-2 complexes (section 2.2.2). Human IGFBP-3 curves are represented by the squares (□), bIGFBP-2 by the circles (○) and hIGFBP-1 by the triangles (△). The amount of tracer bound by a given amount of binding protein is expressed as a percentage of the total number of counts added. The nonspecific background (5% for IGF-I tracer and 10% for IGF-II tracer) was not subtracted. Standard errors are shown where the error exceeds the size of the symbols used.

A



B

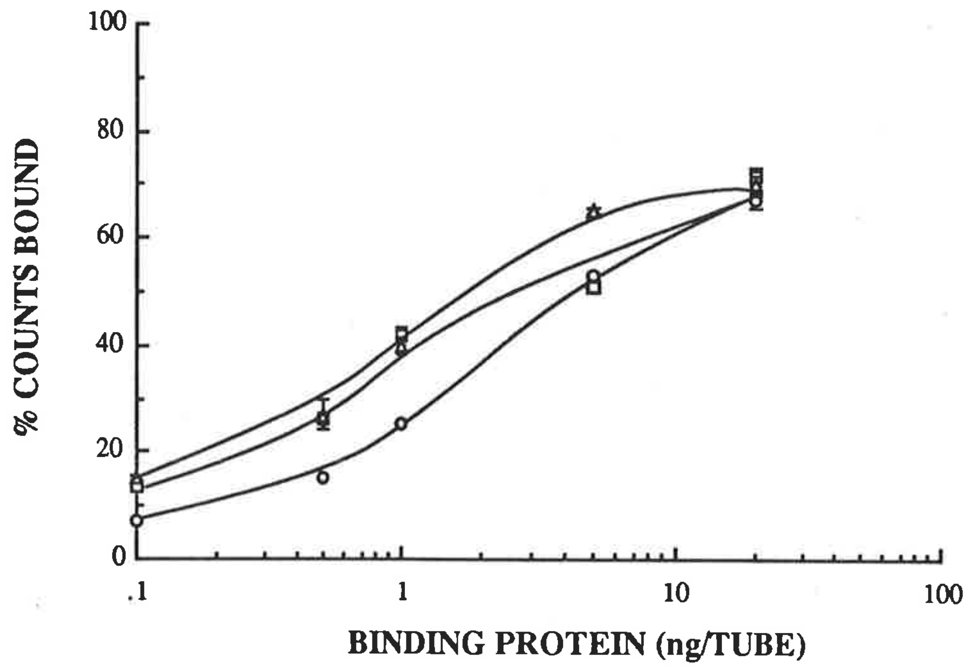


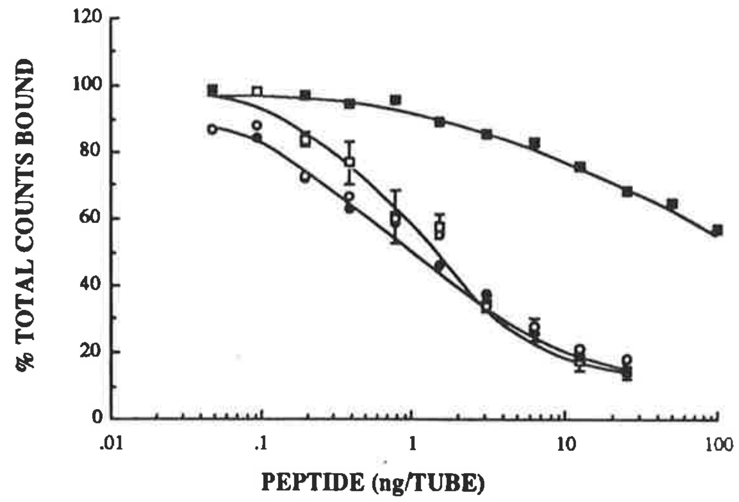
Figure 3.2: Competition binding assays with rhIGF-I tracer and three binding proteins

Competition for binding to various binding proteins was with rhIGF-I (□), bIGF-II (●), hIGF-II (○) and des-(1-3)-IGF-1 (■) as competing ligands. rhIGF-I was a gift from Drs. H. H. Peter and K. Scheibili, Ciba-Geigy, Basle, Switzerland. Synthetic des-(1-3)-IGF-1 was prepared as previously described (Ballard *et al.*, 1987), bIGF-II was purified by the method of Francis *et al.* (1988b) and hIGF-II was from Dr. R. C. Baxter, Camberdown, NSW, Australia.

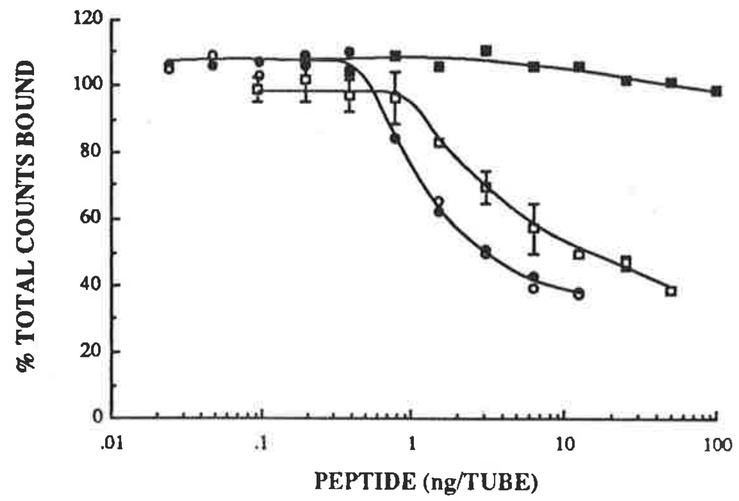
The competition by increasing amounts of ligand is expressed as a percentage of the total number of counts bound in the absence of ligand (B_0). Nonspecific background (NSB) measured in the absence of binding protein and ligand was not subtracted. Standard deviations are shown for curves with rhIGF-I and are representative of the error for each curve. The same assays were performed twice.

A.	Binding to hIGFBP-1	(2.5 ng)	B_0= 35%	NSB = 5.2%
B.	Binding to bIGFBP-2	(10 ng)	B_0 = 31%	NSB = 7.1%
C.	Binding to hIGFBP-3	(1 ng)	B_0 = 58%	NSB = 6.2%

A



B



C

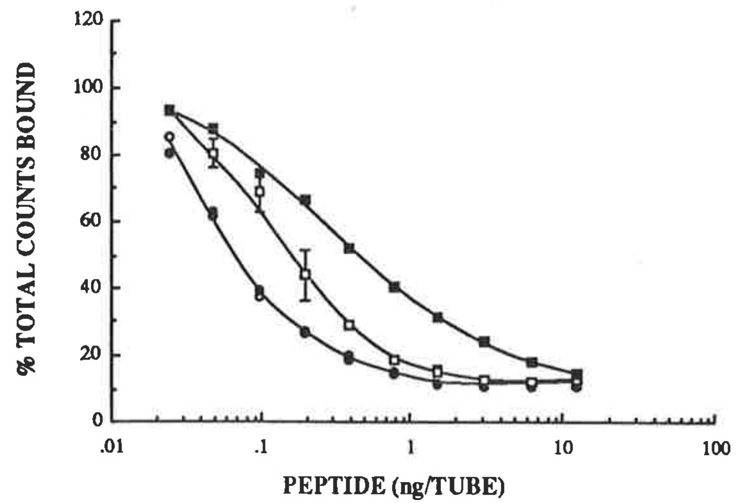


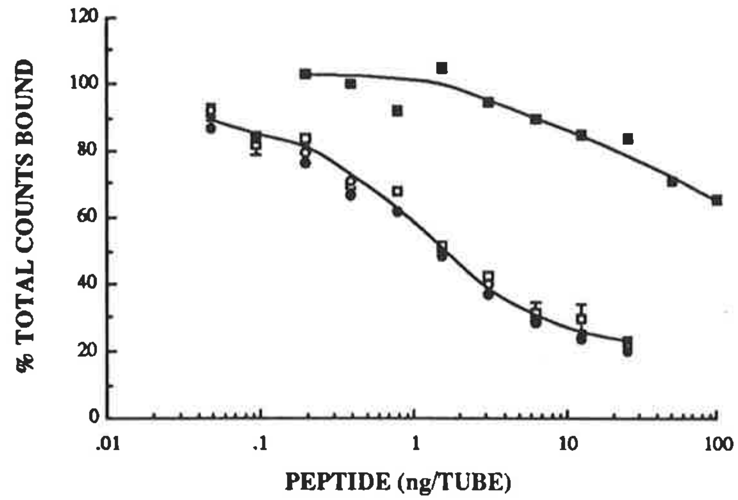
Figure 3.3: Competition binding assays with bIGF-II tracer and three binding proteins

Competition for binding to various binding proteins was with rhIGF-I (□), bIGF-II (●), hIGF-II (○) and des-(1-3)-IGF-1 (■) as competing ligands. rhIGF-I was a gift from Drs. H. H. Peter and K. Scheibili, Ciba-Geigy, Basle, Switzerland. Synthetic des-(1-3)-IGF-1 was prepared as previously described (Ballard *et al.*, 1987), bIGF-II was purified by the method of Francis *et al.* (1988b) and hIGF-II was from Dr. R. C. Baxter, Camberdown, NSW, Australia.

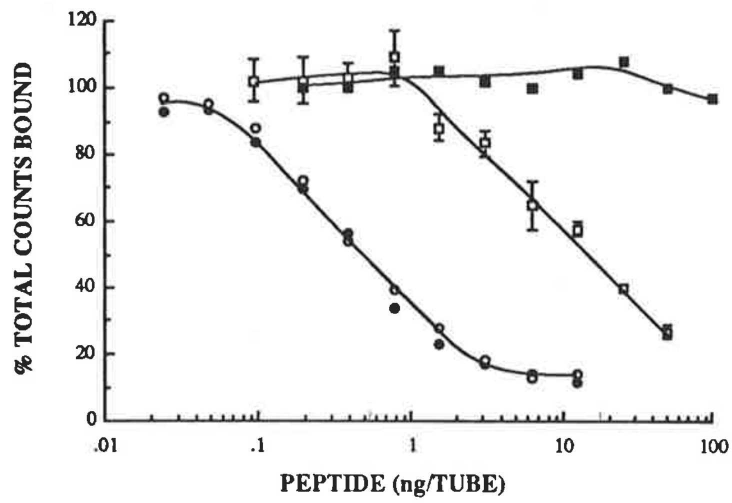
The competition by increasing amounts of ligand is expressed as a percentage of the total number of counts bound in the absence of ligand (B_0). Nonspecific background (NSB) measured in the absence of binding protein and ligand was not subtracted. Standard deviations are shown for curves with rhIGF-I and are representative of the error for each curve. The same experiment has been performed twice.

A.	Binding to hIGFBP-1	(2.5 ng)	B_0= 35%	NSB = 10%
B.	Binding to bIGFBP-2	(2.0 ng)	B_0 = 30%	NSB = 6.4%
C.	Binding to hIGFBP-3	(1 ng)	B_0 = 50%	NSB = 11%

A



B



C

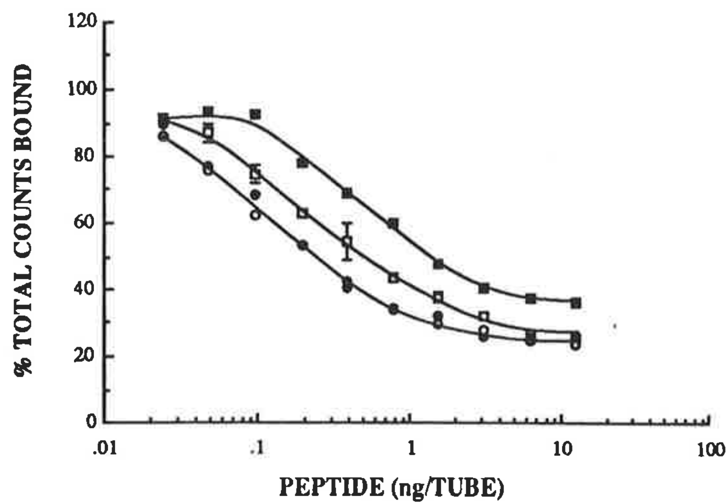


Figure 3.4: Competition by ovine IGFs for binding to bIGFBP-2

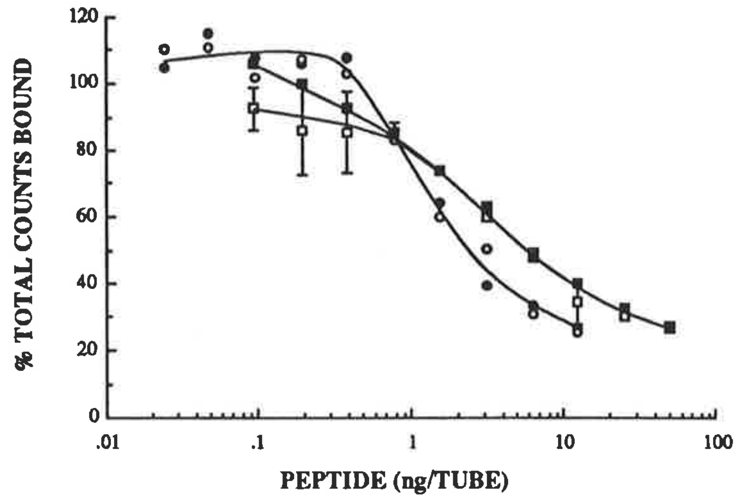
Competition for binding to bIGFBP-2 was with rhIGF-I (□), bIGF-II (●), ovine IGF-I (■) and ovine IGF-II (○) as competing ligands. rhIGF-I was a gift from Drs. H. H. Peter and K. Scheibili, Ciba-Geigy, Basle, Switzerland. bIGF-II was purified by the method of Francis *et al.* (1988b) and ovine IGF-I and IGF-II were purified as previously described (Francis *et al.*, 1989a).

The competition by increasing amounts of ligand is expressed as a percentage of the total number of counts bound in the absence of ligand (B_0). Nonspecific background (NSB) measured in the absence of binding protein and ligand is not subtracted. Standard deviations (bars) are shown for curves with rhIGF-I and are representative of the error for each curve. These assays were repeated twice.

A. Binding to bIGFBP-2 (10ng) and rhIGF-I tracer
B_0 = 28.6% NSB = 6.4%

B. Binding to bIGFBP-2 (2ng) and bIGF-II tracer
B_0 = 57%, NSB = 7.7%

A



B

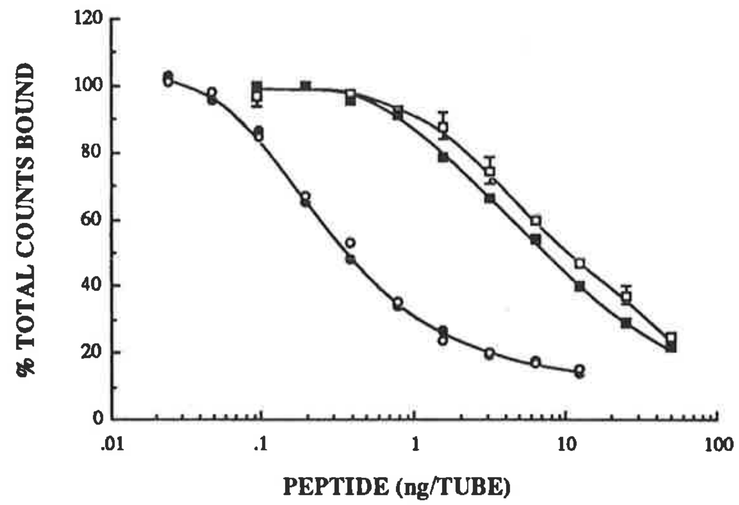


Figure 3.5: Competition binding to bIGFBP-2 human with IGF ligands and tracers

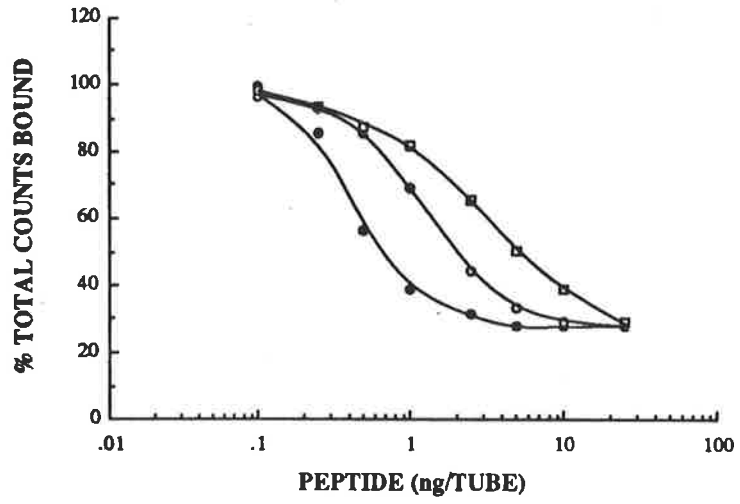
Competition for binding to bIGFBP-2 was with rhIGF-I (□), hIGF-II (○) and bIGF-II (●) as competing ligands. hIGF-I and hIGF-II were purified as previously described (Baxter and De Mellow (1986) and bIGF-II was purified by the method of Francis *et al.* (1988b).

The competition by increasing amounts of ligand is expressed as a percentage of the total number of counts bound in the absence of ligand (B_o). Nonspecific background (NSB) measured in the absence of binding protein and ligand is not subtracted. Standard deviations (bars) are shown for curves with rhIGF-I and are representative of the error for each curve. These assays were repeated twice.

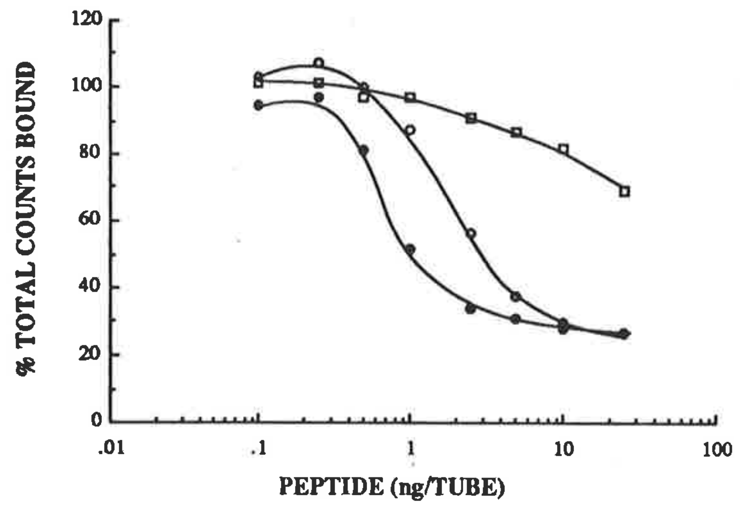
A. Binding to bIGFBP-2 (5ng) with hIGF-I tracer
B_o = 27.7% NSB = 7.7%

B. Binding to bIGFBP-2 (5ng) with hIGF-II tracer
B_o = 45.7% NSB = 12.2%

A



B



CHAPTER 4

CHARACTERIZATION OF IGFBPs PRODUCED BY HUMAN FIBROBLASTS

4.1 INTRODUCTION

Cultured cells have not only been used as sources for binding protein purification but as in vitro models for the study of IGFs and binding proteins. Several aspects have been approached in the determination of the role binding proteins in the modulation of IGF action. Firstly, serum-free conditioned media of cells from different tissues have been analyzed to determine the types of binding proteins produced in particular tissues. Usually cultured cells produce a variety of binding proteins but often there is preferential expression of one species of IGFBP. For example, Western ligand blotting detects only a single IGF-binding species in human hepatoma (Hep G2) cells (Conover and Lee, 1990) and rat calvarial osteoblasts (Gargosky *et. al.*, 1990c).

A second use for cultured cells has been as an experimental system in the determination of factors which influence the production of binding proteins. Growth stimulators, inhibitors, hormones and nutrients have been added or removed from cell culture media and the binding protein production monitored.

Insulin has been shown to inhibit IGFBP-1 production *in vitro* (Lewitt and Baxter, 1989; Conover and Lee, 1990), an effect which is observed *in vivo* upon induction of hyperinsulinemia in diabetic and normal patients (Suikkari *et. al.*, 1988). The same effect of insulin was reported for IGFBP-2 production (Böni-Schnetzler *et. al.*, 1990). Conversely, insulin has a stimulatory affect on other binding proteins in rabbit articular chondrocytes (Froger-Gaillard *et. al.*, 1989). Several other factors including epidermal growth factor (EGF), estradiol and platelet derived growth factor (PDGF) can both stimulate and inhibit binding protein production in different cell culture systems (Mondschein *et. al.*, 1990; Conover (1990); Conover *et. al.*, 1989; Pinzani *et. al.*, 1990).

Model systems have been used to analyze the effect of binding proteins on IGF action. Fibroblasts have been the predominant cell type to be used in experiments involved in the analysis of IGF-binding protein interactions. Knauer and Smith (1980) and Ross *et. al.* (1989) have demonstrated an inhibitory effect of binding proteins on the action of IGF using chicken embryo fibroblasts, a cell type that does not produce binding proteins. However, Clemmons *et. al.* (1986) and De Vroede *et. al.* (1986) demonstrated an enhancement of the effects of IGF on skin fibroblasts by binding proteins in the presence of platelet-poor

plasma. De Mellow and Baxter (1988) also reported an enhancement of IGF action following preincubation of fibroblasts with IGFBP-3.

Until the last year little was known about the binding proteins produced by fibroblasts even though many studies had involved fibroblasts. Human skin fibroblasts had been not only shown to secrete IGFs but were able to respond to IGF and had both type 1 and type 2 receptors (Adams *et. al.*, 1984). The skin fibroblasts secreted several binding proteins (Conover *et. al.*, 1989), one of which was immunologically related to hIGFBP-3 (Martin and Baxter, 1988). Lung fibroblasts produced binding proteins but the nature of these IGFBPs was not known (Adams *et. al.*, 1984).

Considering that fibroblasts have been used extensively in several studies of effects of binding proteins on IGF action, I chose to undertake a detailed analysis of the He[39]L cell line with respect to its binding proteins, IGF production, IGF receptor binding and biological response to IGFs. The aim was to provide more information about the types of binding proteins produced by human lung fibroblasts and fibroblasts in general and to identify which factors may affect the production of those binding proteins.

4.2 RESULTS AND DISCUSSION

4.2.1 Competition binding assays with fibroblast conditioned media

My first approach to the analysis of the He[39]L binding proteins was to analyze the competition binding of binding proteins in He[39]L conditioned medium. The method for the charcoal binding assay is described in section 2.2.1. Assays were performed with rhIGF-I and bIGF-II tracers with competing IGF-I, IGF-II and des-(1-3)-IGF-1 (Figures 4.1a and 4.2a). The binding pattern of He[39]L conditioned medium was then compared with the patterns derived from conditioned media of four other human fibroblast cell lines to determine whether the same binding proteins are produced by all human fibroblasts (Figures 4.1 and 4.2).

The striking result of the competition binding assays was that four of the five fibroblast cell lines assayed exhibited similar binding patterns. Embryonic lung fibroblasts (WI 38), SV 40-transformed IMR 90 fibroblasts (Ag 2804) and skin fibroblasts (SF1972)

all produced binding proteins which competed in a similar manner to He[39]L embryonic lung fibroblast binding proteins (Figures 4.1 and 4.2).

The binding patterns of IMR 90 conditioned medium were, however, very different. This was not only with respect to competition by rhIGF-I and bIGF-II but also in the des-(1-3)-IGF-1 competition curves. Whereas the He[39]L cells produced binding proteins which bind IGF-I and IGF-II with equal potency using IGF-I tracer (Figure 4.1a), IGF-I competed for the binding proteins of IMR 90 cells at lower concentrations than IGF-II (Figure 4.1b). Using IGF-I tracer, competition by des-(1-3)-IGF-1 occurred at similar concentrations in both cell lines (Figures 4.1a and b).

In contrast, with IGF-II tracer there was little competition by des-(1-3)-IGF-1 using He[39]L conditioned medium (Figure 4.2a) but significant competition with IMR 90 conditioned medium (Figure 4.2b). Again, the competition curves with IGF-I and IGF-II were quite different. IGF-II competed with 20 fold greater potency than IGF-I in He[39]L competition curves but both had similar potency in IMR 90 competition curves (Figures 4.2a and b). These results indicate variation in competition binding assay patterns between the three embryonic lung fibroblasts, He[39]L, WI 38 and SF 1972.

A report by Adams *et. al.* (1984) indicates that variation also exists between different isolates of skin fibroblasts. Their skin fibroblast conditioned medium exhibited a different binding pattern to the SF 1972 conditioned medium assayed in this study. Using either IGF-I or IGF-II tracers, IGF-II had greater potency in their competition assays than IGF-I. Unlike the He[39]L and SF 1972 binding proteins which exhibited the preferential binding of IGF-II in the presence of IGF-II tracer (partially a result of iodination, as described in chapter 3) the skin fibroblasts assayed by Adams *et. al.* (1984) did not exhibit this characteristic. Instead IGF-II competed better in assays with IGF-I tracer.

The overall results of the competitive binding assays suggest that all fibroblasts assayed, except IMR 90 cells, produce the same binding proteins leading to similar binding patterns. He[39]L cells produce binding proteins which preferentially bind IGF-II compared to IGF-I and in the presence of IGF-I tracer they bind des-(1-3)-IGF-1. As noted in Chapter 3 this assay demonstrates the sum IGF-binding capacities of all binding proteins in

the conditioned media and, therefore, I would predict the production of a similar array of binding proteins by the cells exhibiting the same binding profiles.

4.2.2 Western ligand blotting of fibroblast conditioned medium

A more precise method for analysis of conditioned medium for the determination of the binding species was adopted to determine whether the prediction was correct that similar cell types produce similar binding proteins. The technique is the Western ligand blot first described by Hossenlopp *et. al.* (1986) and described in section 2.2.10.

Figure 4.3a shows a Western ligand blot of the IGF-binding species of the 5 cell lines described above in competitive binding assays. A striking similarity was seen between the binding patterns of all of the fibroblasts. He[39]L cells mainly produced so called "small molecular weight" binding proteins and there were barely detectable levels of binding protein of the same size as IGFBP-3 (46-52 kDa in Figure 4.3a). All the fibroblasts tested produced very small amounts of IGFBP-3.

In He[39]L conditioned medium there were three IGF-binding species of 25, 29 and 34 kDa as measured on the blot of Figure 4.3a. A 34 kDa binding protein was found in each of the fibroblast media and the 25 kDa binding protein appeared also to be commonly produced.

The 29 kDa species in He[39]L conditioned medium was also present in IMR 90, WI 38 and SF 1972 (very small amounts) conditioned media. This band on the ligand blot corresponded in size with hIGFBP-1 run as a standard on the same gel. Ag 2804 cells did not produce this binding protein. By Northern analysis Conover *et. al.* (1989) failed to detect IGFBP-1 production by a number of different cultured human fibroblasts. Therefore I can not assume that the band migrating at the same position as IGFBP-1 is in fact the same binding protein.

An immunoblot probed with an antibody raised against bIGFBP-2 revealed that none of the fibroblast cell lines produced proteins which could cross react with the antibody (see Figure 4.3b). Unfortunately, human IGFBP-2 was not available as a standard on these blots

but purified bIGFBP-2 was detected by the antibody. The bIGFBP-2 antibody has previously been shown to cross react with IGFBP-2 of other species (Upton *et. al.*, 1990).

WI 38 human embryonic lung fibroblasts were previously shown to produce binding proteins with the molecular weight of approximately 45 kDa as determined by acid gel chromatography (Atkinson *et. al.*, 1980) but further characterization of these IGFBPs has not been reported. The ligand blot presented in this report showed that these cells differ from the two other human embryonic lung fibroblast cells, He[39]L and IMR 90, as WI 38 cells produced an additional IGF-binding species of approximately 31 kDa (Figure 4.3a). The extra IGF-binding species may possibly be a breakdown product of IGFBP-3. A binding species migrating in a similar position in skin fibroblast conditioned medium has recently been proven to be a fragment of IGFBP-3 (Martin and Baxter, 1990). Alternatively, it may be a novel binding protein. It is difficult to speculate on the nature of the binding species on the basis of size alone as different IGFBPs may migrate to the same extent.

Western ligand blotting of fibroblast conditioned media has essentially confirmed the prediction made above that fibroblasts exhibiting similar competitive binding assay profiles may produce the same binding proteins. He[39]L, WI 38 and SF 1972 binding proteins have greatest similarity, with the extra binding protein produced by WI 38 cells not changing the overall binding capability of WI 38 conditioned medium when compared with He[39]L conditioned medium.

In contrast, the IMR 90 cells appear to produce the same types of binding proteins as He[39]L cells but the binding patterns are quite different. There are several reasons the different binding pattern might occur. Firstly, the IMR 90 cells may produce different binding proteins to those of He[39]L cells but this is not evident by comparison of size. However, the SV 40-transformed IMR 90 cells (Ag 2804) exhibit the same binding pattern as He[39]L conditioned medium. The size of the binding proteins of the Ag 2804 are also similar to He[39]L and IMR 90 binding proteins so it is reasonable to predict they are in fact the same binding proteins. This suggests an alternative reason for the difference in binding patterns.

Another explanation may be that the IMR 90 cells are producing the same binding proteins as both He[39]L and Ag 2804 cells but the relative concentrations of each binding species is not the same. IMR 90 cells do secrete a higher proportion of 25 kDa binding protein than the other cells. Alternatively, IMR 90 cells could secrete more IGF thereby altering the relative proportions of binding proteins not bound by exogenous IGFs and available to interact with added IGF in the competition binding assays. Not until all binding proteins of IMR 90 conditioned medium have been purified and characterized could we compare the binding affinities of the individual binding proteins in conditioned medium. Only then would we be able to speculate which are responsible for the total binding pattern demonstrated in the competition binding assays.

It should be pointed out that several reports of the direct identification of binding proteins in the conditioned media by ligand blotting or chemical cross-linking have also revealed heterogeneity between various cell lines of the same cell type. Specifically, unlike the skin fibroblasts mentioned in this study, the human skin fibroblasts described by Martin and Baxter (1988) produced hIGFBP-3 (60-67 kDa) in quantities equalling those of the small molecular weight binding proteins. Binding proteins of 37-43 kDa were present, however, their skin fibroblasts did not produce the equivalent of the 25 kDa species found in all fibroblasts reported here. Clemmons *et. al.* (1986) also reported the production of IGFBP-3 as the predominant IGF-binding species in human skin fibroblasts. Bovine skin fibroblasts, however, did not produce IGFBP-3 (Conover, 1990).

It is not possible to say whether the differences between isolates are indeed due to the nature of the isolates or due to variation in culture conditions between laboratories. However, in this study I conclude that all human fibroblasts analyzed on the basis of size and competition binding assays (except IMR 90 cells in this respect) do produce a remarkably similar range of binding proteins.

Another common characteristic of the fibroblasts studied here is the relatively low abundance of binding proteins in general. In each case the equivalent of 800 µl of conditioned medium was subjected to SDS-polyacrylamide gel electrophoresis. My experience is that 100-300 µl of Hep G2 (Conover and Lee, 1990) or MDBK conditioned

medium is sufficient to easily visualize the binding proteins of those cell lines by ligand blotting.

4.2.3 IGF production by He[39]L cells

Not only do He[39]L cells produce relatively low amounts of binding protein compared with cell lines used previously as sources for IGFBP purification but He[39]L cells do not produce IGFs detectable under our radioimmunoassay (RIA measuring IGF-I; section 2.2.4) and radioreceptor (RRA measuring IGF-II; section 2.2.4) conditions following acid gel chromatography of the conditioned medium. Even when the medium was concentrated 10 fold IGF was undetectable (measured by Dr. P. C. Owens and K. J. Moyse, CSIRO Division of Human Nutrition, South Australia). The limits of detection of the RIA and the RRA are 11pg and 30pg respectively. These amounts are equivalent to a concentration of 27 and 78 pg/fraction/ml medium assayed, respectively.

There are several additional reports of measurement of fibroblast production of IGF. Atkinson *et. al.* (1980) measured approximately 0.2 ng/ml IGF-I in WI 38 cell conditioned medium whereas Clemmons *et. al.* (1981b) measured slightly higher levels (15-20 ng/ml). Adams *et. al.* (1984) also measured low levels of IGF-I and IGF-II produced by skin fibroblasts, although one cell line failed to produce IGF-I. In addition, large molecular weight (precursor) forms of IGF-I have been purified from human skin fibroblasts (Conover *et. al.*, 1989; Clemmons and Shaw, 1986). These forms may also be produced by He[39]L cells but are possibly not detected by the antibody used in our RIA.

Although the He[39]L cells do not appear to be producing IGFs they have type 1 and type 2 receptors (Ballard *et. al.*, 1988) and are able to bind both IGF-I and IGF-II as demonstrated in competitive radioreceptor assays (Figure 4.4 and section 2.2.3). Increasing amounts of IGF-I or IGF-II compete for binding by radiolabelled IGF-I to the type 1 receptor. As seen for many other cell types, IGF-I binds only poorly to the type 2 receptor and therefore competes poorly for radiolabelled IGF-II binding to type 2 receptors.

4.2.4 He[39]L biological response to IGF

Despite the presence of IGF receptors, He[39]L cells respond poorly to IGFs in protein synthesis assays (Figure 4.5; method in section 2.2.6). In fact they are only stimulated by 10% FCS to 120% above basal levels of protein synthesis. In routine protein synthesis assays using rat L6 myoblasts 10 % FCS stimulates up to 300% above control levels and IGF-I stimulates up to 150% (Ballard *et. al.*, 1986) . The lack of response to IGFs by He[39]L cells suggests that He[39]L cell growth is stimulated by another type of growth promoter.

4.2.5 Stimulation of He[39]L binding protein production

A series of stimulation experiments using various peptides to measure the effect on binding protein production demonstrated that IGFs can in fact stimulate IGFBP production (see Figure 4.6). Production of a binding protein doublet of similar size to hIGFBP-3 was stimulated by IGF-I and IGF-II, as was the production of 28-32 kDa binding proteins. It is important to note that no variation was measured in protein content of the different monolayers. By "increased production" I mean increased amounts are found in the conditioned medium. I can not deduce from these experiments whether this is due to an increased rate of synthesis of the protein, decreased breakdown or an increase in secretion rate. Northern analysis of the mRNA encoding IGFBP-3 of stimulated and non-stimulated cells would identify an effect on message levels (presumably corresponding to increased IGFBP-3 synthesis).

The IGF-I variants, des-(1-3)-IGF-1 and long R^3 IGF-I, also stimulated the production of the same binding proteins (Figure 4.6). Long R^3 IGF-I is a recombinant form of IGF-I with Arg at position 3 and a 13 amino acid extension peptide derived from the N-terminal end of porcine growth hormone (pGH). Like des-(1-3)-IGF-1, it has approximately 10 fold increased biological activity above IGF-I and binds extremely poorly to all classes of IGFBPs (Francis *et. al.*, 1991a). The effect of the variant IGF-I peptides on binding protein production has not been previously reported.

Since the variants are unable to bind the small molecular weight binding proteins it would not be possible for the small membrane bound binding proteins detected on the surface of He[39]L cells by chemical cross-linking (Ballard *et. al.*, 1988) to act as receptors for the variants in signalling an increase in binding protein production. Therefore, since not only des-(1-3)-IGF-1 and long R^3 IGF-I but also IGF-I and IGF-II enhance the secretion of the same binding proteins I conclude that all IGFs are acting via the IGF receptors to elicit such an effect.

Thrailkill *et. al.* (1990) observed an inhibition of IGFBP-1 production by human decidual cells. Using a monoclonal antibody to the type 1 receptor (α-IR3) the action of IGF-I was blocked, indicating a type 1 receptor mediated response. This experiment supports my conclusion that an IGF-mediated effect on binding protein production is elicited via the type 1 receptor.

The IGF-I variants actually do not stimulate binding protein production as efficiently as the IGFs. There is a 1.5 fold difference in effect between the variants and IGF-I or IGF-II, determined by laser densitometry of IGFBP-3 on the Western ligand blot of Figure 4.6. This has been confirmed on two subsequent occasions. Des-(1-3)-IGF-1 binds to the type 1 receptors on rat L6 myoblasts with essentially the same affinity as IGF-I (Ballard *et. al.*, 1987), which means that the 1.5 fold difference in ability to stimulate He[39]L binding protein production is not due to receptor binding. The difference between IGF-I and des-(1-3)-IGF-1 stimulation of binding protein production in He[39]L cells may arise due to the ability of IGF-I to bind to He[39]L membrane-bound binding proteins in addition to type 1 receptors. Such membrane bound binding proteins are not detectable on L6 cells by chemical cross-linking (Ballard *et. al.*, 1986).

Some preliminary data from radioreceptor assays with IGF-I tracer suggest that membrane-bound binding proteins do contribute to a higher degree of IGF-I versus des-(1`3)-IGF-1 binding to He[39]L cells (G. L. Francis and M. Ross, personal communication, CSIRO Division of Human Nutrition, Adelaide). In support of this observation, McCusker *et. al.* (1990) reported higher levels of binding of IGF-I than [$Q^3A^4Y^{15}L^{16}$] IGF-I to human fetal fibroblasts. [$Q^3A^4Y^{15}L^{16}$] IGF-I is a mutant which is similar to des-(1-3)-IGF-1 and long R^3 IGF-I in that it has a substantially decreased ability

to bind to binding proteins (Bayne *et. al.*, 1988; Cascieri *et. al.*, 1989). A difference in receptor binding assays between IGF-I and [$Q^3A^4Y^{15}L^{16}$] IGF-I was not detected in cells lacking surface membrane bound binding proteins (McCusker *et. al.*, 1990). This is also observed using L6 cells with IGF-I and des-(1-3)-IGF-1 (Ballard *et. al.*, 1987).

Interestingly, long R^3 IGF-I binds to type 1 receptors on L6 cells with 3-5 fold less potency than IGF-I (Francis *et. al.*, 1991b). Therefore, the slightly poorer effect on binding protein production may partially be attributed to receptor binding in this case.

Insulin, which binds only poorly to the type 1 receptor and does not bind IGFBP, does not have the same stimulatory effect on binding protein production as the IGFs. There is a slight increase in the amount of 32 kDa binding protein in insulin-stimulated cells but no IGFBP-3 is produced. From these experiments it is not possible to determine whether the insulin elicits the effect of stimulation of the 32 kDa binding protein by binding to IGF or insulin receptors on the cell membranes of He[39]L cells.

Using similar concentrations of insulin, Conover (1990) reported the stimulation of IGFBP-3 by bovine fibroblasts. These cells were like He[39]L cells in that they had undetectable IGF levels and did not express IGFBP-3 under normal culture conditions (i.e. not stimulated). Therefore, the concentrations used in my stimulation experiments should have been sufficient to stimulate IGFBP-3 production in He[39]L cells.

4.2.6 Stimulation of SF 1972 binding protein production

Stimulation experiments were repeated with skin fibroblasts to establish whether the effects seen on He[39]L binding protein production apply to fibroblasts of other tissues. The IGF-I, IGF-II and IGF-I variants did behave in a similar manner in that IGFBP-3 production was increased in cells incubated with these peptides (Figure 4.7). The 28-32 kDa binding proteins (corresponding to the 29-34 kDa binding proteins in Figure 4.3a) were also stimulated by all IGF peptides. Again the effect of the variants was slightly smaller than with the IGFs on two separate occaisons.

Unlike the He[39]L stimulation experiments, addition of IGFs to the culture medium of SF 1972 cells caused a dramatic decrease in expression of the smallest 25 kDa binding

protein. A possible explanation for this may be that the 27 kDa protein is still being expressed but IGFs have stimulated the production or activation of a specific protease, resulting in degradation of that IGFBP. It is interesting, however, that the decrease in 27 kDa IGFBP production coincides with increased IGFBP-3 production. Perhaps the smaller binding protein is a fragment of IGFBP-3 and the presence of IGFs protects the cleavage of IGFBP-3 by proteases.

It is also quite possible that in the skin fibroblasts in particular there is a specific mechanism for the regulation of the 25 kDa IGFBP production by IGFs. Inhibition of binding protein production is not unprecedented. Several examples of hormonal/growth factor suppression of binding protein production in tissue culture have been reported (Adashi *et. al.*, 1990; Böni-Schnetzler *et. al.*, 1990; Smith *et. al.*, 1990; Simes *et. al.*, 1991). Until the identity of the 25 kDa binding protein is known (by either direct protein sequencing or cross-reaction with antibody directed against a known binding protein) it is not possible to determine the mechanism by which the IGFs are regulating its expression.

Insulin also had a different effect on skin fibroblast binding protein production. The levels of the binding protein migrating to the same extent as hIGFBP-3 and the 28-32 kDa binding proteins were slightly higher in skin fibroblasts stimulated by insulin. However, the smallest binding protein (25 kDa) remained at the same levels as in conditioned medium of unstimulated skin fibroblasts. This indicates a separate mechanism of stimulation by insulin and perhaps suggests that insulin is acting via its own receptor rather than the type 1 IGF receptor.

Further examples of stimulation experiments with fibroblasts have been reported. In addition to the report of Conover (1990) demonstrating stimulation of IGFBP-3 production by bovine fibroblasts, Martin and Baxter (1990) recorded a 73% increase in IGFBP-3 production by human skin fibroblasts after 72 hours incubation with IGF-I (50 ng/ml). The same skin fibroblasts produced higher concentrations of 29-31 kDa binding proteins as reported in my study. The effect of insulin was not included in their report. In addition, mouse Swiss 3T3 fibroblasts, which mainly produce IGFBP-3, were stimulated to produce more IGFBP-3 by IGF-I and not insulin (Corps and Brown, 1991).

4.2.7 Stimulation of Binding Protein Production by Growth Factors and Hormones

Having established the effect of IGFs and insulin on He[39]L cells I proceeded with stimulation experiments using a range of growth promoters, peptide hormones and the glucocorticoid, dexamethasone. These experiments were performed to further determine factors influencing binding protein production in fibroblasts. The levels of peptide or glucocorticoid chosen to add to the He[39]L cells were based on levels previously shown to have stimulatory effects on binding protein production, although to my knowledge FGF has not been used in such studies before. IGF once again stimulated IGFBP-3 and 30-40 kDa binding proteins in He[39]L cells (Figure 4.8). However, the other factors tested did not alter the production of IGFBPs by He[39]L fibroblasts.

In humans, glucocorticoid excess is associated with growth retardation (Blodgett *et. al.*, 1956; Loeb, 1976). The mechanisms leading to retarded growth have not been determined. However, Luo *et. al.* (1990) have reported increased hepatic IGFBP-1 mRNA and IGFBP-1 serum levels in dexamethasone treated rats. IGF-I levels remained unchanged in these rats and therefore it was suggested that increased IGFBP-1 levels may inhibit IGF action and lead to decreased growth. In contrast, Gourmelen *et. al.* (1982) measured a decrease in serum IGF-binding activity in patients with Cushing's Syndrome receiving glucocorticoid treatment.

In vitro experiments with dexamethasone have also produced a confusing picture of the role of this glucocorticoid in regulation of binding protein production. Concentrations of dexamethasone (1μM) equivalent to physiological concentrations of corticosterone stimulated IGFBP-1 in H4-II-E rat hepatoma cells (Orlowski *et. al.*, 1990b). Stimulation of these cells was not only at the mRNA level detected by Northern analysis but also at the protein level (Western ligand blot). The same levels of dexamethasone were used in stimulation experiments in Figure 4.8. but had no significant effect on binding protein production.

Several researchers have reported an inhibition of binding protein production in response to dexamethasone. At one tenth of the dexamethasone concentration used in this

report many different cell types from several species, including human skin fibroblasts, respond with decreased levels of IGFBP-1 (McCusker and Clemmons, 1988; Conover *et. al.*, 1989; Corps and Brown, 1991). Human fetal liver explants cultured in the presence of 3 μM dexamethasone also secreted lower levels of IGFBP-1 than control cultures (Lewitt and Baxter, 1989).

The effect of glucocorticoids on tissue culture cells and on the body remains unclear. With different cell lines the response varies. He[39]L cells do not respond to dexamethasone in culture medium although the concentrations used in these experiments have previously had effects on a number of cell types.

PDGF and EGF also did not cause any significant effects on production of binding proteins by He[39]L cells (Figure 4.8). PDGF has been shown to be stimulatory or have negligible effects with regard to binding protein production (Pinzani *et. al.*, 1990; Conover *et. al.*, 1989). Estrogen too had little effect on He[39]L binding protein production. Interestingly, two other reports have measured increased IGFBP-3 production *in vitro* in response to estrogen (Schmid *et. al.*, 1989; Mondschein *et. al.*, 1990).

PDGF is a major mitogen in serum and is believed to play a prominent role in wound healing (Ross *et. al.*, 1986). Its mitogenic action has been investigated extensively in mouse Swiss 3T3 fibroblasts (Rozengurt, 1986). Human fibroblasts respond to PDGF by an increased production of IGF (Clemmons *et. al.*, 1981b) which may account for some of the wound healing properties of PDGF. The lack of response of He[39]L fibroblasts and human skin fibroblasts (Conover *et. al.*, 1989) with respect to binding protein production may play a role in the action of PDGF making increasing levels of IGF available to receptors.

EGF has been reported to stimulate IGFBP-3 production in a number of different fibroblasts including human skin fibroblasts (Martin and Baxter, 1988; Mondschein *et. al.*, 1990; Corps and Brown, 1991), although bovine fibroblasts were unaffected (Conover, 1990). IGFBP-3 levels remained virtually undetectable in He[39]L incubated with EGF. Similarly FGF had no affect on binding protein production by He[39]L cells.

It appears from the stimulation experiments with a range of growth factors and hormones that IGFs have the most pronounced affect on He[39]L binding protein production. The lack of response to other growth factors and hormones used in these experiments may be a function of the respective cell surface receptors but the abundance of growth factor/hormone receptors on He[39]L cells is yet to be investigated.

Using information from the characterization of He[39]L cells it is possible to speculate on the mechanisms involved in the modulation of IGF action by fibroblasts in the lung. I envisage that He[39]L fibroblasts are stimulated to grow by some other growth promoter than IGF. However, if IGF is produced by neighbouring cells or is transported into lung tissues it would stimulate the production of the major carrier protein, IGFBP-3, and some of the other smaller molecular weight binding proteins. Release of He[39]L binding proteins into the extracellular space may have the function of sequestering IGF for the stimulation of IGF responsive cells in the lung and so have a role in the paracrine action of IGF. In turn the elevated levels of IGFBP-3 may enhance the action of IGF on He[39]L cells, as preincubation of human skin fibroblasts with IGFBP-3 has previously been shown to enhance the response of those cells to IGF-I (De Mellow and Baxter, 1988).

Figure 4.1: Competition binding assays with fibroblast conditoned media and rhIGF-I tracer

Competition for binding to conditioned media was performed with increasing amounts of rhIGF-I (□), bIGF-II (●) and des-(1-3)-IGF-1 (■). The rhIGF-I and des-(1-3)-IGF-1 were from GroPep Pty. Ltd., South Australia and bIGF-II was a gift from Monsanto Company, St. Louis, MO, U.S.A.

Competition is expressed as a percentage of the number of counts bound by conditioned medium in the absence of competing ligand (B_0). Nonspecific binding (NSB) measured in the absence of conditioned medium and competing ligand was subtracted. Competition assays were performed at least twice and each point on the curves represents the mean of duplicates. Standard deviations (bars) are shown for rhIGF-I curves and are representative of the errors for all competing ligands.

A. Binding to He[39]L conditioned medium (50µl). B_0 = 76%, NSB = 20 %

B. Binding to IMR 90 conditioned medium (15µl). B_0 = 70%, NSB = 12.8%

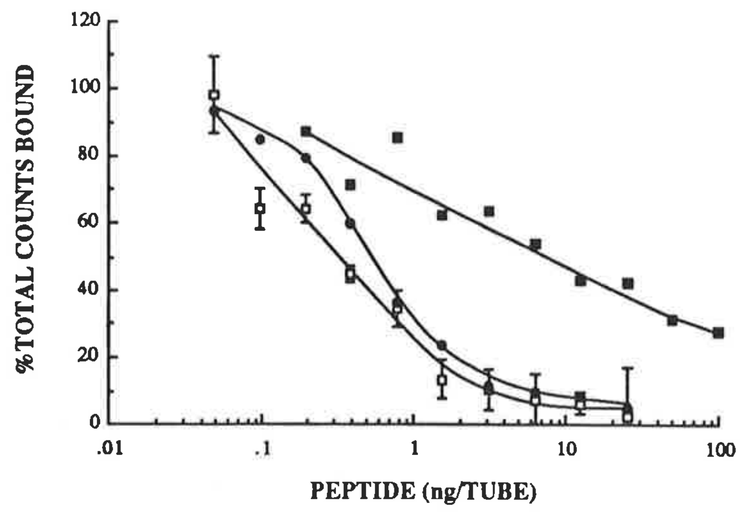
C. Binding to Ag 2804 conditioned medium (15µl). B_0 = 51%, NSB = 12.8%

D. Binding to SF 1972 condtioned medium (15µl). B_0 = 40.7%, NSB = 18.2%

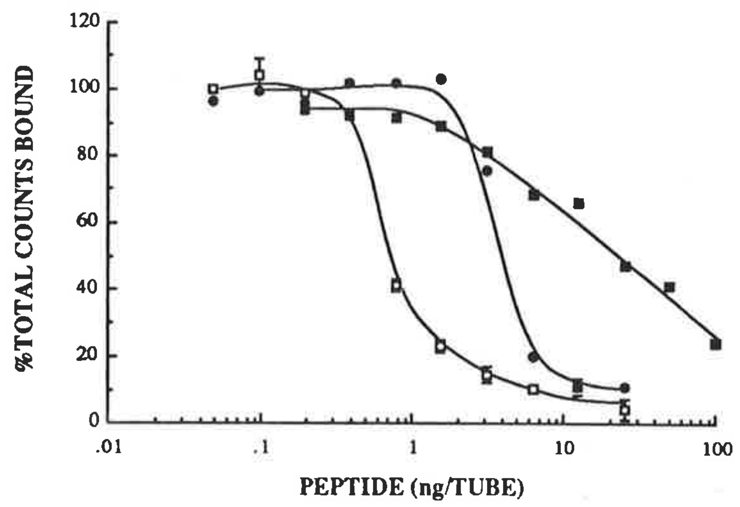
E. Binding to WI 38 conditioned medium (50µl). B_0 = 69.3%, NSB = 20%

He[39]L cells	human embryonic lung fibroblasts
IMR 90 cells	human embryonic lung fibroblasts
Ag 2804 cells	SV 40-transformed IMR 90 cells
SF 1972 cells	human skin fibroblasts
WI 38 cells	human embryonic lung fibroblasts

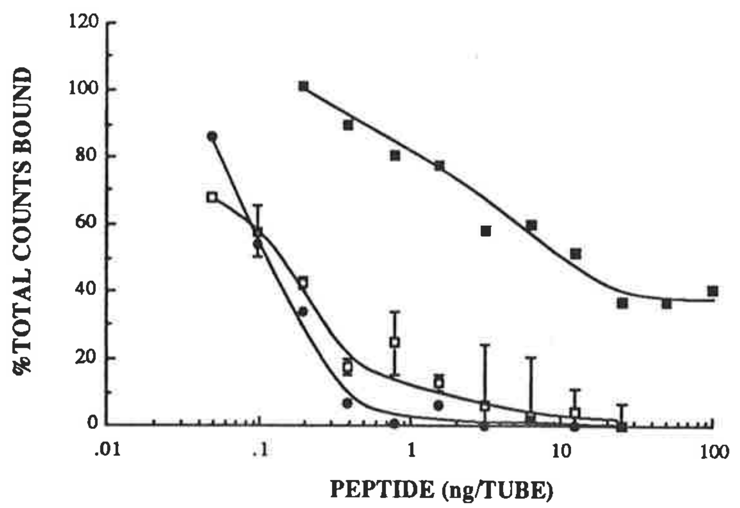
A



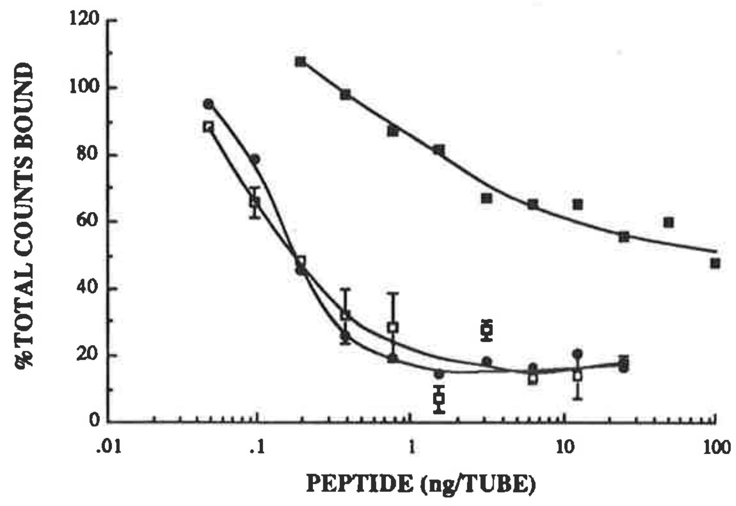
B



C



D



E

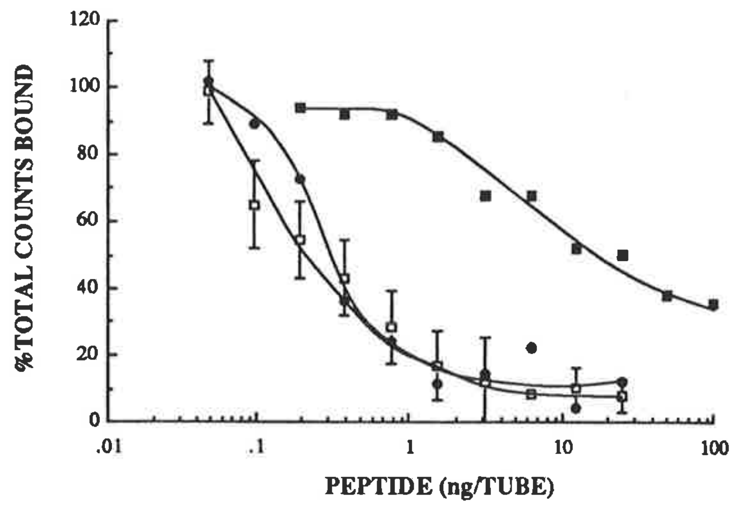


Figure 4.2: Competition binding assays with fibroblast conditoned media and bIGF-II tracer

Competition for binding to conditioned media was performed with increasing amounts of rhIGF-I (□), bIGF-II (●) and des-(1-3)-IGF-1 (■). The rhIGF-I and des-(1-3)-IGF-1 were from GroPep Pty. Ltd., South Australia and recombinant bIGF-II was a gift from Monsanto Company, St. Louis, MO, U.S.A.

Competition is expressed as a percentage of the number of counts bound by conditioned medium in the absence of competing ligand (B_0). Nonspecific binding (NSB) measured in the absence of conditioned medium and competing ligand was subtracted. Competition assays were performed at least twice and each point on the curves represents the mean of duplicates. Standard deviations (bars) are shown for rhIGF-I curves and are representative of the errors for all competing ligands.

A. Binding to He[39]L conditioned medium (15µl). B_0 = 52%, NSB = 23.4 %

B. Binding to IMR 90 conditioned medium (50µl). B_0 = 40%, NSB = 23.4%

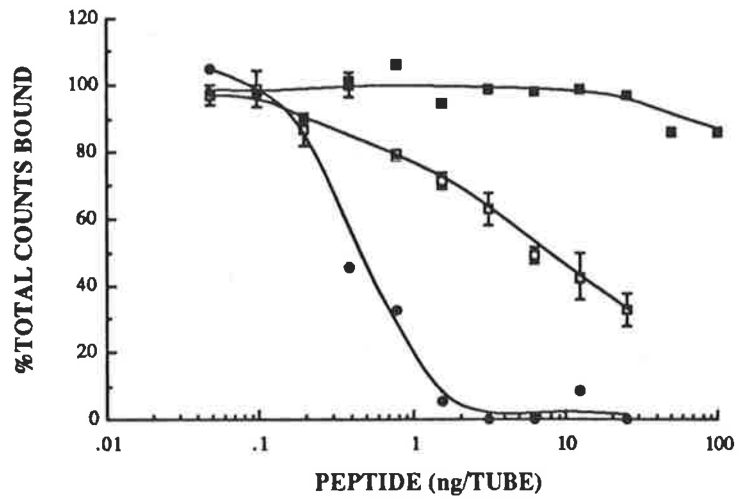
C. Binding to Ag 2804 conditioned medium (15µl). B_0 = 57.7%, NSB = 23.4%

D. Binding to SF 1972 condtioned medium (15µl). B_0 = 53%, NSB = 20.2%

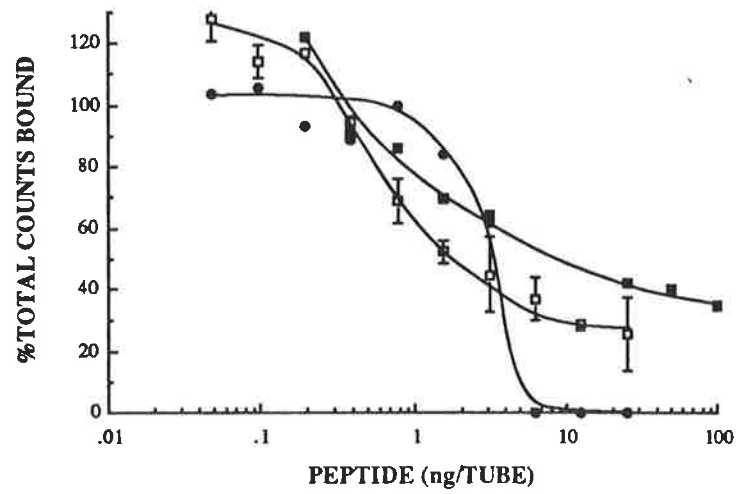
E. Binding to WI 38 conditioned medium (50µl). B_0 = 54.7%, NSB = 20.2%

He[39]L cells	human embryonic lung fibroblasts
IMR 90 cells	human embryonic lung fibroblasts
Ag 2804 cells	SV 40-transformed IMR 90 cells
SF 1972 cells	human skin fibroblasts
WI 38 cells	human embryonic lung fibroblasts

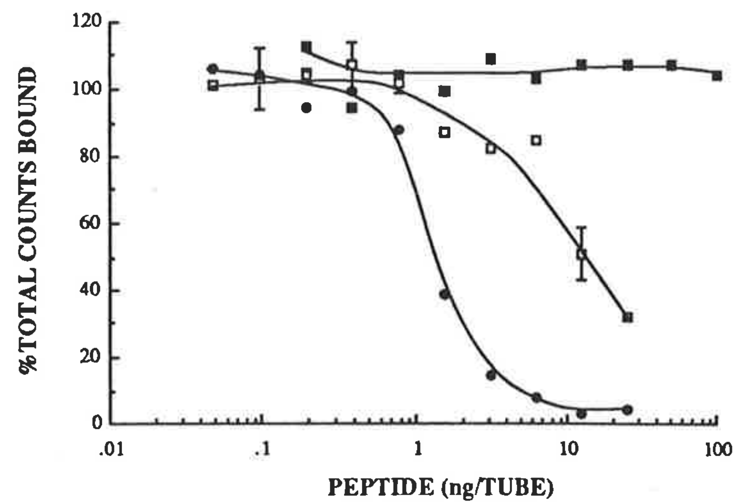
A



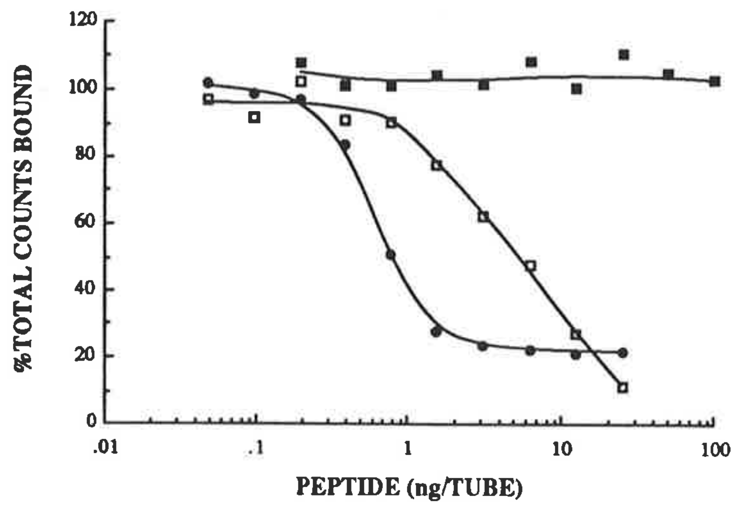
B



C



D



E

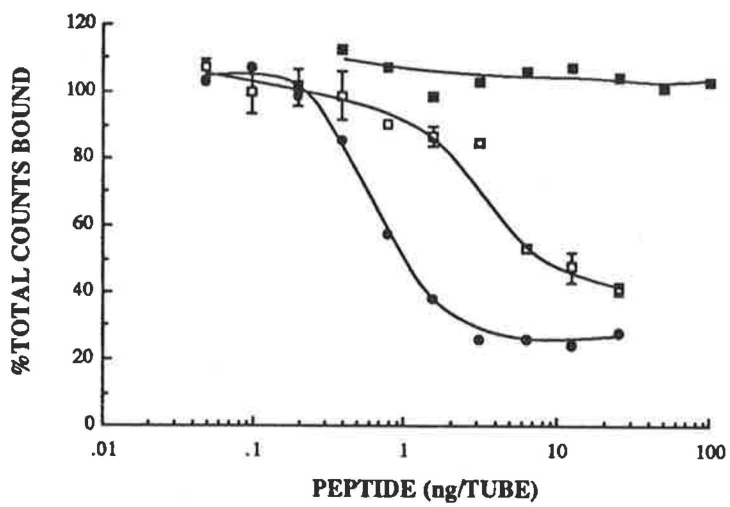


Figure 4.3: Analysis of fibroblast binding proteins

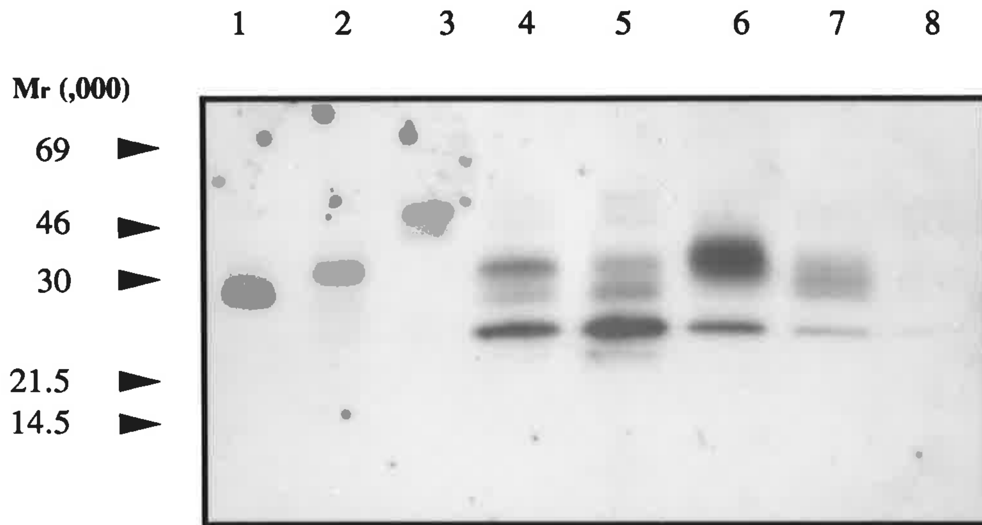
A. Fibroblast conditioned medium was concentrated using Centricon microconcentrators prior to separation on 12.5 % non-reducing SDS-polyacrylamide gels. 800µl equivalent of each conditioned medium was analyzed for the presence of IGF-binding protein by Western ligand blotting (section 2.2.11) following transfer to nitrocellulose. Filters were probed with ^{125}I IGF-II (10^6 cpm/filter) and exposed to autoradiograph film for 2 days.

Lanes	
1	Purified hIGFBP-1 (10ng)
2	Purified bIGFBP-2 (10ng)
3	Purified hIGFBP-3 (25ng)
4	He[39]L conditioned medium
5	IMR 90 conditioned medium
6	Ag 2804 conditioned medium
7	WI 38 conditoned medium
8	SF 1972 conditioned medium

The migration of molecular weight standards is indicated by arrows.

B. The same filter in a) was probed with an antibody directed against bIGFBP-2 (1/100, overnight at 4°C; see section 2.2.11).

A



B

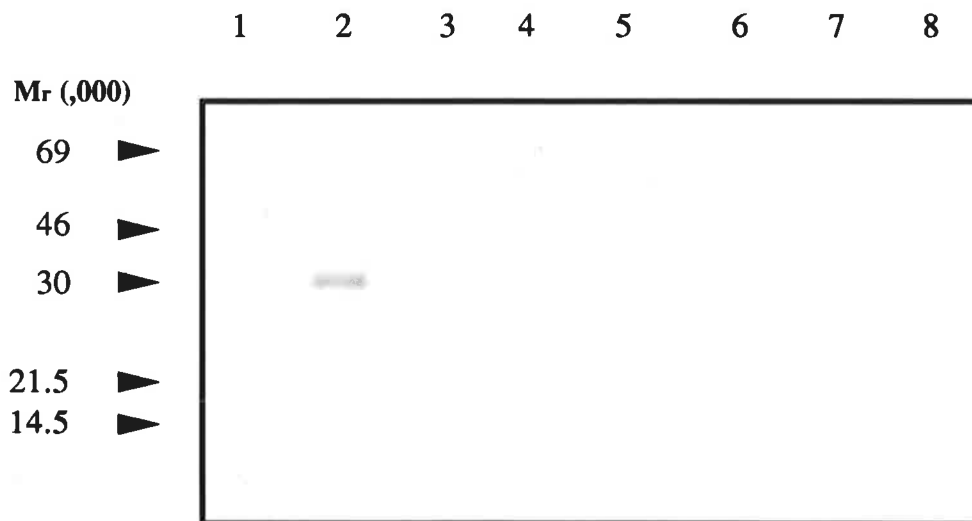
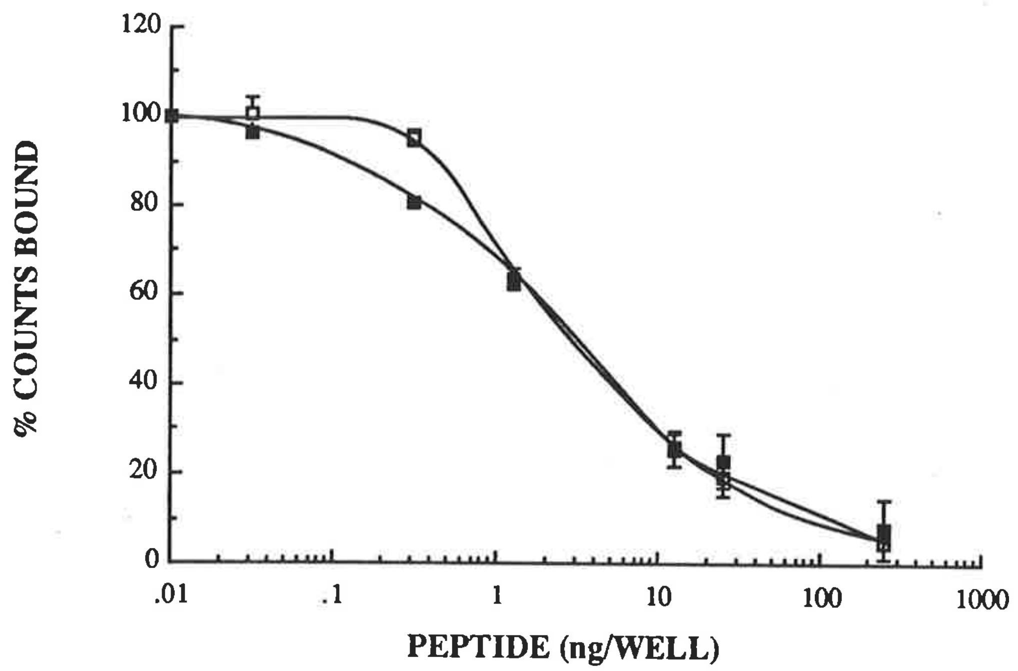


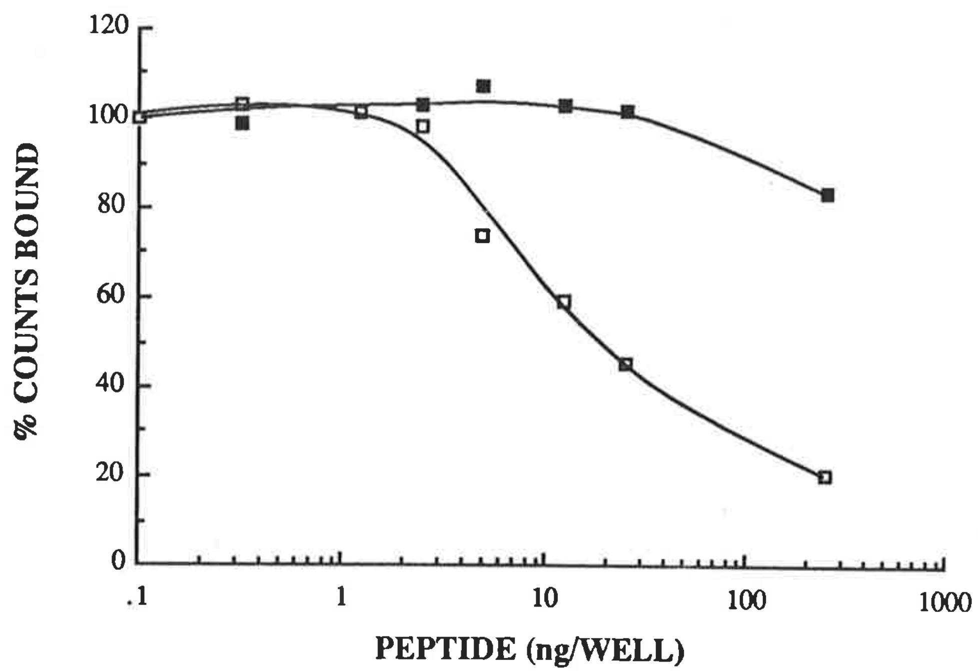
Figure 4.4: Competitive He[39]L radioreceptor assays

Increasing amounts of competing ligand were added in the presence of **A** rhIGF-I tracer and **B** bIGF-II tracer and binding to He[39]L IGF cell membrane receptors was measured. Competing rhIGF-I (□) was from GroPep Pty. Ltd., South Australia and bIGF-II (●) was a gift from Monsanto Company, St. Louis, MO, U.S.A. Binding to receptors was measured after overnight at 4°C. Competition is expressed as a percentage of the counts bound in the absence of competing ligand. Each point on the competition curves represents mean of triplicate wells. Standard errors are shown where the error exceeds the size of the symbols on the curves. In both IGF-I and IGF-II radioreceptor assays insulin (500ng) did not compete for receptor binding.

A



B



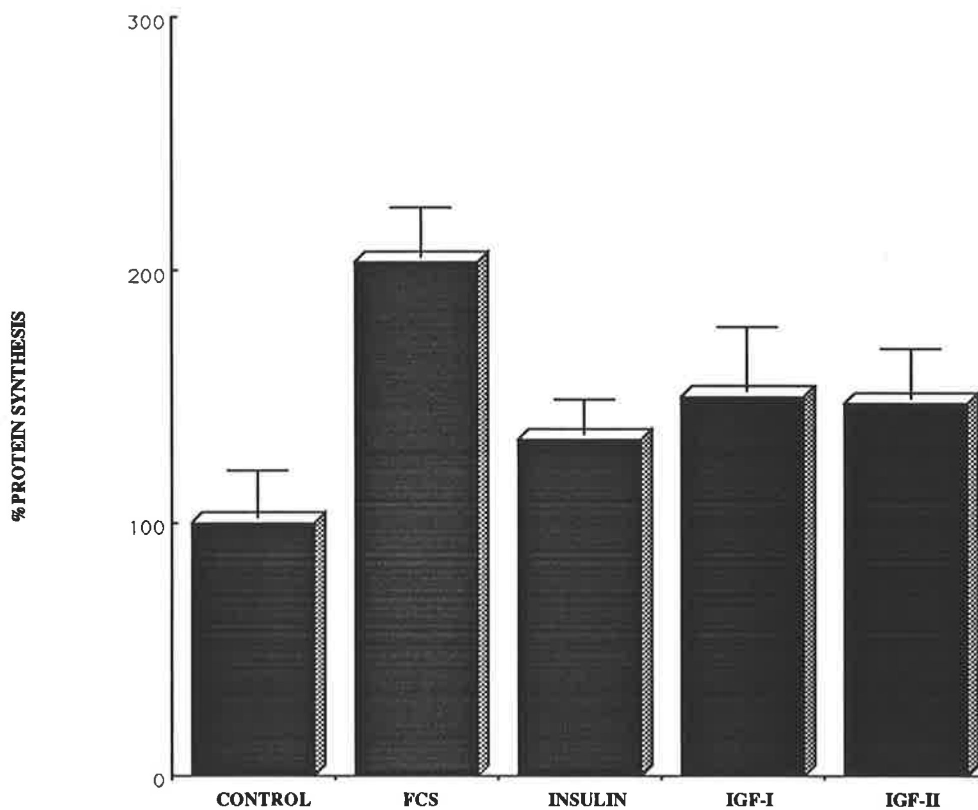


Figure 4.5: Biological response of He[39]L cells to IGF-I. Protein synthesis assays were used to measure the biological response of He[39]L cells to IGF-I as described in section 2.2.5. Cells were incubated overnight in the presence of 100 ng/ml rhIGF-I (Gropep Pty. Ltd., Adelaide, South Australia) or 10% fetal calf serum (FCS). Control wells contained serum-free medium only. The incorporation of ^{3}[H] leucine into newly synthesized proteins was measured and the results were expressed as a percentage of the counts incorporated where 100% represents the incorporation into control cells.

Figure 4.6: Stimulation of He[39]L binding protein production by IGFs and insulin

He[39]L cells were grown to confluence before washing in serum-free medium to remove endogenous binding proteins. Peptides in serum-free medium were added to the washed cells for 48 hours. Medium was collected, concentrated and then separated on 12.5 % SDS polyacrylamide gels (800μl equivalent/lane). Proteins were transferred to nitrocellulose which was probed with bIGF-II tracer (10^6 cpm/filter) and exposed to autoradiograph film for 3 days. These experiments were repeated 3 times.

Recombinant peptides were from GroPep Pty Ltd., South Australia except for recombinant bIGF-II, a gift from Monsanto Company, St. Louis, MO, U.S.A, and insulin (Commonwealth serum laboratories, Melbourne, Australia)

Lanes	
1	He[39]L cells incubated with serum-free medium
2	IGF-I (100ng/ml)
3	IGF-II (100ng/ml)
4	Des-(1-3)-IGF-1 (100ng/ml)
5	Long R^3 IGF-I (100ng/ml)
6	Insulin (500ng/ml)

The extent of migration of molecular weight standards (left) and purified binding proteins (right) is indicated by arrows.

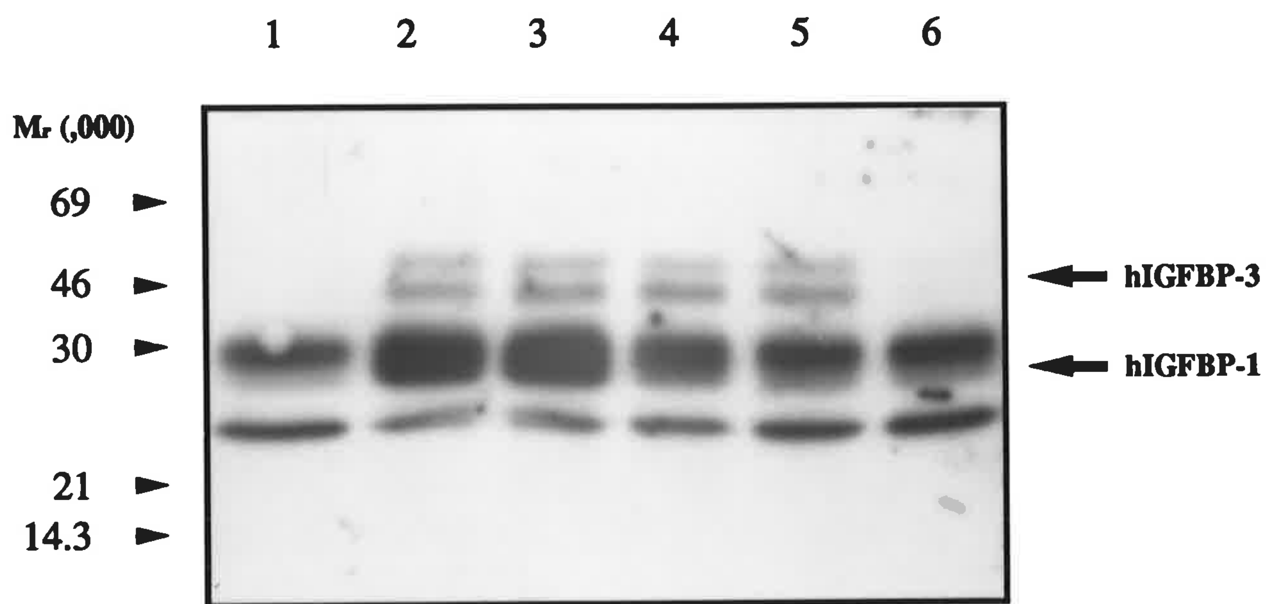

1
2
3
4
5
6
Mr (,000)
69
46
30
21
14.3
hIGFBP-3
hIGFBP-1

Figure 4.7: Stimulation of SF 1972 binding protein production by IGFs and insulin

SF 1972 cells were grown to confluence before washing in serum-free medium to remove endogenous binding proteins. Peptides in serum-free medium were added to the washed cells for 48 hours. Medium was collected, concentrated and then separated on 12.5 % SDS polyacrylamide gels (800μl equivalent/lane). Proteins were transferred to nitrocellulose which was probed with bIGF-II tracer (10^6 cpm/filter) and exposed to autoradiograph film for 4 days. These experiments were performed twice.

Recombinant peptides were from GroPep Pty. Ltd., South Australia except for recombinant bIGF-II, a gift from Monsanto Company, St. Louis, MO, U.S.A, and insulin (Commonwealth Serum Laboratories, Melbourne, Australia).

Lanes	
1	SF 1972 cells incubated with serum-free medium
2	IGF-I (100ng/ml)
3	IGF-II (100ng/ml)
4	Des-(1-3)-IGF-1 (100ng/ml)
5	Long R^3 IGF-I (100ng/ml)
6	Insulin (500ng/ml)

The extent of migration of molecular weight standards (left) and purified binding proteins (right) is indicated by arrows.

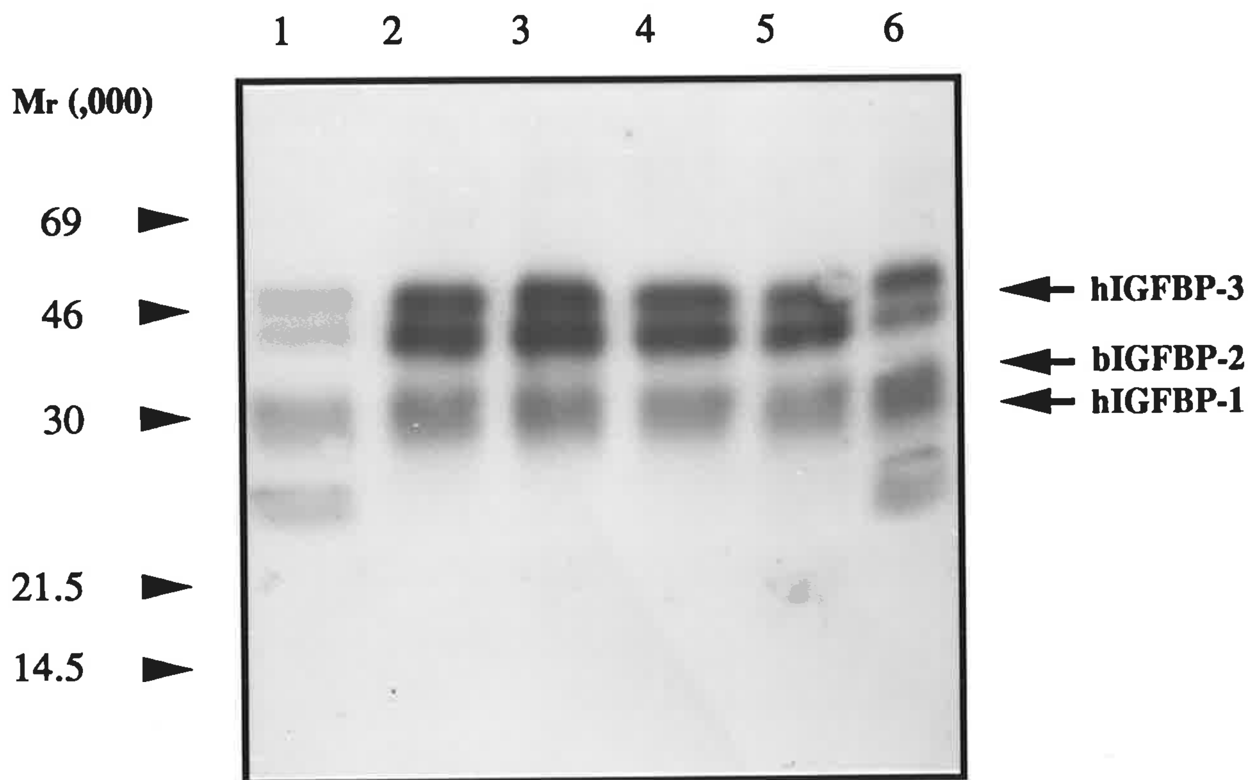
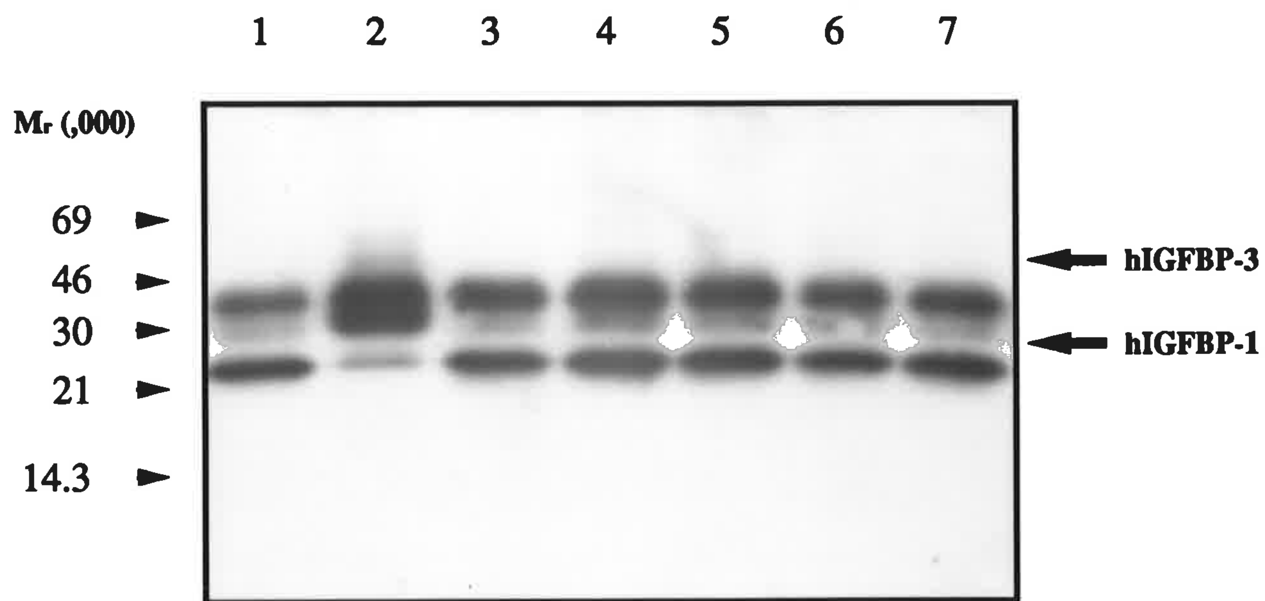

1
2
3
4
5
6
Mr (,000)
69
46
30
21.5
14.5
hIGFBP-3
bIGFBP-2
hIGFBP-1

Figure 4.8: Stimulation of He[39]L binding protein production by growth promoters and glucocorticoid

Confluent He[39]L cell monolayers were incubated in the presence of various growth factors, dexamethasone and estrogen using the procedure as described in section 2.2.8 and in Figures 4.6 and 4.7. Conditioned medium was collected after a 48 hour incubation, concentrated and separated on 12.5% SDS polyacrylamide gels. Proteins were transferred to nitrocellulose which was probed with ^{125}I IGF-II (10^6 cpm/filter) and exposed to autoradiograph film for 3 days. These experiments were performed twice.

Lanes	
1	He[39]L cells incubated with serum-free medium
2	IGF-I (100ng/ml)
3	Epidermal growth factor (30ng/ml)
4	Fibroblast growth factor (30ng/ml)
5	Platelet-derived growth factor (30ng/ml)
6	Dexamethasone (1μM)
7	Estrogen (10nM)

The extent of migration of molecular weight markers is indicated by arrows.


1
2
3
4
5
6
7
Mr (,000)
69
46
30
21
14.3
hIGFBP-3
hIGFBP-1

CHAPTER 5

PURIFICATION AND CHARACTERIZATION OF He[39]L IGFBPs

5.1 INTRODUCTION

Before the commencement of this study an observation was made in our laboratory which formed the basis for the present investigation. A comparative study was conducted using the serum-free, conditioned media from a variety of cell lines in competitive charcoal binding assays. The human lung fibroblast cell line (He[39]L) produced binding proteins which behaved differently in those assays to the binding proteins of the cell lines formerly used in IGFBP purification, namely the human hepatoma cell line (Hep G2) and the bovine kidney cell line (MDBK). Like cerebrospinal fluid binding proteins (Binoux *et. al.*, 1986), He[39]L conditioned medium preferentially bound IGF-II compared with IGF-I. On the basis of that observation the He[39]L cell line seemed to be a likely source of novel binding proteins.

The serum-free conditioned medium of cultured cells containing binding proteins has been used as an abundant source for purification of several IGFBPs. Moses *et. al.* (1979) first identified rat liver cells (BRL 3A) as producers of small molecular weight binding proteins in a comparative study of rat serum and BRL 3A serum-free conditioned medium. These cells were later the source of rat IGFBP-2 (Mottola *et. al.*, 1986; Lyons and Smith, 1986; Romanus *et. al.*, 1987). Human hepatoma cells (Hep G2) were shown to secrete high levels of IGF binding protein (Moses *et. al.*, 1983) and IGFBP-1 was subsequently purified from the serum-free medium of these cells (Povoa *et. al.*, 1985).

More recently, the bovine form of IGFBP-2 was isolated from bovine kidney cells (MDBK, Szabo *et. al.*, 1988), mouse Swiss 3T3 cells were the source of IGFBP-3 (Blat *et. al.*, 1989a, 1989b) and IGFBP-4 was isolated from human osteocarcinoma cells (TE89, Mohan *et. al.*, 1989). Apart from the earliest purification methods, all binding proteins have been purified using IGF affinity chromatography. The approach I undertook to isolate novel binding proteins from the serum-free conditioned medium of He[39]L cells was based on the method used for the purification of bIGFBP-2 developed in our laboratory (Szabo *et. al.*, 1988) and also involved IGF affinity chromatography.

The aim of the study outlined in this chapter was to purify novel binding proteins from the serum-free conditioned medium of He[39]L cells. One of the two binding proteins purified was characterized on an immunological basis as well as by size, relative binding

affinity and lectin affinity chromatography. The isolation and description of novel binding proteins will ultimately result in adding to our knowledge of the types of binding proteins produced by cultured cells and human embryonic lung fibroblasts in particular.

5.2 RESULTS

A flow chart in Figure 5.1A outlines the steps taken in the purification of He[39]L binding proteins and the detailed method is described in section 2.3. I will first discuss the purification of the He[39]L binding protein which I have extensively characterized.

5.2.1 Purification of the 32 kDa He[39]L binding protein

Following acidification serum-free He[39]L conditioned medium was applied to an S-Sepharose cation exchange column. Binding protein activity was monitored throughout the purification by charcoal binding assays and by interference in receptor binding assays (see sections 2.2.1 and 2.2.3). Active fractions eluted from the cation exchange column between 0.25M and 0.8M ammonium acetate using a linear gradient of 0.05M to 1M ammonium acetate (pH 6.0-7.5). They were pooled and directly applied to an IGF-I affinity column. Binding protein was specifically eluted with 0.5M acetic acid.

The final purification step involving reverse phase high performance liquid chromatography (rpHPLC) analysis of the He[39]L binding protein resulted in a single peak eluting at approximately 28% acetonitrile in 0.1% trifluoroacetic acid (Figure 5.1B.i). The maximum binding protein activity coincided with the protein peak (Absorbance = 215nm; Figure 5.1B.ii). Approximately 60 μg of the 32 kDa He[39]L binding protein were purified from a 15 litre purification of He[39]L conditioned medium.

The rpHPLC-purified protein was separated on a 15% nonreducing SDS-polyacrylamide gel into three bands as detected by silver staining (see section 2.2.9 and Figure 5.2). The major species was estimated as having the molecular weight of 32,000 and the two minor bands were 20,000 and 16,000.

Using a method termed electroblotting (Matsudaira, 1987; described in section 2.3.5) the He[39]L binding protein was separated on a nonreducing gel and transferred to a polyvinylidene difluoride membrane (PVDF). The 32 kDa protein on the PVDF was placed directly in the sample chamber of an Applied Biosystems 470 A Sequenator. This method

COLLECT SERUM-FREE CONDITIONED
MEDIUM FROM He[39]L CELL FACTORY

ACIDIFY AND FILTER He[39]L
CONDITIONED MEDIUM

CATION EXCHANGE CHROMATOGRAPHY

S-Sepharose, 50mM to 1M ammonium acetate
gradient (pH 6.0 to 7.5)

IGF AFFINITY CHROMATOGRAPHY

elution with 0.5M acetic acid

REVERSE PHASE HPLC
20 to 40 % acetonitrile/0.1% TFA gradient

LYOPHILIZE AND STORE AT -80 C

Figure 5.1A: Steps in purification of He[39]L binding proteins. The series of chromatography steps used in the purification of He[39]L binding proteins are shown. The detailed method of purification is described in section 2.3. Following each step fractions collected were assayed using charcoal binding and radioreceptor binding assays described in sections 2.2.1 and 2.2.3 respectively.

has two distinct advantages over the traditional sequencing methods. Firstly, it allowed me to sequence the major binding protein species without the presence of the smaller proteins. Also sequences can be derived from 20 pmoles of protein rather than 100 pmoles used in more traditional methods with this sequencer.

The He[39]L protein yielded an N-terminal sequence of 22 amino acids (Figure 5.3). Two of the 22 residues were not identified (positions 13 and 17). The lack of signal suggests that these may represent cysteine residues. The yields of phenylthio-hydantoin (PTH)-amino acids in each round of a second sequencing experiment is shown in Figure 5.4. This sequence confirmed the first 22 amino acids sequenced and yielded a the sequence of a further 3 amino acids.

When the He[39]L binding protein was transferred to PVDF for sequencing a second membrane was placed behind the one applied to the sequencer. A small proportion of the protein transferred to this second membrane. After Western ligand blotting the major 32 kDa band was shown to bind IGF-II tracer. The smaller molecular weight fragments did not bind IGF (data not shown).

The second amino acid sequence described above was derived from the mixture of all three He[39]L proteins. Neither sequence had contamination by other sequences implying that the three bands obtained after separation on SDS-polyacrylamide gels most probably had identical N-terminal sequences or possibly that the two smaller species were N-terminally blocked and therefore did not sequence. If the smaller peptides in the 32 kDa He[39]L binding protein preparation are indeed breakdown products they must arise from C-terminal truncation.

5.2.2 Characterization of the 32 kDa He[39]L binding protein

The He[39]L binding protein was compared with other binding proteins by Western ligand blot (described in section 2.2.11 and Figure 5.5). Clearly this protein differs in size (32 kDa) from hIGFBP-1 (28 kDa), bIGFBP-2 (34 kDa) and hIGFBP-3 (41.5 kDa and 38.5 kDa). It is important to point out that the 32 kDa He[39]L binding protein described in this chapter is the same protein described as being 34 kDa in Chapter, Figure 4.3a, identified by Western ligand blotting in He[39]L conditioned medium. The difference in sizes arises due to different electrophoresis set ups used in these separate experiments.

Immunoblotting (see section 2.2.11 and Figure 5.6) showed that an antibody raised against bIGFBP-2 did not cross-react with He[39]L conditioned medium. Purified bIGFBP-2 did cross-react with the antibody and is shown in lane 2 Figure 5.6. The antibody also identifies the same protein in MDBK conditioned medium (Upton *et. al.*, 1990), which was the original source for purification of bIGFBP-2 (Szabo *et. al.*, 1988). The amount of He[39]L conditioned medium blotted (800μl) was sufficient for the detection of binding proteins by Western ligand blotting.

Furthermore, in immunoprecipitation experiments the He[39]L binding protein did not cross-react with either the antibody raised against bIGFBP-2 or another antibody directed to hIGFBP-1 (method in section 2.2.2; see Figure 5.7). These results suggest that He[39]L binding protein is unrelated to both IGFBP-1 and IGFBP-2.

Northern analysis (section 2.4.2) was performed with He[39]L and MDBK total cellular RNA (Figure 5.8) by Ms Z. Upton. A strong signal was seen with MDBK RNA when the Northern transfer was probed with a 1.2 kb bIGFBP-2 cDNA probe (Upton *et. al.*, 1990). However, the He[39]L RNA did not contain sequences complementary to IGFBP-2 cDNA as no signal was seen on the same Northern blot, even after extended exposure of 2 weeks.

Lectin affinity chromatography (section 2.2.12) was used to analyze the He[39]L binding protein for glycosylation. Binding protein was loaded and the column was washed in load buffer prior to elution. Fractions containing binding protein were identified by charcoal binding assay. Using wheat germ agglutinin the He[39]L binding protein was shown to be glycosylated (Figure 5.9). Porcine IGFBP-3 also bound to the same lectin.

A second lectin, concanavalin A, which also detects N-linked glycosylation, failed to bind the He[39]L binding protein. However, porcine IGFBP-3 did bind to concanavalin A and was specifically eluted with 0.5 M α-D-mannopyranoside. Neither hIGFBP-1 or bIGFBP-2 bound to wheat germ or concanavalin A columns.

Further characterization of the He[39]L binding protein was on the basis of relative IGF-binding affinity (Figure 5.10). Competition binding assays were performed with the various forms of IGF, IGF-I, IGF-II and des-(1-3)-IGF-1, as competing ligands using

rhIGF-I and bIGF-II tracers. Lower concentrations of IGF-II were needed for the same extent of competition achieved with IGF-I using IGF-I tracer. Five fold less binding protein was used in competition assays with IGF-II tracer and even lower concentrations of IGF-II were needed to give the same degree of competition as IGF-I. Des-(1-3)-IGF-1 did not compete significantly for binding with either tracer.

5.2.3 Purification of 17-19 kDa binding protein

Several observations were made in refining the method for the purification of the 32 kDa He[39]L binding protein. Initially the purification procedure involved the use of an IGF-II affinity column also used in the purification of bIGFBP-2 (Szabo *et. al.*, 1988) rather than the IGF-I column described above. The original purification resulted in a single apparently pure protein peak as analysed by rpHPLC. The N-terminal peptide sequence, however, indicated the presence of more than one peptide. An attempt was made in the following purification to separate the two proteins which were apparently very similar as they both eluted from the IGF-II affinity column and had similar hydrophobicities.

Two detection methods were used throughout He[39]L binding protein purifications. These were the charcoal binding assay and the radioreceptor assay. The latter detects binding protein as it interferes with the binding of IGF to IGF receptors. Most interestingly, in the original purification the leading fractions of the binding protein peak following rpHPLC separation were active only in the radioreceptor assay and not in the charcoal binding assay. The rest of the fractions were active in both assays. In this respect the 17-19 kDa He[39]L binding protein is similar to IGFBP-1 in that both are undetectable by charcoal binding assay.

Following a particularly shallow acetonitrile/0.1% TFA gradient I was able to separate the fractions containing the 17-19kDa He[39]L binding protein active only in the radioreceptor assay (Figure 5.11). The protein was sequenced using conventional sequencing techniques rather than electroblotting. The sequence is shown in Figure 5.12. In fact two sequences were present in the peptide preparation. The yields of the PTH-derivatized amino acids in each round of sequencing are shown in Figure 5.13. The sequence with slightly lower yields appeared to be a truncated form of the more abundant peptide but lacked 3 amino acids at the N-terminus.

Analysis of the protein by silver staining showed that it was in fact separated into two species of 17.5 and 19 kDa (Figure 5.14). Unfortunately, further analysis of the peptide was not possible. Upon storage the binding protein was degraded and lost IGF-binding activity.

Subsequent purifications were carried out with an IGF-I affinity column in an attempt to make the separation of the two binding protein species easier. The IGF-II column used in the initial purifications was rather old and had a much lower binding capacity than the new column. Following purifications then yielded only the 32 kDa binding protein.

5.3 DISCUSSION

Characterization of binding proteins is important in understanding the modulation of IGF action as binding proteins have been shown to both inhibit and enhance the action of IGF (Ross *et. al.*, 1989; Ritvos *et. al.*, 1988; Clemmons *et. al.*, 1986). Three binding proteins have been extensively characterized (IGFBP-1, IGFBP-2 and IGFBP-3) and cDNA sequences have been determined from several species for each of these (reviewed in section 1.2). The genes for IGFBP-1, IGFBP-2 and IGFBP-3 have been isolated (Cubbage *et. al.*, 1989; Brown and Rechler, 1990; Cubbage *et. al.*, 1990; Ehrenborg *et. al.*, 1991). A fourth binding protein (IGFBP-4) has recently been identified and cDNA clones have been isolated (Shimasaki *et. al.*, 1990b; LaTour *et. al.*, 1990). This binding protein has not yet been characterized to the extent of the other three. Serum from humans and rats contain each of these four binding proteins and they are also found in other body fluids. In this chapter I have described the purification of two novel binding proteins which differ from IGFBPs 1, 2, 3 and 4.

Three classes of binding protein have been previously defined on the basis of IGF-binding affinity as demonstrated in competition binding assays (Forbes *et. al.*, 1988 and Chapter 3). The first class is represented by hIGFBP-3 which binds IGF-I and IGF-II with equal potency. The same competition seen with IGF-I and IGF-II is achieved with two fold higher concentrations of des-(1-3)-IGF-1. Human IGFBP-1 represents a second class of binding protein where both IGF-I and IGF-II bind equally but des-(1-3)-IGF-1 is bound poorly, if at all. The third class is represented by bIGFBP-2 which does not bind des-(1-3)-IGF-1 and prefers IGF-II. Competition assays described in this chapter

(Figure 5.10) demonstrate that the 32 kDa He[39]L binding protein does not bind des-(1-3)-IGF-1 and has greater affinity for IGF-II than IGF-I. In this respect it is similar to bIGFBP-2.

Despite this similarity further analysis of the binding protein suggested that it is not the human form of IGFBP-2. Both immunoblots and Northern analysis showed that the 32 kDa He[39]L binding protein is unrelated to IGFBP-2. The antibody directed against bIGFBP-2 only cross-reacted with bIGFBP-2 (Figure 5.6). This antibody can not only cross-react with bovine IGFBP-2 but has also been used to identify related proteins from other species, in particular in fetal rat serum and medium conditioned by rat liver cells, BRL 3A (Upton *et. al.*, 1990). However, the bIGFBP-2 antibody did not detect any proteins in He[39]L conditioned medium, indicating that this protein is not a human form of IGFBP-2.

Similarly, a 1092 bp cDNA clone of bIGFBP-2 was used to probe a Northern blot with 10 μg He[39]L total RNA and no hybridization was detected (Figure 5.8). However, a single mRNA species (approximately 1.4 kb) was detected in total RNA from bovine kidney cells (MDBK).

Immunoprecipitation experiments using polyclonal antibodies directed against hIGFBP-1 and bIGFBP-2 failed to cross-react with the 32 kDa He[39]L binding protein (Figure 5.8). This is further evidence that the He[39]L binding protein is not related to bIGFBP-2. In addition, not only do the binding patterns of IGFBP-1 and He[39]L binding protein differ but they are also immunologically unrelated. Finally, all three proteins differ in molecular weight (Figure 5.5) as determined by Western ligand blotting.

Analysis of glycosylation revealed that both the 32 kDa He[39]L binding protein and pIGFBP-3 are glycosylated. The He[39]L binding protein binds to wheat germ agglutinin and pIGFBP-3 binds to both wheat germ agglutinin and concanavalin A (Figure 5.9). This indicates that the 32 kDa He[39]L binding protein has N-acetyl glucosaminyl sugars but no α-mannosyl sugars, whereas both are detected on the pIGFBP-3. Based on glycosylation, relative binding affinities and size the two binding proteins are clearly unrelated.

Lectin affinity chromatography in this chapter supports cDNA sequence analysis of pIGFBP-3 revealing 3 potential N-linked glycosylation sites (Shimasaki *et. al.*, 1990a).

IGFBP-3 from other species are also glycosylated. Not only are the He[39]L binding protein and IGFBP-3 glycosylated but rat and human IGFBP-4 cDNA sequences encode one potential N-linked glycosylation site (Shimasaki *et. al.*, 1990b). IGFBP-1 has no N-linked sites but has 5 potential O-linked sites (Brinkman *et. al.*, 1988a). In contrast, IGFBP-2 has neither N- nor O-linked glycosylations. Neither IGFBP-1 nor IGFBP-2 bound to wheat germ agglutinin or concanavalin A.

N-terminal protein sequencing revealed that the 32 kDa He[39]L binding protein has a unique amino acid sequence (Figure 5.3). A computer search, including the GENBANK (release 67, 1991), National Biomedical Research Foundation protein identification resource (release 21, 1989) and nucleic acid (release 36, 1990) databases, revealed no significant homology to other proteins, in particular the type 1 and type 2 IGF receptors.

An outstanding feature of the binding proteins sequenced before the isolation of the 32 kDa binding protein was the conservation of 18 cysteines across all sequences for IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 from various species. Two residues of the He[39]L sequence have not been identified but are suspected to be cysteines. However, these do not align with the cysteines of sequences of these four well characterized binding proteins (see Figure 5.3). Glycosylated Asn or Thr residues are often undetectable in the analysis of sequence data and could account for unassigned residues. However, this is unlikely in the case of the He[39]L sequence as no glycosylation motifs (Asn-X-Ser/Thr for N-linked glycosylation; Marshall, R. D., 1972) are evident in the sequence flanking the unassigned residues.

Since isolation of the 32 kDa He[39]L binding protein several additional N-terminal sequences of the same protein have been reported (see Figure 5.15; Roghani *et. al.*, 1989; Martin *et. al.*, 1990; Zapf *et. al.*, 1990a; Andress and Birnbaum, 1991; Shimasaki *et. al.*, 1991b). The transformed fibroblast binding protein (TFBP) described by Martin *et. al.* (1990) and the cerebrospinal fluid binding protein (Roghani *et. al.*, 1989) exhibit essentially the same properties as the 32 kDa He[39]L binding protein. Their isolates also show preference for binding to IGF-II than IGF-I and do not bind des-(1-3)-IGF-1. The TFBP cross-reacts with an antibody directed against hIGFBP-3 which is interesting when all of the differences between the two binding proteins described in this chapter are considered. It is

possible that the antibody is directed against the glycosylated region of hIGFBP-3 and cross-reaction with TFBP results from the common feature of glycosylation.

Figure 5.15 shows a comparison of sequences similar to the 32 kDa He[39]L binding protein and highlights the heterogeneity between these. Considerable variation is seen in the amino acids corresponding to the first and fifth residues of the He[39]L binding protein sequence. Interestingly, although both the TFBP and human serum protein sequences were derived from carboxymethylated protein (Martin *et. al.*, 1990; Zapf *et. al.*, 1990a) the human serum isolate does not have a cysteine at the position corresponding to He[39]L residue 5, whereas TFBP does. However, the positions of two cysteines present in the human serum binding protein sequence do correspond to those of the two unassigned residues in the He[39]L sequence, suggesting that the unassigned residues are indeed cysteines.

During the writing of this thesis I received a preprint of a report of the full length cDNA sequence of this protein from Dr. N. Ling (Shimasaki *et. al.*, 1991a). It is now known as IGFBP-6 (Ballard *et. al.*, 1991b). The N-terminal sequence derived from the cDNA sequence reveals that in fact the first three cysteines of this protein do align to cysteines of IGFBPs 1, 2, 3, and 4. A significant difference is seen between several of the N-terminal sequences and the deduced protein sequence as shown in Figure 5.15 (see Chapter 6, Figure 6.6 for the full deduced protein sequence). There are three extra amino acids at the N-terminus of the protein sequence deduced from the cDNA sequence when compared with all the human isolates of the same protein (Figure 5.15), which suggests alternative processing of the leader peptide from the mature peptide.

The position of the cysteine residues is most interesting. The fifth residue encoded by the hIGFBP-6 cDNA corresponds to the second residue in all human isolates of the protein and is a cysteine. However, 4 of the 6 human protein sequences had assigned an Ala to this position, with the remaining two sequences not having assigned sequence to that position. In addition, the hIGFBP-6 cDNA encodes a cysteine in the position corresponding to residue 5 of the He[39]L binding protein sequence, confirming the sequence of Martin *et. al.* (1990). Using these two cysteines Shimasaki *et. al.*, (1991a) were able to align the hIGFBP-6 sequence with IGFBP 1, 2, 3 and 4 sequences (see Figure 6.6 for sequence alignments).

Even more surprising was the sequence of rat IGFBP-6 (rIGFBP-6). This cDNA sequence encoded a protein similar to that sequenced by Shimasaki *et. al.* (1991b) in Figure 5.15. However, the cysteines in the human IGFBP-6 sequence corresponding to positions 13 and 17 of the He[39]L sequence are absent in the rat sequence. Both rat and human sequences also lack two further cysteines slightly further towards the C-terminus. This information is particularly relevant to experiments performed in Chapter 6 and will be discussed in detail in that chapter.

Several isolates of the same protein as the 17-19 kDa He[39]L binding protein have recently been reported (Andress and Birnbaum, 1991; Bautista *et. al.*, 1991; Shimasaki *et. al.* 1991b). The N-terminal amino acid sequences are compared in Figure 5.15. If the unassigned residues in the He[39]L binding protein sequence are in fact cysteines, as suspected, then the two cysteines in the motif Cys, Gln, Pro, Cys (residues 7 to 10) would match the identical motif at the N-terminal end of IGFBP-3 (residues 13 to 16; see Figure 5.12). The U-2 osteosarcoma binding protein has cysteines at positions 4 and 7 which correspond to the unassigned residues in the He[39]L binding protein. These sequences suggest that the cysteines may be conserved between this binding protein and IGFBPs 1, 2, 3 and 4.

Interestingly, the protein isolated by Andress and Birnbaum (1991) had a molecular weight of 23,000 but Bautista *et. al.* (1991) saw a molecular weight of 29,000. The latter protein was particularly unstable and degraded overnight at 5°C to 24 kDa and smaller fragments. The 24 kDa protein had the same N-terminal sequence which is not surprising in the light of the results presented here and reported by Andress and Birnbaum (1991). With degradation Bautista's protein lost binding activity. This binding protein appears to be particularly sensitive to proteases.

The 32 kDa He[39]L binding protein is also susceptible to degradation and is particularly labile in acid conditions (personal observation and communication with Dr. J. L. Martin, Camperdown, NSW, Australia). Despite the similarity in molecular weight between the 17-19 kDa He[39]L binding protein and the breakdown products purified in the preparation of the 32 kDa He[39]L binding protein these peptides are not related. No secondary sequence was detected in sequencing of the 32 kDa preparation, suggesting that

the smaller peptides in this preparation had the same N-terminal sequence as the 32 kDa He[39]L binding protein or they were N-terminally blocked. Also, the breakdown products of the 32 kDa binding protein did not bind IGF-II in Western ligand blotting. However, the 17-19 kDa He[39]L binding protein preparation was active in inhibiting the binding of IGF-II to cell surface receptors and was sequenced, as described above.

In accord with my report, Bautista *et. al.* (1991) also mentioned the purification of truncated forms of the binding protein lacking one or two amino acids at the N-terminus. Truncated forms of bIGFBP-2 lacking 3 N-terminal amino acids and with carboxy terminal truncations have previously been purified in our group (Ms A.McCoy, personal communication). The sequences of IGFBP-5 and IGFBP-6 isolates presented in Figure 5.15 indicate the marked heterogeneity at the N-terminus. Alternative splicing of the leader peptide from the mature protein must result in the extensive variation of binding protein sequences. The biological significance of these truncated binding proteins is unknown and they may only be a result of the purification procedure.

Apparently both Andress and Birnbaum (1991) and Bautista *et. al.* (1991) experienced the same difficulty of detection of the binding protein with the standard charcoal binding assay as reported in this chapter. They both detected the binding protein throughout purification by precipitation methods similar to those used in the competition assays with IGFBP-1 described in chapter 3. IGF-binding protein complexes were precipitated in the presence of 2% immune serum globulin and 25% PEG. It should be noted that in my hands IGFBP-1 at high concentrations can be detected by charcoal binding assays whereas the 17-19 kDa He[39]L binding protein is undetectable.

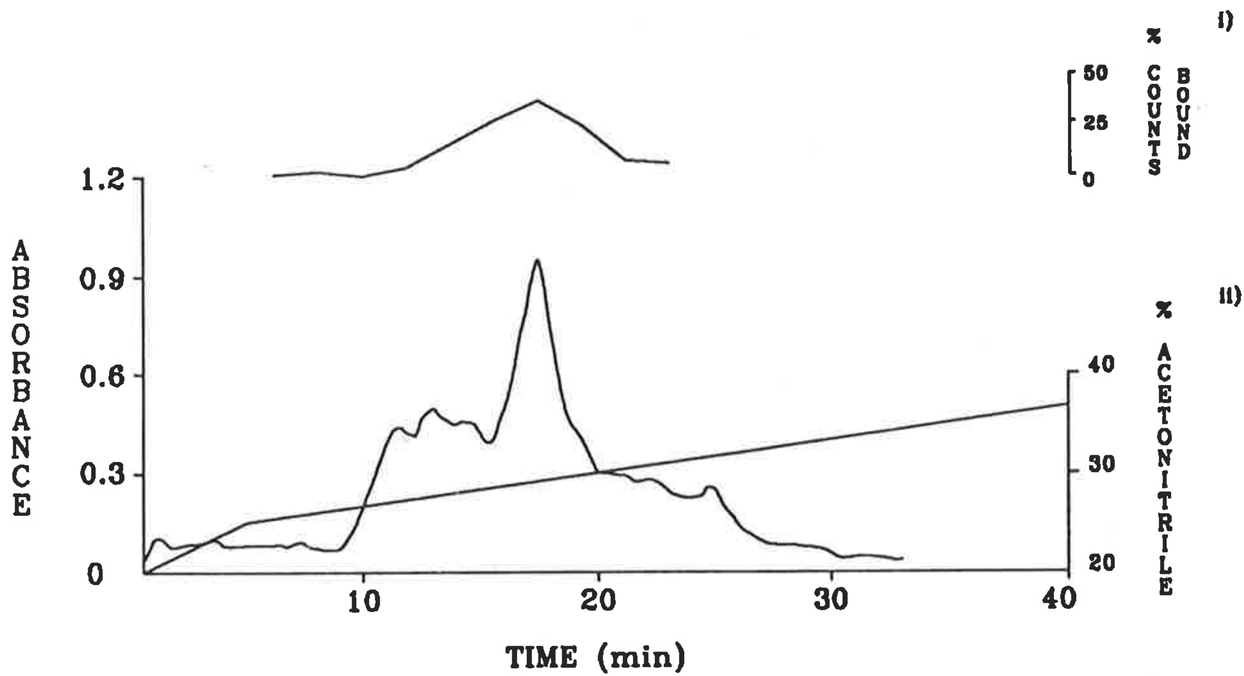
The sequence of a cDNA clone encoding a protein with the same N-terminal amino acid sequence as the 17-19 kDa He[39]L protein has been reported by Kiefer *et. al.*(1991), see Figure 5.15. Without isolating the protein they isolated the cDNA clone from human osteosarcoma cell-derived cDNA using the polymerase chain reaction (PCR) with primers derived from common sequences found for all binding proteins. Shimasaki *et. al.*(1991a) also reported the rat and human sequences of this binding protein. Full length cDNA clones encode a protein with a molecular weight of 28,500. The binding protein has now been classified as IGFBP-5 (Ballard *et. al.*, 1991b).

The cDNA sequences of IGFBP-5 confirm the conservation of the 18 cysteines between IGFBPs 1 to 4 (See Figure 6.6 for full sequence comparison). As seen in previous sequence comparisons the greatest degree of similarity of IGFBP-5 with other binding protein amino acid sequences lies at the amino and carboxy ends of the protein. A non-conserved region lies in the middle of the protein sequence.

The only other information available about this new binding protein is that it has greater affinity for IGF-II than IGF-I (Bautista *et. al.*, 1991). With the availability of the cDNA clone as a probe it will be interesting to learn of the factors influencing the expression of this binding protein. Evidently IGFBP-5 has not been detected in human serum (Kiefer *et. al.*, 1991) but it has been isolated from three cell lines so far, two of which were bone cells. Perhaps this binding protein plays a regulatory role in mediating the IGF action in the extracellular space within various tissues rather than acting as a carrier protein.

Figure 5.1B: Reverse phase HPLC analysis of the 32 kDa He[39]L binding protein

The final step in the purification of the 32 kDa He[39]L binding protein involved reverse phase high-performance liquid chromatography (HPLC). Pooled active fractions from IGF affinity chromatography were applied to a reverse-phase HPLC aquapore butyl cartridge (4.6 mm x 3.0 cm; Applied Biosystems, Santa Clara, CA. USA.) in 0.1% (v/v) trifluoroacetic acid. A 25-45% (v/v) acetonitrile gradient over 40 minute was used to elute the IGFBP.The absorbance at 215nm is shown in (i;). The acetonitrile gradient (0.2 ml/minute) is indicated by the straight line. Active fractions were identified by charcoal binding assays (i:) and activity is expressed as the percentage of the total number of counts of ^{125}I IGF-II (2000 cpm) added to each tube (10µl/fraction assayed).



i)
% COUNTS BOUND
50
25
0
1.2
0.9
0.6
0.3
0
ABSORBANCE
ii)
% ACETONITRILE
40
30
20
10
20
30
40
TIME (min)

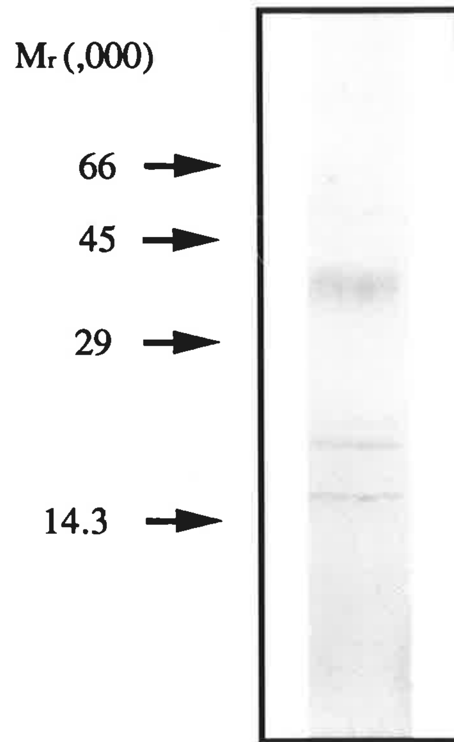


Figure 5.2: He[39]L binding protein molecular weight determination. The HPLC-purified binding protein (200 ng) was separated by 15 % SDS polyacrylamide gel electrophoresis under non-reducing conditions and visualized by silver staining. Arrows indicate migration of molecular weight markers.

He[39]L BP	L A P G P G Q G V Q A G X P G G X V E E E D R G G

hIGF BP-1	A P W Q C A P C S A E K L A L C P P V S - -
rIGF BP-2	E V L F R C P P C T P E R L A A C G P P P V P
hIGF BP-3	G A S S G G L G P V V R C E P C D A R A L A Q C A P P P - -
hIGF BP-4	D E A I H C P P C S E E K L A R C R P P V G -

Figure 5.3: The N-terminal amino acid sequence of the 32 kDa He[39]L binding protein. The first 25 amino acids of the 32 kDa He[39]L binding protein were determined following separation of the protein on a 5-15% linear gradient gel, transfer to polyvinylidene difluoride (PVDF) and direct application of the membrane to the sequencer. The method of electroblotting for sequencing was described by Matsudiara (1987) and is outlined in section 2.3.5.

The sequence of the He[39]L binding protein is compared to the N-terminal sequences of the four binding proteins for which full length cDNA sequences are available. These are hIGFBP-1 (Brinkman et. al., 1988a), hIGFBP-2 (Binkert et. al., 1989; Zapf et. al., 1990a), hIGFBP-3 (Wood et. al., 1988) and hIGFBP-4 (Shimasaki et. al., 1990b; LaTour et. al., 1990). Unidentified residues are shown as crosses and gaps created when sequences are aligned are shown as dashes.

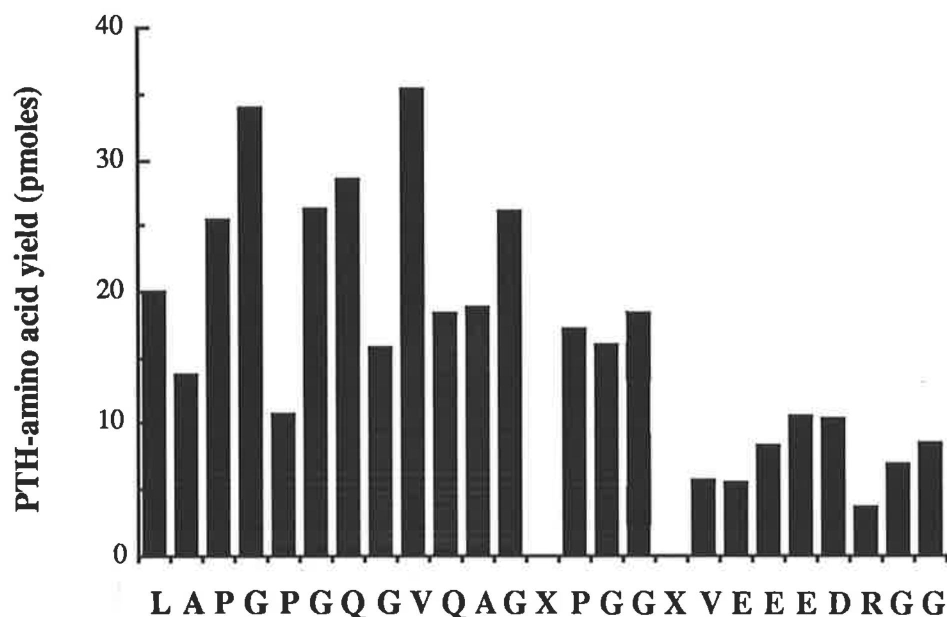
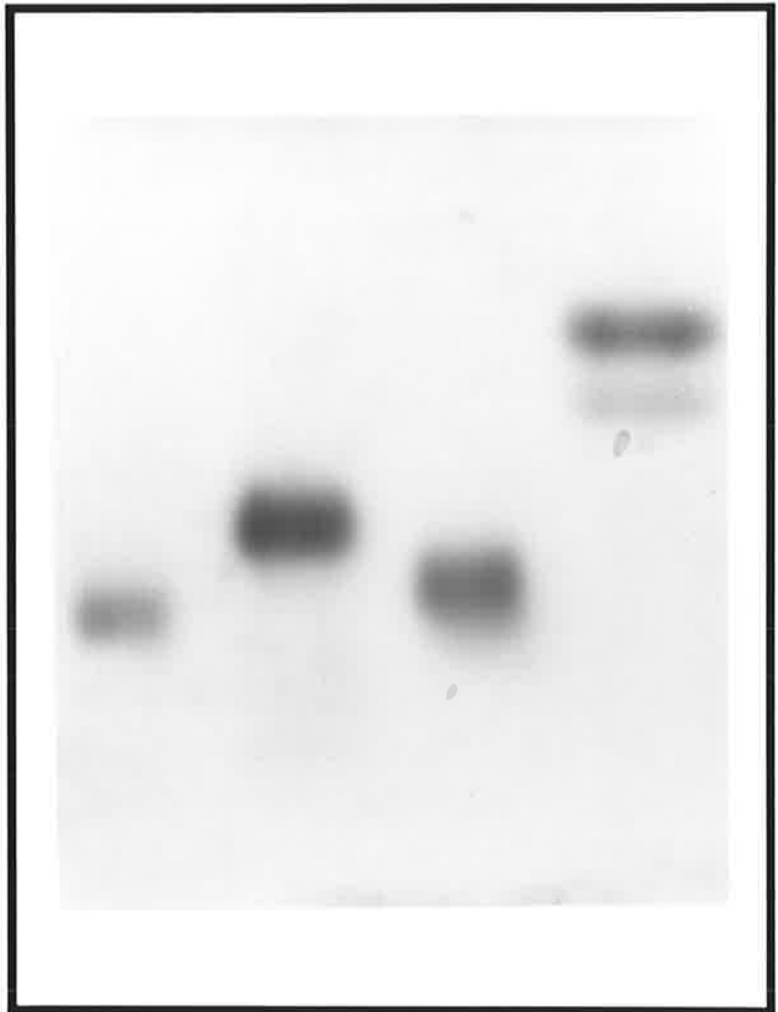


Figure 5.4: Yield of PTH-derivatives from the He[39]L binding protein during N-terminal amino acid sequencing. Following separation of the He[39]L binding protein and electroblotting onto polyvinylidene difluoride (Matsudaira, 1987) the sample was sequenced with an Applied Biosystems gas-phase automated peptide sequencer (Model 470). The PTH-amino acids derived during the yield (pmoles) was quantified by direct comparison with derivatised standards analysed under the same conditions. The sequence of the He[39]L binding protein is shown in the one letter code and the yields for each cycle are expressed as a histogram.

Figure 5.5: Western ligand blot of the purified 32 kDa He[39]L binding protein

Purified binding proteins were separated on 12.5% SDS polyacrylamide gels under non-reducing conditions. Proteins were transferred to nitrocellulose. The filter was probed with ^{125}I IGF-II (10^6 cpm/filter) overnight and exposed to autoradiograph film for 5 days at -80°C. The extent of migration of molecular weight standards is indicated by arrows.

Lanes	
1	hIGFBP-1 (25ng)
2	bIGFBP-2 (25ng)
3	He[39]L BP (25ng)
4	hIGFBP-3 (10ng)


1
2
3
4
Mr (,000)
66
45
29
14.3

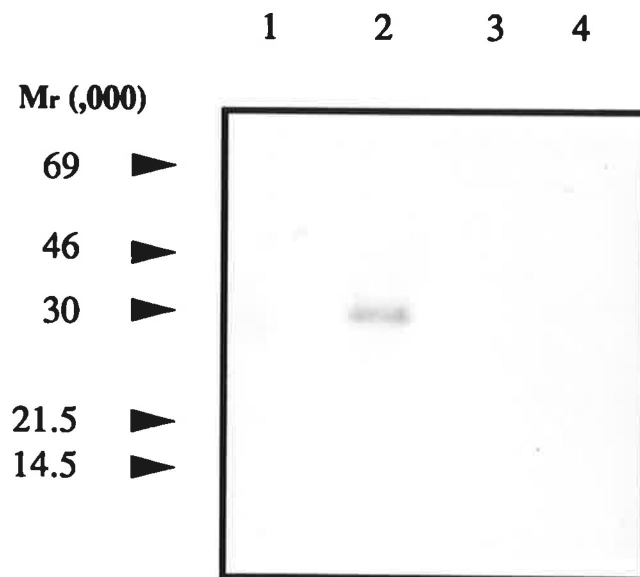


Figure 5.6: Cross-reaction of binding proteins and He[39]L conditioned medium with an antibody against bIGFBP-2.

Proteins were separated on a12.5% SDS polyacrylamide gel and transferred to nitrocellulose. The blot was probed with an antibody raised agaust bIGFBP-2 (1/100, see section 2.2.10) followed by an anti-rabbit biotinylated antibody and avidin-alkaline phosphatase. Specific binding of bIGFBP-2 antibody was visualized by developing with the substrates for avidin alkaline phosphatse. The extent of migration of molecular weight markers is indicated by arrows.

Lanes

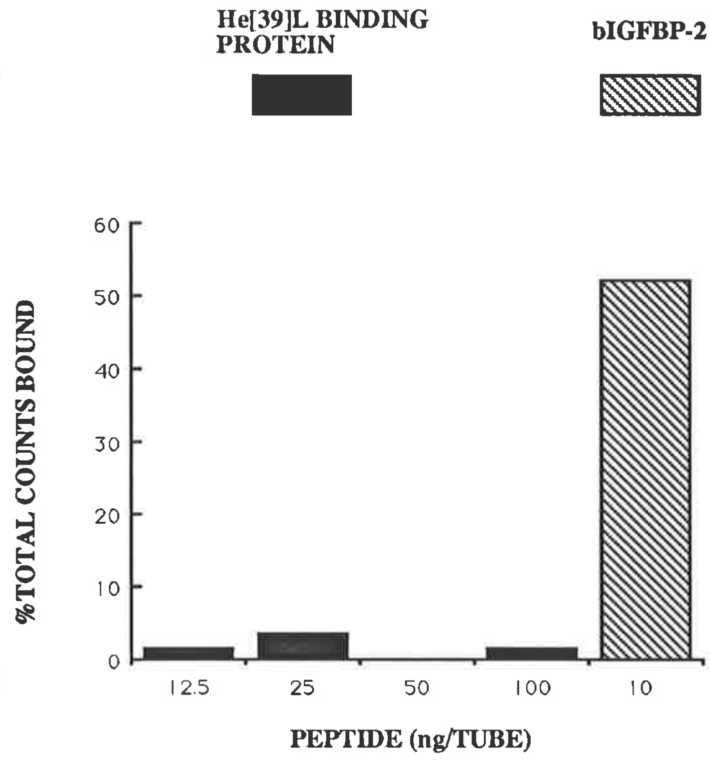
1	Purified hIGFBP-1 (10 ng)
2	Purified bIGFBP-2 (10 ng)
3	Purified hIGFBP-3 (25 ng)
4	He[39]L conditioned medium (800µl equivalent)

Figure 5.7: Immunoprecipitation of the 32 kDa He[39]L binding protein

Binding proteins were incubated with ^{125}I IGF-I for 1 hour and precipitated with antibody overnight at 4°C using the method described in section 2.2.2. Cross-reaction with antibody was detected by counting precipitates in a gamma counter and is expressed as a percentage of the total counts added. Counts nonspecifically precipitated were subtracted (6%).

^{125}I IGF-I-IGFBP complexes were precipitated with an antibody raised against bIGFBP-2 (a) or hIGFBP-1 (b). Precipitation of increasing amounts of He[39]L binding protein was compared with 10ng bIGFBP-2 or 2.5ng hIGFBP-1.

A



B

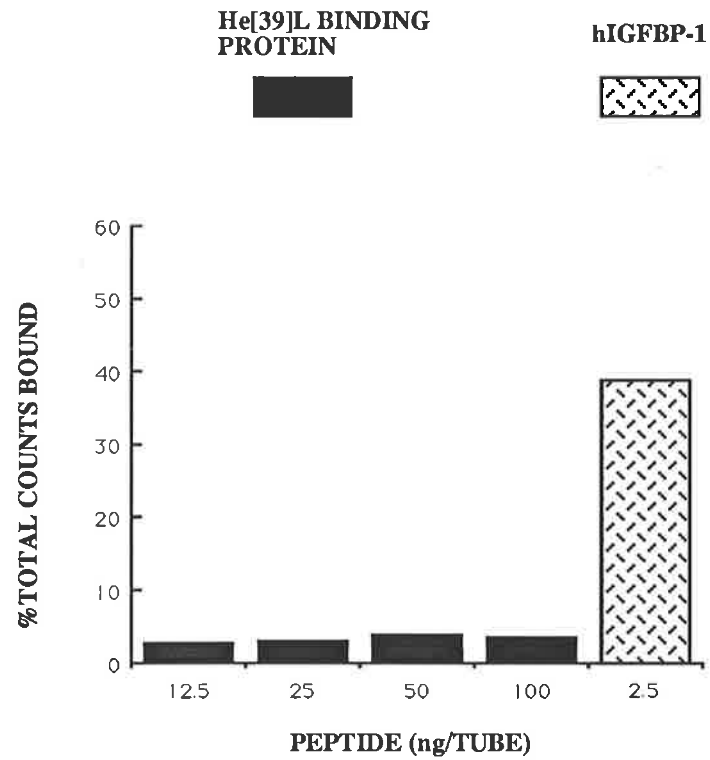


Figure 5.8: Northern analysis of He[39]L RNA using a bIGFBP-2 probe

Total cellular RNA was prepared using a caesium chloride method described in section 2.4. The RNA (10-15µg) was separated on a 1% agarose/6.5% formaldehyde gel and transferred to Nytran. The Northern filter was probed with a ^{32}P nick translated 1092bp bIGFBP-2 cDNA clone (Upton *et al.*, 1990). Both MDBK total RNA (**1**) and He[39]L total RNA (**2**) were analyzed on this filter. The extent of migration of radiolabelled RNA markers is shown by arrows.

1
2
kb
9.49
7.46
4.40
2.37
1.35
0.24

Figure 5.9: Lectin affinity chromatography of the He[39]L binding protein

Wheatgerm agglutinin (a) and concanavalin A (b) were used in the detection of glycoproteins. Each column was washed with starting buffer (section 2.3.3) for 15 minutes (3ml) before loading He[39]L binding protein (500ng). Glycoproteins were eluted with (a) N-acetyl-glucosamine and (b) methyl-α-mannopyranoside for 25 minutes (5ml) and the column was washed again in starting buffer for 20 minutes (4ml). Fractions (1ml) were assayed for binding protein activity using the charcoal binding assay (12μl assayed/fraction). The activity in each fraction shown as a histogram is expressed as a percentage of the total number of counts added.

Human IGFBP-1 (hIGFBP-1), bIGFBP-2 and hIGFBP-3 (500ng each) were also analysed for glycosylation. Active fractions were detected using the charcoal binding assay for bIGFBP-2 and hIGFBP-3 and the antibody/PEG precipitation method for hIGFBP-1 (section 2.2.2). Solid arrows indicate the active fractions from lectin affinity chromatography. Broken arrows indicate fractions with low levels of activity.

WHEAT GERM LECTIN

pIGFBP3

hIGFBP1
bIGFBP2

20
10
0

% COUNTS BOUND

1 2 3 4 5 6 7 8 9 10 11 12

VOLUME (ml)

CONCANAVALIN A

80
60
40
20
0

% COUNTS BOUND

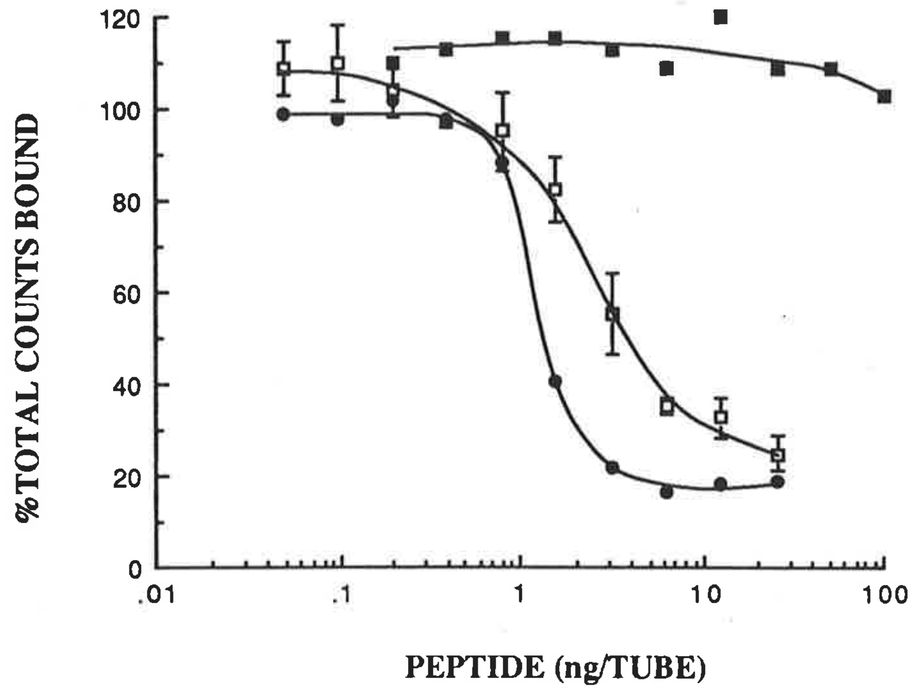
1 2 3 4 5 6 7 8 9 10 11 12

VOLUME (ml)

Figure 5.10: Competition binding assays with the 32 kDa He[39]L binding protein

Competition for binding to the 32 kDa He[39]L binding protein was with rhIGF-I (□), bIGF-II (●) and des-(1-3)-IGF-1 (■). rhIGF-I was a gift from Drs. H. H. Peter and K. Scheibli, Ciba-Geigy, Basle, Switzerland. Synthetic des-(1-3)-IGF-1 was prepared as previously described (Ballard *et al.*, 1987) and bIGF-II was purified by the method of Francis *et al.* (1988b). Increasing amounts of competing ligand were used in the presence of rhIGF-I tracer **A.** or bIGF-II tracer **B.** Competitive binding was expressed as a percentage of the counts bound in the absence of competing ligand (B_0). The nonspecific binding measured in the absence of binding protein or ligand was not subtracted. Competition for IGF-I tracer **A.** was performed with 10ng He[39]L binding protein (B_0 = 41.2%, NSB = 6.8%) and for IGF-II tracer **B.** with 2ng He[39]L binding protein (B_0 = 62.6%, NSB = 13%). Each point on the competition curves represents the mean of duplicate tubes. Standard deviations are shown for rhIGF-I curves and are representative of the error for all curves.

A



B

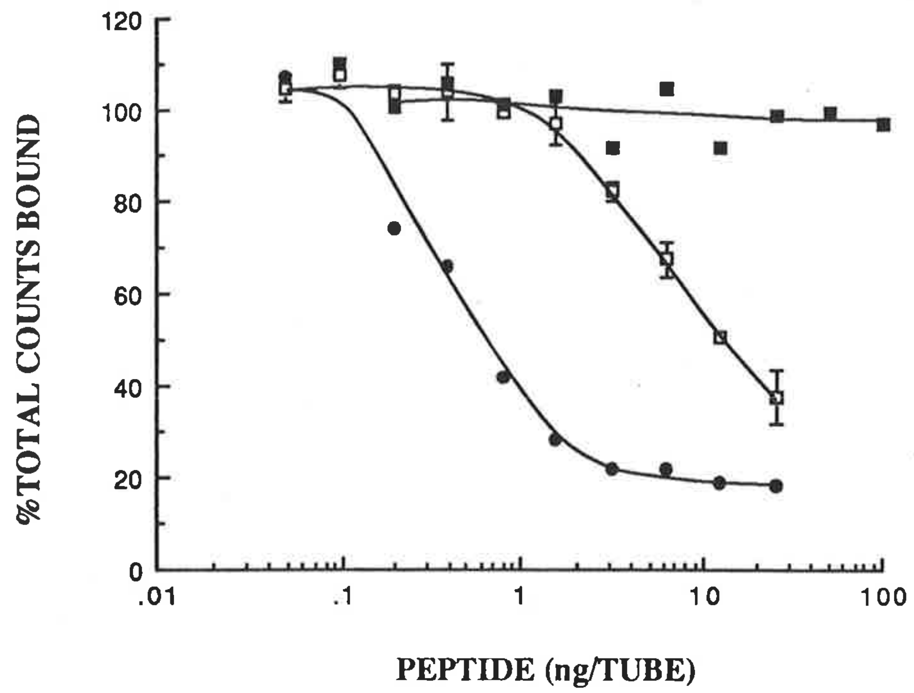
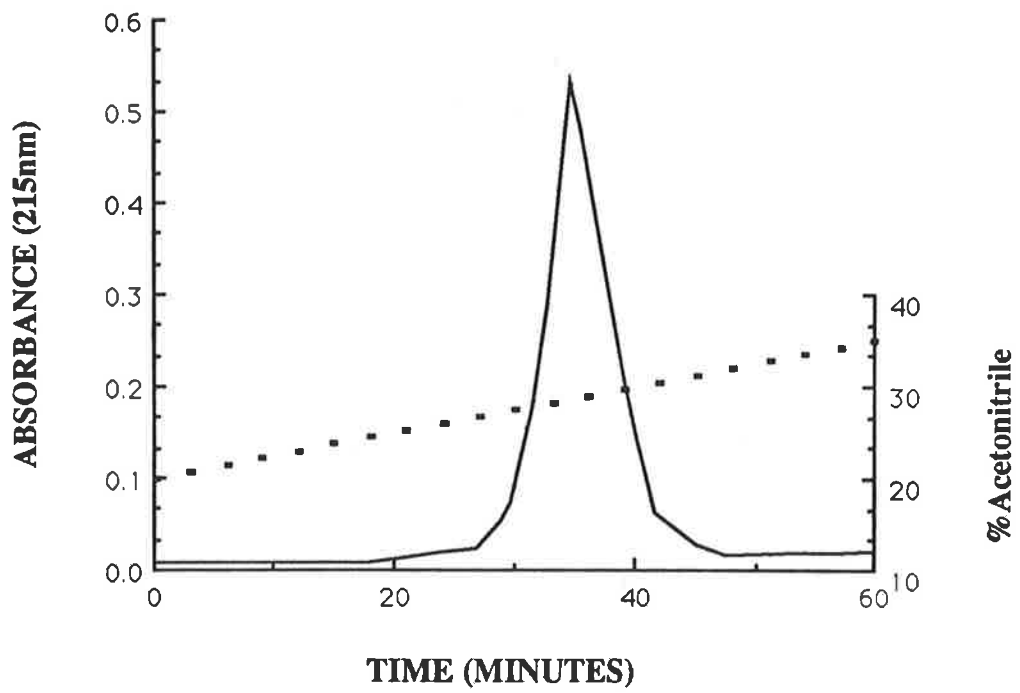


Figure 5.11: Reverse phase HPLC analysis of the 17-19 kDa He[39]L binding protein

The final step in the purification of the 17-19 kDa He[39]L binding protein involved reverse phase high-performance liquid chromatography (HPLC). The absorbance at 215nm is shown in (b). The acetonitrile gradient (0.2 ml/minute) is indicated by the dashed line. Active fractions were identified by interference in radioreceptor assays (a) and activity is expressed as the percentage of counts of ^{125}I IGF-II bound in the absence of added sample (Control=100%, 5μl/fraction assayed).

A
% CONTROL
100
80
60
40
20
0


B
ABSORBANCE (215nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
40
30
20
10
%Acetonitrile
TIME (MINUTES)

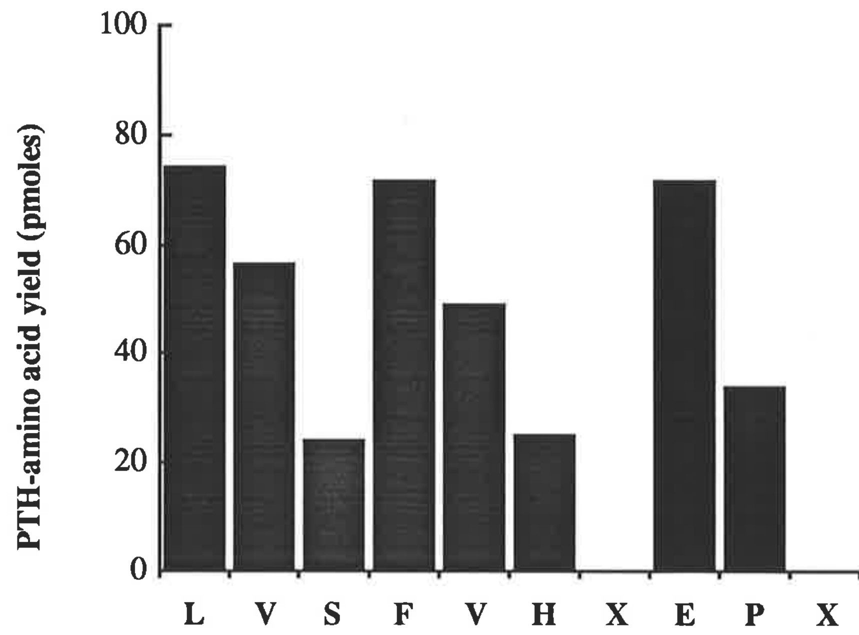
```
He[39]L BP                    L V S F V H X E P X D E K

hIGF BP-1                         A P W Q C A P C S A E K L A L C P P V S - -
hIGF BP-2                         E V L F R C P P C T P E R L A A C G P P P V P
hIGF BP-3           G A S S G G L G P V V R C E P C D A R A L A Q C A P P P - -
hIGF BP-4                         D E A I H C P P C S E E K L A R C R P P V G -
```

Figure 5.12: The N-terminal amino acid sequence of the 17-19 kDa He[39]L binding protein. The first 13 amino acids of the 17-19 kDa He[39]L binding protein were determined by automated Edman degradation. The sequence of the He[39]L binding protein is compared to the N-terminal sequences of the four binding proteins for which full length cDNA sequences are available. These are hIGFBP-1 (Brinkman et. al., 1988), hIGFBP-2 (Binkert et. al., 1989; Zapf et. al., 1990a), hIGFBP-3 (Wood et. al., 1988) and hIGFBP-4 (Shimasaki et. al., 1990b; LaTour et. al., 1990). Unidentified residues are shown as crosses and gaps created when sequences are aligned are shown as dashes.

Figure 5.13: Yield of PTH-derivatives from the 17-19 He[39]L binding protein during N-terminal amino acid sequencing

The He[39]L binding protein sample was directly applied to an Applied Biosystems gas-phase automated peptide sequencer (Model 470). The PTH-amino acids derived during the yield of each cycle of Edman degradation were analysed by rpHPLC. The yield (pmoles) was quantified by direct comparison with derivatised standards analysed under the same conditions. The sequence of the He[39]L binding protein is shown in the one letter code and the yields for each cycle are expressed as a histogram. Two related sequences were derived from Edman degration. The slightly more abundant sequence (a) has 3 additional amino acids at the N-terminal end when compared with the second sequence (b).


A
PTH-amino acid yield (pmoles)
100
80
60
40
20
0
L
V
S
F
V
H
X
E
P
X

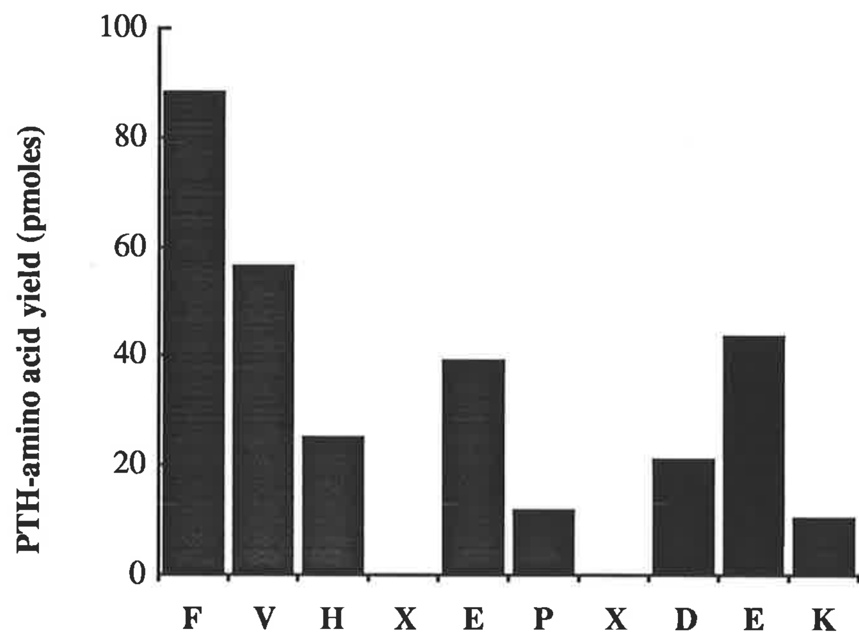

B
PTH-amino acid yield (pmoles)
100
80
60
40
20
0
F
V
H
X
E
P
X
D
E
K

Figure 5.14: Silverstaining of the 17-19 kDa He[39]L binding protein

The rpHPLC purified He[39]L binding protein was separated on a 15% SDS-polyacrylamide gel under non-reducing conditions. Protein was visualized using the silverstaining technique described by Heueshoven and Dernick (1985) and also outlined in section 2.2.10. The extent of migration of molecular weight standards is indicated by arrows.

A
B
Mr (,000)
66
45
29
14.3

Figure 5.15: N-terminal sequence comparison of He[39]L binding proteins with other isolates of the same IGFBPs

1. The 32 kDa He[39]L binding protein N-terminal sequence is compared with sequences of other isolates of the same protein. Boxes indicate positions with sequence differences. The amino acid sequence derived from the cDNA clone corresponding to these proteins is shown below the sequences of different isolates. Sequences are from the following references:

CSF BPs	Roghani *et. al.*, 1989
TFBP	Martin *et. al.*, 1990 (transformed fibroblast binding protein)
U-2 osteoblast cell BP	Andress and Birnbaum, 1991
Human serum BP	Zapf *et. al.*, 1990a
Rat serum	Shimasaki *et. al.*, 1991b
Porcine follicular fluid	Shimasaki *et. al.*, 1991b
Human IGFBP-6	Shimasaki *et. al.*, 1991a

2. The 17-19 kDa He[39]L binding protein N-terminal sequence is compared with sequences of other isolates and the amino acid sequence derived from a cDNA clone encoding the same proteins. Sequences are from the following references:

U-2 osteoblast cell BP	Andress and Birnbaum, 1991
hBP-IGF BP	Bautista et. al., 1991 (human bone derived IGF BP)
Rat serum	Shimasaki *et. al.*, 1991b
Porcine follicular fluid	Shimasaki *et. al.*, 1991b
Human IGFBP-5	Shimasaki *et. al.*, 1991a

1. N-terminal Sequence of the 32 kDa He[39]L BP and Related Proteins

Protein	Sequence
He[39]L BP	L A P G P G Q G V Q A G X P G G X V E E E D A G G
CSF BP (CHILD)	L A P G X G Q G V Q A G A P G
CSF BP (ADULT)	X X P G K G Q G V Q A L L P G
Tex Ag BP	A A P G C G Q G V Q A G
U-2 Osteoblast cell BP	X X P G P G Q G V Q A G X P G
	R H
Human Serum	L A P G L G Q G V Q A G C P G G C V E E E D G G S P A Q X G
	A
Rat serum	A L A G X P G X G P G V Q
Porcine follicular fluid	A Q X P G X G Q G V Q T G X P G G X A E E E D G G
Human IGFBP-6 (from cDNA sequence)	A L A A C P G C G Q G V Q A G C P G G C V E E E D G G

2. N-Terminal Sequence of the 17-19 kDa He[39]L BP and Related Proteins

Protein	Sequence
17-19 kDa He[39]L BP	L V S F V H X E P X D E K
U-2 Osteosarcoma cell BP	F V H C E P C D E K A L S M X P P S X L
hBD-IGF BP	L G F F V X V E P D D K A A L
Rat serum	L G S F V H X E P X D E K A L S M X P P S P L G X E L V K E P X G
Porcine follicular fluid	L G S F V H X E P X D E K A L S M X P P
Human IGFBP-5 (from cDNA sequence)	L G S F V H C E P C D E K A L S M C P P S P L G C E L V K E P C G

CHAPTER 6

CLONING OF THE 32 kDa He[39]L IGFBP

6.1 INTRODUCTION

The cDNA clones encoding IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-4 have been extensively characterized (see Table 1.2 for references). Clones encoding hIGFBP-1 were the first to be described and they are approximately 1.5-1.6 kb encoding a 259 amino acid peptide, with a 25 amino acid leader peptide. The leader peptide directs secretion of the binding protein and is cleaved from the mature protein. Subsequent analysis of IGFBP cDNA clones has revealed a similar clone length as seen for hIGFBP-1. Bovine, rat and human IGFBP-2 cDNA clones are approximately 1.4 kb and encode 284, 270 and 289 amino acid peptides with leader peptides of 26 to 39 residues (Upton *et. al.*, 1990; Brown *et. al.*, 1989; Margot *et. al.*, 1989; Binkert *et. al.*, 1989).

While the major serum IGF carrier protein, IGFBP-3, has been cloned from human, rat and porcine sources (see Table 1.2 for references), the acid stable subunit of the large molecular weight complex has not been cloned. The IGFBP-3 clones encode peptides much smaller (28.7kDa) than predicted by SDS polyacrylamide gel electrophoresis (38-47 kDa), the difference in size due to glycosylation. The leader sequence for hIGFBP-3 is 27 residues long.

By using specific molecular probes a great deal has been learnt about the levels of expression, tissue distribution and control of expression of mRNA encoded by IGFBP genes. Northern analysis of rat tissues has demonstrated higher levels of expression of IGFBP-2 in fetal tissues than in adult tissues, particularly in the liver (Margot *et. al.*, 1989). In addition, IGFBP-1 is expressed in higher levels in fetal human liver than adult human liver (Brinkman *et. al.*, 1988a) whereas IGFBP-3 is found predominantly in adult liver but is also present in most other adult tissues (Shimasaki *et. al.*, 1989).

The same probes have been used to specifically localize which cells within certain tissues produce particular binding proteins. Using *in situ* hybridization with cDNA probes the site of IGFBP-1 production in the endometrium has been localized (Julkunen *et. al.*, 1990; Croze *et. al.*, 1990). Interestingly, these studies have revealed separate sites of production of IGFBP-1 and IGF-I, with IGFBP-1 localized in stromal cells and IGF-I in the smooth muscle cell layer (Croze *et. al.*, 1990). The way in which IGFBP-1 acts to control

IGF-I action to allow proliferation and differentiation of the endometrium in the uterus is not understood, but it may involve the inhibitory action of binding proteins or the ability to sequester IGF-I to its site of action.

cDNA probes have not only allowed the localization of the sites of binding protein production but have also provided a precise means of studying the control of binding protein expression. In studies similar to those outlined in Chapter 4, hormones and growth factors have been added to cell cultures and the levels of mRNA determined in response to these factors. Estrogen and progesterone influence the levels of binding protein mRNA in endometrial stromal cells (Guidice *et. al.*, 1991). Coincubation of rat hepatocytes with insulin leads to a decrease in rat IGFBP-2 (rIGFBP-2) mRNA (Böni-Schnetzler *et. al.*, 1990). In animal models tissue RNA has been probed to measure the response to various factors. Both rIGFBP-1 and rIGFBP-2 hepatic mRNA levels are decreased in response to the presence of growth hormone (GH) in the hypophysectomized or fasted rat, respectively (Glassock *et. al.*, 1990; Seneviratne *et. al.*, 1990; Murphy *et. al.*, 1990). Such studies complement the earlier available methods of measuring binding protein levels and add greatly to our understanding of the regulation of binding protein production.

Undoubtedly the availability of specific probes for the 32 kDa He[39]L binding protein would be significant in determining the distribution and regulation of expression of this binding protein as well as for measuring specific He[39]L binding protein levels. Ultimately such information would aid in understanding the process of regulation of IGF action by binding proteins. This chapter outlines the approaches taken in the attempt to obtain a specific probe for the 32 kDa He[39]L binding protein.

The production of a specific antibody for the He[39]L binding protein would provide such a useful probe. However, as described in Chapter 5, the levels of binding protein purified from the conditioned medium of He[39]L cells (approximately 60μg from 15 litres of starting material) have not been sufficient for the immunization procedures required for antibody production.

Stimulation experiments outlined in Chapter 4 demonstrated increased production of IGFBP-3 in response to IGFs by He[39]L cells but failed to identify any factors which

could specifically stimulate the production of the 32 kDa He[39]L binding protein. I concluded that He[39]L cells were not a suitable source of large enough amounts of He[39]L binding protein for the production of a specific antibody. Therefore, I turned to the isolation of a cDNA clone encoding the He[39]L binding protein in an attempt to produce a specific probe.

Two strategies were undertaken to isolate a He[39]L binding protein cDNA clone. The first approach taken was the conventional method of library screening with oligonucleotides. The cDNA library chosen for screening was made from RNA of the IMR 90 human embryonic lung fibroblast cell line. I chose this library for several reasons. Firstly, both cell lines are of a similar origin and are therefore likely to produce similar binding proteins. In fact Western ligand blotting revealed they do produce binding proteins of similar sizes (see section 4.2.2). In addition, the SV 40 transformed IMR 90 cell line (Ag 2804) secretes a binding protein with an almost identical N-terminal amino acid sequence as the He[39]L binding protein (Martin *et. al.*, 1990). In section 4.2.1 I reported that the IMR 90 cells have a different competition binding pattern to other fibroblasts tested. However, this does not mean that the cells are not producing the He[39]L binding protein. Therefore, the information available suggests that this cell line is likely to produce a binding protein similar to the He[39]L binding protein.

A number of the N-terminal amino acids of the He[39]L binding protein sequence could be encoded by several alternative codons. The level of degeneracy in sequences encoding the He[39]L amino acids caused significant problems in the design of specific oligonucleotides and this will be discussed in section 6.2. Although successful screening of libraries with degenerate oligonucleotides has been reported (Sambrook *et. al.*, 1989) this procedure is certainly more difficult than screening with longer, homologous probes. Hence the need for an alternative, parallel approach to the isolation of He[39]L binding protein cDNA clones.

The alternative strategy was to use the polymerase chain reaction (PCR) in an attempt to amplify He[39]L cDNA sequences from cDNA synthesized from He[39]L cellular RNA. Having amplified a long, homologous He[39]L sequence it would then be used in screening the cDNA library. PCR involves the amplification of a particular sequence by a heat stable

DNA polymerase isolated from the thermophilic bacterial species *Thermus aquaticus* (*Taq*; Saiki *et. al.*, 1985; Mullis and Faloona, 1987) and is schematically described in Figure 6.1. Two oligonucleotides able to hybridize to opposite strands of that sequence act as primers for the DNA polymerase. By repeated cycles of denaturation, annealing and elongation at appropriate temperatures a specific sequence can be amplified 2^n times, where n equals the number of cycles completed. At the commencement of this work the use of PCR was a novel approach not yet used in the isolation of binding protein clones.

Having already designed an oligonucleotide specific to the N-terminal amino acid sequence I had to design a second oligonucleotide for use in PCR and which would be specific for the He[39]L sequence. As purification yields from large volumes of He[39]L conditioned medium were relatively low and the He[39]L binding protein was particularly unstable (see Chapter 5) I chose not to isolate internal peptides for use in design of the second oligonucleotide. Therefore, it was necessary to make some important assumptions when designing the second oligonucleotide for PCR. The most striking feature of IGFBP cDNA clones sequenced at the time (IGFBPs 1, 2, 3 and 4 from several species) was the conservation of 18 cysteines throughout. Human IGFBP-4 has two additional cysteines in the middle of the cDNA sequence and possibly one at the C-terminus. When comparing all IGFBP cDNA sequences, the central region contains the least conserved sequences whereas the N- and C-terminal ends show striking homology between all IGFBPs (see Figure 1.1 for sequence comparison of the human IGFBPs 1, 2, 3 and 4). From this information it seemed reasonable to assume that the 18 cysteines found in all the IGFBP sequences play a role in the formation of a common IGF-binding structure and would therefore be conserved in newly isolated binding protein sequences.

The N-terminal He[39]L sequence indicated a possible difference to other binding proteins. The sequence contained unassigned residues which were potentially cysteines at positions 13 and 17. These could not be aligned with the N-terminal cysteines of known binding protein sequences. This suggested an extension in the N-terminal region of the He[39]L binding protein, possibly containing two extra cysteines. Despite the different N-terminal region, the He[39]L binding protein had a similar IGF-binding pattern to bIGFBP-2 (section 5.3) which inferred the two IGFBPs have a similar binding structure and suggested the 18 cysteines found in previously sequenced binding proteins were maintained

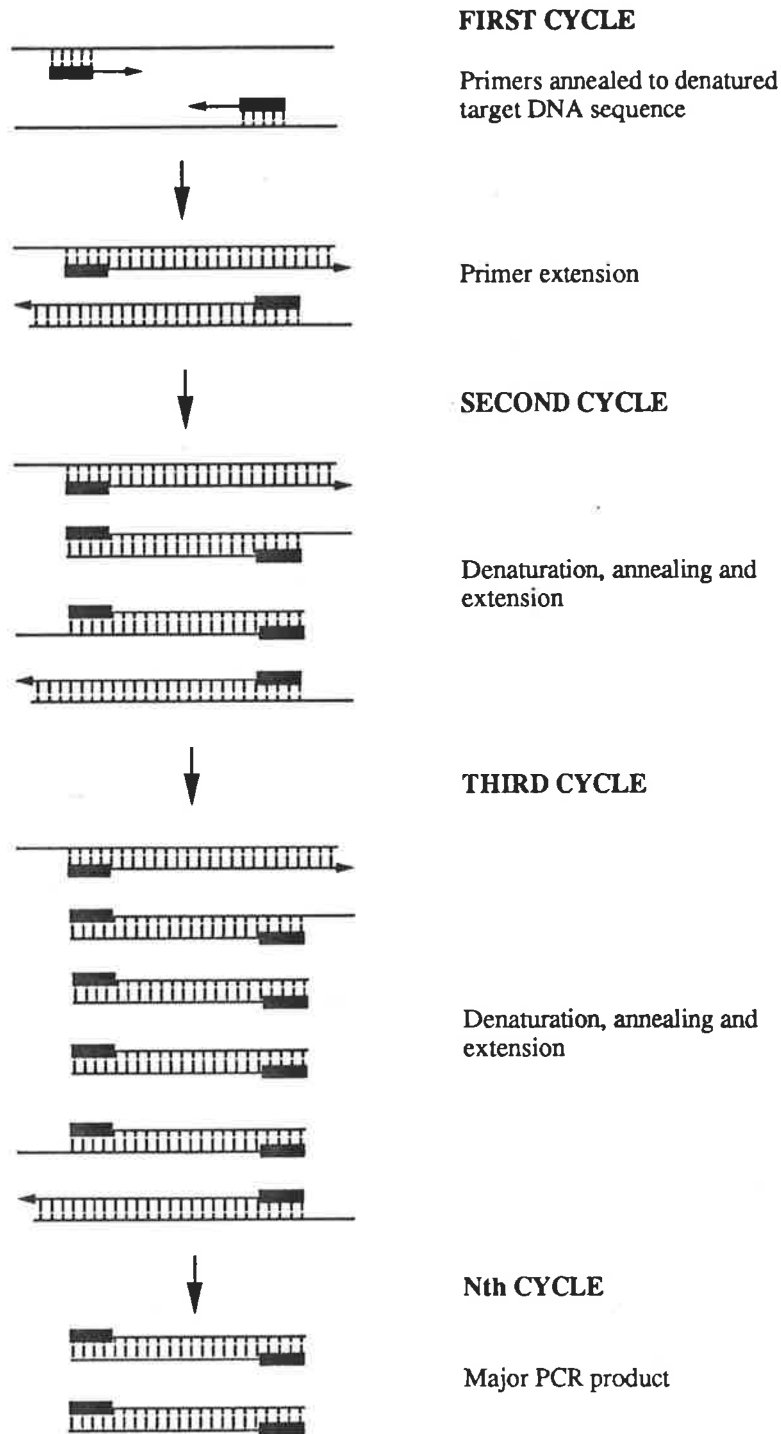


Figure 6.1: Diagramatic representation of the polymerase chain reaction (PCR). Specific primers (▬) are annealed to denatured template DNA and *Taq* polymerase synthesizes sequences in a 5' to 3' direction, complementary to the template. In subsequent cycles products already generated can act as template for further amplification. Therefore, in N cycles 2^N products will be amplified by this procedure. Double stranded DNA is represented as IIIIIII.

by the He[39]L binding protein sequence. On this assumption the second oligonucleotide for PCR reactions was designed using binding protein sequences encoding cysteines and other conserved residues.

Having designed the second binding protein specific oligonucleotide the aim was to combine this primer with the oligonucleotide encoding the least degenerate region of the He[39]L sequence to amplify a specific probe for screening the IMR 90 cDNA library.

The aim of this chapter, therefore, was to isolate a cDNA clone encoding the He[39]L binding protein using two approaches. Having such a probe would allow the analysis of tissues for the abundance and specific localization of the binding protein as well as providing a tool for examining factors influencing the expression of the He[39]L binding protein.

6.2 RESULTS AND DISCUSSION

Although both the PCR and direct library screening approaches were carried out simultaneously I will describe the different series of experiments separately. I will discuss the PCR experiments first.

6.2.1 Original Polymerase Chain Reaction (PCR) Approach

There are many variables in the conditions of a PCR reaction which can be altered to achieve optimal conditions. In the following section I will describe the basic approach undertaken to systematically test various reaction conditions with the aim of finding optimal amplification conditions. I will describe the first reaction in detail as an example of the design process of further reactions, since each PCR amplification of a particular sequence needs to be optimized separately.

Generally, in designing a new PCR reaction amounts of template and concentrations of the other components were chosen from examples in the literature. Innis and Gelfand, (1990) present a set of useful guidelines for setting up PCR reactions. The following Table outlines the PCR reaction conditions used in the original PCR reaction. The basic reaction is also described in section 2.4.1. Concentrations of reaction components adhere to the

recommendations of the manufacturer of the *Taq* polymerase (Bresatech Ltd., Adelaide, Australia).

REACTION COMPONENTS	**ORIGINAL REACTION** (concentrations/100µl)
Template	IMR 90 cDNA library: 1µl or Human genomic DNA: 100ng
Primer concentrations	1.5-2µM each
dNTP concentrations	200µM
Tris-HCl pH 8.8	67mM
$MgCl_2$	2mM
Ammonium sulphate	16.6mM
Triton X-100	0.45%
gelatin	200µg/ml
Taq polymerase	5units

REACTION CONDITIONS	
1. Temperatures	
Denaturation	94 °C
Annealing	37 °C
Extension	72 °C
2. Times	
Denaturation	1 minute
Annealing	2 minutes
Extension	1 minutes

Template DNA was denatured at 94 °C for 5 minutes prior to amplification.

The first primer designed to the N-terminal amino acid sequence of the He[39]L binding protein is shown in Figure 6.2 (P23). The oligonucleotide of 23 bases was actually a mixture of all possible sequences encoding the amino acids 15 to 22. There were 1024 different sequences represented in this mixture. Residues 15 to 22 were chosen as the most suitable when designing this oligonucleotide as they represented the least degenerate stretch of sequence (see Figure 6.2). This primer is the equivalent sequence to mRNA i.e. represents the coding sequence.

A second primer (P15), complementrary to the coding sequence, was designed from all binding protein sequences available at the time (see Figure 6.3). These included human IGFBP-1 (hIGFBP-1), hIGFBP-2, bovine IGFBP-2 (bIGFBP-2), rIGFBP-2 and hIGFBP-3. The position of the sequence from which the primer was derived is marked in Figure 6.4, which shows the entire protein sequence of bIGFBP-2. The 15 bases encoding these 5 amino acids are highly conserved throughout all 5 binding protein sequences and the oligonucleotide had only four degeneracies. A schematic representation of the PCR reaction involving P23 and P15 is presented in Figure 6.5.

For the P23/P15 reaction I chose to use human genomic DNA or IMR 90 cDNA library as template DNA. The IMR 90 library was chosen for conventional oligonucleotide screening for reasons described in section 6.1. It therefore was an obvious choice also for a template in this PCR. However, it was also possible that the library would not contain clones encoding the He[39]L binding protein. In case of this eventuality I chose to use genomic DNA as a template in parallel experiments. At the time of designing this reaction the hIGFBP-3 gene had been sequenced. No introns were present in the first 404 bp of the coding region for hIGFBP-3 (includes the leader peptide). On the basis of this information and also knowing that previously sequenced binding protein cDNA clones showed remarkable homology in this region, I thought it unlikely that the He[39]L sequence would have an intron within the first 200 nucleotides encoding the N-terminal amino acids.

PCR products are generally identified on the basis of expected size. On comparison with known binding protein sequences I estimated that the product resulting from PCR with P15 and P23 would be in the order of 100 to 200 base pairs, taking into account the possibility that the He[39]L binding protein may have an N-terminal extension (see section 6.1). PCR with P15 and primers encoding the first N-terminal amino acids of the IGFBPs sequenced at the time would result in products with the following sizes:

IGFBP	base pairs
hIGFBP-1	105
hIGFBP-2	153
bIGFBP-2	138
rIGFBP-2	111
hIGFBP-3	129

Another more accurate means of identifying a PCR product is to probe with an oligonucleotide derived from sequence between the two PCR primers. In this case I did not have any internal sequence data or suitable sequences common to all binding proteins to make a probe against.

The annealing temperature (Tm) of the most A/T rich sequence in the P23 mixture is 61°C, based on the formula Tm = 2°C (A/T) + 4°C (G/C) -5 °C (Miyada and Wallace, 1987). The lowest annealing temperature of the binding protein specific P15 is 45°C. However, my strategy was to start with conditions which would definitely allow amplification of the desired product even if some nonspecific products resulted from the low stringency conditions. Once a product of the expected size was visualized then reaction conditions could be optimized to remove any unwanted products.

A length of 200 bases would under optimal conditions be synthesized by *Taq* polymerase in several seconds. Innis *et. al.* (1988) have reported an extension rate of >60 nucleotides per second at 70°C. However in practise longer extension times are chosen (Innis and Gelfand, 1990).

Under these conditions I failed to obtain any PCR products in the size range expected, although the buffer, dNTPs, *Taq* polymerase and conditions were suitable for amplification of other DNA and primers used as a control (see section 6.2.2).

Even after sequential variation of the components, as specifically indicated in the table following, no products were evident. For each template DNA (genomic DNA or IMR 90 library cDNA) the other components were systematically varied in separate reactions. The primer concentration was raised in one reaction in case the concentration of the correct sequence in the degenerate primer was limiting. Two different concentrations of library DNA were used in case the abundance of clones was too low for amplification at the lower concentration. Also several annealing temperatures up to the Tm for P15 (45°C) were tested. None of these variations lead to the amplification of a PCR product.

REACTION COMPONENTS

Template	IMR 90 cDNA library: 1μl or 5μl
Primer concentrations	1.5-2μM each or 3-4μM each

REACTION CONDITIONS

1. Temperatures	
Denaturation	94 °C
Annealing	37 °C or 42 °C or 45 °C
Extension	72 °C
2. Times	
Denaturation	1 minute
Annealing	30 seconds or 1minute
Extension	1 minute or 2 minutes

The reason for this result was not evident at the time. ^{32}P labelled P15 had been used as a probe in Southern analysis of bIGFBP-2 cDNA and it specifically detected the binding protein DNA (data not shown). However, I was unable to amplify an He[39]L binding protein sequence. I decided that perhaps the 15mer and 24mer oligonucleotides were too short for specific annealing when such degeneracies were involved. Two new oligonucleotides were designed to overcome the problems of length and degeneracy. Also, another PCR was designed using bIGFBP-2 sequences as a model system for the He[39]L PCR (see section 6.2.2).

I was to discover later in the process of writing this thesis that neither the size of the binding protein specific P15 primer nor the degeneracy of P23 were in fact the cause of the problems with the above reaction. I was provided with a preprint of the complete cDNA sequence of hIGFBP-6 (Shimasaki *et. al.*, 1991a) which encodes a binding protein with the identical N-terminal amino acid sequence to the He[39]L binding protein. The cDNA sequence revealed that, unlike any other binding proteins sequenced so far, hIGFBP-6 has only 16 cysteine residues. The two missing cysteines are found at the end of the Gly Cys Gly Cys Cys motif chosen as the conserved sequence encoded by P15 (see Figure 6.6 for a comparison of the hIGFBP-6 protein sequence with other human IGFBP sequences). This explains why the P23/P15 PCR would not produce the expected product. Shimasaki *et. al.* (1991a) had also been unsuccessful with a PCR using oligonucleotides encoding the same

sequence as P15 and the N-terminal hIGFBP-6 sequence. The implications of the lack of the two cysteines in hIGFBP-6 will be discussed later in this chapter.

6.2.2 PCR of bIGFBP-2 sequences

Without the knowledge of the hIGFBP-6 sequence I continued to presume the problems with the first He[39]L PCR lay in the degeneracy and length of the primers. I decided to set up a model bIGFBP-2 PCR system to optimize the conditions for the amplification of binding protein sequences. A bIGFBP-2 cDNA clone was previously isolated in our laboratory (Upton *et. al.*, 1990) and was used as the template for the model reaction.

Availability of the bIGFBP-2 cDNA sequence made design of the primers easy. The primers used for the amplification of bIGFBP-2 sequences are shown in Figure 6.3 (P30) and 6.4 (P24). P30 was complementary to mRNA and was directed to a sequence common to all binding protein sequences available at the time. It was a mixture of 32 oligonucleotides encoding the same sequence. This area of common sequence was significantly longer than the P15 sequence. It also allowed for a longer product to be amplified which would assist in the ultimate aim of deducing the cDNA sequence of the He[39]L binding protein. This PCR would amplify sequence in the centre of the He[39]L binding protein, a region normally not conserved between different binding proteins (see Figure 6.6). An Eco RI restriction endonuclease site was included at the 5' end to allow easy cloning of PCR products.

The second primer for the bIGFBP-2 PCR (P24) encoded the first 8 amino acids of the bIGFBP-2 sequence. It was a homologous primer and was the same sequence as mRNA (Figure 6.4). The product I expected from this reaction using the full length bIGFBP-2 cDNA clone as a template (Upton *et. al.*, 1990) was 672 bp. Figure 6.5b represents the bIGFBP-2 PCR.

I chose to amplify the bIGFBP-2 sequence at 55°C as the Tm's of P30 and P24 were 55°C and 79°C, respectively. The reaction conditions were basically the same as described above except the reaction was carried out in a smaller volume (20µl). A product of the correct size was amplified under these conditions (see Figure 6.7). The product was digested with Sma I and Not I restriction endonucleases to give the sizes of digestion fragments

expected from the known bIGFBP-2 cDNA sequence. This was convincing evidence that the product was in fact the desired bIGFBP-2 sequence.

6.2.3 The second He[39]L PCR reaction

Having established the conditions for the amplification of a binding protein sequence I could apply these conditions to the amplification of the He[39]L sequence. In this way I could at least be sure that the P30 was a suitable primer for amplification of binding protein sequences. In addition another He[39]L specific primer was designed. This primer (P29) encoded the amino acids 13 to 22 and was complementary to the noncoding strand (Figure 6.2). This time codons having 3 or 4 alternatives for bases in the third position were substituted with inosine in these positions. Inosine is a neutral nucleotide and can form base pairs with all dNTPs. In this way some of the problems of using a degenerate primer are eliminated (Patil and Dekker, 1989). Only one oligonucleotide was synthesized rather than a mixture. Therefore the primer concentration should not be a limiting factor. Also, the base pairing of inosine with each conventional base is of equal strength allowing more stringent hybridization conditions to be used. The addition of inosines in this sequence made this primer also appropriate for screening the IMR 90 cDNA library as described later in section 6.3.

As P30 lies 3' of two intron/exon boundaries of the hIGFBP-3 gene there was a possibility that an intron might also be present in similar locations in the gene encoding the He[39]L binding protein. Therefore, it was decided that genomic DNA was inappropriate for the amplification of He[39]L sequences using P29 and P30. Instead, cDNA synthesized from He[39]L total cellular RNA was chosen as the template DNA for the P29/P30 He[39]L PCR. The synthesis of cDNA from RNA is outlined in section 2.4.1.

Using the information available from the binding protein cDNA sequences I predicted the He[39]L PCR product with P29 and P30 would be between 500 and 700bp. The predicted length of products resulting if similar PCRs were performed with P30 and

primers encoding N-terminal amino acids of the binding proteins sequenced at the time are as follows:

IGFBP	base pairs
hIGFBP-1	553
hIGFBP-2	687
bIGFBP-2	672
rIGFBP-2	629
hIGFBP-3	650

A diagram of the P29/P30 PCR is shown in Figure 6.5c.

Like the bIGFBP-2 specific primer (P24), the He[39]L specific primer had a Tm of 79°C. Since the Tm's of the primers in both reactions were identical I could use the same reaction conditions for the He[39]L PCR as used for the amplification of bIGFBP-2 sequences, particularly as the length of the He[39]L product was expected to be similar to the bIGFBP-2 PCR product.

I was unsuccessful in the He[39]L PCR in amplifying any products using identical conditions as used for the bIGFBP-2 PCR. Even when the amounts of cDNA template (2, 5 and 10μl of a 20μl cDNA reaction) were varied, products were not formed. In an attempt to encourage the formation of PCR products three rounds of annealing at 37°C preceded the usual 30 cycles of 55°C. However, no products were formed under these conditions either.

An important control reaction was included in these experiments. Using the cDNA from He[39]L RNA as a template the δ-aminolevulinate synthetase (ALAS) cDNA was amplified with two 25bp primers resulting in a 415 base pair product (see Figure 6.7). The primers for this reaction were kindly provided by T. Cox, Department of Biochemistry, University of Adelaide, Australia. The ALAS product could be amplified from 5μl of He[39]L cDNA under both annealing conditions described above. The fact that a product of 415bp could be amplified indicated that the cDNA synthesis was working. The distance from the poly A tail of the cDNA encoding ALAS to the 5' primer site is 890bp. Therefore, the cDNA synthesis had produced products of the desired size for the amplification of He[39]L sequences. In fact, when a ^{32}P dATP was incorporated into the cDNA synthesis

reaction and products were separated on a 1% agarose/TBE gel (section 2.4.5) the length of cDNA synthesized appeared to be from <300bp up to several kb.

The bIGFBP-2 PCR was also repeated with cDNA made from MDBK bovine kidney cells (the original source of bIGFBP-2; Szabo *et. al.*, 1988). Incorporation of a ^{32}P dATP into MDBK cDNA synthesis also indicated products from <300bp up to several kb. Again the PCR reaction conditions chosen were identical to those for the amplification of the same bIGFBP-2 using the cloned DNA as a template (section 6.2.2). Interestingly, no products were obtained under these conditions. Variation of template amounts and reaction conditions as for the He[39]L PCR described above did not result in amplification of bIGFBP-2 sequences.

In summary, bIGFBP-2 sequences could be amplified using the full length bIGFBP-2 cDNA clone as a template. However, when cDNA made from RNA was used as a template for either the bIGFBP-2 or the He[39]L PCRs no products were amplified. A control PCR with ALAS primers and He[39]L cDNA as a template resulted in the amplification of a specific product.

At this stage I received the hIGFBP-6 cDNA sequence which proved useful in the analysis of these PCR experiments. Although the P15 (Gly, Cys, Gly, Cys, Cys) sequence was not present in hIGFBP-6 the P30 sequence was conserved in this sequence. Comparison of the cDNA sequence and P30 showed identity in 16 of the 21bp encoding the Pro, Asn, Cys, Asp, His, Arg, Gly motif. Most importantly the first 5bp at the 3' end of P30 are identical to the corresponding cDNA sequence. This is the priming end of the oligonucleotide and at least the first two bases must match for priming to be successful (Sommer and Tautz, 1989). This information confirms that P30 was an appropriate choice of primer for the amplification of He[39]L sequences. In addition, the degenerate He[39]L binding protein specific probe, P29, contained the sequence homologous to the clone sequence encoding Cys, Pro, Gly, Gly, Cys, Val, Gln, Gln, Gln, Asp. The combination of P29 and P30 should result in amplification of He[39]L sequences.

The total hIGFBP-6 clone length was only 918bp which is considerably shorter than other binding protein clones, the major difference being in the nonconserved region in the

centre of the sequence (see Figure 6.6). Analysis of the hIGFBP-6 sequence showed that the product I should be amplifying with P29 and P30 is 465bp, which is slightly smaller than I was expecting. Thus in theory the shorter length product should be easier to amplify.

The first consideration when trying to explain the puzzling PCR results was perhaps that the message levels produced by these cell lines were too low for the amplification of binding protein sequences using this method. However, there are several arguments against such an explanation. Firstly, bIGFBP-2 mRNA can be easily detected by Northern analysis of MDBK RNA (see Chapter 5, Figure 5.8). Also, the ALAS gene is of relatively low abundance and is difficult to detect by Northern analysis (Dr. H. Healey, personal communication). The amount of RNA used in cDNA synthesis (2μg) was sufficient for the amplification of rare mRNA species of 1 to 10 copies per cell (Kawasaki, 1990). When the amounts of RNA per cell are considered (approximately 10pg/mammalian cell), then 2μg RNA is equivalent to 200,000 cells or the same number of mRNA molecules of a rare transcript. This number of target molecules should be easily amplified, even using highly degenerate primers (Knoth *et. al.*, 1988).

An explanation for the inability to amplify bIGFBP-2 and He[39]L binding protein sequences from cDNA synthesized from cellular RNA may lie in the secondary structure formed by the RNA encoding these sequences. It is possible that the RNA forms such a stable structure that the incubation at 65°C for 5 minutes is insufficient to unfold the structure completely. This would mean that the synthesis of cDNA encoding the bIGFBP-2 or He[39]L sequences would be inefficient or perhaps impossible under these conditions. In turn, levels of full length cDNA for amplification by PCR would be extremely low and therefore problems would arise in detecting the PCR products using the primers described above. In contrast, it is possible to amplify ALAS cDNA synthesized from ALAS mRNA, indicating that RNA secondary structure is not a problem in this case.

Predicitions of secondary structures formed by the RNA of hIGFBP-6, bIGFBP-2 and ALAS were performed using the FOLD program (Zucker and Steigler, 1981). The results of the hIGFBP-6 structure prediction are shown in Figure 6.8. The free-energy (ΔG) value calculated for the 918base hIGFBP-6 RNA secondary structure is -1432 kJ/mole (-342 kcal/mole) which is comparable to an example presented by Cech et. al. (1983) for the

proposed intron-splicing structure of *Tetrahymena* ribosomal RNA, suggesting that the formation of such a structure by hIGFBP-6 RNA is at least possible. An even lower free-energy value was calculated for the 1395base bIGFBP-2 RNA (-2384 kJ/mole or -569 kcal/mole). In comparison the ALAS RNA (2056bp) is less likely to form significant secondary structure as the calculated ΔG value is substantially higher for this length of RNA (-2706 kJ/mole or -646 kcal/mole). Therefore, the predictions indicate that RNA secondary structure could be forming in both IGFBP RNAs whereas this is less likely for ALAS. However, these predictions must be interpreted with reservation as they do not take into account any enviromental factors which may be affecting the formation of secondary structure or the influence of tertiary structure. Despite the recognized limitations of this prediction it appears that secondary structure may be a problem in the synthesis of cDNA from hIGFBP-6 and bIGFBP-2 RNAs.

Kiefer *et. al.*, (1991) recently reported the amplification of an approximately 80bp hIGFBP-6 sequence commencing at the P30 site described above but with the second primer site towards the end encoding the C-terminal amino acids Cys, Trp, Cys, Val. This PCR protocol was also used to amplify sequences of IGFBP 2, 4 and 5. The template for the PCRs was cDNA synthesized from poly A^+ RNA. 2.5µg poly A^+ RNA was used in cDNA synthesis and 1/100 of the products were used in subsequent PCRs. It appears that under these conditions cDNA of sufficient length and in high enough concentrations can be synthesized for the amplification of detectable PCR products.

Albiston and Herington (1990) also recently amplified cDNA encoding C-terminal rIGFBP-3 sequences. Again poly A^+ RNA was used in the synthesis of cDNA (5µg) and this time one tenth of the products were used in PCR reactions. According to the above calculations and assuming that poly A^+ RNA represents 1-2% of the total RNA, these amounts of template are in significant excess. It is possible that some difficulty in producing high enough levels of cDNA due to similar reasons I have suggested may have warranted the use of such high levels of template RNA.

Neither of the reports described above amplified sequences across the central region of binding protein sequences. It is possible that this region of the RNA is involved in tight secondary structure (see Figure 6.8).

Having received the sequence of the cDNA encoding the hIGFBP-6 I decided not to continue with this approach in the attempt to isolate a He[39]L cDNA clone. In the light of the problems discussed above in the amplification of binding protein sequences spanning the central nonconserved region, my next approach to the PCR of binding protein sequences would be to design PCRs resulting in short product lengths derived from sequences encoding the carboxy terminal end.

6.2.4 Oligonucleotide Screening of the IMR 90 cDNA library

Using the oligonucleotide P29 described in section 6.2.3 the lambda gt11 IMR 90 library was screened for He[39]L binding protein clones at the same time as conducting the PCR reactions described above. The screening method is outlined in detail in section 2.4.3. The hybridization and washing conditions were similar to those used previously in the isolation of cDNA clones from libraries using inosine-containing oligonucleotides (Sambrook *et. al.*, 1989). Half a million plaques (equivalent to 1/4 of the total original library) were transferred to nylon membranes and screened in duplicate in the first round with ^{32}P labelled P29. The same filters were screened with ^{32}P labelled P30 (the binding protein specific probe also used in PCR) and 6 plaques were positive with both probes. Plugs (5mm in diameter) containing the 6 plaques were picked from the agar plates on which the phage were grown and replated at a lower density. Phage were transferred again to nylon membranes and subjected to a second round of screening with P29. Four positives were rescreened for a third time. Three of the 4 plaques remained positive with the P29 probe.

Lambda DNA was isolated from the 3 clones hybridizing with P29 in the third round of library screening (method described in section 2.4.4). Eco RI released all three clones from the vector, as this enzyme was used in the original construction of the lambda library. The three clones were approximately 700bp, 1.25kb and 1.75kb. The longer clones were chosen as likely candidates for full length cDNA clones on the basis of the sizes of previously sequenced small molecular weight binding protein cDNA clones (IGFBP-1 and IGFBP-2 clones are 1.4-1.6kb). Following restriction endonuclease digestion the lambda DNA of the 1.25 and 1.75kb clones was analyzed by Southern blotting as described in

section 2.4 5 (Figure 6.9). The clone inserts were identified by probing with ^{32}P labelled P29 (see Figure 6.9b).

A restriction map constructed from the sized fragments following digestion with a number of enzymes is shown in Figure 6.10. The map reveals that the 1.25 and 1.75kb clones are of different cDNAs. The restriction fragments containing sequences complementary to the P29 probe are indicated on the map for each clone.

After reprobing the Southern blot and the third round library screen filters with the binding protein specific probe (P30) it was revealed that the clones selected with P29 did not contain the P30 site but P30 did hybridize specifically with bIGFBP-2 cDNA on the same Southern blot. These results suggested that the lambda clones may not be He[39]L binding protein cDNA clones (see Figure 6.9c). This was confirmed by sequencing of the 550bp Pst I/Bgl II fragment of the 1.25kb clone which had been subcloned into the Bluescript BKS+ vector. The sequence is shown in Figure 6.11. The site to which P29 binds is 82% homologous to the P29 sequence if a single base insertion is made. Flanking sequence does not shown any similarity to known binding protein sequences as determined by searching for localized homologies with the SEQHP programme from the ANALYSEQ suite of programmes (Staden, 1982).

Both the 700bp and 1.75kb lambda inserts were subcloned into the Bluescript BKS+ vector and were sequenced from both ends. Again, no sequence homologous to other binding protein sequences was identified. The distance sequenced (approximately 250bp in each case) should have been sufficient to identify at least the leader sequence of the binding protein clones. By comparison with other binding protein clones, the leader sequence lies within approximately the first 80-200bp of the 5' end of the clones. Considerable homology is seen between the amino acid sequences of the binding protein leader peptides. Leucine is particularly common in these sequences.

As the 700bp and 1.75kb clones are completely different to the 1.25 clone as determined by restriction analysis (data not shown for 700bp fragment) and they did not contain P30 sequences it was concluded that none of the clones encoded the He[39]L binding protein.

It is strange that in the first round of screening of the IMR 90 library all 6 clones chosen for further screening were positive with both P29 and P30, but in the third round those remaining positive with P29 did not hybridize to P30. In between probings the filters were stripped and exposed to make sure no signals remained. Following hybridization with P30 filters were washed in 5xSSC (1 x =150 mM NaCl, 15 mM sodium citrate pH 7.0), 0.1% (v/v) SDS at 42°C for 2x30 minutes. The 6 positives in the first round screen were not the only duplicate positives with P30. There were 15 duplicate positives in total. This was the expected result as P30 should detect all binding protein clones and IMR 90 cells produce more than one species of binding protein (see section 4.2.2). I have no explanation for the binding of P30 to the first round positives when subsequent selection and purification yielded clones unrelated to IGFBPs and unable to bind P30.

It is quite possible that the IMR 90 cDNA library does contain He[39]L binding protein clones but more phage have to be screened to isolate them. As discussed above, comparison of P29 with the hIGFBP-6 cDNA sequence indicates that this oligonucleotide is homologous to the corresponding sequence in the clone. It should therefore be a suitable probe for screening the library. From this round of screening it is evident that the probe does cross hybridize with other clones in the library which means that more positives need to be screened until the correct clone is identified. It is encouraging to note that Shimasaki *et. al.* (1991a) measured the highest levels of IGFBP-6 mRNA in the lung when probing rat tissues with a rat IGFBP-6 clone. IGFBP-6 was widely expressed in almost all tissues. Therefore further screening of the IMR 90 cDNA library with the same probe or a homologous probe designed using the available cDNA sequence would be the next step towards isolating a He[39]L probe.

6.4 CONCLUSION

The reasons for the failure of PCR to amplify both He[39]L and bIGFBP-2 sequences from cDNA synthesized from RNA are unclear. However, the control reactions indicated that the PCR reaction conditions were suitable for amplification of bIGFBP-2 and ALAS sequences. Also, He[39]L cDNA from RNA was used as a template in the latter PCR indicating that the cDNA synthesis was working. As rare transcripts have been amplified

from the same amounts of RNA used in these experiments it seemed unlikely that the PCR reactions were not working due to lack of sufficient template. These controls seem to suggest that the problem of amplification actually arose specifically in the synthesis of binding protein cDNA. It is possible that the secondary structure of the binding protein RNAs is inhibiting the synthesis of full length cDNA. RNA secondary structure predictions indicated that this indeed might be a problem. However, it is possible that I have not found the ideal conditions for the amplification of binding protein sequences from cDNA. This seems less likely as the bIGFBP-2 control PCR using a plasmid containing the bIGFBP-2 clone was successful.

Attempts to isolate He[39]L cDNA clones from the IMR 90 cDNA library have as yet been unsuccessful. As the oligonucleotide used in screening procedures was degenerate I isolated clones containing related sequences. Therefore, further screening may result in isolation of the desired clone. With the availability of the hIGFBP-6 cDNA sequence I have not continued with either PCR or library screening at this stage.

The cDNA sequences of the human and rat forms of IGFBP-6 are unusual when compared with the sequences of the five other IGFBP cDNA sequences (see Figure 6.6). Both hIGFBP-6 and rIGFBP-6 lack 2 cysteine residues within the Gly, Cys, Gly, Cys, Cys motif, which is highly conserved in the other binding protein sequences not only at the amino acid sequence level but also within cDNA sequences. A further 2 cysteines are missing in the rIGFBP-6 sequence (the 3rd and 4th cysteines in all other IGFBP sequences). Also of significance is the fact that 4 independently derived protein sequences of hIGFBP-6 isolated from various sources have an Ala in the position corresponding to the first cysteine encoded by the hIGFBP-6 cDNA (see Figure 5.15). This information questions the significance of these 5 cysteines in the formation of the tertiary structure of the binding proteins and will be extremely useful in future assignment of disulphide bonds forming the IGFBP tertiary structure.

```
He[39]L BP N-TERMINAL SEQUENCE   L   A   P   G   P   G   Q   G   V   Q   A   G   X   P   G   G   X   V   E   E   E   D   R   G   G

DNA SEQUENCE                  5' -TGA CCA GGA GGA CCA GGA CAA GGA GTA CAA CCA GGA TGC CCA GGA GGA TGC GTA GAA GAA GAA GAC CGA GGA GGA- 3'
                                  C C   C   C   C   C   C   G   C   C   G   C   C   T   C   C   C   T   C   G   G   G   C A C   C   C
                                    G   G   G   G   G   G       G   G       G   G       G   G   G       G               G   G   G
                                    T   T   T   T   T   T       T   T       T   T       T   T   T       T               T   T   T

PRIMER P23
(TOTAL DEGENERACIES=1024)
                              5' -GGA GGA TGC GTA GAA GAA GAA GA- 3'
                                    C   C   T   C   G   G   G
                                    G   G       G
                                    T   T       T

PRIMER P29
(TOTAL DEGENERACIES=32)
                              5' -TGC CCI GGI GGI TGC GTI GAA GAA GAA GA- 3'
                                    T               T       G   G   G
```

Figure 6.2: Oligonucleotides encoding He[39]L binding protein sequences. All possible DNA sequences encoding the first 25 amino acids of the He[39]L binding protein are shown below the protein sequence. Primers P23 and P29 were designed to correspond to the least degenerate region of that sequence (underlined). The P23 primer preparation contains all possible sequences (1024 different primer sequences) whereas the P29 preparation consists of 32 sequences with inosine (I) in positions where 3 or 4 base pairs may be used. Both P23 and P29 are complementary to noncoding sequences.

```
                      G    C    G    C    C

hIGFBP-1        5' -GGC TGC GGC TGT TGC- 3'
hIGFBP-2        5' -GGC TGC GGC TGC TGC- 3'
bIGFBP-2        5' -GGC TGC GGC TGC TGC- 3'
rIGFBP-2        5' -GGC TGC GGT TGC TGC- 3'
hIGFBP-3        5' -GGC TGC GGC TGC TGC- 3'

PRIMER P15      3' -CCG ACG CCA ACA ACG- 5'
(DEGENERACIES=4)              G   G
```

```
hIGFBP-1             P   N   C  [N]  K  [N]  G
hIGFBP-2             P   N   C   D   K  [H]  G
bIGFBP-2             P   N   C   D   K  [H]  G
rIGFBP-2             P   N   C   D   K  [H]  G
hIGFBP-3             P   N   C   D   K  [K]  G

hIGFBP-1        5' -CCA AAC TGC AAC AAG AAT GGA- 3'
hIGFBP-2        5' -CCC AAC TGT GAC AAG CAT GGC- 3'
bIGFBP-2        5' -CCC AAC TGT GAC AAG CAT GGC- 3'
rIGFBP-2        5' -CCC AAC TGT GAC AAG CAT GGC- 3'
hIGFBP-3        5' -CCC AAC TGT GAC AAG AAG GGA- 3'
                                                 EcoR I
PRIMER P30      3' -GGG TTG ACA CTG TTC GTA CCG CTT AAG GAG- 5'
(DEGENERACIES=32)               G T     T C   T
```

Figure 6.3: Sequence of oligonucleotides encoding sequences common to all IGFBP. The cDNA sequences encoding amino acid sequences common to IGFBP 1, 2 and 3 of various species are shown. Residues or bases which vary between the sequences are boxed or shaded, respectively. Oligonucleotides were designed to incorporate all possible sequences arising from the degeneracies indicated. The upper and lower panels show the sequences used in the design of P15 which has 4 degeneracies and P30 which has 32 degeneracies. P30 has an EcoR I restriction enzyme site at the 5' end.

Figure 6.4: Location of polymerase chain reaction (PCR) primers in the bIGFBP-2 sequence

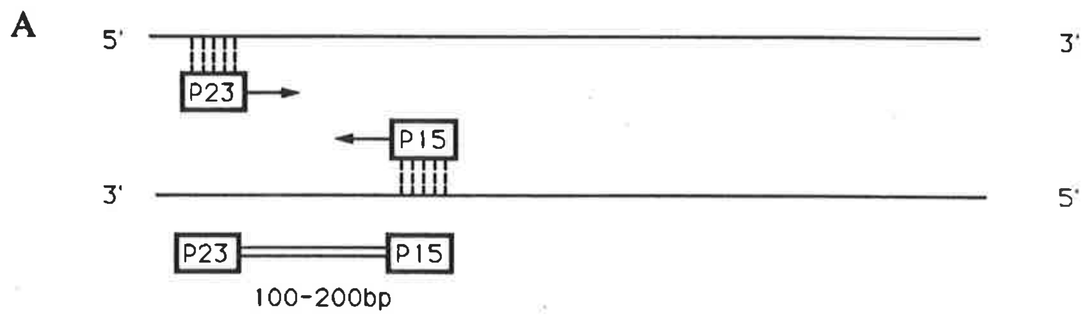
The amino acid sequence of bIGFBP-2 is shown above the cDNA sequence, reported by Upton *et. al.* (1990). Cysteine residues are indicated by stars. The position of PCR primers P24, P15 and P30 are indicated by arrows with the pointer showing the direction of synthesis from the respective primers. The DNA sequence encoding the first 8 amino acids of the mature peptide was chosen as the bIGFBP-2 specific primer (P24) and is highlighted below the bIGFBP-2 sequence. This figure is adapted from Upton *et. al.*, 1990.

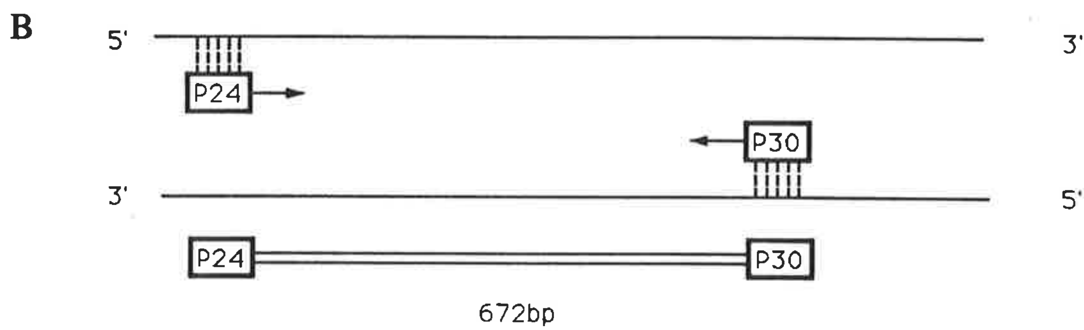
```
        -26                            -14                        -7
        ProAlaLeuLeuLeuLeuProProLeuLeuLeuLeuLeuLeuGlyAlaGlyGlyGly
        CCCGCGCTGCTGCTGCTGCCGCCGCTGCTGCTGCTGCTGCTGGGCGCGGGCGGCGGC   (60)
        -6  *                +1       4      *         *       14
P24     AspCysGlyAlaAlaSerGluValLeuPheArgCysProProCysThrProGluSerLeu
        GACTGCGGGGCGGCCAGCGAGGTGCTGTTCCGCTGCCCGCCCTGCACGCCCGAGAGCCTG  (120)
        15     *                  24                              34
        AlaAlaCysLysProProProGlyAlaAlaAlaGlyAlaProGlyAspAlaArgValPro
        GCCGCCTGCAAGCCCCCGCCCGGCGCCGCGGCCGGCGCGCCCGGCGACGCCCGCGTGCCC  (180)
        35*                     *  44 *   *        *              54
P15     CysGluLeuValArgGluProGlyCysGlyCysCysSerValCysAlaArgLeuGluGly
        TGCGAGCTGGTCCGCGAGCCGGGCTGCGGCTGCTGCTCCGTGTGCGCCCGGCTGGAGGGA  (240)
        55     *                 *64                   *          74
        GluArgCysGlyValTyrThrProArgCysGlyGlnGlyLeuArgCysTyrProAsnPro
        GAGCGGTGCGGCGTGTACACCCCCCGCTGCGGTCAGGGGCTGCGGTGCTATCCAAACCCG  (300)
        75                        84                   *          94
        GlySerGluLeuProLeuHisAlaLeuValHisGlyGluGlyThrCysGluLysHisGly
        GGGTCCGAGCTGCCCCTGCACGCGCTGGTCCACGGCGAGGGCACTTGCGAAAAGCACGGC  (360)
        95                       104                             114
        SerAlaGluTyrSerAlaThrProGluGlnValAlaAspAsnGlyGluGluHisSerGlu
        AGTGCTGAGTACAGCGCCACGCCCGAGCAGGTTGCAGACAACGGCGAGGAGCACTCTGAG  (420)
        115                      124                             134
        GlyGlyLeuValGluAsnHisValAspGlyAsnValAsnLeuMetGlyGlyGlyGlyGly
        GGAGGCCTGGTCGAGAACCACGTGGACGGGAACGTGAACTTGATGGGAGGTGGAGGTGGT  (480)
        135                      144                             154
        AlaGlyArgLysProLeuLysSerGlyMetLysGluLeuAlaValPheArgGluLysVal
        GCCGGTCGGAAGCCCCTCAAGTCCGGCATGAAGGAGCTGGCCGTGTTCCGGGAGAAGGTC  (540)
        155                      164                             174
        ThrGluGlnHisArgGlnMetGlyLysGlyGlyLysHisHisLeuGlyLeuGluGluPro
        ACGGAGCAGCACCGGCAGATGGGCAAGGGTGGCAAACATCACCTCGGCCTGGAGGAGCCC  (600)
        175                      184     *                       194
        LysLysLeuArgProProProAlaArgThrProCysGlnGlnGluLeuAspGlnValLeu
        AAGAAGCTGCGGCCGCCACCTGCCAGGACCCCCTGCCAGCAGGAATTGGACCAGGTCCTG  (660)
        195                      204                             214
        GluArgIleSerThrMetArgLeuProAspGluArgGlyProLeuGluHisLeuTyrSer
        GAGCGGATCTCCACCATGCGCCTTCCGGATGAGCGGGGTCCCCTGGAGCACCTCTACTCC  (720)
        215           *          224                       *     234
P30     LeuHisIleProAsnCysAspLysHisGlyLeuTyrAsnLeuLysGlnCysLysMetSer
        CTACACATCCCCAACTGTGACAAGCATGGCCTGTACAACCTCAAACAGTGCAAGATGTCT  (780)
        235                  *   *244                              254
        LeuAsnGlyGlnArgGlyGluCysTrpCysValAsnProAsnThrGlyLysLeuIleGln
        CTGAACGGGCAGCGTGGGGAGTGCTGGTGTGTGAACCCTAACACCGGGAAGCTGATCCAG  (840)
        255                      264 *                           274
        GlyAlaProThrIleArgGlyAspProGluCysHisLeuPheTyrAsnGluGlnGlnGly
        GGAGCCCCCACCATCCGGGGAGACCCCGAGTGTCATCTCTTCTACAACGAGCAGCAGGGG  (900)
        275                      284
        AlaArgGlyValHisAsnGlnArgMetGln
        GCTCGAGGGGTGCACAACCAGCGGATGCAGTAAACATAGCCAGCCGGTGCCTGGCACCCC  (960)
        CACCATCCGGGGAGACCCGAGTGTCACTCTCTTCTACAACGAGCAGCAGGGGGCTCGAGG (1020)
        GGTGCACACCAGCGGATGCAGTAAACCATAGCCAGCCGGTCCTGGCACCCCCCACCTCTT (1080)
        CGGAGCGGGCAGAGCATGGAGAGCGCATGCGTGGTGGGTGGCGGGGCAGAGCTTCCAGGA (1140)
        GTTCTGACATGTGTATTTATATTTGGAAAGAGACCGGCACCGAGCTCGGCACACACCCCC (1200)
        CCACCCCCTTCCCCACCTTCCCCCTCCCCAGCCGGAGATGCCTGCACCCTCCCTGCTCGA (1260)
        GGCCTGCTGGGCAGGAAGGGGATGGTTGCAGTGGCAGAGCTGGGGTAAATGTTTGGGAGA (1320)
        GGGAAAAAAAGAAATTTTTATTTTTGAACCCCTGTGTCTCTTTTGCTTAAGATTAAAGGA (1380)
        AGGAAAAATAAGGAA (1395)
```

```
                        +1                           +8
                        Glu Val Leu Phe Arg Cys Pro Pro
PRIMER 24           5' -GAG GTG CTG TTC CGC TGC CCG CCC- 3'
```

Figure 6.5: Diagramatic representation of the three IGFBP polymerase chain reactions (PCR)

Primers (boxes) are shown annealed to target DNA sequences. The direction of synthesis by *Taq* polymerase is indicated by arrows and the expected length of PCR product is below each diagram. The original PCR reaction (A) involved the use of the primers P23 and P15 with genomic DNA or IMR 90 library cDNA as a template. The product was expected to be 100-200 bp, although Shimasaki et. al. (1991a) have since reported that the P15 sequence is not present in hIGFBP-6, which corresponds to the He[39]L IGFBP. The amplification of bIGFBP-2 sequences (B) involved the primers P24 and P30 with a plasmid containing bIGFBP-2 cDNA as template DNA. A 672bp product was amplified. The primers P29 and P30 were used in the second He[39]L PCR (C) in an attempt to amplify a 500-700 bp sequence using cDNA synthesized from cellular RNA. The hIGFBP-6 cDNA sequence (Shimasaki et. al., 1991a) indicates this product should in fact be 465bp.


A
5'
3'
P23
P15
3'
5'
P23
P15
100-200bp


B
5'
3'
P24
P30
3'
5'
P24
P30
672bp

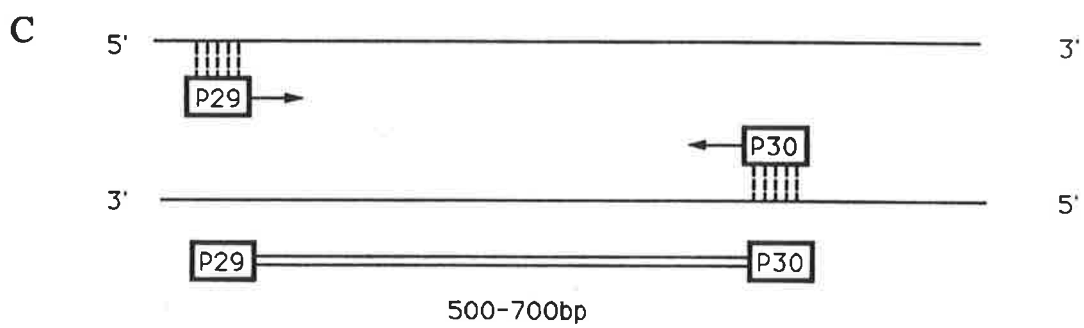

C
5'
3'
P29
P30
3'
5'
P29
P30
500-700bp

Figure 6.6: Sequences of the six human IGFBPs

The amino acid sequences of six human IGFBPs derived from cDNA clones so far sequenced are represented in this figure. Cysteine residues are highlighted. Sixteen cysteines are conserved throughout all human IGFBP sequences. Human IGFBP-4 has two extra cysteines in the central nonconserved region. There is a discrepancy between the two reported hIGFBP-2 sequences over the cysteine at the C-terminal end (Binkert et. al., 1989; Zapf, et. al., 1990). The two residues of hIGFBP-6 replacing the cysteines conserved in other sequences are highlighted by a star. The sequences corresponding to binding protein specific primers (P15 and P30) used in polymerase chain reactions are underlined.

```
                                                                  **
IGFBP-1         AP--WQCAPCSAEKLALCPPVSA---------------SCSEVTRS--A--GCGCCPMCALPLGAACG
IGFBP-2          EVLFRCPPCTPERLAACGPPPVAPPAAVAAVAGGARMPCAELVRE--P--GCGCCSVCARLEGEACG
IGFBP-3   GASSGGLGPVVRCEPCDARALAQCAPPPA---------------VCAELVRE--P--GCGCCLTCALSEGQPCG
IGFBP-4          DEAIHCPPCSEEKLARCRPP----------------VGCEELVRE--P--GCGCCATCALGLGMPCG
IGFBP-5         LGSFVHCEPCDEKALSMCPPSP---------------LGCE-LVKE--P--GCGCCMTCALAEGQSCG
IGFBP-6           ALARCPGCGQGVQAGCP------------------GGCVEEEDGGSPAEGCAEAEGCLRREGQECG
                                                                 <-- P15

IGFBP-1   VATARCARGLSCRALPGEQQPLHALTRGQGACVQES-----------DASAPHAAEAGSPESPESTEITE
IGFBP-2   VYTPRCGQGLRCYPHPGSELPLQALVMGEGTCEKRR-----------DAEYGASPEQVADNGDDHSE---
IGFBP-3   IYTERCGSGLRCQPSPDEARPLQALLDGRGLCVNAS-----------AVSRLRAYLPAPPAP-------L
IGFBP-4   VYTPRCGSGLRCYPPRGVEKPLHTLMHGQGVCMELAEIEAIQESLQPSDKDEGDHPNNSFSPCSAHDRRC
IGFBP-5   VYTERCAQGLACLPRQAEEKPLHALLHGRGVCLNEKSYREQVKIERDSREHEEPTTSEMAEETYSPKIFR
IGFBP-6   VYTPNCAPGLQCHPPKDDEAPLRALLLGRGRCLPARAPAVAEENPKESKPQAGTARPQDVNRRDQQRNPG

IGFBP-1   EELLDNFHLMAPSEEDHS----ILWDAIST------YDGSKALHVTNIKKWKE-----------------
IGFBP-2   -------GGLVENHVD---STMNMLGGGGSAGRKPLKSGMKELAVFREKVT-EQHRQMGKGGKHHLGLEE
IGFBP-3   -------GNASESEEDRSAGSVESPSVSSTHRVSDPKFHPLHSKIIIIKKGHAKDSQRYKVDYESQSTDT
IGFBP-4   ------LQKHFAKIRDRSTSGGKMKVNGAPREDARPVPQG------------------------------
IGFBP-5   PKHTRISELKAEAVKKDRRKKLTQSKFVGGAENTAHPRIISAPEMRQESQ--------------------
IGFBP-6   TSTTPSQPNSAGVQDTEM----------------------------------------------------

IGFBP-1   --------------PCRIELYRVVESLAKAQETSGEEI----SKFYLPNCNKNGFYHSRQCETSMDGEAG
IGFBP-2   PKKLRPPPART---PCQQELDQVLERISTMRLPDERGPLEHLYSLHIPNCDKHGLYNLKQCKMSLNGQRG
IGFBP-3   QNFSSESKRETEYGPCRREMEDTLNHLKFLNVLSPRGV-------HIPNCDKKGFYKKKQCRPSKGRKRG
IGFBP-4   --------------SCQSELHRALERLAASQ--S-RTH-EDLYIIPIPNCDRNGNFHPKQCHPALDGQRG
IGFBP-5   -------------GPCRRHMEASLQELKASPRMVPRAV----YL---PNCDRLGFYKRKQCKPSRGRKRG
IGFBP-6   -------------GPCRRHLDSVLQQLQTEVYRGAQTL----YV---PNCDHRGFYRKRQCRSSQGQRRG
                                                   <-- P30

IGFBP-1   LCWCVYPWNGKRIPGSPEIRGDPNCQMYFNVQN
IGFBP-2   ECWCVNPNTGKLIQGAPTIRGDPECHLFYNEQQEACGVHTQRMQ
IGFBP-3   FCWCVDKYGQPLPGYTTKGKEDVHCYSMQSK
IGFBP-4   KCWCVDRKTGVKLPGGLEPKGELDCHQLADSFRE
IGFBP-5   ICWCVD-KYGMKLPGMEYVDGDFQCHTFDSSNVE
IGFBP-6   PCWCVDR-MGKSLPGSPDGNGSSSCPTGSSG
```

Figure 6.7: Analysis of polymerase chain reaction (PCR) products

PCR was used to amplify bIGFBP-2 and aminolevulinate synthetase (ALAS) sequences. **A.** The bIGFBP-2 product was digested (A) with the restriction enzymes Not I and Sma I. Undigested product (B) was in neighbouring tracks. Not I digested the bIGFBP-2 PCR product to give smaller fragments of approximately 534bp and 138bp as expected. Sma I digested the bIGFBP-2 DNA reducing the product to approximately 457bp. The predicted 215bp smaller band is not visible on this photograph. These DNA fragments were compared against SPP-1 DNA markers digested with EcoR I. The ALAS product (415bp) is shown in **B** and its size was compared against pUC DNA digested with Hpa I.

The reaction conditions used in the amplification of both bIGFBP-2 and ALAS sequences were as follows:

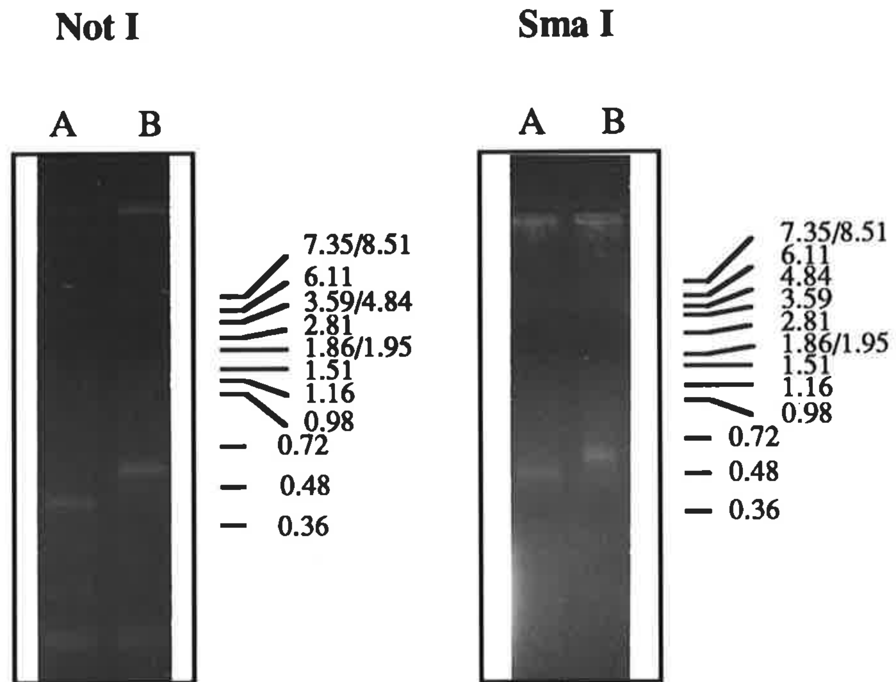
<u>Reaction Components</u>

Template DNA	bIGFBP-2 clone, 10ng plasmid with 1.39kb insert or 5μl He[39]L cDNA (for ALAS PCR)
Primers	100ng each (1-2μM)
dNTPs	100μM each
1X Bresa *Taq* Buffer	
Taq polymerase	0.5 units

<u>Reaction Conditions</u>

Denaturation	94°C, 45 seconds
Annealing	55°C, 3 minutes
Extenstion	72°C, 3 minutes

A **bIGFBP-2 PCR**



B **ALAS PCR**

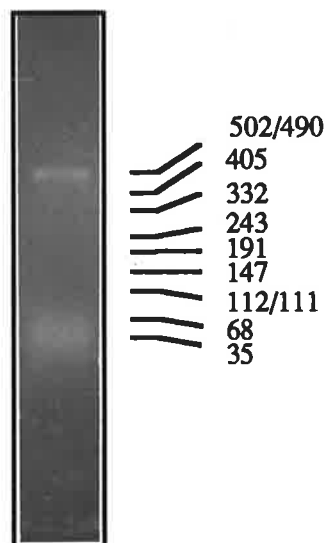


Figure 6.8 Prediction of secondary structure in the hIGFBP-6 mRNA

The possibility of the formation of secondary structures in the hIGFBP-6 mRNA was predicted using the FOLD program (Zucker and Steigler, 1981). The graphic output was formed using the program SQUIGGLES. The free-energy (ΔG) value for the 918base structure was calculated to be -1432 kJ/mole (-342 kcal/mole). Residue one corresponds to the 5' end of the hIGFBP-6 cDNA clone (Shimasaki *et.al.*, 1991a) and residue 918 is at the 3' end. Paired bases are joined by short lines and base numbers are indicated in 10 base intervals.

420 430 410 400 390 860 440 850 450 870 460 380 840 470 510 880 500 520 370 830 480 890 810 820 490 530 800 540 760 750 740 580 910 290 300 310 320 330 340 350 360 230 210 200 190 180 170 160 150 130 5' 3' 730 590 570 550 560 260 270 250 280 240 220 720 600 10 120 710 610 110 20 700 620 690 640 650 100 30 680 660 630 670 40 90 50 80 60 70

Figure 6.9 Southern analysis of 1.25kb and 1.75kb lambda gt11 clones

The two clones isolated from the IMR 90 cDNA library were digested with EcoR I, Bgl II and Pst I restriction endonucleases and in various combinations of those enzymes. **A.** Digested DNA was separated on a 1% agarose/TBE gel and visualized following staining with ethidium bromide. **B.** Southern analysis of the clones was performed as described in section 2.4.5. DNA was transferred to Nytran membrane and the filter was probed with ^{32}P labelled P29 which encodes the He[39]L binding protein N-terminal sequence. Fragments containing sequences hybridizing with the probe were identified after exposure to X-ray film overnight. **C.** The filter was stripped by placing in boiling water and allowed to cool to room temperature. Exposure overnight to X-ray film confirmed the removal of the first probe. Following prehybridization the filter was probed with ^{32}P labelled P30. Specific hybridization was visualized after exposure overnight to X-ray film. Fragment sizes were determined by comparison with SPP-1 phage/EcoR I markers.

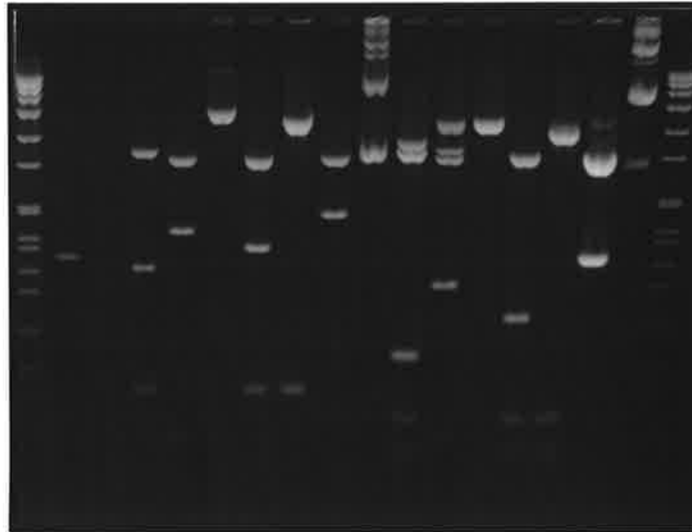
The DNA in each track was as follows:

Mr. SPP-1 markers
1. bIGFBP-2 cDNA (1.39kb clone)
2. Lambda with 1.75 kb insert digested with Bgl II/Pst I
3. Lambda with 1.75 kb insert digested with Bgl II/EcoR I
4. Lambda with 1.75 kb insert digested with Bgl II
5. Lambda with 1.75 kb insert digested with EcoR I/Pst I
6. Lambda with 1.75 kb insert digested with Pst I
7. Lambda with 1.75 kb insert digested with EcoR I
8. Lambda with 1.75 kb insert, undigested
9. Lambda with 1.25 kb insert digested with Bgl II/Pst I
10. Lambda with 1.25 kb insert digested with Bgl II/EcoR I
11. Lambda with 1.25 kb insert digested with Bgl II
12. Lambda with 1.25 kb insert digested with EcoR I/Pst I
13. Lambda with 1.25 kb insert digested with Pst I
14. Lambda with 1.25 kb insert digested with EcoR I
15. Lambda with 1.25 kb insert, undigested

Mr. SPP-1 markers

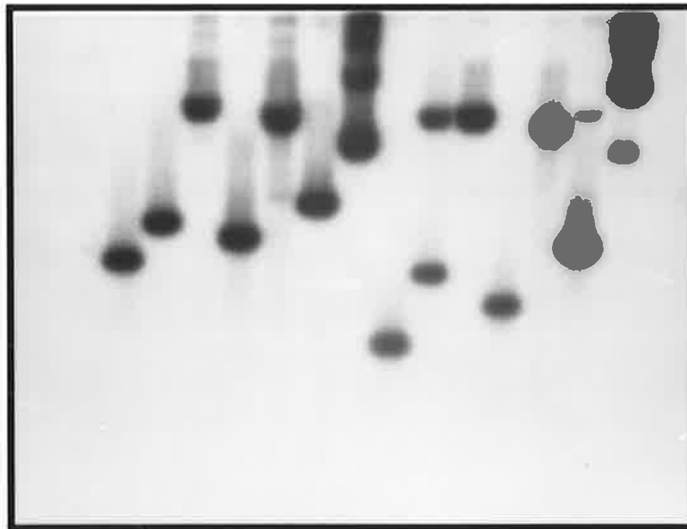
A

Mr 1 - 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Mr



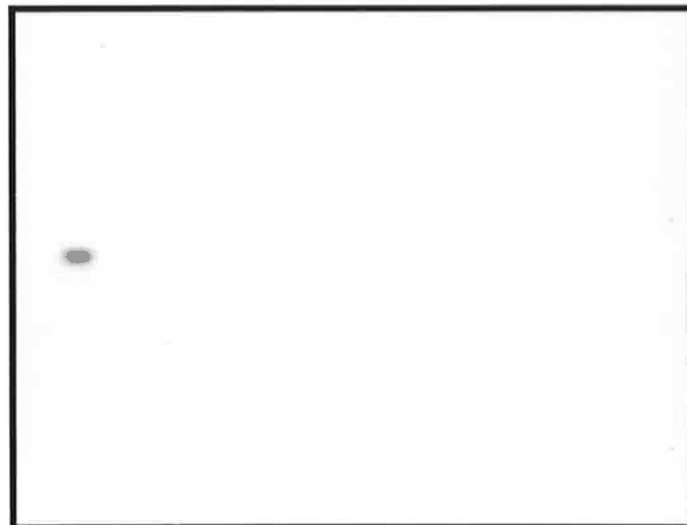
B

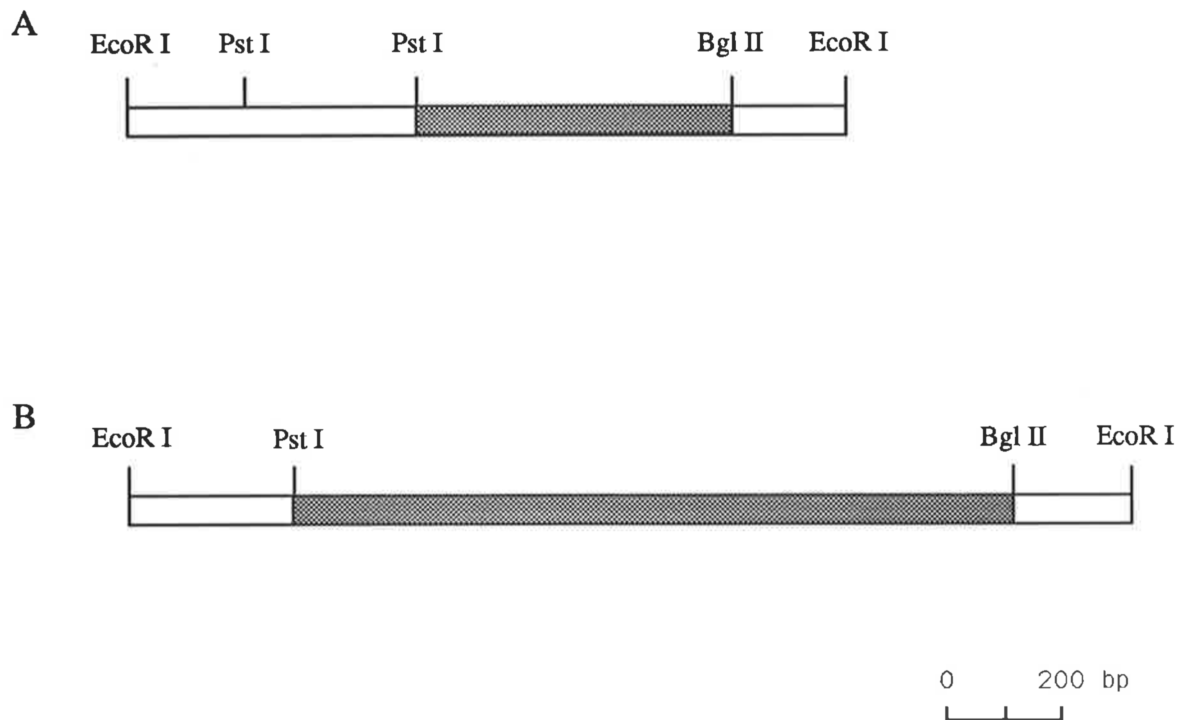
Mr 1 - 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Mr



C

Mr 1 - 2 3 4 5 6 7 8 9 10 11 12 13 14 15 Mr





Figrue 6.10: Restriction maps of 1.25kb and 1.75kb clones isolated from IMR 90 cDNA library. Lambda cDNA clones isolated from the IMR 90 cDNA library were digested with restriction enzymes (EcoR I, Pst I and Bgl II) and separated on a 1% agarose/TBE gel. Restriction fragments were sized against SPP1 markers (Bresatec Pty. Ltd.). Shaded areas are restriction fragments which hybridized to the P29 oligonucleotide used for screening the cDNA library (see Figure 6.8). **A.** The 1.25kb clone. **B.** The 1.75kb clone.

```
AAC TAG TGG ATC TCC CCC CAT AAC ATT TAT AGT TTA TTT GAC AAT AAA ACT GGT TAA GAC TTT GAA CTG AAC ACA AAA CAA
ACA ATA ATA CAA AAA AGT TTT AAA AAC TTA AAA ACT ATG TGT TTG GAG CCA TTT TCC TTC TCT TCA GCC TTG CTC TCC AAT
CCT CAG GAA GGG CTT CAA AAT TAG CAT TTC TCT CTG CCA TGC CAC TAA CCA GCT GCT GCT GTT TCC ACT CTT CAA TTC GCT
TCT GTG CAG TTG ATT CCC GAT CCT CTT CTC TGG AAC TAG AAT TGT CCT CTT CAT CTA ACT CAC GCT GGA TAC TCT GCC ACT

                                                             T               C       A   A   A
                                                            TGC CCI GGI GGI TGT GTI -GA GGA GGA GGA      P29
                                                            |    || ||| |||  || | |  || ||| ||| |||
TTT TTA CCA AAG ATG GCA TTT TGG TCT TAC TCT TCT TTG CTT TTG GGA GCT GGT GGT GGT GGA GGA GGA GGA GGA GGA GGT

GGT GGT GGT GGT TCT ATA ATG GTA GCG GTA GGC ACA GCA CCT CGG GCC TGA ACT GGC TGC AAT GAG GGA GCA GTC ACT CCA
ATT GGG ACA GAA CAT TCT GCA TAA CTC ATA ATT GCA GGT CGT GCC GCT AGT CCT TGG TAA TTT GAC TGC AGG
```

Figure 6.11: Identification of the P29 site in cDNA sequence of a Bgl II/Pst I fragment isolated from the 1.25 kb clone. The Bgl II/Pst I fragment of the 1.25kb lambda clone isolated from the IMR 90 cDNA library was subcloned into a Bluescript BKS+ vector. Sequencing revealed an insert of 542bp. A sequence corresponding to P29 matching in 24/29 positions was identified in the clone. Matches are indicated by vertical bars. This match is possible if a gap is introduced into the P29 sequence (-). The vector sequence is underlined.

CHAPTER 7

GENERAL DISCUSSION

The last few years has seen an enormous expansion in the IGF binding protein field. The complexity of the regulation of IGF action by binding proteins was not realized until the initial reports of the purification of binding proteins. Also, with the development of the Western ligand blot technique (Hossenlopp *et al.*, 1986) the numbers of binding proteins could be estimated in various tissues and fluids.

The importance of understanding the modulation of IGF action by binding proteins has been stressed in recent times by the interest in the clinical use of IGFs. Several *in vivo* studies have reported increased growth in response to IGF-I infusion not only in growth hormone (GH) deficient (hypophysectomized) but also fasted rats (Schoenle *et al.*, 1985; Maiter *et al.*, 1988). A similar response was also observed in normal animals (Hizuka *et al.*, 1986; Philipps *et al.*, 1988). Increased food conversion rates have been observed in nitrogen restricted rats (Tomas *et al.*, 1991), rats with gut resection (Lemmey *et al.*, 1991), streptozotocin-induced diabetes or treated with glucocorticoid (Ballard *et al.*, 1991c). These studies have also reported the positive effect of des-(1-3)-IGF-1 on growth rate. The results seem encouraging in terms of future administration of IGFs in the treatment of polytrauma. Understanding the mechanisms of regulation of IGF action by IGF binding proteins will be of extreme importance when considering the clinical applications of IGFs. Already the beneficial effects of co-administration of IGF-I and IGFBP-3 versus IGF-I alone have been observed in wound healing. However, the mechanisms leading to the improved wound healing are not as yet understood (Sommer *et.al.*, 1991).

In this thesis I have approached the problem of defining the role of IGF binding proteins in two ways. The first was to examine the relative IGF-binding affinities of three different binding proteins and to thereby define three classes of IGF binding proteins. The second approach was to purify and characterize two novel binding proteins. A detailed study of one of these (the 32kDa He[39]L binding protein) related the properties of this binding protein to other known binding proteins. The isolation of novel binding proteins provides vital information necessary for investigating structural determinants involved in the binding of IGFs, a specific aim of our laboratory.

The studies outlined in Chapter 3 relating to the relative binding affinities of hIGFBP-1, bIGFBP-2 and hIGFBP-3 were a key to identifying the importance of the first three amino acids of IGF-I in binding to the small molecular weight binding proteins. The

truncated form of IGF-I, des-(1-3)-IGF-1, is 6-10 times more biologically active *in vitro* (Francis *et al.*, 1988b) as a result of its inability to bind the small molecular weight binding proteins.

The definition of three classes of IGFBP with respect to relative IGF-binding affinity suggests that each class of binding protein has a distinct function in the modulation of the IGF action. Previous studies have measured the majority of IGF in the circulation to be in association with the large molecular weight binding protein complex (Martin and Baxter, 1985). The ability of IGFBP-3 to bind not only IGF-I, IGF-II but also des-(1-3)-IGF-1 supports those observations.

The fact that the smaller binding proteins bind des-(1-3)-IGF-1 poorly, if at all, suggests that they are involved in an alternative function. To date cDNA clones of a total of 5 small molecular weight binding proteins have been purified. The need for so many different types of small binding proteins is unclear. They may play a role in directing IGFs to specific tissues. For instance, the predominance of IGFBP-1 in placental tissue suggests a major role in the decidualization of the endometrium (Bell, 1989). Thus, IGF associated with the large molecular weight carrier complex in the circulation represents the proportion of IGF having an endocrine role, whereas the smaller binding proteins are most likely involved in the action of IGFs at the tissue level.

Sara and Hall (1990) have proposed that the two forms of IGF-I also play different roles. They suggest that IGF-I and des-(1-3)-IGF-1 arise from the two precursor forms, IGF-Ia and IGF-Ib respectively. A protease would specifically cleave the IGF-Ia form to des-(1-3)-IGF-1. As des-(1-3)-IGF-1 has only been purified from tissues or a fluid (colostrum) rich in cells or cell debris Sara and Hall (1990) suggest it acts in an autocrine manner, whereas IGF-I is found in the circulation and most likely has a paracrine action. The inability of the small binding proteins to bind des-(1-3)-IGF-1 would therefore facilitate the autocrine action.

Several other mechanisms have been recently proposed to be involved in the control of the IGF-binding protein interaction. Proteases have been identified which cleave particular binding proteins (predominantly IGFBP-3) in pregnancy and also as a result of trauma in surgery (Gargosky *et al.*, 1990a; Guidice *et al.*, 1990; Davenport *et al.*, 1990; Holly *et al.*,

UNIVERSITY OF ADELAIDE LIBRARY

1991). Proteolytic cleavage of binding proteins has also been identified in seminal plasma and has been attributed to seminal plasma antigen (Cohen *et. al.*, 1991).

The identification of binding protein proteases opens a new area of research. Their presence may be providing a mechanism for the release of IGF from the carrier protein. Such mechanisms have been demonstrated to play a role in certain hormone carrier systems. CBG (Corticosteriod binding globulin) and T_4 transport protein (TBG) are carrier proteins which have structural homologies with serine protease inhibitors known as SERPINs. Most SERPINs interact with specific proteases and are then cleaved themselves by the protease. In the case of CBG, its cleavage by elastase results in a dramatic reduction in steroid binding activity (Hammond *et al.*, 1990).

The involvement of proteases in the IGF-binding protein interaction also presents the possibility of an alternative model for the generation of the truncated form of IGF-I. Although in the case of CBG the steroid itself is not affected by the proteolytic cleavage mechanism, it is possible that during release of IGF-I from IGFBPs by proteolysis a concurrent cleavage of IGF-I occurs to form des-(1-3)-IGF-1. Thus, the purification and characterization of the binding protein specific proteases will certainly be vital in understanding the control of IGF action.

Further properties of binding proteins isolated so far may also be involved in altering the IGF-binding protein interaction. IGFBP-1 secreted by tissue culture cells has been shown to be phosphorylated (Clemmons *et al.*, 1991). The phosphorylated form has a greater affinity for IGF-I than the non-phosphorylated form. Also, multimeric forms of IGFBP-1 resulting from intermolecular disulphide bond formation are biologically active (Busby *et al.*, 1989). However, the significance of these forms is not clear as yet .

The modification by glycosylation may also be significant in the action of binding proteins. To date IGFBP-3 (Martin and Baxter, 1986), IGFBP-4 (Dr P. E. Walton, personal communication) and the 32 kDa He[39]L binding protein (hIGFBP-6, see Chapter 5) have been shown to be glycosylated. An interesting report by Cornell *et al.* (1987) identifies that glycosylation may infer different properties to binding proteins. A glycosylated form of the 150 kDa large molecular weight IGF carrier is biologically active when complexed with IGF-II, whereas the nonglycosylated form bound to IGF-I is inactive in the same assay.

Several researchers have reported that IGF bound to binding proteins is unable to bind to IGF receptors (Knauer and Smith, 1980; Ritvos *et al.*, 1987; Ross *et al.*, 1989). The enhancement of IGF action by binding proteins has also been reported (Clemmons *et al.*, 1986; Elgin *et al.*, 1987; De Mellow and Baxter, 1988). It is possible that modification by glycosylation, phosphorylation or other means may confer the stimulatory or inhibitory role to binding proteins observed by different researchers.

The presence of Arg-Gly-Asp sequences in IGFBP-1 and IGFBP-2 sequences have been implicated in the possible association of those binding proteins with cellular membranes. Binding proteins have been identified on the surface of several cell types including fibroblasts. McCusker *et al.* (1990) have suggested the membrane bound IGF binding proteins are involved in the stimulatory action of binding proteins, enhancing the action of IGF by aiding the interaction with IGF receptors. Interestingly, He[39]L lung fibroblasts have membrane bound binding proteins but the 32 kDa He[39]L binding protein (hIGFBP-6) does not contain an Arg-Gly-Asp sequence. This may mean that the surface associated binding protein is not related to IGFBP-6.

Initial characterization of the 32 kDa He[39]L binding protein has not identified any further modifications to the mature protein such as phosphorylation which might alter its interaction with IGFs. In Chapter 5 I described that the He[39]L binding protein is immunologically unrelated to bIGFBP-2 and is also distinct from IGFBPs 1, 3 and 4. However, the binding pattern of the 32 kDa He[39]L binding protein is similar to that of bIGFBP-2 (see Chapter 5), which belongs to one of the three classes defined in this thesis. Comparison of the amino acid sequences of these two binding proteins indicates the greatest similarities at either end (Figure 6.6). These similarities must confer a structure to bIGFBP-2 and the He[39]L IGFBP resulting in common binding patterns. Therefore the basic determinant of IGF binding affinity lies in the amino acid sequence of the two binding proteins. These observations are particularly interesting considering that hIGFBP-6 lacks two cysteines previously thought to be important in the formation of a common binding protein structure.

With the availability of so many binding protein sequences it will be possible to make predictions of residues which might be involved in the IGF binding site. Site directed mutagenesis of IGFBP cDNA clones and expression of mutant proteins will be vital in

determining specific residues of IGFBPs involved in the IGF-binding protein interaction. Systematic mutation of the cysteine residues would be the first important changes to analyze. In addition, mutation of the common sequence used as an oligonucleotide in the PCR reactions described in Chapter 7 would prove interesting.

Chemical modification of particular residues in the IGFBPs using a strategy similar to the one described by Moss *et al.* (1991) would also provide useful information about the IGF binding site. This would involve modification of tyrosine residues by Chloramine T in the presence of IGF. Protection from modification of particular tyrosines in the presence of IGF (determined by the distribution of ^{125}I incorporation) would indicate the involvement of that residue in the IGF binding site.

In Chapter 4 I examined factors and hormones which might have an influence on binding protein expression by He[39]L cells and SF 1972 human skin fibroblasts. Altering the local production of binding proteins would in turn affect the availability of IGF to bind the cellular receptors. Both fibroblast types responded to IGF-I, IGF-II, des-(1-3)-IGF-1 and long R^3 IGF-I with an increased production of IGFBP-3. A slight increase in production of the binding proteins with a similar size to hIGFBP-6 was also observed. Perhaps the production of binding proteins in response to higher levels of IGF is a mechanism of "fine tuning" the levels of IGF available in the tissues containing these cell types.

With the availability of the cDNA clone encoding hIGFBP-6 it will be interesting to determine the tissue distribution of this binding protein. As yet only rat tissues have been analyzed for the presence of rIGFBP-6 by Northern analysis (Shimasaki *et. al.*, 1991a). To date it appears that the fibroblasts analysed by Western ligand blotting (Chapter 4) all express a binding protein of similar size to hIGFBP-6. It is possible that all fibroblasts express this protein. hIGFBP-6 has also been localized in serum, cerebrospinal fluid and is produced by cultured osteoblasts (Roghani *et al.*, 1989; Zapf *et al.*, 1990a; Andress and Birnbaum, 1991). Determination of the levels of expression of IGFBP-6 in diseased states or in cases of trauma will ultimately help us understand the role of IGFBP-6 in the modulation of IGF action.

The observations presented in this thesis will useful in the identification and classification of novel binding proteins in the future. The isolation of two novel binding proteins adds to the complexity of the control mechanisms regulating the action of IGFs. Further characterization of these binding proteins on molecular and structural levels will be vital for future understanding of the mechanism of IGF-binding protein interactions.

BIBLIOGRAPHY

Adams, S. O., Kapadia, M., Mills, B. and Daughaday, W. H. (1984) *Endocrinology* **115**, 520-526, Release of insulin-like growth factors and binding protein activity into serum-free medium of cultured human fibroblasts.

Adams, S.O., Nissley, S.P., Handwerger, S. and Rechler, M.M. (1983) *Nature* **302**, 150-152, Developmental patterns of insulin-like growth factor -I and -II. Synthesis and regulation in rat fibroblasts.

Adashi, E. Y., Resmick, C. E., Hernandez, E. R., Hurwitz, A. and Rosenfeld, R. G. (1990) *Mol. Cell Endocrinol.* **74**, 175-184, Ovarian granulosa cell-derived insulin-like growth factor (IGF) binding proteins: Release of low molecular weight, high affinity IGF-selective species.

Albiston, A. L. and Herington, A. C. (1990) *Biochem. Biophys. Res. Comm.* **166**, 892-897, Cloning and characterization of the growth hormone-dependent insulin-like growth factor binding protein (IGFBP-3) in the rat.

Andress, D. L. and Birnbaum, R. S. (1991) *Biochem. Biophys. Res. Comm.* **176**, 213-218, A novel human insulin-like growth factor binding protein secreted by osteoblast like cells.

Armstrong, D. G., McKay, C. O., Morrell, D. J. and Goddard, C. (1989) *J. Endocrinol.* **120**, 373-378, Insulin-like growth factor-I binding proteins in serum from the domestic fowl.

Atkinson, P. R., Weidman, E. R., Bhaumick, B. and Bala, R. M. (1980) *Endocrinology* **106**, 2006-2012, Release of somatomedin-like activity by cultured WI-38 human fibroblasts.

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

Bala, R. M., Lopatka, J., McCoy, E. and McArthur, R. G. (1981) *J. Clin. Endocrinol. Metab.* **52**, 508-512, Serum immunoreactive somatomedin levels in normal adults, pregnant women at term, children at various ages, and children with constitutionally delayed growth.

Ballard, F. J., Baxter, R. C., Binoux, M., Clemmons, D. R., Drop, S. L. S., Hall, K., Hintz, R. L., Rechler, M. M., Rutanen, E. M. and Schwander, J. C. (1990b) *J. Clin. Endocrinol. Metab.* **70**, 817-818, Report on the nomenclature of the IGF binding proteins.

Ballard, F. J., Baxter, R. C., Binoux, M., Clemmons, D. R., Drop, S. L. S., Hall, K., Hintz, R. L., Ling, N., Rechler, M. M., Schwander, J. C., Spencer, E. M. and Zapf, J. (1991b) On the nomenclature of the IGFBPs. In *Modern concepts of insulin-like growth factors*. E. M. Spencer (Editor) Elsevier, N.Y., USA, pp 731-732.

Ballard, F.J., Francis, G.L., Ross,M., Bagley, C.J., May, B.L. and Wallace, J.C. (1987) *Biochem. Biophys. Res. Comm.* **149**, 398-404, Natural and synthetic forms of insulin-like growth factor-1 (IGF-1) and the potent derivative, destripeptide IGF-1: biological activities and receptor binding.

Ballard, F. J., Johnson, R. J., Owens, P. C., Francis, G. L. Upton, F. M., McMurtry, J. P. and Wallace, J. C. (1990a) *Gen. Comp. Endocrinol.* **79**, 459-468, Chicken insulin-like growth factor-I: Amino acid sequence, radioimmunoassay, and plasma levels between strains and during growth.

Ballard, F. J., Knowles, S. E., Walton, P. E., Edson, K., Owens, P. C., Mohler, M. A. and Ferraiolo, B. L. (1991a) *J. Endocrinol.* **128**, 197-204, Plasma clearance and tissue distribution of labelled insulin-like growth factor-I (IGF-I), IGF-II and des-(1-3) IGF-1 in rats.

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

Ballard, F. J., Ross, M., Upton, F. M. and Francis, G. L. (1988) *Biochem. J.* **249**, 721-726, Specific binding of insulin-like growth factors 1 and 2 to the type 1 and type 2 receptors respectively.

Ballard, F. J., Tomas, F. M., Read, L. C., Knowles, S. E., Owens, P. C., Lemmey, A. B., Martin, A. A., Wells, J. R. E., Wallace, J. C. and Francis, G. L. (1991c) Effects of IGF-I and IGF analogs on growth during catabolic states in rats. In *Modern concepts of insulin-like growth factors*. E. M. Spencer (Editor) Elsevier, N.Y., USA, pp 617-627.

Bar, R. S., Harrison, L. C., Baxter, R. C., Boes, M., Dake, B. L., Booth, B. and Cox, A. (1987) *Biochem. Biophys. Res. Comm.* **148**, 734-739, Production of IGF-binding proteins by vascular endothelial cells.

Bautista, C. M., Baylink, D. J. and Mohan, S. (1991) *Biochem. Biophys. Res. Comm.* **176**, 756-763, Isolation of a novel insulin-like growth factor (IGF) binding protein from human bone: A potential candidate for fixing IGF-II in human bone.

Baxter R. C. (1986) *Adv. Clin. Chem.* **25**, 49-115, The Somatomedins: Insulin-like growth factors.

Baxter, R. C. (1988) *J. Clin. Endocrinol. Metab.* **67**, 265-272, Characterization of the acid-labile subunit of the growth hormone-dependent insulin-like growth factor binding protein complex.

Baxter, R. C. (1989) *J. Clin. Endocrinol. Metab.* **68**, 638-646, Structural and immunological comparison of insulin-like growth factor binding proteins of cerebrospinal and amniotic fluids.

Baxter, R. C. (1990) *J. Clin. Endocrinol. Metab.* **70**, 1347-1353, Circulating levels and molecular distribution of the acid-labile (α) subunit of the high molecular weight insulin-like growth factor-binding protein complex.

Baxter, R.C. and Cowell, C.T. (1987) *J. Clin. Endocrinol. Metab.* **65**, 432-440, Diurnal rhythm of growth hormone-independent binding protein for insulin-like growth factors in human plasma.

Baxter, R. C. and De Mellow, J. S. M. (1986) *Clin. Endocrinol.* ***24***, 267-278, Measurement of insulin-like growth factor-II by radioreceptor assay using ovine placental membranes.

Baxter, R. C. and Martin, J. L. (1986) *J. Clin. Invest.* **78**, 1504-1512, Radioimmunoassay of growth hormone-dependent insulinlike growth factor binding protein in human plasma.

Baxter, R. C. and Martin, J. L. (1987) *Biochem. Biophys. Res. Comm.* **147**, 408-415, Binding proteins for insulin-like growth factors in adult rat serum. Comparison with other human and rat binding proteins.

Baxter, R. C. and Martin, J. L. (1989) *Prog. Growth Factor Res.* **1**, 49-68, Binding proteins for the insulin-like growth factors: Structure, regulation and function.

Baxter, R. C., Martin, J. L. and Beniac, V. A. (1989) *J. Biol. Chem.* **264**, 11843-11848, High molecular weight insulin-like growth factor binding protein complex. Purification and properties of the acid-labile subunit from human serum.

Baxter, R.C., Martin, J.L. and Wood, M.E. (1987) *J. Clin. Endocrinol. Metab.* **65**, 423-431, Two immunoreactive binding proteins for insulin-like growth factors in human amniotic fluid: relationship to fetal maturity.

Baxter, R. C., Martin, J. L., Tyler, M. I. and Howden, M. E. H. (1986) *Biochem. Biophys. Res. Comm.* **139**, 1256-1261, Growth hormone-dependent insulin-like growth factor (IGF) binding protein from human plasma differs from other human IGF binding proteins.

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

Bayne, M. L., Applebaum, J., Underwood, D., Cicchi, G. G., Green, B. G., Hayes, N. S. and Cascieri, M. A. (1989) *J. Biol. Chem.* **264**, 11004-11008, The C region of human insulin-like growth factor (IGF) I is required for high affinity binding to the type I IGF receptor.Bell, G. I., Stempien, M. M., Fong, N. M. and Seino, S. (1990) *Nucleic Acids Res.* **18**, 4275, Sequence of a cDNA encoding guinea pig IGF-I.

Bell, S. C. (1989) *Human Reproduction* **4**, 125-130, Decidualization and insulin-like growth factor (IGF) binding protein: implications for its role in stromal cell differentiation and the decidual cell in haemochorial placentation.

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

Bennett, A., Wilson, D.M., Liu, F., Nagashima, R., Rosenfeld, R.G. and Hintz, R.L. (1983) *J. Clin. Endocrinol. Metab.* **57**, 609-612, Levels of insulin-like growth factors I and II in human cord blood.

Bers, G. and Garfin, D. (1985) *Biotechniques* **3**, 275-289, Protein and nucleic acid blotting and immunobiochemical detection.

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

Binoux, M. and Hossenlopp, P. (1988) *J. Clin. Endocrinol. Metab.* **67**, 509-514, Insulin-like growth factor (IGF) and IGF-binding proteins: Comparison of human serum and lymph.

Binoux, M., Lassare, C. and Gourmelen, M. (1986) *J. Clin. Endocrinol. Metab.* **63**, 1151-1155, Specific assay for insulin-like growth factor (IGF) II using the IGF binding proteins extracted from cerebrospinal fluid.

Binoux, M., Hardouin, S., Lassarre, C. and Hossenlopp, P. (1982) *J. Clin. Endocrinol. Metab.* **55**, 600-602, Evidence for production by the liver of two IGF binding proteins with similar molecular weights but different affinities for IGF-I and IGF-II. Their relations with serum and cerebrospinal fluid IGF binding proteins.

Binoux, M., Seurin, D., Lassarre, C. and Gourmelen, M. (1984) *J. Clin. Endocrinol. Metab.* **59**, 453-462, Preferential measurement of insulin-like growth factor (IGF) I-related peptides in serum with the aid of IGF-binding proteins (IGF BPs) produced by rat liver in culture. Estimation of serum IGF BP levels.

Blat, C., Bohlen, P., Villaudy, J., Chatelain, G., Golde, A. and Harel, L. (1989a) *J. Biol. Chem.* **264**, 6021-6024, Isolation and amino-terminal sequence of a novel cellular growth inhibitor (inhibitory diffusible factor 45) secreted by 3T3 fibroblasts.

Blat, C., Delbe, J., Villaudy, J., Chatelain, G., Golde, A. and Harel, L. (1989b) *J. Biol. Chem.* **264**, 12449-12454, Inhibitory diffusible factor 45 bifunctional activity. As a cell growth inhibitor and as an insulin-like growth factor I-binding protein.

Blodgett, F. M., Burgin, L., Iezzoni, D., Gribetz, D. and Talbot, N. B. (1956) *N. Engl. J. Med.* **254**, 636-641, Effects of prolonged cortisone therapy on the statural growth, skeletal maturation and metabolic status of children.

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

Blundell, T.L., Bedarkar, S, and Humbel, R.E. (1983) *Fed. Proc.* **2**, 2592-2598, Tertiary structures, receptor binding, and antigenicity of insulinlike growth factors.

Bondy, C. A., Werner, W., Roberts, Jr., C. T. and LeRoith, D. (1990) *Mol. Endocrinol.* **4**, 1386-1398, Cellular pattern of insulin-like growth factor-I (IGF-I) and type I IGF receptor gene expression in early organogenesis: Comparison with IGF-II gene expression.

Böni-Schnetzler, M., Schmid, C., Mary, J.-L., Zimmerli, B., Meier, P. J., Zapf, J., Schwander, J. and Froesch, E. R. (1990) *Mol. Endocrinol.* **4**, 1320-1326, Insulin regulates the expression of the insulin-like growth factor binding protein 2 mRNA in rat hepatocytes.

Bornfeldt, K. E., Arnqvist, H. J., Engberg, B., Mathews, L. S. and Norstedt, G. (1989) *J. Endocrinol.* **122**, 651-656, Regulation of insulin-like growth factor-I and growth hormone receptor gene expression by diabetes and nutritional state in rat tissues.

Brewer, M. T., Stetler, G. L., Squires, C. H., Thompson, R. C., Busby, W. H. and Clemmons, D. R. (1988) *Biochem. Biophys. Res. Comm.* **152**, 1289-1297, Cloning, Characterization and expression of a human insulin-like growth factor binding protein.

Brinkman, A., Groffen, C., Kortleve, D. J., Geurts van Kessel, A. and Drop, S. L. S. (1988a) *EMBO J.* **7**, 2417-2423, Isolation and characterization of a cDNA encoding the low molecular weight insulin-like growth factor binding protein (IBP-1).

Brinkman, A., Groffen, C. A. H., Kortleve, D. J. and Drop, S. L. S. (1988b) *Biochem. Biophys. Res. Comm.* **157**, 898-907, Organization of the gene encoding the insulin-like growth factor binding protein IBP-1.

Brinkman, A., Kortleve, D. J., Groffen, C. A. H. and Drop, S. L. S. (1989) *In Insulin-like growth factor binding proteins*. S. L. S. Drop. and R. L. Hintz, (Editors) pp 11-20, The C-terminal region of the low molecular weight IGF binding protein, IGFBP-1, is essential for IGF-I binding.

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

Brown, A. L. and Rechler, E. M. (1990) *Mol. Endocrinol.* **4**, 2039-2051, Cloning of the rat insulin-like growth factor-binding protein-2 gene and identification of a functional promoter lacking a TATA box.

Brown, A. L., Chiariotti, L., Orlowski, C. C., Mehlman, T., Burgess, W. H., Ackerman, E. J., Bruni, C. B. and Rechler, M. M. (1989) *J. Biol. Chem.* **264**, 5148-5154, Nucleotide sequence and expression of a cDNA clone encoding a fetal rat binding protein for insulin-like growth factors.

Bürgi, H., Müller, W. A., Humbel, R. E., Labhart, A. and Froesch, E. R. (1966) *Biochem. Biophys. Acta* **121**, 349-359, Non-suppressible insulin-like activity of human serum. I. Physicochemical properties, extraction and partial purification.

Busby, W. H., Klapper, D. G. and Clemmons, D. R. (1988a) *J. Biol. Chem.* **263**, 14203-14210, Purification of a 31,000-dalton insulin-like growth factor binding protein from human amniotic fluid.

Busby, W. H., Snyder, D. K. and Clemmons, D. R. (1988b) *J. Clin. Endocrinol. Metab.* **67**, 1225-1230, Radioimmunoassay of a 26,000-dalton plasma insulin-like growth factor-binding protein: Control by nutritional variables.

Busby, W. H., Hossenlopp, P., Binoux, M. and Clemmons, D. R. (1989) *Endocrinology* **125**, 773-777, Purified preparations of the amniotic fluid-derived insulin-like growth factor-binding protein contain multimeric forms that are biologically active.

Butler, J. H. and Gluckman, P. D. (1986) *J. Endocrinol.* **109**, 333-338, Circulating insulin-like growth factor-binding proteins in fetal, neonatal and adult sheep.

Canalis, E., McCarthy, T. and Centrella, M. (1988) *Endocrinology* **122**, 22-27, Isolation and characterization of insulin-like growth factor I (somatomedin-C) from cultures of fetal rat calvariae.

Cao, Q-P., Duguay, S. J., Plisetskaya, E., Steiner, D. F. and Chan, S. J. (1989) *Mol. Endocrinol.* **3**, 2005-2010, Nucleotide sequence and growth hormone-regulated expression of salmon insulin-like growth factor 1 mRNA.

Carlsson-Skwirut, C., Jörnvall, H., Holmgren, A., Andersson, C., Bergman, T., Lundquist, G., Sjögren, B. and Sara, V.R. (1986) *FEBS Lett.* **201**, 46-50, Isolation and characterization of variant IGF-1 as well as IGF-2 from adult human brain.

Cascieri, M.A., Chicchi, G.G., Applebaum, J., Hayes, N.S., Green, B.G. and Bayne, M.L. (1988a) *Biochemistry* **27**, 3229-3233, Mutants of human insulin-like growth factor I with reduced affinity for the type 1 insulin-like growth factor receptor.

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

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

Cech, T. R., Tanner, N. K., Tinoco, I., Weir, B. R., Zucker, M. and Perlman, P.S. (1983) *Proc. Natl. Acad. Sci.* (USA) **34**, 3903-3907, Secondary structure of the Tetrahymena ribosomal RNA intervening sequnece: structural homology with fungal mitochondrial intervening sequences.

Chatelain, P. G., Van Wyk, J. J., Copeland, K. C., Blethen, S. L. and Underwood, L. E. (1983) *J. Clin. Endocrinol. Metab.* **56**, 376-383, Effect of *in vitro* action of serum proteases or exposure to acid on measurable immunoreactive somatomedin-C in serum.

Chen, E. J. and Seeburg, P. H. (1985) *DNA* **4**, 165-170, Supercoil sequencing: a fast simple method for sequencing plasmid DNA.

Clemmons, D. R. and Gardner, L. I. (1990) *J. Cell. Physiol.* **145**, 129-135, A factor contained in plasma is required for IGF binding protein-1 to potentiate the effect of IGF-I on smooth muscle cell DNA synthesis.

Clemmons, D.R. and Shaw, D.S. (1986) *J. Biol. Chem.* **261**, 10293-10298, Purification and biological properties of fibroblast somatomedin.

Clemmons, D. R., Underwood, L. E. and Van Wyk, J. J. (1981b) *J. Clin. Invest.* **67**, 10-19, Hormonal control of immunoreactive somatomedin production by cultured human fibroblasts.

Clemmons, D. R., Camacho-Hubner, C., Jones, J. I., McCusker, R. H. and Busby, W. H. (1991) Insulin-like growth factor binding proteins: Mechanisms of action at the cellular level. In *Modern concepts of insulin-like growth factors*. E. M. Spencer (Editor) Elsevier, N.Y., USA, pp 475-486.

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

Clemmons, D.R., Elgin, R.G., Han, V.K.M., Casella, S.J., D'Ercole, J.A. and Van Wyk, J.J. (1986) *J. Clin. Invest.* **77**, 1548-1556, Cultured fibroblast monolayers secrete a protein that alters the cellular binding of somatomedin-C/insulinlike growth factor I.

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

Clemmons, D. R., Thissen, J. P., Maes, M., Ketelslegers, J. M. and Underwood, L. E. (1989) *Endocrinology* **125**, 2967-2972, Insulin-like growth factor-I (IGF-I) infusion into hypophysectomized or protein-deprived rats induces specific IGF-binding proteins in serum.

Clemmons, D. R., Underwood, L. E., Chatelain, P. G. and Van Wyk, J. J. (1983) *J. Clin. Endocrinol. Metab.* **56**, 384-389, Liberation of immunoreactive somatomedin C from its binding proteins by proteolytic enzymes and heparin.

Clemmons, D. R., Van Wyk, J. J., Ridgway, E. C., Kilman B., Kjellberg, R. N. and Underwood, L. J. (1979) *New Engl. J. Med.* **22**, 1138-1142, Evaluation of acromegaly by radioimmunoassay of somatomedin-C.

Cohen, K. L. and Nissley, S. P. (1976) *Acta Endocrinol.* **83**, 243-258, The serum half-life of somatomedin activity: Evidence for growth hormone dependence.

Cohen, P., Graves, H. C.B., Kamarei, M., Peehl, D. M. and Rosenfeld, R. G. (1991) *73rd Annual meeting of the Endocrine Society*, June 19-22, Washington DC, Prostate specific antigen (PSA) is an IGF binding protein (IGFBP-3) protease found in seminal plasma. Abstract 1365.

Conover, C. A. (1990) *Endocrinology* **126**, 3139-3145, Regulation of insulin-like growth factor (IGF)-binding protein synthesis by insulin and IGF-I in cultured bovine fibroblasts.

Conover, C. A. and Lee, P. D. (1990) *J. Clin. Endocrinol. Metab.* **70**, 1062-1067, Insulin regulation of insulin-like growth factor-binding protein production in cultured Hep G2 cells.

Conover, C. A., Lui, F., Powell, D., Rosenfeld, R. G. and Hintz, R. L. (1989) *J. Clin. Invest.* **83**, 852-859, Insulin-like growth factor binding proteins from cultured human fibroblasts. Characterization and hormonal regulation.

Cornell, H. J., Engberg, G. and Herington, A. C. (1987) *Biochem. J.* **241**, 745-750, Preferential association of the insulin-like growth factors I and II with metabolically inactive and active carrier-bound complexes in serum.

Corps, A. N. and Brown, K. D. (1991) *Endocrinology* **128**, 1057-1064, Mitogens regulate the production of insulin-like growth factor-binding protein by Swiss 3T3 cells.

Costigan, D.C., Guyda, H.J. and Posner, B.I. (1988) *J. Clin. Endocrinol. Metab.* **66**, 1014-1018, Free insulin-like growth factor I (IGF-I) and IGF-II in human saliva.

Cotterill, A.M., Cowell, C.T., and Baxter, R.C., McNeill, D. & Silinik, M. (1988) *J. Clin. Endocrinol. Metab.* **67**, 882-887, Regulation of the growth hormone-independent growth factor-binding protein in children.

Cotterill, A. M., Cowell, C. T. and Silinik, M. (1989) *J. Endocrinol.* **123**, R17-R20, Insulin and variation in glucose levels modify the secretion rates of the growth hormone-independent insulin-like growth factor binding protein-1 in the human heptablastoma cell line Hep G2.

Croze, F., Kennedy, T. G., Schroedter, I. C., Friesen, H. G. and Murphy, L. J. (1990) *Endocrinology* **127**, 1995-2000, Expression of insulin-like growth factor-I and insulin-like growth factor-binding protein-1 in the rat uterus during decidualization.

Cubbage, M. L., Suwanichkul, A. and Powell, D. R. (1989) *Mol. Endocrinol.* **3**, 846-851, Structure of the human chromosomal gene for the 25 kilodalton insulin-like growth factor binding protein.

Cubbage, M. L., Suwanichkul, A. and Powell, D. R. (1990) *J. Biol. Chem.* **265**, 12642-12649, Insulin-like growth factor binding protein-3. Organization of the human chromosomal gene and demonstration of promoter activity.

Czech, M. P. (1989) *Cell* **59**, 235-238, Signal transmission by the insulin-like growth factors.

D'Ercole, A. J., and Wilkins, J. R. (1984) *Endocrinology* **114**, 1141-1144, Affinity labeled somatomedin-C-binding proteins in rat sera.

D'Ercole, A. J., Stiles, A. D. and Underwood, L. E. (1984) *Proc. Natl. Acad. Sci.* (USA) **81**, 935-939, Tissue concentrations of somatomedin C: Further evidence for multiple sites of synthesis and paracrine or autocrine mechanisms of action.

D'Ercole, A. J., Willson, D. F. and Underwood, L.E. (1980) *J. Clin. Endocrinol. Metab.* **51**, 674-676, Changes in the circulating form of serum somatomedin-C during fetal life.

D'Ercole, A. J., Hill, D. J., Strain, A. J. and Underwood, L. E. (1986) *Pediatr. Res.* **20**, 253-255, Tissue and plasma somatomedin-c/insulin-like growth factor I concentrations in the human fetus during the first half of gestation.

Ehrenborg, E., Vilhelmsdotter, S., Bajalica, S., Larsson, C., Stern, I., Koch, J., Brondum-Nielsen, K. and Luthman, H. (1991) *Biochem. Biophys. Res. Comm.* **176**, 1250-1255, Structure and localization of the human insulin-like growth factor-binding protein 2 gene.

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

Daughaday, W.H., Hall,K., Raber, M.S., Salmon, W.D., Jr., Van den Brande, J.L. and Van Wyk, J.J. (1972) *Nature* **235**, 107, Somatomedin: proposed designation for sulphation factor.

Daughaday, W. H., Hall, K., Salmon, W. D., Van den Brande, J. L. and Van Wyk, J. J. (1987) *J. Clin. Endocrinol. Metab.* **65**, 1075-1076, On the nomenclature of the somatomedins and insulin-like growth factors.

Daughaday, W. H., Parker, K. A., Borowsky, S., Trivedi, B. and Kapadia, M. (1982a) *Endocrinology* **110**, 575-581, Measurement of Somatomedin-related peptides in fetal, neonatal, and maternal rat serum by insulin-like growth factor (IGF) I radioimmunoassay, IGF-II radioreceptor assay (RRA), and multiplication-stimulating activity RRA after acid-ethanol extraction.

Daughaday, W. H., Ward, A. P., Goldberg, A. C., Trivedi, B. and Kapadia, M. (1982b) *J. Clin. Endocrinol. Metab.* **55**, 916-921, Characterization of somatomedin binding in human serum by ultracentrifugation and gel filtration.

Davenport, M. L., Clemmons, D. R., Miles, M. V., Camacho-Hubner, C., D'Ercole, A. J. and Underwood, L. E. (1990) *Endocrinology* **127**, 1278-1286, Regulation of serum insulin-like growth factor-I (IGF-I) and IGF binding proteins during rat pregnancy.

Dawe, S.R., Francis, G.L., McNamara, P.J., Wallace, J.C. and Ballard, F.J. (1988) *J. Endocrinol.* **117**, 173-181, Purification, partial sequences and properties of chicken insulin-like growth factors.

Dayhoff, M. O., Schwartz, R. M. and Ocrutt, B. C. (1978) *Atlas of Protein Sequence and Structure* , National Biomedical Research Foundation, Washington, DC, USA. 5th Edition.

De Mellow, J. S. and Baxter, R. C. (1988) *Biochem. Biophys. Res. Comm.* **156**, 199-204, Growth hormone-dependent insulin-like growth factor (IGF) binding protein both inhibits and potentiates IGF-I-stimulated DNA synthesis in human skin fibroblasts.

De Pablo, F., Scott, L. A. and Roth, J. (1990) *Endocrine Rev.* **11**, 558-577, Insulin and insulin-like growth factor I in early development: peptides, receptors and biological events.

De Vroede, M.A., Rechler, M.M., Nissley, S.P., Joshi, S., Burke, G.T. and Katsoyannis, P.G. (1985) *Proc. Natl. Acad. Sci.* (USA) **82**, 3010-3014, Hybrid molecules containing the B-domain of insulin-like growth factor I are recognized by carrier proteins of the growth factor.

De Vroede, M.A., Tseng, L.Y-H., Katsoyannis, P.G., Nissley, S.P., and Rechler, M.M. (1986) *J. Clin. Invest.* **77**, 602-613, Modulation of insulinlike growth factor I binding to human fibroblast monolayer cultures by insulinlike growth factor carrier proteins released to the incubation media.

Devereux, J., Haeberli, P. and Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387-395, A comprehensive set of sequence analysis programs for the VAX.

Donahue, L. R., Hunter, S. J., Sherblom, A. P. and Rosen, C. (1990) *J. Clin. Endocrinol. Metab.* **71**, 575-579, Age-related changes in serum insulin-like growth factor-binding proteins in women.

Donovan, S. M., Oh, Y., Pham, H. and Rosenfeld, R. G. (1989) *Endocrinology* **125**, 2621-2627, Ontogeny of serum insulin-like growth factor binding proteins in the rat.

Drop, S. L. S., Kortleve, D. J. and Guyda, H. J. (1984a) *J. Clin. Endocrinol. Metab.* **59**, 899-907, Isolation of a somatomedin-binding protein from preterm amniotic fluid. Development of a radioimmunoassay.

Drop, S. L. S., Kortleve, D. J., Guyda,H. J. and Posner, B. I. (1984b) *J. Clin. Endocrinol. Metab.* **59**, 908-915, Immunoassay of a somatomedin-binding protein from human amniotic fluid: Levels in fetal, neonatal, and adult sera.

Drop, S. L. S., Valiquette, G., Guyda, H. J., Corvol, M. T. and Posner, B. I. (1979) *Acta Endocrinol.* **90**, 505-518, Partial purification and characterization of a binding protein for insulin-like activity (ILAs) in human amniotic fluid: A possible inhibitor of insulin-like activity.

Dulak, N.C. and Temin, H.M. (1973) *J. Cell. Physiol.* **81**, 153-160, A partially purified polypeptide fraction from rat liver cell conditioned medium with multiplication-stimulating activity for embryo fibroblasts.

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

Elgin, R.G., Busby, W.H. Jr. and Clemmons, D.R. (1987) *Proc.Natl. Acad. Sci.* (USA) **84**, 3254-3258, An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I.

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

Ewton, D. Z., Fallen, S. L. and Florini, J. R. (1987) *Endocrinology* **120**, 115-123, The type II insulin-like growth factor (IGF) receptor has low affinity for IGF-I analogs: pleiotypic actions of IGFs on myoblasts are apparently mediated by the type 1 receptor.

Forbes, B., Ballard, F. J. and Wallace, J. C. (1990) *J. Endocrinol.* **126**, 497-506, An insulin-like growth factor-binding protein purified from medium conditioned by a human lung fibroblast cell line (He[39]L) has a novel N-terminal sequence.

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

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

Francis, G. L. McMurtry, J. P. Johnaon, R. J. and Ballard, F. J. (1990) *J. Endocrinol.* **124**, 361-370, Plasma clearance of chicken and human insulin-like growth factor-I and their association with circulating binding proteins in chickens.

Francis, G. L., McNamara, P. J., Filsell, O. H. and Ballard, F. J. (1988a) *J. Endocrinol.* **117**, 183-189, Plasma half-lives of native and modified insulin-like growth factor-I in lambs.

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

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

Francis, G. L., Ross, M., Brazier, J. A., Walton, P. E., Wallace, J. C. and Ballard, F. J. (1991a) *Proceedings 2nd International IGF Symposium*, San Francisco, California, January 12-16, Abstract A7.

Francis, G. L., Ross, M., Ballard, F. J., Milner, S. J., Senn, C., McNeil, K. M., Wallace, J. C., King, R. M. and Wells, J. R. E. (1991b) *J. Biol. Chem.* submitted for publication.

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

Froesch, E.R., Bürgi, H., Ramseier, E.B., Bally, P. and Labhart, A (1963) *J. Clin. Invest.* **42**, 1816-1834, Antibody-suppressible and nonsuppressible insulin-like activities in human serum and their physiologic significance. An insulin assay with adipose tissue of increased precision and specificity.

Froger-Gaillard, B., Hossenlopp, P., Adolphe, M. and Binoux, M. (1989) *Endocrinology* **124**, 2365-2372, Production of insulin-like growth factors and their binding proteins by rabbit articular chondrocytes: Relationships with cell multiplication.

Furlanetto, R.W. (1980) *J. Clin. Endocrinol. Metab.* **51**, 12-19, The somatomedin C binding protein: Evidence for a heterologous subunit structure.

Gargosky, S. E., Moyse, K. J., Walton, P. E., Owens, J. A., Wallace, J. C., Robinson, J. S. and Owens, P. C. (1990a) *Biochem. Biophys. Res. Comm.* **170**, 1157-1163, Circulating levels of insulin-like growth factors increase and molecular forms of their serum binding proteins change with human pregnancy.

Gargosky, S. E., Walton, P. E., Owens, P. C., Wallace, J. C. and Ballard, F. J. (1990b) *J. Endocrinol.* **127**, 183-290, Insulin-like growth factor-I (IGF-I) and IGF binding proteins both decline in the rat during late pregnancy.

Gargosky, S. E., Walton, P. E., Wallace, J. C. and Ballard, F. J. (1990c) *J. Endocrinol.* **127**, 391-400, Characterization of insulin-like growth factor-binding proteins in rat serum, lymph, cerebrospinal and amniotic fluids, and in media conditioned by liver, bone and muscle cells.

Gluckman, P. D. and Butler, J. H. (1983) *J. Endocrinol.* **99**, 223-232, Parturition-related changes in insulin-like growth factors-I and -II in the prenatal lamb.

Glassock, G. F., Gelber, S. E., Lamson, G., McGee-Tekula, R. and Rosenfeld, R. G. (1990) *Endocrinology* **127**, 1792-1803, Pituitary control of growth in the neonatal rat: effects of neonatal hypophysectomy on somatic and organ growth, serum insulin-like growth factors (IGD)-I and -II levels, and expression of IGF binding proteins.

Goad, W. B. and Kanehisa, M. (1982) *Nucleic Acids Res.* **10**, 247-263, Pattern recognition in nucleic acid sequences: A general method for finding local homologies and symmetries.

Goldberg, A. C., Trivedi, B., Delmez, J. A., Harter, H. R. and Daughaday, W. H. (1982) *J. Clin. Endocrinol. Metab.* **55**, 1040-1045, Uremia reduces serum insulin-like growth factor I, increases insulin-like growth factor II, and modifies their serum protein binding.

Gopinath, R., Walton, P. E. and Etherton, T. D. (1989) *J. Endocrinol.* **120**, 231-236, An acid stable insulin-like growth factor (IGF)-binding protein from pig serum inhibits binding of IGF-I and IGF-II to vascular endothelial cells.

Gourmelen, M., Girard, F. and Binoux, M. (1982) *J. Clin. Endocrinol. Metab.* **54**, 885-891, Serum somatomedin/insulin-like growth factor (IGF) and IGF carrier levels in patient with Cushing's syndrome or receiving glucocorticoid therapy.

Grant, M. B., Russell, B., Harwood, H. J. and Merimee, T. J. (1987) *J. Clin. Endocrinol. Metab.* **64**, 1060-1065, Separation of the insulin-like growth factor-binding proteins in plasma and purification of the larger molecular weight species.

Guidice, L. C., Farrell, E. M., Pham, H., Lamson, G., Rosenfeld, R. G. (1990) *J. Clin. Endocrinol. Metab.* **71**, 806-816, Insulin-like growth factor binding proteins in maternal serum throughout gestation and in the puerperium: Effects of a pregnancy-associated serum protease activity.

Guidice, L. C., Milkowski, D. A., Lamson, G., Rosenfeld, R. G. and Irwin, J. C. (1991) *J. Clin. Endocrinol. Metab.* **72**, 779-787, Insulin-like growth factor binding proteins in human endometrium: steroid-dependent messenger ribonucleic acid expression and protein synthesis.

Guler, H-P., Scmid, C., Zapf, J. and Froesch, E. R. (1989) *Proc. Natl. Acad. Sci.* (USA) **86**, 2868-2872, Effects of recombinant insulin-like growth factor I on insulin secretion and renal function in normal human subjects.

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

Hammond, G. L. (1990) *Endocrine Reviews* **11**, 65-79, Molecular properties of corticosteroid binding globulin and the sex-steroid binding proteins.

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

Han, V. K. M., Lund, P. K., Lee, D. D. and D'Ercole, A. J. (1988) *J. Clin. Endocrinol. Metab.* **66**, 422-429, Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution.

Hardouin, S., Gourmelen, M., Nguiez, P., Seurin, D., Roghani, M., Le Bouc, Y., Povoa, G., Merimee, T. J., Hossenlopp, P., and Binoux, M. (1989) *J. Clin. Endocrinol. Metab.* **69**, 1291-1301, Molecular forms of serum insulin-like growth factor (IGF)-binding proteins in man: relationships with growth hormone and IGFs and physiological significance.

Hardouin, S., Hossenlopp, P., Segovia, B., Seurin, D., Portolan, G., Lassarre, C. and Binoux, M. (1987) *Eur. J. Biochem.* **170**, 121-132, Heterogeneity of insulin-like growth factor binding proteins and relationships between structure and affinity 1. Circulating forms in man.

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

Heldin, C-K. and Westermark, B. (1989) *Eur. J. Biochem.* **184**, 487-496, Growth factors as transforming proteins.

Hepler, J. E., Van Wyk, J. J. and Lund, P. K. (1990) *Endocrinology* **127**, 1550-1552, Different half-lives of insulin-like growth factor I mRNAs that differ in length of 3' untranslated sequence.

Herington, A. C. and Kuffer, A. D. (1981) *Endocrinology* **109**, 1634-1640, Identification of a specific inhibitor of nonsuppressible insulin-like activity in a partially purified human serum fraction.

Heueshoven, J. and Dernick, R. (1985) *Electrophoresis* **6**, 103-112, Simplified method for silver staining of proteins in polyacrylamide gels and the mechanisms of silver staining.

Heyner, S., Smith, R. M. and Schultz, G. A. (1989) *Bioessays* **11**, 171-175, Temporally regulated expression of insulin and insulin-like growth factors and their receptors in early mammalian development.

Hill, D. J., Clemmons, D. R., Wilson, S., Han, V. K. M., Strain, A. J. and Milner, R. D. G. (1989) *J. Mol. Endocrinol.* **2**, 31-38, Immunological distribution of one form of insulin-like growth factor (IGF)-binding protein and IGF peptides in human fetal tissues.

Hintz, R. L. and Liu, F. (1976) *J. Clin. Enocrinol. Metab.* **45**, 988-995, Demonstration of specific plasma protein binding sites for somatomedin.

Hizuka, N., Takano, KK., Shizume, K., Asakawa, K., Miyakawa, M., Tanaka, I. and Horikawa, R. (1986) *Eur. J.Pharmacol.* **125**, 143-146, Insulin-like growth factor I stimulates growth in normal growing rats.

Hodgkinson, S. C., Moore, L., Napier, J. R., Davis, S. R., Bass, J. J. and Gluckman, P. D. (1989) *J. Endocrinol.* **120**, 429-438, Characterization of insulin-like growth factor binding proteins in ovine tissue fluids.

Holly, J. M. P., Davies, S. C., Cotterill, A. M., Coulson, V. J., Ross, R. J. M., Miell, J. P., Abdulla, A. F. and Wass, J. A. H. (1991) *Proceedings 2nd International IGF Symposium*, San Francisco, California, January 12-16, Abstract C48.

Honegger, A. and Humbel, R. E. (1986) *J. Biol. Chem.* **261**, 569-575, Insulin-like growth factors I and II in fetal and adult bovine serum. Purification, primary structures, and immunological cross-reactivities.

Hossenlopp, P., Segovia, B., Lassarre, C., Roghani, M., Bredon, M. and Binoux, M. (1990) *J. Clin. Endocrinol. Metab.* **71**, 797-805, Evidence of enzymatic degradation of insulin-like growth factor-binding proteins in the 150K complex during pregnancy.

Hossenlopp, P., Seurin, D., Segovia-Quinson, B., Hardouin, S. and Binoux, M. (1986) *Anal. Biochem.* **154**, 138-143, Analysis of serum insulin-like growth factor binding proteins using Western blotting: Use of the method for titration of the binding proteins and competitive binding studies.

Hossenlopp, P., Seurin, D., Segovia, B., Portolan, G. and Binoux, M. (1987) *Eur. J. Biochem.* **170**, 133-142, Heterogeneity of insulin-like growth factor binding proteins between structure and affinity 2. Forms released by human and rat liver in culture.

Hossner, K. L., Link, G. A. and Yemm, R. S. (1988) *J. Anim. Sci.* **66**, 1401-1408, Comparison of insulin-like growth factor I serum binding proteins in sheep and cattle: Species differences in size and endogenous binding capacities.

Hoyt, E. C., Van Wyk, J. J. and Lund, P. K. (1988) *Mol. Endocrinol.* **2**, 1077-1086, Tissue and development specific regulation of a complex family of rat insulin-like growth factor I messenger ribonucleic acids.

Huhtala, M.-L., Koistinen, R., Palomaki, P., Partanen, P., Bohn, H. and Seppala, M. (1986) *Biochem. Biophys. Res. Comm.* **141**, 263-270, Biologically active domain in somatomedin-binding protein.

Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J. and Hood, L. E. (1983) *Methods Enzymol.* **91**, 399-413, High sensitivity sequencing with a gas-phase sequenator.

Humbel, R. E. (1990) *Eur. J. Biochem.* **190**, 445-462, Insulin-like growth factors I and II.

Hykla, V. W., Teplow, D. B., Kent, S. B. H. and Straus, D. S. (1985) *J. Biol. Chem.* **260**, 14417-14420, Identification of a peptide fragment from the carboxyl-terminal extension region (E domain) of rat proinsulin-like growth factor-II.

Innis, M. A. and Gelfand, D. H. (1990) Optimization of PCRs. In *PCR protocols. A guide to methods and applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (Editors), Academic Press, Inc., San Diego, CA, USA, pp3-12.

Innis, M. A., Myambo, K. B., Gelfand, D. H. and Brow, M. A. D. (1988) *Proc. Natl. Acad. Sci.* (USA) **85**, 9436-9440, DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplified DNA.

Jacobs, S., Kull, F.C. Jr., Earp, H.S., Svoboda, M.E., Van Wyk, J.J. and Cuatrecasas, P. (1983) *J. Biol. Chem.* **58**, 9581-9584, Somatomedin-C stimulates the phosporylation of the β-subunit of its own receptor.

Jakob, A., Hauri, C. and Froesch, E. R. (1968) *J. Clin. Invest.* **47**, 2678-2688, Nonsuppressible insulin-like activity in human serum. III. Differentiation of two distinct molecules with nonsuppressible ILA.

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

Julkunen, M., Koistinen, R., Aalto-Setala, K., Seppala, M., Janne, O. A. and Kontula, K. (1988) *FEBS lett.* **236**, 295-302, Primary structure of human insulin-like growth factor-binding protein/placental protein 12 and tissue-specific expression of its mRNA.

Julkunen, M., Koistinen, R., Suikkari, A-M., Seppala, M. and Janne, O. A. (1990) *Mol. Endocrinol.* **4**, 700-707, Identification by hybridization histochemistry of human endometrial cells expressing mRNAs encoding a uterine β-lactoglobulin homologue and insulin-like growth factor-binding protein-1.

Kajimoto, Y. and Rotwein, P. (1989) *Mol. Endocrinol.* **3**, 1907-1913, Structure and expression of a chicken insulin-like growth factor I precursor.

Kajimoto, Y. and Rotwein, P. (1990) *Mol. Endocrinol.* **4**, 217-226, Evolution of insulin-like growth factor I (IGF-I): structure and expression of an IGF-I precursor from *Xenopus laevis.*

Kallincos, N. C., Wallace, J. C., Francis G. L. and Ballard, F. J. (1990) *J. Endocrinol.* **124**, 89-97, Chemical and biological characterization of chicken insulin-like growth factor-II.

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

Kaufmann, U., Zapf, J., Torretti, B. and Froesch, E. R. (1977) *J. Clin. Endocrinol. Metab.* **44**, 160-166, Demonstration of a specific serum carrier protein of nonsuppressible insulin-like activity in vivo.

Kawasaki, E. S. (1990) Amplification of RNA. In *PCR protocols. A guide to methods and applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky and T. J. White (Editors), Academic Press, Inc., San Diego, CA, USA, pp 21-27.

Kiefer, M. C., Ioh, R. S., Bauer, D. M. and Zapf, J. (1991) *Biochem. Biophys. Res. Comm.* **176**, 219-225, Molecular cloning of a new human insulin-like growth factor binding protein.

Knauer, D. J. and Smith, G. L. (1980) *Proc. Natl. Acad. Sci.* (USA) **77**, 7252-7256, Inhibition of biological activity of multiplication-stimulating activity by binding to its carrier protein.

Knoth, K., Roberts, S., Poteet, C. and Tamkun, M. (1988) *Nucleic Acids Res.* **16**, 10932, Highly degenerate, inosine containing primers specifically amplify rare cDNA using the polymerase chain reaction.

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

Kurtz, A., Haerti, W., Jelkmann, W., Zapf, J. and Bauer, C. (1985) *J. Clin. Invest.* **76**, 1643-1648, Activity in fetal bovine serum that stimulates erythroid colony formation in fetal mouse livers is insulinlike growth factor I.

Kurtz, A., Zapf, J., Eckardt, K-U., Clemmons, G., Froesch, E. R. and Bauer, C. (1988) *Proc. Natl. Acad. Sci.* (USA) **85**, 7825-7829, Insulin-like growth factor I stimulates erythropoiesis in hypophysectomized rats.

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

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

Laureys, G., Barton, D. E., Ullrich, A. and Franke, U. (1988) *Genomics* **3**, 224-229, Chromosomal mapping of the gene for the type II insulin-like growth factor receptor/cation-independent mannose 6-phophate receptor in man and mouse.

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

Lemmey, A. B., Martin, A. A., Read, L. C., Tomas, F. M., Owens, P. C. and Ballard, F. J. (1991) *Am. J. Physiol.* **260**, 213-E219, IGF-I and the truncated analogue des-(1-3)-IGF-1 enhance growth in rats after gut resection.

Lewitt, M. S. and Baxter, R. C. (1989) *J. Clin. Endocrinol. Metab.* **69**, 246-252, Regulation of growth hormone-independent insulin-like growth factor-binding protein (BP-28) in cultured human fetal liver explants.

Lineham, J. D. Smith, R. M., Dahlenburg, G. W., King, R. A., Haslam, R. R., Stuart, M. C. and Faull, L. (1986) *Early Human Develop.* **13**, 37-46, Circulating insulin-like growth factor I levels in newborn premature and full-term infants followed longitudinally.

Lobel, P., Dahms, N.M. and Kornfeld, S. (1988) *J. Biol. Chem.* **63**, 2563-2570, Cloning and sequence analysis of the cation-independent mannose 6-phospate.

Loeb, J. N. (1976) *N. Engl. J. Med.* **295**, 547-552, Corticosteroids and growth.

Lord, A. P. D., Martin, A. A., Walton, P. E., Ballard, F. J. and Read, L. C. (1991) *J. Endocrinol.* **129**, 59-68, Insulin-like growth factor binding proteins in tissue fluids from the lamb.

Luo, J., Reid, R.E. and Murphy, L. J. (1990) *Endocrinology* **127**, 1456-1462, Dexamethasone increases hepatic insulin-like growth factor binding protein-1 (IGFBP-1) mRNA and serum IGFBP-1 concentrations in the rat.

Luthman, H., Soderling-Barros, J., Persson, B., Engerg, C., Stern, I., Lake, M., Franzen, S., Israelsson, M., Raden, B., Hjelmqvist, L., Enerback, S., Carlsson, P., Bjursell, G., Povoa, G., Hall, K. and Jornvall, H. (1989) *Eur. J. Biochem.* **180,** 259-265, Human insulin-like growth factor-binding protein. Low-molecular-mass form: protein sequence and cDNA cloning.

Lyons, R. M. and Smith, G. L. (1986) *Mol. Cell. Endocrinol.* **45**, 263-270, Characterization of multiplication-stimulating activity (MSA) carrier protein.

Maiter, D., Fliesen, T., Underwood, L. E., Maes, M., Gerard, G., Davenport, M. L. and Ketelslegers, J. M. (1989) *Endocrinology* **124**, 2604-2611, Dietary protein restriction decreases insulin-like growth factor I independent of insulin and liver growth hormone binding.

Maiter, D., Maes, M., Underwood, L. E., Fliesen, T., Gerard, G. and Ketelslegers, J.-M. (1988) *J. Endocrinol.* **118**, 113-120, Early changes in serum concentrations of somatomedin-C induced by dietary protein deprivation in rats: contributions of growth hormone receptor and post-receptor defects.

Margot, J. B., Binkert, C., Landwehr, J., Heinrich, G. and Schwander, J. (1989) *Mol. Endocrinol.* **3**, 1053-1060, A low molecular weight insulin-like growth factor binding protein from rat: cDNA cloning and tissue distribution of its messenger RNA.

Marquardt, H., Todaro, G.J., Henderson, L.E. and Oroszlan, (1981) *J. Biol. Chem.* **256**, 6859-6865, Purification and primary structure of a polypeptide with multiplication-stimulating activity from rat liver cell cultures.

Marshall, R. D. (1972) *Ann. Rev. Biochem.* **41**, 673-702, Glycoproteins.

Martin, J. L. and Baxter, R. C. (1985) *J. Clin. Endocrinol. Metab.* **61**, 799-801, Antibody against acid-stable insulin-like growth factor binding protein detects 150,000 MOL WT growth hormone-dependent complex in human plasma.

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

Martin, J. L. and Baxter, R. C. (1988) *Endocrinology* **123**, 1907-1915, Insulin-like growth factor-binding protein (IGF-BPs) produced by human skin fibroblasts: Immunological relationship to other human IGF-BPs.

Martin, J. L. and Baxter, R. C. (1990) *Endocrinology* **127**, 781-788, Production of an insulin-like growth factor (IGF)-inducible IGF-binding protein by human skin fibroblasts.

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

Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035-10038, Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes.

McCusker, R. H. and Clemmons, D. R. (1988) *J. Cell. Physiol.* **137**, 505-512, Insulin-like growth factor binding protein secretion by muscle cells: Effect of cellular differentiation and proliferation.

McCusker, R. H., Campion, D. R. and Clemmons, D. R. (1988) *Endocrinology* **122**, 2071-2079, The ontogeny and regulation of a 31,000 molecular weight insulin-like growth factor-binding protein in fetal porcine plasma and sera.

McCusker, R. H., Camacho-Hubner, C., Bayne, M. L., Cascieri, M. A. and Clemmons, D. R. (1990) *J. Cell. Physiol.* **144**, 244-253, Insulin-like growth factor (IGF) binding to human fibroblast and glioblastoma cells: The modulating effect of cell released IGF binding proteins (IGFBPs).

McMorris, F. A., Smith, T. M., DeSalvo, S. and Furnaletto, R. W. (1986) *Proc. Natl. Acad. Sci* (USA) **83**, 822-826, Insulin-like growth factor I/somatomedin C: A potent inducer of oligodendrocyte development.

Merimee, T. J., Zapf, J. and Froesch, E. R. (1982) *J. Clin. Endocrinol. Metab.* **55**, 999-1002, Insulin-like growth factors in the fed and fasted states.

Merimee, T. J., Grant, M. and Tyson, E. (1984) *J. Clin. Endocrinol. Metab.* **59**, 752-755, Insulin-like growth factors in amniotic fluid.

Mesiano, S., Young I. R., Browne, C. A. and Thornburn, G. D. (1988) *J. Endocrinol.* **119**, 453-460, Failure of acid-ethanol treatment to prevent interference by binding proteins in radioligand assays for the insulin-like growth factors.

Meuli, C., Zapf, J. and Froesch, E. R. (1978) *Diabetologia* **14**, 255-259, NISLA-carrier protein abolishes the action of nonsuppressible insulin-like activity (NSILA-S) on perfused rat heart.

Miyada, C. G. and Wallace, R. B. (1987) *Methods Enzymol.* **154**, 94-107, Oligonucleotide hybridization techniques.

Mohan, S., Bautista, C. M., Wergedal, J. and Baylink, D. J. (1989) *Proc. Natl. Acad. Sci. (USA)* **86**, 8338-8342, Isolation of an inhibitory insulin-like growth factor (IGF) binding protein from bone cell-conditioned medium: A potential local regulator of IGF action.

Mondschein, J. S., Smith, S. A. and Hammond, J. M. (1990) *Endocrinology* **127**, 2298-2306, Production of insulin-like growth factor binding proteins (IGFBPs) by porcine granulosa cells: identification of IGFBP-2 and -3 and regulation by hormones and growth factors.

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

Moses, A. C., Nissley, S. P. and Cohen, K. L. (1976) *Nature* **263**, 137-141, Specific binding of a somatomedin-like polypeptide in rat serum depends on growth hormone.

Moses, A. C., Cohen, K. L., Johnsonbaugh, R. and Nissley, S. P. (1978) *J. Clin. Endocrinol. Metab.* **46**, 937-946, Contribution of human somatomedin activity to the serum growth requirement of human skin fibroblasts and chick embryo fibroblasts in culture.

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

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

Moss, J. A., Francis, G. L., Ross, M., Wallace, J. C. and Ballard, F. J. (1991) *J. Biol. Chem.* **266**, 909-914, Insulin-like growth factor (IGF)-I and IGF-II binding to an IGF binding protein. An investigation using chemical modification of tyrosine residues as a structural probe for the sites of interaction.

Mottola, C. and Czech, M. P. (1984) *J. Biol. Chem.* **259**, 12705-12713, The type II insulin-like growth factor receptor does not mediate increased DNA synthesis in H-35 hepatoma cells.

Mottola, C., MacDonald, R. G., Brackett, J. L., Mole, J. E., Anderson, J. K. and Czech, M. P. (1986) *J. Biol. Chem.* **61**, 11180-11188, Purification and amino-terminal sequence of an insulin-like growth factor-binding protein secreted by rat liver BRL-3A cells.

Mullis, K. B. and Faloona, F. A. (1987) *Methods Enzymol.* **155**, 335-350, Specific synthesis of DNA *in vitro* via a polymerase-catalyzed chain reaction.

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

Murphy, L. J. Murphy, L. C. and Friesen, H. G. (1987b) *Mol. Endocrinol.* **1**, 445-450, Estrogen induces insulin-like growth factor-I expression in the rat uterus.

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

Nagamatsu, S., Chan, S. J., Falkmer S. and Steiner D. F. (1991) *J. Biol. Chem.* **266**, 2397-2402, Evolution of the insulin gene superfamily. Sequence of a preproinsulin-like growth factor cDNA from the atlantic hagfish.

Ocrant, I., Pham, H., Oh, Y. and Rosenfeld, R. G. (1989) *Biochem. Biophys. Res. Comm.* **159**, 1316-1322, Characterization of insulin-like growth factor binding proteins of cultured rat astroglial and neuronal cells.

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

Ooi, G. T. and Herington, A. C. (1986) *Biochem. Biophys. Res. Comm.* **137**, 411-417, Covalent cross-linking of insulin-like growth factor-I to a specific inhibitor from human serum.

Ooi, G. T. and Herington, A. C. (1990) *Biochem. J.* **260**, 615-620, Recognition of insulin-like-growth-factor-binding proteins in serum and amniotic fluid by an antiserum against a low-molecular-mass insulin-like-growth-factor / binding protein.

Ooi, G. T., Aguilar, M., Hearn, M. T. W. and Herington, A. C. (1989) Relationship of a low molecular weight (16k) IGF-inhibitor/binding protein to the native IGF-binding proteins of human serum. In *Insulin-like growth factor binding proteins.* Drop, S. L. S. and Hintz, R. L. (Editors) Elsevier, N.Y., USA, pp 231-238.

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

Orlowski, C. C., Ooi, G. T. and Rechler, M. M. (1990b) *Mol. Endocrinol.* **4**, 1592-1599, Dexamethasone stimulates transcription of the insulin-like growth factor-binding protein-1 gene in H4-II-E rat hepatoma cells.

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

Owens, P. C., Johnson, R. J., Cambell, R. G. and Ballard, F. J. (1990) *J. Endocrinol.* **124**, 269-275, Growth hormone increases insulin-like growth factor-I (IGF-I) and decreases IGF-II in plasma of growing pigs.

Patil, R. V. and Dekker, E. E. (1989) *Nucleic Acids Res.* **18**, 3080, PCR amplification of an *Escherichia coli* gene using mixed primers containing deoxyinosine at ambiguous positions in degenerate amino acid codons.

Pearson, W. R. and Lipman, D. J. (1988) *Proc. Natl. Acad. Sci.* (USA) **85**, 2444-2448, Improved tools for biological sequence analysis.

Penhoat, A., Naville, N., Jaillard, C., Chatelain, P. G. and Saez, J. M. (1989) *J. Biol. Chem.* **264**, 6858-6862, Hormonal regulation of insulin-like growth factor I secretion by bovine adrenal cells.

Philipps, A. F., Persson, B., Hall, K., Lake, M., Skottner, A., Sanengen, T. and Sara, V. R. (1988) *Pediatr. Res.* **23**, 298-305, The effects of biosynthetic insulin-like growth factor-I supplementation on somatic growth, maturation, and erythropoiesis on the neonatal rat.

Pinzani, M., Abboud, H. E. and Aron, D. C. (1990) *Endocrinology* **127**, 2343-2349, Secretion of insulin-like growth factor-I and binding proteins by rat liver fat-storing cells: Regulatory role of platelet-derived growth factor.

Povoa, G., Roovete, A. and Hall, K. (1984b) *Acta Endocrinol.* **107**, 563-570, Cross-reaction of serum somatomedin-binding protein isolated from human amniotic fluid.

Povoa, G., Enberg, G., Jörnvall, H. and Hall, K. (1984a) *Eur. J. Biochem.* **44**, 199-204, Isolation and characterization of a somatomedin-binding protein from mid-term human amniotic fluid.

Povoa, G., Isaksson, M., Jörnvall, H. and Hall, K. (1985) *Biochem. Biophys. Res. Comm.* **128**, 1071-1078, The somatomedin-binding protein isolated from a human hepatoma cell line is identical to the human amniotic fluid somatomedin-binding protein.

Powell, D. R., Suwanichkul, A. and Cubbage, M. L. (1989) The 25 kilodalton insulin-like growth factor binding protein: Analysis of chromosomal gene structure and demonstration of promotor activity. In *Insulin-like growth factor binding proteins.* Drop. S. L. S. and Hintz, R. L. (Editors) Elsevier, N.Y., USA, pp 3-10, .

Read, L.C., Ballard, F.J., Francis, G.L., Baxter, R.C., Bagley, C.J. and Wallace, J.C. (1986) *Biochem. J.* **233**, 215-221, Comparative binding of bovine, human and rat insulin-like growth factors to membrane receptors and to antibodies against human insulin-like growth factor-1.

Rechler, M. M. and Nissley, S. P. (1985) *Ann. Rev. Physiol.* **47**, 425-442, The nature and regulation of the receptors for insulin-like growth factors.

Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. E. and Humbel, R. E. (1980) *Endocrinology* **107**, 1451-1459, Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins.

Rinderknecht, E. and Humbel, R.E. (1976a) *Proc. Natl. Acad. Sci.* (USA) **73**, 2365-2369, Polypeptides with nonsuppressible insulin-like and cell-growth promoting activities in human serum: Isolation, chemical characterization and some biological properties of forms I and II.

Rinderknecht, E. and Humbel, R.E. (1976b) *Proc. Natl. Acad. Sci.* (USA) **73**, 4379-4381, Amino-terminal sequences of two polypeptides from human serum with nonsuppressible insulin-like and cell-growth-promoting activities: Evidence for structural homology with insulin B chain.

Rinderknecht, E. and Humbel, R.E. (1978a) *J. Biol. Chem.* **253**, 2769-2776, The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin.

Rinderknecht, E. and Humbel, R.E. (1978b) *FEBS Lett.* **89**, 283-286, Primary structure of human insulin-like growth factor II.

Ritvos, O., Ranta, T., Jalkanen, J., Suikkari, A-M., Voultainen, R., Bohn, H. and Rutanen. E-M. (1988) *Endocrinology* **122**, 2150-2157, Insulin-like growth factor (IGF) binding protein from human decidua inhibits the binding and biological action of IGF-I in cultured choriocarcinoma cells.

Roberts, C. T. Jr., Lasky, S. R., Lowe, W. L., Seaman, W. T. and LeRoith, D. (1987) *Mol. Endocrinol.* **1**, 243-248, Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids: Differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues.

Roghani, M., Hossenlopp, P., Lepage, P., Balland, A. and Binoux, M. (1989) *FEBS Lett.*, **55**, 253-258, Isolation from human cerebrospinal fluid of a new insulin-like growth factor-binding protein with a selective affinity for IGF-II.

Romanus, J. A., Tseng, L. Y.-H., Yang, Y. W.-H. and Rechler, M. M. (1989) *Biochem. Biophys. Res. Comm.* **163**, 875-881, The 34 kilodalton insulin-like growth factor binding proteins in human cerebrospinal fluid and the A673 rhabdomyosarcoma cell line are human homologues of the rat BRL-3A binding protein.

Romanus, J. A., Yang, Y. W.-H., Nissley, S. P. and Rechler, M. M. (1987) *Endocrinology* **121**, 1041-1050, Biosynthesis of the low molecular weight carrier protein for insulin-like growth factors in rat liver and fibroblasts.

Romanus, J. A., Terrel, J. E., Yang, Y. W.-H., Nissley, S. P. and Rechler, M. M. (1986) *Endocrinology* **118**, 1743-1758, Insulin-like growth factor carrier proteins in neonatal and adult rat serum are immunologically different: demonstration using a new radioimmunoassay for the carrier protein from BRL-3A rat liver cells.

Rosenfeld, R. G. Pham, H., Conover, C. A., Hintz, R. L. and Baxter, R. C. (1989) *J. Clin. Endocrinol. Metab.* **68**, 638-646, Structural and immunological comparison of insulin-like growth factor binding proteins of cerebrospinal and amniotic fluids.

Rosenfeld, R. G., Pham, H., Oh, Y., Lamson, G. and Guidice, L. C. (1990) *J. Clin. Endocrinol. Metab.* **70**, 551-553, Identification of insulin-like growth factor-binding protein-2 (IGFBP-2) and a low molecular weight IGFBP in human seminal plasma.

Rosenfeld R. G., Wilson D. M., Lee P. D. K., Hintz R. L. (1986) *J. Pediatr.* **109**, 428-433, Insulin-like growth factors I and II in evaluation of growth retardation.

Ross, R., Raines, E. W. and Bowen-Pope, D. F. (1986) *Cell* **46**, 155-169, The biology of platelet-derived growth factor.

Ross, M., Francis, G. L., Szabo, L., Wallace, J. C. and Ballard, F. J. (1989) *Biochem. J.* **258**, 267-272, Insulin-like growth (IGF) -binding proteins inhibit the biological activities of IGF-1 and IGF-2 but not des-(1-3) -IGF-1.

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

Rotwein, P., Pollock, K.M., Didier, D.K. and Krivi, G.G. (1986) *J. Biol. Chem.* **261**, 4828-4832, Organization and sequence of the human insulin-like growth factor I gene. Alternative RNA processing produces two insulin-like growth factor I precursor peptides.

Rozengurt, E. (1986) *Science* **234**, 161-166, Early signals in the mitogenic response.

Ruoslahti, E. and Pierschbacher, M. D. (1986) *Cell* **44**, 517-518, Arg-Gly-Asp: A versatile cell recognition signal.

Rutanen, E-M., Pekonen, F. and Makinen, T. (1988) *J. Clin. Endocrinol. Metab.* **66**, 173-180, Soluble 34 K binding protein inhibits the binding of insulin-like growth factor I to its cell receptors in human secretory phase endometrium: Evidence for autocrine/paracrine regulation of growth factor action.

Rutanen, E-M., Koistinen, R., Wahlstrom, T., Bohn, H., Ranta, T. and Seppala, M. (1985) *Endocrinology* **116**, 1304-1309, Synthesis of placental protein 12 by human decidua.

Saiki, R. K., Scharf, S., Faloona, F., Mullis, K. B., Horn, G. T., Erlich, H. A. and Arnheim, N. (1985) *Science* **230**, 1350-1354, Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia.

Salmon, W.D., Jr. and Daughaday, W.H. (1957) *J. Lab. Clin. Med.* **48**, 825-836, A hormonally controlled serum factor which stimulates sulfate incorporation by cartilage in vitro.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *A laboratory manual*, Molecular Cloning 2nd Edition, Cold Spring Harbour Laboratory, Cold Spring Harbour N.Y., USA.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci.* (USA) **74**, 5463-5467, DNA sequencing with chain terminating inhibitors.

Sara, V. R. and Hall, K. (1990) *Physiological Reviews* **70**, 591-614, Insulin-like growth factors and their binding proteins.

Sara, V.R., Carlsson-Skwirut, C., Andersson, C., Hall, E., Sjögren, B., Holmgren, A. and Jörnvall, H. (1986) *Proc. Natl. Acad. Sci.* (USA) **83**, 4904-4907, Characterization of somatomedins from human fetal brain: Identification of a variant form of insulin-like growth factor I.

Schalch, D. S., Heinrich, U. E., Koch, J. G., Johnson, C. J and Schlueter, R. J. (1978) *J. Clin. Endocrinol. Metab.* **46**, 664-671, Nonsuppressible insulin-like activity (NSILA). I. Development of a new sensitive competitive protein-binding assay for determination of serum levels.

Schmid, C., Steiner, T. and Froesch, E. R. (1983) *FEBS Lett.* **161**, 117-121, Preferential enhancement of myoblast differentiation by insulin-like growth factors (IGF I and IGF II) in primary cultures of chicken embryonic cells.

Schmid, C., Steiner, T. and Froesch, E. R. (1984) *FEBS Lett.* **173**, 48-52, Insulin-like growth factor I supports differentiation of cultured osteoblast-like cells.

Schmid, C., Ernst, M., Zapf, J. and Froesch, E. R. (1989) *Biochem. Biophys. Res. Comm.* **160**, 788-794, Release of insulin-like growth factor carrier proteins by osteoblasts: Stimulation by estradiol and growth hormone.

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

Seneviratne, C., Luo, J. and Murphy, L. J. (1990) *Mol. Endocrinol.* **4**, 1199-1204, Transcriptional regulation of rat insulin-like growth factor-binding protein-1 expression by growth hormone.

Shimasaki, S., Gao, L., Shimonaka, M. and Ling, N. (1991a) *Mol. Endocrinol.* Isolation and molecular cloning of insulin-like growth factor-binding protein-6 (IGFBP-6). In press.

Shimasaki, S., Koba, A., Mercado, M., Shimonaka, M. and Ling, N. (1989) *Biochem. Biophys. Res. Comm.* **165**, 907-912, Complementary DNA Structure of the High Molecular Weight Rat Insulin-Like Growth Factor Binding Protein (IGF-BP3) and Tissue Distribution of Its Messenger RNA

Shimasaki, S., Shimonaka, M., Ui, M., Inouye, S., Shibata, F. and Ling, N. (1990a) *J. Biol. Chem.* **265**, 2198-2202, Structural characterization of a follicle-stimulating hormone action inhibitor in porcine ovarian follicular fluid. Its identification as the insulin-like growth factor-binding protein.

Shimasaki, S., Shimonaka, M., Bicsak, T. and Ling, N. (1991b) *Proceedings 2nd International IGF Symposium*, San Francisco, California, January 12-16, Abstract C54.

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

Shimonaka, M., Schroeder,R., Shimasaki, S. and Ling, N. (1989) *Biochem. Biophys. Res. Comm.* **165**, 189-195, Identification of a novel binding protein for insulin-like growth factors in adult rat serum.

Simes, J. M., Wallace, J. C. and Walton, P. E. (1991) *J. Endocrinol.* **130**, 909-914, The effects of insulin-like growth factor-I (IGF-I), (IGF-II) and des-(1-3)-IGF-1, a potent IGF analogue, on growth hormone and IGF-binding protein secretion from cultured rat anterior pituitary cells.

Smith, E. P., Dickson, B. A. and Chernausek, S. D. (1990) *Endocrinology* **127**, 2744-2751, Insulin-like growth factor binding protein-3 secretion from cultured rat sertoli cells: dual recognition by follicle stimulating hormone and insulin-like growth factor-I.

Smith, E. P., Svoboda, M. E., Van Wyk, J. J., Kierszenbaum, A. L. and Tres, L. L. (1987) *Endocrinology* **120**, 186-193, Partial characterization of a somatomedin-like peptide from the medium of cultured rat sertoli cells.

Sommer, R. and Tauz, D. (1989) *Nucleic Acids Res.* **17**, 6749, Minimal homology requirements for PCR primers.

Sommer, A., Maak, C. A., Spratt, S. K., Mascarenhas, D., Tressel, T. J., Rhodes, E. T., Lee, R., Roumas, M., Tatsuno, G. P., Flynn, J. A., Gerber, N., Taylor, J., Cundy, H., Nannet, L., Hunt, T. K. and Spencer, E. M. (1991) Molecular genetics and actions of recombinant insulin-like growth factor binding protein-3. In *Modern concepts of insulin-like growth factors*, E. M. Spencer (Editor), Elsevier, N.Y. USA, pp 715-728.

Spratt, S. K., Tatsuno, G. P., Yamanaka, M. K., Ark, B. C., Detmer, J., Mascarenhas, D., Flynn, J., Talkington-Verser, C. and Spencer, E. M. (1990) *Growth Factors* **3**, 63-72, Cloning and expression of human insulin-like growth factor binding protein 3.

Staden, R. (1982) *Nucleic Acids Res.* **10**, 2951-2961, An interactive graphics program for comparing and aligning nucleic acid and amino acid sequences.

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

Straus, D. S. and Takemoto, C. D. (1988) *J. Biol. Chem.* **263**, 18404-18410, Amino acid limitation negatively regulates insulin-like growth factor-II mRNA levels and E-domain peptide secretion at a post-transcriptional step in BRL-3A rat liver cells.

Straus, D. S. and Takemoto, C. D. (1990) *Endocrinology* **127**, 1849-1860, Effect of dietary protein deprivation on insulin-like growth factor (IGF) -I and -II, binding protein-2, and serum albumin gene expression in rat.

Suikkari, A-M. (1989) *Eur. J. Obstet. Gynecol. Reprod. Biol.* **30**, 19-25, Insulin-like growth factor (IGF) and its low molecular weight binding protein in human milk.

Suikkari, A-M., Koivisto, V. A., Rutanen, E-M., Yki-Järvinen, H., Karonen, S-L. and Seppälä, M. (1988) *J. Clin. Endocrinol. Metab.* **66**, 266-272, Insulin regulates the serum levels of low molecular weight insulin-like growth factor-binding protein.

Sussenbach, J. S. (1989) *Prog. Growth Factor Res.* **1**, 33-40, The gene structure of the insulin-like growth factor family.

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

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

Travvakol, A., Simmen, F.A. and Simmen, R.C.M. (1988) *Mol. Endocrinol.* **2**, 674-681, Porcine insulin-like growth factor-I (pIGF-I): complementary deoxyribonucleic acid cloning and uterine expression of messenger ribonucleic acid encoding evolutionarily conserved IGF-I peptides.

Thraikill, K. M., Clemmons, D. R., Busby, W. H. and Handwerger, S. (1990) *J. Clin. Invest.* **86**, 878-883, Differential regulation of insulin-like growth factor binding protein secretion from human decidual cells by IGF-I, insulin, and relaxin.

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

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

Ui, M., Shimonaka, M., Shimasaki, S. and Ling, N. (1989) *Endocrinology* **125**, 912-916, An insulin-like growth factor-binding protein in ovarian follicular fluid blocks follicle-stimulating hormone-stimulated steroid production by ovarian granulosa cells.

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

Unterman, T. G., Patel, K., Mahathre, V. K., Rajamohan, G., Oehler, D. T. and Becker, R. E. (1990) *Endocrinology* **126**, 2614-2624, Regulation of low molecular weight insulin-like growth factor binding proteins in experimental diabetes mellitus.

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

Veldhuis, J. D. and Demers, L. M. (1985) *Biochem. Biophys. Res. Comm.* **130**, 234-240, A role for somatomedin C as a differentiating hormone and amplifier of hormone action on ovarian cells: Studies with synthetically pure human somatomedin C and swine granulosa cells.

Waites, G. T., James, R. F. L., Walker, R. A. and Bell, S. C. (1990) *J. Endocrinol.* **124**, 333-339, Human 'pregnancy-associated endometrial α1-globulin', a 32 kDa insulin-like growth factor-binding protein: Immunohistological distribution and localization in the adult and fetus.

Walton, P. E. and Etherton, T. D. (1989) *J. Endocrinol.* **120**, 153-160, Effects of porcine growth hormone and insulin-like growth factor-I (IGF-I) on immunoreactive IGF-binding protein concentration in pigs.

Walton, P. E., Baxter, R. C., Burleigh, B. D. and Etherton, T. D. (1989) *Comp. Biochem. Physiol.* **92B**, 153-160, Purification of the serum acid-stable insulin-like growth factor binding protein from the pig (*sus scrofa*).

Walton, P. E., Grant, P., Owens, J. A., Wallace, J. C. and Ballard, F. J. (1990) *Proc. Endocrine Soc.* (Abst.) **180**, 69, Isolation and characterization of ovine plasma IGF binding proteins 1, 2 and a 24 kDa species.

Walton, P. E., Grant, P. A., Francis, G. L., Wallace, J. C. and Ballard, F. J. (1991) *Biochem. J.* Isolation and characterization of ovine plasma insulin-like growth factor binding protein (IGFBP)-3 two molecular weight size variations of IGFBP-4. Submitted for publication.

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

White, R. M. Nissley, S. P., Moses, A. C., Rechler, M. M. and Johnsonbaugh, R. E. (1981) *J. Clin. Endocrinol. Metab.* **53**, 49-57, The growth hormone dependence of a somatomedin-binding protein in human serum.

White, R. M., Nissley, S. P., Short, P. A., Rechler, M. M. and Fennoy, I. (1982) *J. Clin. Invest.* **69**, 1239-1252, Developmental pattern of a serum binding protein for multiplication stimulating activity in the rat.

Wilbur, W. J. and Lipman, D. J. (1983) *Proc. Natl. Acad. Sci* (USA) **80**, 726-730, Rapid similarity searches of nucleic acids and protein data banks.

Wilkins, J.R. and D'Ercole, A.J. (1985) *J. Clin. Invest.* **75**, 1350-1358, Affinity-labeled plasma somatomedin-C/insulin-like growth factor I binding proteins. Evidence for growth hormone dependence and subunit structure.

Wood, T. L., Brown, A. L., Rechler, M. M. and Pintar, J. E. (1990) *Mol. Endocrinol.* **4**, 1257-1263, The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in midgestational rat embryo.

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

Yang-Feng, T. L., Franke, U. and Ullrich, A. (1985) *Science* **228**, 728-731, Gene for human insulin receptor: Localization to site on chromosome 19 involved in pre-B-cell leukemia.

Zapf, J., Schoenle, E. and Froesch, E. R. (1978) *Eur. J. Biochem.* **87**, 285-296, Insulin-like growth factors I and II: Some biological actions and receptor binding characteristics of two purified constituents of nonsuppressible insulin-like activity of human serum.

Zapf, J., Waldvogel, M. and Froesch, E. R. (1975a) *Arch. Biochem. Biophys.* **168**, 638-645, Binding of nonsuppressible insulinlike activity to human serum. Evidence for a carrier protein.

Zapf, J., Walter, H. and Froesch, E. R. (1981) *J. Clin. Invest.* **68**, 1321-1330, Radioimmunological determination of insulinlike growth factors I and II in normal subjects and in patients with growth disorders and extrapancreatic tumor hypoglycemia.

Zapf, J., Born, W., Chang, J-Y., James, P. and Froesch, E.R. (1975b) *Biochem. Biophys. Res. Comm.* **156**, 1187-1194, Isolation and NH_2-terminal amino acid sequences of rat serum carrier proteins for insulin-like growth factors.

Zapf, J., Born, W., Chang, J.-Y., James, P., Froesch, E. R. and Fischer, J. A. (1988) *Biochem. Biophys. Res. Comm.* **156**, 1187-1194, Isolation and NH_2-terminal amino acid sequences of rat serum carrier proteins for insulin-like growth factors.

Zapf, J. Hauri, C., Waldvogel, M., Futo, E., Häsler, H., Binz, K., Guler, H. P., Schmid, C. and Froesch, E. R. (1989) *Proc. Natl. Acad. Sci.* (USA) **86**, 3813-3817, Recombinant human insulin-like growth factor I induces its own specific carrier protein in hypophysectomized and diabetic rats.

Zapf, J., Kaufmann, U., Eigenmann, E. J. and Froesch, E. R. (1977) *Clin. Chem.* **23**, 677-682, Determination of nonsuppressible insulin-like activity in human serum by a sensitive protein-binding assay.

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

Zapf, J., Schmid, H. P., Guler, M., Waldvogel, M., Hauri, C., Futo, E., Hossenlopp, P, Binoux, M. and Froesch, E. R. (1990b) *J. Clin. Invest.* **86**, 952-961, Regulation of binding proteins for insulin-like growth factors (IGF) in humans. Increased expression of IGF binding protein 2 during treatment of healthy adults and in patients with extrahepatic tumor hypoglycemia.

Zumstein, P.P., Luthi, C. and Humbel, R.E. (1985) *Proc. Natl. Acad. Sci.* (USA) 82, 3169-3172, Amino acid sequence of a variant pro-form of insulin-like growth factor II.

Zucker, M. and Steigler, P. (1981) *Nucleic Acids Res.* **9**, 133-148, Optimal computer folding of large RNA sequences using thermodynamics and auxiliary information.